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Enzymatic pre-treatment of cellulose rich biomasses for use in the biogas process

Enzymatisk förbehandling av cellulosarika substrat för användning i biogasprocessen

Erik Stenströmer Moglia

ABSTRACT

Enzymatic pre-treatment of cellulose rich biomasses for use in the biogas process

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An experimental study was performed in order to investigate the effect of an enzymatic pre-treatment step for the biogas production potential from cellulose rich material. The enzymes used for the pre-treatments were a mix of cellulases and hemicellulases. The biogas production was determined by an anaerobic batch test set-up. The substrates used were wheat, distiller's waste liquid and wheat straw. The results showed that the enzymatic pre-treatment increased the methane production rate during an initial step of the anaerobic digestion. An increase of methane yield in a continuous biogas reactor can therefore be expected. However the level of this increase will be dependent on the retention time of the biogas process. The increase in methane yield will decrease with increasing retention times. The biogas potentials, measured when the gas production had levelled out, were not influenced by an "optimal" dose (calculated for corn) of enzymes. However the results indicate that an increase of biogas potential may be obtained by a higher dose of enzymes during pre-treatment. An alkaline peroxide pre-treatment followed by enzymatic pre-treatment were also performed in order to see if a breakage of the lignin structure could enhance the effect of the enzyme treatment. This experiment showed that a degradation of the fibre structure was an effective pre-treatment by it-self as this increased the biogas potential. The alkaline treatment also increased the effect of the preceding enzymatic pre-treatment at an initial stage but it did not in the end increase the biogas potential. The experiments also showed that the biogas system naturally has a quite good capacity to degrade cellulose.

Keyword: Biogas production potential, pre-treatment, enzymes, cellulose, lignin

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REFERAT

Enzymatisk förbehandling av cellulosa rika substrat för användning i biogasprocessen

Erik Stenströmer Moglia

En experimentell studie genomfördes för att undersöka effekterna på biogasproduktionen av en enzymatisk förbehandling. Enzymerna som användes var en blandning av cellulaser och hemicellulaser. Biogasproduktionen från ett specifikt substrat bestämdes genom anaeroba satsvisa försök. Substraten som användes var vete, drankvatten och halm. Experimenten visade på en höjning av metanproduktionshastigheten under en inledande fas av de satsvisa försöken. Detta betyder att en enzymatisk förbehandling av ett substrat innan en kontinuerlig process har en potential att ge en ökning i metanutbytet. Graden av denna ökning kommer dock att vara kopplad till processens uppehållstid. Resultatet från beräkningar visade att ökningen i metanutbytet minskar med en ökande uppehållstid i biogasprocessen. Den teoretiska metangasbildningspotentialen för ett substrat ökade inte då en ”optimal” dos av enzym (beräknad för majs) tillsattes. Resultaten visar dock att en högre dos av enzymer under förbehandling av halm kan höja potentialen. Vidare genomfördes en alkalisk väteperoxid behandling följt av en enzymatisk förbehandling. Detta för att se om enzymernas effekt kunde förbättras genom att förstöra cellulosaens kristallina fiberstruktur och därmed göra den mer tillgänglig för nedbrytning. Experimentet visade att en strukturknäckande behandling är en effektiv behandling i sig själv som ökar biogaspotentialen hos ett substrat, i detta fall halm, och att den initialt också ökade effekten av den efterföljande enzymatiska behandlingen. Emellertid kunde inte heller här den enzymatiska behandlingen öka biogaspotentialen. Slutsatser som kan dras av denna studie är att det troligen går att öka metanbildningspotentialen genom enzymatisk-/alkalisk väteperoxidbehandling men att det i biogassystemet finns en relativt god inneboende förmåga att bryta ned cellulosa.

Nyckelord: Biogas potential, förbehandling, enzymer, cellulosa, lignin

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PREFACE

This is a master thesis performed in the project *MicroDrive*, microbially derived energy, at the faculty of natural resources and agriculture (NL), Swedish University of Agricultural Sciences. Supervisor was Dr. Mats Sandgren, Department of Molecular Biology, SLU and reviewer were Ass. Prof. Anna Schnürer, Department of Microbiology, SLU.

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Uppsala, Juni 2008
Erik Stenströmer Moglia

POPULÄRVETENSKAPLIG SAMMANFATTNING

Enzymatiskförbehandling av halm och vete för användning i biogasprocessen

Erik Stenströmer Moglia

En av de stora utmaningarna för samhället i framtiden är att ersätta den fossila oljan med förnyelsebar energi och fordonsbränsle. Stora satsningar på forskning och produktion av bioenergi måste genomföras för att möta detta krav. Många olika biodrivmedel finns idag tillgängliga såsom biodiesel, etanol, metanol och biogas, som alla har sina fördelar och nackdelar. En mycket viktig del i produktionen av bioenergi är omvandlingen av celluloserika material till energi och fordonsbränsle. Cellulosa är den mest vanliga organiska föreningen på jorden och stora mängder energi från fotosyntesen är bundet i cellulosa. Cellulosa är biologiskt svårnedbrytbart på grund av sin struktur och mycket forskning sker med avseende på förbehandlingar av celluloserika biomassor för användning i bioenergiindustrin. Speciellt har det genomförts mycket forskning på förbehandling inför produktion av bioetanol. Här används t.ex. tekniker som syra- och ångexplosions- behandling i kombination med enzymatisk förbehandling. I framtiden ses förgasning av torra celluloserika biomassor som ett mycket intressant alternativ för energiproduktion.

Få undersökningar med förbehandling av celluloserika biomassor för användning i biogasprocessen har genomförts, detta trots att dessa visat att biogasprocessen är ett effektivt alternativ för framställning av fordonsgas från denna typ av material. Biogas är troligen också det enda alternativet för energiframställning av blöta celluloserika biomassor som t.ex. nötgödsel, potatisblast och annat jordbruksavfall. I biogasprocessen sker nedbrytning av cellulosa genom en rad olika mekanismer med många olika mikroorganismer inblandade och slutprodukten är metan och koldioxid (biogas). Metan kan användas som drivmedel eller för kraft och/eller värmeproduktion. Nedbrytningen av cellulosa ses allmänt som en vanlig hastighets begränsande faktor i en biogasprocess och mycket kan vinnas genom att öka hastigheten på nedbrytningen. Ett alternativ som ses som mycket intressant är enzymatisk förbehandling av cellulosa inför biogasprocessen. Enzymatisk förbehandling inför biogasprocessen har tidigare, med framgång, testats på slam från reningsverk.

I detta arbete genomfördes en experimentell studie av effekten av enzymatisk förbehandling på några olika celluloserika material för biogasproduktion. Undersökningen genomfördes med enzymer följt av nedbrytningsförsök i enkla småskaliga biogasreaktorer av förbehandlat material. Enzymerna som används för förbehandlingen var en blandning av cellulaser och hemicellulaser och var skänkta av Genencore. Undersökningen var till viss del ett led i en optimeringsansats av biogasprocessen i en anläggning i Norrköping (Tekniska Verken AB, Linköping). Studien, som gjordes som ett examensarbete i civilingenjörsprogrammet miljö- och vattenteknik på Uppsala tekniska högskola, var en del av ex-jobbsskolan *MicroDrivE* (Microbially Derived Energy) vid Institutionen för Mikrobiologi, SLU, Uppsala.

Resultaten från undersökningen visade att det går att öka biogasproduktionen i en kontinuerlig biogasprocess med hjälp av en enzymatisk förbehandling av ett celluloserikt material, t ex vetestrå. Hur stor ökningen blir beror bl.a. på halten av

cellulosa samt på materialets uppehållstid i biogas reaktorn. I en kontinuerlig process tillsätts och tas ut material kontinuerlig och hastigheten på inmatningen inverkar på materialets s.k. uppehållstid i reaktorn. Upphållstiden är mycket viktig då den bestämmer hur länge materialet är i reaktorn och därmed hur mycket av startmaterialet som hinner brytas ner. Den enzymatiska förbehandlingen leder till ett snabbare nedbrytningsförlopp i biogasprocessen och därmed kommer en större del av material omvandlas till biogas. Undersökningen visade även att en hög dos av enzymer vid förbehandlingen ökade biogas potentialen av materialet dvs. hur mycket som blev nedbrutet och slutligen omvandlat till biogas. Vid enzymatisk förbehandling av substratet som används vid biogas anläggningen i Norrköping, drankvatten och vete, kunde ingen ökning av biogasproduktionen ses. Den troliga anledningen var att cellulosahalten var för låg för att en effekt skulle kunna mätas.

Försöken visade också att biogasprocessen i sig själv har en hög förmåga att bryta ned cellulosa och att en kemisk förbehandling som enbart syftar till att bryta upp cellulosa strukturen, och göra den mer tillgänglig för biologisk nedbrytning, är tillräckligt effektiv för att ge en ökning av biogasproduktionen. En sådan förbehandling ökar till viss del även effekten av en efterföljande enzymatisk behandling på så sätt att nedbrytningen av materialet i ett initialt skede är hög. Den enzymatiska förbehandlingen hade emellertid inte i dessa försök någon effekt på den slutgiltiga biogas potentialen. Försöken visade även att en direkt enzymatisk tillsatts till en biogasreaktor ej har någon effekt på biogasproduktionen. Detta berodde troligen på att pH i processen var för högt för de cellulosanedbrytande enzymerna.

Mer forskning behövs för att klargöra effekten av samt optimera en enzymatisk förbehandling innan biogasprocessen. Det skulle även vara intressant att undersöka hur biogasprocessen kan optimeras för att de i biogasprocessen naturligt förekommande cellulosanedbrytande enzymerna skall kunna bryta ned cellulosarika biomassor fortare och mer effektivt.

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1 INTRODUCTION

Anaerobic digestion of wastewater, agricultural waste and other organic waste is nothing new (Leven, 2006). This method has been used for a long time in for example China and India. Here simple anaerobic reactors are used for the purpose of producing methane for cooking and for light. Anaerobic digestion of municipal sludge, in a bigger scale, was introduced already in 1860. However not until 1970:s an increase in research about biogas production and technological development could be seen! Today there are biogas reactors that digest many different kinds of substrates and there are many techniques available depending on the substrate to be used in the process. In Sweden during 2006, a total of 1.2 TWh of energy as biogas were produced (Statens energimyndighet, 2008)

By using the anaerobic digestion technology it is possible to biologically treat different organic wastes and at the same time produce energy and a nutrient rich residue. Since almost no nutrients are lost in a biogas reactor the digestion residue has high concentrations of for example phosphorus and nitrogen and have the potential to be used as an agricultural fertilizer (Odlare, 2005). This “bio manure” should be regarded as a mineral fertilizer because of its low organic content and high concentration of nutrients (Svensson et.al. 2004). The use of biogas residues as fertilizer is also positive in an economic and environmental point of view as the production and the distribution may be performed at a lower energy cost as compared to the production of artificial fertilizers (Berglund and Börjesson, 2005).

It is also important not to regard the biogas process only as a waste treatment step but also as a bio energy/fuel production alternative. Biogas can be produced from energy crops such as silage and corn as well as from different agricultural and municipal wastes. The wide range of substrates to be used in the biogas process makes biogas as vehicle fuel efficient with regard to land use and to the use of organic by-products and wastes (Börjesson and Mattiasson, 2007). Anaerobic digestion is also an important tool to decrease the emissions of greenhouse gases (Börjesson and Mattiasson, 2007). The trend in Sweden today is that the production of biogas as vehicle fuel is increasing. During the first half of 2006 ~10 Mm³ (~475 GWh) upgraded biogas was sold as vehicle fuel and the goal is to produce 1000 GWh by 2010. The energy gained in biogas production, when producing vehicle fuel, depends on the substrates used. Estimation for 4 different crops gave a quota of outgoing- / ingoing energy between 3.3 and 4. This quota takes into account the energy used for production of crops, transport, production and upgrading of biogas. The production cost is between 3-5.5 Swedish kronor / m³ for a co-digestion biogas plant (1 m³ biogas ≈ 1 l petrol; Nordberg, 2006). Biogas can be seen as a promising bio energy alternative together with the second generation of vehicle fuels based on lignocelluloses, for example thermal gasification (Börjesson and Mattiasson, 2007). Wet cellulose substrates such as cow manure, corn and silage will however probably only be used as a substrate in biogas production (Nordberg, 2006). However, one problem when using such substrates is that the hydrolysis of cellulose then can be rate limiting for the whole biogas process (Gerardi, 2003)

1.2 AIM

The main purpose of this master thesis was to examine the effect of an enzymatic pre-treatment on the biogas yield from cellulose rich substrates. A commercial mix of cellulases and hemicellulases was evaluated. The enzymes are expressed by a commercial modified species of *Hypocrea jecorina* and provided by Genencor.

The sub-purpose was to examine if the biogas production at a biogas plant in Norrköping (owner: Tekniska Verken AB in Linköping) could be enhanced by an enzymatic pre-treatment of the substrates (wheat and distillers waste) used. Tekniska Verken AB is an industrial partner to the research program *MicroDrivE*, SLU, in which this thesis has been a part.

2 BACKGROUND

2.1 THE BASICS ABOUT BIOGAS

Biogas is mainly composed of carbon dioxide and methane and is produced during anaerobic (i.e. without oxygen) digestion of organic material (Gerardi, 2003). The biogas can also contain small amounts of e.g. hydrogen sulphide, nitrous oxide and ammonia (Gerardi, 2003). The biogas process occurs naturally in for example swamps and dunghills but can also be established in constructed biogas reactors (Marchaim, 1992). Methane is rich in energy and can be used as fuel in e.g. combustion engines and turbines. Combustion of methane results in heat, water and carbon dioxide. The process which leads to the degradation of organic material to carbon dioxide and methane is carried out by a number of different microorganisms, using the organic material as substrate for their metabolism (Gerardi, 2003).

2.2 THE MICROBIOLOGICAL PROCESSES DURING BIOGAS PRODUCTION

A number of different microorganism groups are critical for the degradation of organic material in the biogas process. The transformation of complex organic material to carbon dioxide and methane occurs through a number of steps where different microorganism groups with different metabolic functions play an important role (Zinder, 1984). The transformation is visualized in the flow scheme below (figure 1).

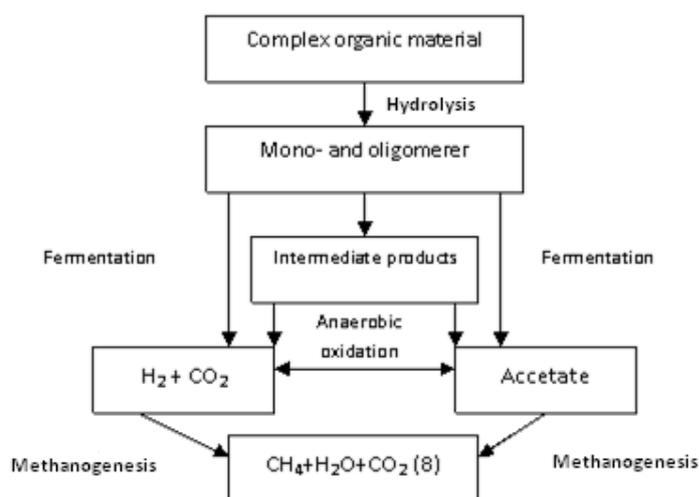


Figure 1 The carbon flow in the methane production process (Schnürer, 1995)

In the first step, complex material such as proteins fats and polysaccharides, e.g. cellulose and starch, are split into smaller units such as amino acids, cellobiose and glucose. This so called hydrolysis is performed by extracellular enzymes excreted by different bacteria. The rate by which a substrate is hydrolyzed can vary a lot depending on the composition of the material, i.e. the concentrations of proteins, fats, carbohydrates and lignin. When a substrate has a high content of cellulose the

enzymatic hydrolysis often becomes the rate limiting step in the biogas process. Cellulose has a molecular structure making it relatively resistant to degradation (2.6 CELLULOSE).

In the next step of the anaerobic degradation process, smaller soluble molecules (amino acids, sugars etc.) are used by fermentative bacteria as a carbon and energy source. In this step it is mainly the same bacteria as those active in the first hydrolysis step which is involved. However, they also compete with other non enzyme excreting bacteria. The fermentation step is performed by a variety of bacterial groups and consists of many different reactions. The products formed during fermentation depend on the character of the original substrate i.e. the content of protein, lipid and carbohydrates. The end products in the different fermentation steps, the so called intermediate products, are alcohols, short chain volatile fatty acids e.g. acetate and propionate, long fatty acids, hydrogen gas and carbon dioxide.

The products from the fermentative steps are used in the next step of the biogas process, the so-called anaerobic oxidation step, by a group of bacteria called the proton-reducing bacteria. During the anaerobic oxidation, acetate, carbon dioxide and hydrogen are the main products formed. The reactions in this step can, for thermodynamically reasons, only occur when a low hydrogen pressure persists. Therefore these organisms are dependent on the hydrogen consuming partner organisms active in the following degradations step. These hydrogenotrophic microorganisms use hydrogen and carbon dioxide as energy and carbon source, respectively, and the end product can be methane, hydrogen sulphide or nitrogen gas depending on the active organism and which electron acceptor it uses. In the biogas process, the active hydrogenotrophic microorganisms are mainly the methane producing bacteria. This interaction between the hydrogen producing and the hydrogen consuming organisms, which makes them dependent on each other for their substrate, is called syntrophy (Schink and Stams, 2006). During anaerobic oxidation, and also during some fermentation reactions, a lot of acetate is produced. This acetate is an important substrate for some of the methane producing microorganisms and stand for the main part of methane produced in the biogas process (Gerardi, 2003).

If the hydrogenotrophic organisms cannot keep the hydrogen pressure at sufficient low levels an accumulation of fatty acids will occur and the process will as a consequence go “sour” (Gerardi, 2003). This scenario can occur after an overload of substrate or if the methane producing organisms have been disturbed or a combination of both. It takes a long time for the methane producers to recover after a disturbance; due to slow growth rate. Examples on factors that the methane producing organisms are sensitive for are e.g. high ammonia concentrations, low pH and fast changes in temperature (Gerardi, 2003; Edmond-Jacques, 1986).

2.3 BASIC OPERATIONAL PARAMETERS AND PROCESS TECHNIQUES OF A BIOGAS PROCESS

There are several different reactor designs and process types for treatment of organic material in a biogas process. Also different processes use different process parameters in order to optimize and stabilize the process. For example, depending on the input of substrate, stirring degree, and temperature interval etc the biogas process will behave differently and different methane yields will be obtained. Today there is no dominating design of biogas reactors in Europe (Agrobiogas, 2006). Some of the different options of process techniques are continuous-, batch-, fed-batch-, plug-flow-, continuous series connected reactors and anaerobic filter (Nordberg, 2006). In this thesis wet digestion in one step continuous processes and batch reactors will be further discussed.

Batch process: In a batch process all substrate is put into the reactor at start. The biogas process is initiated, after closing the reactor, and the biogas is collected from the process until the methane production has ceased. Then the reactor is opened and emptied/cleaned and a new batch is started (Bains, 1998). This type of process is common at dump gas facilities where big amounts of waste are digested without stirring (Agrobiogas, 2006). Small scale batch reactors are also common in e.g. China and India for production of biogas to be used in households (Marchaim, 1992). The degree of degradation of the substrate in a batch process is very high and a low content of organic matter is left in the biogas residues after that the process has been terminated (Edmond-Jacques, 1986).

Continuous or semi-continuous one step process: In this process the substrate is fed into the reactor in a steady flow or in small doses; simultaneously the same volume is taken out of the process. The hydraulic retention time (HRT) is an important parameter in this process and it is a measure of how long time the substrate stays in the reactor. The HRT is approximated as the volume of the reactor divided by the outflow [s] (Edmond-Jacques, 1986). The continuous reactor is the most common large scale biogas process in Sweden (Agrobiogas, 2006). In these continuous processes the most common process type is the mixed reactor (Nordberg, 2006). The degree of degradation will usually be lower in a continuous process compared to the batch process (Gerardi, 2003). This lower degree of degradation in a continuous process can be explained by the fact that some organic material that newly has been added to the reactor will be taken out. The amount of non-degraded organic material that is taken out of the reactor will differ depending on the hydraulic retention time. The remaining organic material is often allowed to be converted to biogas in a post digestion and/or a biogas sludge storage tank.

In the biogas process two temperature intervals are commonly used, 30-40° C (mesophilic) and 50-60°C (termophilic) (Gerardi, 2003; Edmond-Jacques, 1986). These temperature intervals represent areas for optimal growth and activity of microorganisms. Depending on the temperature used, the composition of the microbial community will vary. The termophilic temperature interval generally generates a higher yield of methane gas and a higher destruction degree of pathogens as compared to a lower temperature. The higher temperature however also results in a lower microbial diversity, which will make the process more sensitive for disturbances compared to a mesophilic process. The termophilic process will also result in a faster process speed and can therefore be more difficult to operate. The methane producing microorganisms are sensitive to temperature changes and to obtain a stabile biogas process the

temperature should not vary too much; in a thermophilic process not more than 1 degree per day and in a mesophilic reactor not more than 2-3 degrees / day (Gerardi, 2003).

2.4 SUBSTRATES FOR USE IN BIOGAS PRODUCTION

Many different types of organic material can be used in the biogas process for production of methane. Due to the variations in composition and structure, different organic materials will give different biogas yields and gas compositions (Agrobiogas, 2006). The biogas potential for a specific substrate can be determined by a batch experiment (3.4 BATCH-EXPERIMENT) and is usually given as ml (1 atm, 0°C) methane / g volatile solids, where volatile solids is a measurement on the amount of organic material in the substrate. The biogas potential for some substrates can be seen in table 1. Commonly, co-digestion of two substrates gives a higher biogas potential than a single substrate (Agrobiogas, 2006). Therefore, to clearly reveal the potential of substrate mixtures, separate investigations are necessary to estimate the potential when co-digesting. Investigations have been carried out in Denmark which shows that co-digestion is necessary to get an economical viable biogas plant (Agrobiogas, 2006). The increase in biogas yield in co-digestion can partly be explained by a better composition of micronutrients. For example, the use of household waste, when co-digesting with silage, raises the concentration of cobalt, a trace element of importance for growth of microorganisms (Jarvis et. al, 1997). Energy crops typically contain relatively low concentrations of micronutrients, which can limit the microorganisms responsible for the biogas production (Agrobiogas, 2006).

Table 1 Methane produced from different substrates, tested in batch experiments (ml [1 atm, 0°C] / g Volatile solids) (Agrobiogas, 2006).

Substrate:	Produced ml CH₄/ g Volatile solids
Pig manure	290
Cow manure	200
Chicken manure	300
Wheat straw	150
Corn straw	200

The composition of the substrate will also determine the structure of the microbial community in the biogas process. Some species in the process are specialised for certain types of organic compounds e.g. proteins and enzymes from these organisms might not have the ability to degrade e.g. polysaccharides (Edmond-Jaques, 1986). As a consequence, changes in substrate composition will therefore also lead to changes in the bacterial composition. The concentrations in the substrate of proteins or ammonia will also affect the biogas process. High concentration of these compounds can lead to an inhibition of the methane producing microorganisms (Schnürer, 1995). But at the same time the incoming concentration of nitrogen should not be so low that it limits the microbial growth. A carbon-nitrogen quota of 16-19 is generally considered as being optimal (Edmond-Jaques, 1986) for the biogas process. Also, biogas processes can be run at relatively high ammonia levels if allowed to slowly adapt (Schnürer, 1995). When digesting cellulose rich material, the nitrogen quota will be low and therefore limiting for the biogas process (Gerardi, 2003).

2.5 POTENTIAL SUBSTRATES AND PRODUCTION OF BIOGAS

Several calculations concerning the biogas potential in Sweden has been carried out and the results vary from 1.1 to 17 TWh per year (Nordberg, 2006). This can be compared

to the nuclear plant Forsmark which produces around 25 TWh per year (Vattenfall, 2007). The different values of theoretical biogas potential are caused by differences on type and amount of substrates included in the calculations. The highest calculated potential (17 TWh) take wheat straw into account while the lower potentials disregard wheat straw as a substrate, due to its low biogas potential. Furthermore, the lowest estimation only take into account organic waste and substrates produced close to the bigger cities (Nordberg, 2006). None of the investigations includes residues from the forest industry. The forestry industry has estimated that they can increase the outtake of forestry residues by 20 TWh/year. Also, the Swedish forest biomass is estimated to increase by 0.5-0.7% / year. It is calculated that a realistic yearly increase in outtake of this biomass is 18 Mm³, which can be converted to 36 TWh when counted as energy (The Royal Swedish Academy of sciences, 2007). This increase of biomass outtake from the forestry has a potential to be used in the production of bio-fuel. The main part of this biomass consists of cellulosic material, which is relatively hard to degrade. It stands clear that bio energy from cellulosic biomasses such as wheat straw, forestry residues and energy crops such as silage, salix and corn has a great potential for use in the bio energy industry. Today thermal gasification is seen as the most interesting alternative for these lignocellulosic biomasses (The Royal Swedish Academy of sciences, 2007; Börjesson and Mattiason, 2007). However, a lot of research concerning cellulosic material for ethanol production has also been performed, only a few investigations concerning biogas production from these types of materials have been made (Nordberg, 2007).

2.6 CELLULOSE

Cellulose is the most common renewable organic compound on earth and on a global scale plants produces almost 100 billion tons of cellulose every year (Campbell and Reece, 2002). Cellulose is very stable and hard to degrade due to the dense packing of the cellulose chains in the crystalline cellulose microfibrils. Cellulose is a polymer of glucose units, and on an average one cellulose molecule contains 5000 glucose units (Campbell and Reece, 2002). Depending on the position of the hydroxyl group at carbon atom nr 1, glucose can have two forms, α and β (figure 2). In cellulose all the glucose molecules are in the β form and the glucose units are linked by β -1, 4-glycoside bonds (figure 3). If the glucose units instead are in α form, we get starch. This means that in cellulose every other glucose molecule alternate in “back and forward” position. Because of this alternation the cellulose molecules are un-branched, linear and not soluble (Hart and Craine, 2003). The smallest repeating unit in cellulose consists of two glucose molecules and is called cellobiose (figure 3).

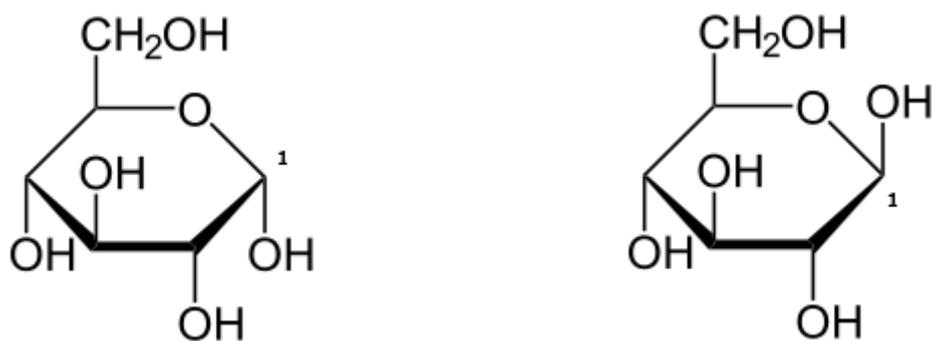


Figure 2 The two isomers of glucose, to the left α -glucose, to the right β glucose. (Wikipedia, 2007)

Because of the linear structure of cellulose the hydroxyl groups are free to form a hydrogen-bond with the hydroxyl groups of another cellulose molecule lying parallel to it. Parallel cellulose molecules can in this way form strong units which are called microfibrils. These units are strong building material for plants. Wood, linen and cotton are mainly built from cellulose. Other common organic carbons in plant cell walls are hemicelluloses and lignin. Hemicellulose is a heterogeneous polysaccharide composed of a wide range of polysaccharides and is shorter than cellulose. Lignin is a heterogeneous aromatic polymer which is forming a very complex and stable structure (Betts, 1991). Lignin cannot be degraded by microorganisms during anaerobic conditions (Dinsdale et al, 1994) but under aerobic conditions degradation of lignin is performed by white rot fungi and moulds (Betts, 1991; Fountoulakis et. al, 2002).

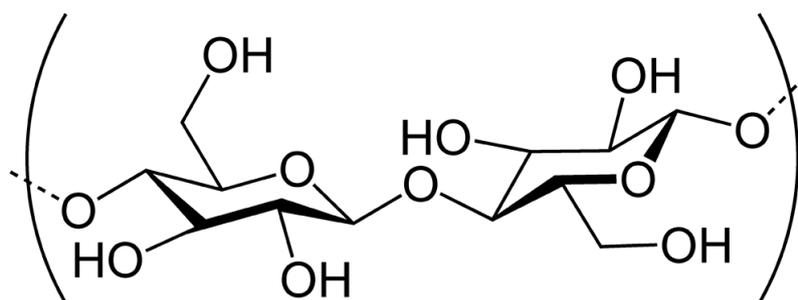


Figure 3 Two glucose molecules in a cellulose chain, cellobiose (Wikipedia, 2007)

Few organisms are able to produce enzymes which are able to break the β bond in cellulose. For example humans are not able to digest cellulose. But there are some microbes that can hydrolyze cellulose and degrade it into glucose and cellobiose. This degradation is performed by production of extracellular- and membrane bound enzymes that split the cellulose molecule into shorter glucan polymers such as e.g. cellobiose (Campbell and Reece, 2002).

2.7 PRE-TREATMENT METHODS

The goal, when pre-treating a substrate for use in the biogas process, is to maximize the methane production and to minimize the time required to get the maximum yield. A fast degradation process results in a higher total degradation of the incoming substrate in a continuous biogas process and therefore also generates a higher methane yield.

Commonly used pre-treatment alternatives are heat/thermal treatment, centrifugation, chemical- and ultrasonic treatment and grinding (Nordberg and Edström, 1997; Agrobiogas, 2006). A combination of these pre-treatments has also proven efficient, for example a combination of chemical and thermal pre-treatment (Vlyssides, 2004). These pre-treatment methods are all aiming at destroying the structure of the starting material and make it more soluble, thereby making it more accessible for degradation by the microorganism. As a consequence, the hydrolysis reactions proceed faster in the biogas process. Smaller particles are easier to make soluble and has a bigger area on which the microorganism and extracellular enzymes can act on. Heating, centrifugation and chemical- and ultrasonic treatments are usually performed on wastewater sludge, while grinding is usually used when energy crops are used for biogas production. In a historic perspective these methods has not been very economical with the exception of grinding of crops (Agrobiogas, 2006).

A coming and interesting pre-treatment alternative during biogas production is pre-treatment with hydrolytic enzymes. The enzymes can either be used in a pre-treatment step or directly added to the biogas process. Different enzymes specific for certain types of material can be used. For an optimal treatment, different enzymes should for example be used when pre-treating waste water sludge compared to cellulose rich raw material. When used in waste water sludge, the enzymes do not only hydrolyze the organic material they also destroy bacteria flocks and membranes. Disruption of flocks and membranes makes the bacteria more accessible for degradation and decrease problems with foaming in the biogas reactor at the waste water treatment plant (Davidsson et. al., 2007; Recktenwald et. al., 2007). Enzymatic pre-treatment on cellulose rich material for use in the biogas process has not yet been tested in large-scale (Nordberg, 2006). However enzymes are commonly used to hydrolyze cellulose rich materials during ethanol production and a lot of pre-treatments methods trying to enhance the efficiency of the enzymatic treatment have been evaluated. Some examples of pre-treatments are; dilute acid pre-treatment (Saha et. al., 2005); thermal steam treatment followed by enzymatic hydrolysis with both hemicellulases and cellulases (Öhgren et. al., 2006); alkaline peroxide treatment (Saha and Cotta, 2006), and thermal treatment (Palmarola-Adrados et. al., 2004). These pre-treatments before enzymatic treatment are performed to destroy the crystalline fibre structure and make the cellulose more accessible for the enzymes in the following hydrolytic step. Hemicelluloses and lignin form a physical barrier around the cellulose and hinders cellulose degrading enzymes to reach the cellulose (Adney et. al., 1991). For example, by adding hemicellulases together with cellulases, higher cellulose degradation efficiency can be obtained (Öhgren et. el, 2006). It has also been shown that lignin can bind cellulases and thereby hinder the enzymatic hydrolysis. This binding can be inhibited by simultaneous addition of polymers and detergents (Jørgensen, 2003). Ethanol may also be used as a pre-treatment option to degrade lignin (Öhgren et. al, 2006). An alternative way of optimising the hydrolysis of cellulose rich materials is to separate the biogas process into a two step process, with the hydrolysis and the fermentation taking place in one reactor and the methanogenesis in another. By doing this it is possible to optimize the environment for the different functional bacterial groups separately. Higher concentrations of hydrolytic enzymes have been shown to be possible to reach in the hydrolysis step of a two step process as compared to a one-stage system (Fox and Pohland, 1994).

2.8 CELLULOSE DEGRADING ENZYMES

Enzymes are a group of proteins that function as biological catalyst (Hart and Craine, 2003). The compound the enzyme acts on is referred to as the substrate of the enzyme. The enzyme binds to its substrate and the catalytic function of the enzyme converts the substrate to other product(s) (Campbell and Reece, 2002). Most enzymes are named after its substrate and with the ending –ase. For example the extracellular enzymes, which are able to split the cellulose molecule into cellobiose and glucose are called cellulases. The cellulases can be divided into two main classes, endoglucanases (EG) and cellobihydrolases (CBH). EG hydrolyze internal β -1.4-glycosidic bonds in cellulose chains and CBH hydrolyze β -1.4-glycosidic bonds processively from the ends of the cellulose molecule. Many cellulolytic organisms degrade the crystalline cellulose by secreting a set of different EGs and CBHs (Sandgren, 2003). These sets of cellulose degrading enzymes commonly work synergistically together, which means that together they are more effective than the sum of every enzyme by itself. A more rapid and

efficient hydrolysis of cellulose is thereby obtained (Nidetzky et al. 1994). Synergy can be explained by the endo-exo model which says that the EGs cut inside the cellulose molecule and produces more ends for the CBHs to work on. One example of an organism able to produce an efficient set of cellulases is the fungi *Hypocrea jecorina* (Sandgren, 2003).

The activity of the enzymes is affected by general environmental factors such as temperature and pH (Campell and Reece, 2003). These factors determine the velocity of the enzymatic reaction. For example with an increasing temperature the hydrolysis velocity increases up to a certain point at which it drops very sharply. The temperature has then exceeded the point at which the enzyme starts to degrade and change form. Each enzyme has an optimal temperature at which its reaction is fastest. The optimal temperature is the highest possible temperature before the enzymes starts to denature and thereby losing its enzymatic properties.

Cellulases and hemicellulases are commercially produced and used in different industrial applications to modify biological materials containing cellulose and hemicelluloses e.g. in detergents and fabric softening (Hart and Craine, 2003, Madigan and Martinko, 2006).

3 MATERIALS AND METHODS

Enzymatic pre-treatment were performed with two different substrates; a mixture of distiller's waste liquid and wheat and a mixture of distiller's waste liquid and wheat straw. These enzymatic pre-treatments were done in order to hydrolyze cellulose and hemicelluloses into monomers and thereby increase the rate of degradation and the biogas potential of the substrates. The effect of the enzymatic pre-treatment of the substrates was evaluated by an anaerobic batch digestion set-up in which the biogas production and its composition were measured over time. Experiments in which enzymes were added straight into a biogas process were also carried out as well as experiments with a chemical pre treatment.

3.1 SUBSTRATES

Milled wheat and distiller's waste liquid were provided by Tekniska Verken AB in Linköping. This is the substrates used in the biogas plant in Norrköping. Wheat straw and boss were provided by Anders Eriksson, a farmer who is also an employee at SLU.

The wheat straw was milled by using a kitchen mixer and filtered afterwards. The fraction consisting of particles in the size of 0.5-1.0 mm were used in the experiments. The substrates dry substance (DS) and volatile substances (VS) were determined by standard methods (American public health association/water environment federation 1995). Shortly, DS is the percentage of mass left after drying the sample at 105°C as compared to the total mass. VS, a measure of the organic content in a sample, is the percentage of mass loss after making the sample red hot at 550°C. The remaining ash, after burning the sample at 550°C, corresponds to the mineral content of the sample. Triplicate samples of each substrate were investigated and standard deviations were calculated by using the standard deviation function in the Microsoft office program Excel. The cellulose concentrations were assumed to be 12.5 % of TS for wheat according to Åman (1987) and 80 % of TS for wheat straw according to Nilsson (1994). The cellulose concentration in distiller's waste liquid was assumed to be around 6 % of DS.

3.2 ENZYMATIC PRE-TREATMENT

Enzymatic pretreatments were performed with a mix of hemicellulases and cellulases expressed by *Hypocrea jecorina* and provided by Genencor (Palo Alto, USA). The concentrations of enzyme used in the pre-treatment experiments were 1 X and 4 X, where X equals 10 mg enzymes / g added cellulose. X is the enzyme concentration recommended by the enzyme manufacturer for hydrolysis of corn stover.

Each parallel sample, of every substrate, was mixed with 19 ml deoxidized water in separate bottles (309 ml). The load of substrate in every bottle in the pre-treatment was calculated from the load later to be used in the anaerobic batch digestion experiment (4 g VS/l). The pH was controlled and if necessary adjusted to pH 5 with 1M NaOH. The enzyme was added and the bottles were sealed with rubber stoppers and incubated on a shake table for three days in 37°C or 55°C. 37°C is a optimal temperature for the enzymes used, and 55°C was tested as it is the mean temperature in the substrate mixing tank at Norrköping biogas plant. Three main enzymatic pre-treatment experiments were performed to test different parameters (figure 4).

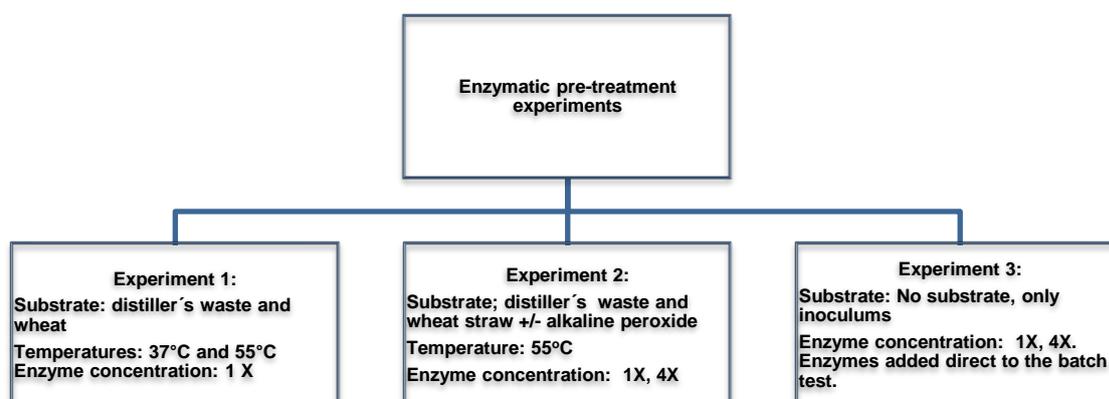


Figure 4 Set-up of enzymatic pre-treatment experiments

In experiment 1, enzymatic pre-treatment of distillers waste liquid mixed with wheat was tested. The mixture of substrates was wheat (90 % VS) and distillers waste liquid (10 % VS). One enzyme concentration was used, 1X, and in addition also a control with non treated substrate was started. The pre-treatments were carried out at two temperatures, 37°C and 55°C. Every treatment was started in replica of five separate bottles. In experiment 2, a alkaline peroxide pre-treatment was performed before the enzyme pre-treatment. The substrate mixture was wheat straw and boss (90 % VS) and distillers waste liquid (10 % VS). Enzyme treatment was also performed directly on this substrate mixture without the preceding chemical treatment. Two different enzyme concentrations were used, 1X and 4X, and the incubation temperature was, 55°C. A control with no addition of enzymes was also prepared and treated in a similar way as the material being treated with enzyme. Controls were started with both non-treated substrate and substrate subjected to the alkaline peroxide treatment. Every treatment was started in replica of five separate bottles. In experiment 3, no pre-treatment were performed and instead enzymes were directly added to the anaerobic batch test, containing only inoculums (see 3.4). Two different enzyme concentrations were used, 1X and 4X, and as in experiment 1 and 2 five separate bottles of each treatment were started. Controls, without addition of enzymes, were also started.

3.3 ALKALINE PEROXIDE TREATMENT

An alkaline peroxide pre-treatment (Saha et. al, 2006) was used to enhance the effect of the enzymatic treatment. The pre-treatments were performed in separate bottles (309ml). The substrate was mixed with water (8.6 % weight / volume water) and H₂O₂ (2.15 % volume / volume) and the pH were adjusted to pH 11.5 by adding 7 M NaOH. The amount of substrate treated were equivalent to 4 g VS / l calculated for a volume of 150 ml, the volume later used in the batch experiment. The bottles were incubated at 37°C on a shake table for 3 hours. After 3 hours of incubation, water was added to reach the same volume as used in the enzymatic pre-treatment experiment (20 ml). The pH was then adjusted to approximately 5 by adding 1M H₂SO₄. After the pH adjustment the enzymatic pre-treatment were started by addition of appropriate amount of enzyme.

3.4 BATCH EXPERIMENT

The biogas potential, and the methane production rate, was determined by using a batch method (Hansen et. al, 2004) modified by (Leksell, 2005). During flushing with N_2 / CO_2 (80/20), the bottles (309 ml) from the pre-treatment experiments were filled with 130 ml of inoculums to a total volume of 150 ml by addition of degassed water. The final volume of 150 ml was chosen to reach a total load of substrate corresponding to 4 g VS / l. The inoculums used were reactor fluid from the biogas plant in Norrköping. The VS and DS was determined once for one batch of these inoculums, before the start of experiment 1. The batches of inoculums later used in experiment 2 and 3 were assumed to be nearly identical to the first batch. Controls, containing only inoculum and no substrate, were started in all experiments. The batch bottles were incubated at 37°C on a shake table (figure 5). During incubation, the gas pressure was measured by using a digital pressure meter (GMH 3110, Greisinger electronic, Regenstauf, Germany). Gas samples for determination of methane concentration and carbon dioxide concentration were taken at the same time. The carbon dioxide concentration of the samples was measured straight away, the concentration of methane gas was determined at a later stage. The batch bottles were then adjusted to atmospheric pressure by collecting the biogas into a gas bag. VFA samples were taken at three different occasions, at start, after 3 days and after 15 days, during experiment 1. During experiment 3, VFA samples were taken at start and after day 1 of incubation. VFA samples were taken after adjustment to atmospheric pressure. VFA, methane and carbon dioxide determinations were carried out as described in section 3.6.



Figure 5 Batch bottles (1 l) on a shake table in a constant temperature room. Photo taken by Erik S. Moglia, 2007.

3.5 MNP COUNTING OF SULPHATE REDUCING BACTERIA

The number of sulphate reducing bacteria present in the inoculum from Norrköpings biogas plant was counted by serial dilution (in triplicate) of 1 ml inoculum in 9 ml anaerobic medium. The medium used was a bicarbonate-buffered basal medium described by (Zehnder et al, 1984) and modified by (Schnürer et.al., 1994), with the exception that the hydrogen sulphide was omitted as reducing agent. Two different substrates were used, either H₂/CO₂ (80/20) or sodium acetate (20mM). As electron acceptor, 10mM SO₄⁻ was added. Anaerobic conditions were established by flushing the tubes at all times with N₂ and closing the tubes with rubber stoppers and aluminium caps. All transfers and injections of liquid were performed with a syringe. The tubes were then incubated for ~ 3 weeks at 37°C. H₂S, produced from reduction of SO₄⁻ and indicating presence of sulphate reducing bacteria, were detected by dropping liquid onto hydrogen sulphide lead acetate paper strip (Fluka, Industriestrasse 25 CH-9471 Buchs).

3.6 ANALYZES

3.6.1 Methane

The gas samples were analyzed for methane by gas chromatography (Chrompack model CP 9001, Varian associated Inc., 3120 Hansens Way, Palo Alto CA 94304, USA), with helium as the carrier gas at a flow rate of 18 ml min⁻¹. The column used was Hayesep-R “80-100 Mesh, 2.5 m 1/8 in 2mm SS” (Varian associated Inc), the oven temperature was set to 125⁰C. Methane was detected with a flame-ionization detector, 901A FID (Varian associated Inc). The detector temperature was set to 250⁰C and the flow rates of H₂ and N₂ were 24 ml min⁻¹ and 30 ml min⁻¹ respectively. External methane standard was prepared by injecting 100 % methane gas into sealed bottles of the same type as the gas sampling bottles. For example, injection of 0.1 ml 100 % methane into one bottle resulted in a standard of a concentration corresponding to 10 %. Standards, made to have approximately the same concentration as the samples, were injected in the beginning and at the end of every run of gas sample analyzes. When many gas samples were analyzed at the same time, standards were also injected during the session, the gas samples areas were later compared to the in time nearest standard. The injected gas volume was 0.3 ml and every sample was injected in triplicate. In order to decrease the risks of leakage, the gas was analyzed within two weeks after sampling of batch bottles.

3.6.2 Carbon dioxide

Carbon dioxide was measured by using a fermentation tube (VWR International; figure 6). This tube contained 7 M NaOH. When biogas (5ml) is bubbled through the tube, carbon dioxide is solved into the lye while methane remains in the gas phase. The concentration of carbon dioxide in the biogas can then be read directly by subtracting the gas volume trapped above from the original lye pillar in the tube. A bended needle was used to inject the gas into the fermentation tube (figure 6).

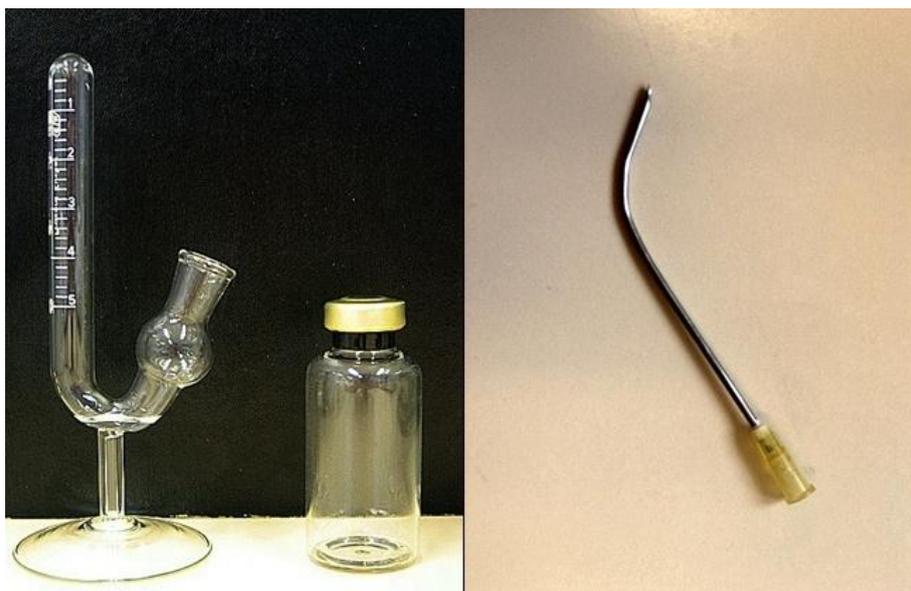


Figure 6. A fermentation tube, a gas sample bottle and a bended needle used for measuring carbon dioxide. Photo taken by Erik S. Moglia, 2007.

3.6.3 Volatile fatty acids

Liquid samples (0.3 ml) were taken from the batch bottles with a syringe. The samples were frozen for approx. 24 hours, then thawed and centrifuged for 10 minutes at 10500 g. The liquid phase was removed and filtered through a 2 μm filter before analyzed with high pressure liquid chromatography (HPLC). The HPLC used was a Agilent 1100 (Agilent Technologies, Inc. Headquarters 5301 Stevens Creek Blvd Santa Clara , CA 95051 USA). The acids were separated on an ion exchange column (Rezex ROA-Organic Acid H⁺; Skandinaviska Genetec AB, Sweden) and detected with a refractive index detector. The effluent was 5 mM H₂SO₄ with a flow rate 0.6 ml minute⁻¹. Pure external standards were used to identify and determine the concentration of volatile fatty acids. The standard contained glucose, acetate, propionate, butyrate, i-butyrate, valerate, i-valerate, capronate and i-Capronate.

3.7 STATISTICS AND FORMULAS

All handling of data and calculation were performed in excel, an example of a programmed excel data sheet can be seen in appendix 7.2 *EXCEL DATA SHEET*. If not stated otherwise all confidence intervals were calculated using a student t distribution and a 95 % confidence level. For comparison between series student t test has been performed with a significance level of 95%.

3.5.1 Student t-test and student-t confidence intervals

Methane production rate was estimated from experimental results. The gas production results from the experiments X_1, X_2, X_3, X_5 were assumed to be a random sample from a normal distribution $N(\mu, \sigma)$ with a student t distribution with four degrees of freedom. σ were estimated from the standard deviation and a 95 % confidence interval was used.

If the 95 % confidence intervals were close to each other or overlapped, a two sample student's t-tests were performed in order to find out if the samples could be separated. The populations were assumed to be a random sample from $N(\mu_1, \sigma)$ and $N(\mu_2, \sigma)$, σ was assumed to be the same for both distributions. This could be done as the same experimental setup and analytical methods were used for every series. X_1, X_2, \dots, X_n and Y_1, Y_2, \dots, Y_n are separate systems independent from each other. (Johnson and Tsui, 1998).

$$T = \frac{\bar{X} - \bar{Y}}{S_{pooled} \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \quad d.f. = n_1 + n_2 - 2$$

$$S_{pooled} = \frac{\sum_{i=1}^{n_1} (X_i - \bar{X})^2 + \sum_{i=1}^{n_2} (Y_i - \bar{Y})^2}{n_1 + n_2 - 2}$$

$H_0: \mu_1 - \mu_2 = 0$ Versus $H_1: \mu_1 - \mu_2 > 0$ Rejection region $T \geq t_\alpha$ or $H_1: \mu_1 - \mu_2 < 0$ with rejection region $T \leq -t_\alpha$

N = the sample size, S_{pooled}^2 = the pooled estimator of the common variance σ^2 , μ = the true mean, \bar{X} = the sample means, t_α = is from the student t distribution.

3.5.2 Calculation of theoretical energy yield

Theoretical energy in methane produced methane in the batch test was calculated by using;

- 1) $\Delta H_{\text{reaction}}^0 = \sum(\Delta H_f)(\text{products}) - \sum(\Delta H_f)(\text{reactants})$, H = enthalpy (Zumdahl, 2002).
- 2) $pV = nRT$, the ideal gas law, p = pressure, V = volume, n = amount of substance, R = the ideal gas constant, T = temperature in degrees Kelvin (Zumdahl, 2002).

The gas law was used to calculate the amount of methane produced. To obtain a theoretical value of energy bound in the methane, the enthalpy for the combustion of methane to carbon dioxide and water was calculated and multiplied with the amount of produced methane.

4 RESULTS

4.1 SUBSTRATES

Results from the determination of DS and VS of the different substrates used in this study can be seen in table 2. Both the wheat and the wheat straw had high DS values while the distiller's waste liquid had very high water content. Notable is the high amount of inorganic matter in wheat straw, the difference in DS and VS.

Table 2 VS and DS content for substrates and inoculum used in this study.

Substrate:	DS (% weight of wet weight)	VS (% weight of wet weight)
Wheat	87.3 ±0.1	86.1 ±0.1
Wheat straw	91.5 ±0.8	79.9 ±0.6
Distillers waste liquid	8.7 ±0	8.0 ±0.1
Inoculum	3.5±0.0	2.7±0.0

4.2 PRE-TREATMENT OF WHEAT AND DISTILLES WASTE LIQUID, EXPERIMENT 1

The methane production rates for the different pre-treatment performed in experiment 1 can be seen in figure 7. The rates are given as ml normal volume (NV; volume at 0°C and 1 atm)/ day. The different curves represent methane production rates from enzymatic pre-treated substrate, and not pre-treated substrate, incubated at 37°C and 55°C. For comparison, the rates from batches with only inoculum, and no addition of substrate, is also included in the figure.

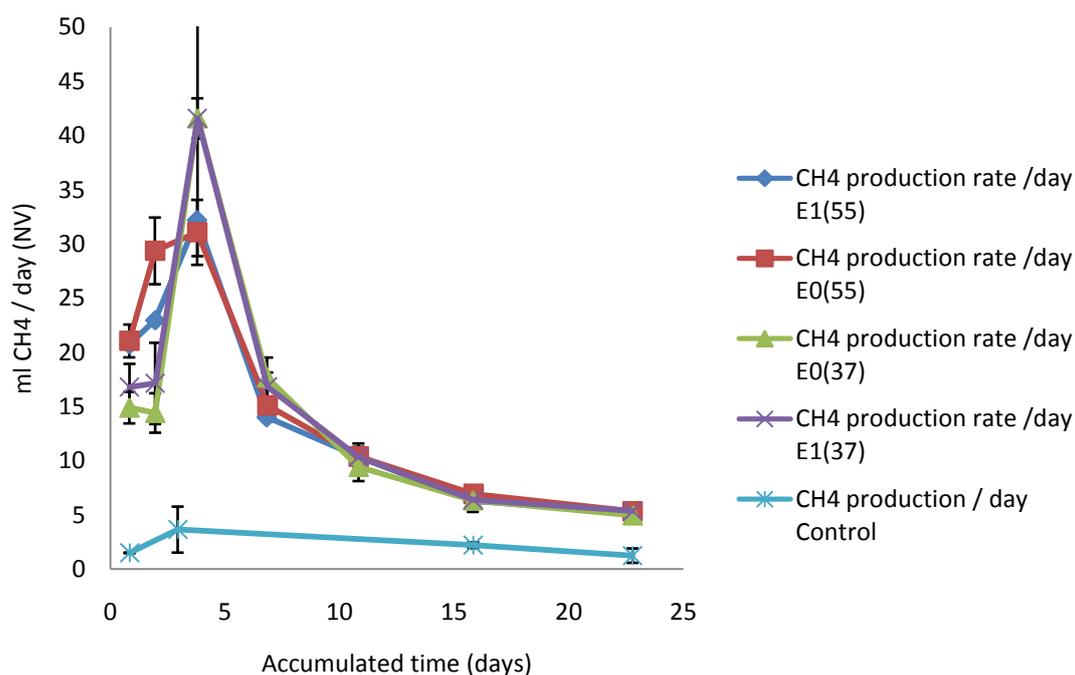


Figure 7 Methane production rates [ml CH₄ (NV) / day] obtained during experiment 1. E indicates enzyme treatment and the following number shows enzyme dosage. The number in the parenthesis shows the incubation temperature during enzymatic treatment. For example, E1(55) represents a treatment with the enzyme dosage 1X (X=10 mg enzymes/g cellulose) and an incubation temperature of 55°C during the pre-treatment.

A lag phase of approximately 2 days, where the methane production was low, can be seen for all the batch bottles in experiment 1 (figure 7). After this lag phase a fast increase of the methane production occurred, followed by a decrease in rate after 5 days of incubation. For the two different pre-treatment temperatures, a difference in gas production rates could be seen during the first 10 days of incubation, with a higher rate at the lower temperature. Furthermore, substrate pre-treated with enzyme at 37°C (E1(37)) gave, during the first two days, a significantly higher methane production rate than non treated substrate (E0(37)). The high deviation seen for the non treated material (37°C), after approximately 4 days, can be explained by the fact that one bottle started to produce methane slightly later than the other bottles. This bottle has a lower methane production during 0.8-3.8 days and a higher production rate from 3.8-6.8 days. In comparison, no difference in the methane production from substrate pre treated at 55°C could be seen.

The methane production rates from experiment 1 was analysed by a student t-test, significance level of $\alpha = 0.05$ (table 3).

Table 3 Results from student t-test, $\alpha=0.05$, of data (methane production rates) from experiment 1. Yes or No represents separation possible or not.

Incubation time (days)	E1(55) versus E0(55)	E1(37) versus E0(37)
0.8	No	Yes
2	Yes	Yes
3.8	No	No
6.8	Yes	No
10.8	No	No
15.8	Yes	No
22.79	No	No

This analysis shows that no trend can be seen when comparing treatment E1(55) versus E0(55), i.e. there is no significant differences in methane production rates between enzyme treated and non enzyme treated substrate. At the lower incubation temperature, enzyme treated material E1(37) gave significant higher rates than non treated material E0(37) during the first 2 days of incubation.

The gas potential of the different pre-treatments in experiment 1, measured at the end of the batch experiments, showed no significant differences. The mean for all bottles were 306 ± 14 ml (NV) methane / g added VS. No accumulation of VFA was noticed in any of the bottles and the total concentrations of all analyzed acids were below 0.6 g / l.

4.3 WHEAT STRAW PRE-TREATMENT, EXPERIMENT 2

The results from the pre-treatment experiments with wheat straw in experiment 2 are divided into two sub groups, and presented in two different graphs (figure 8 and figure 9). The first graph (figure 8) shows the methane production rate calculated for the substrates which had been pre-treated with enzymes solely and the not pre-treated substrate. The second graph (figure 9) shows the methane production rate for substrate that was treated by the alkaline peroxide pre-treatment and with or without a preceding enzyme treatment.

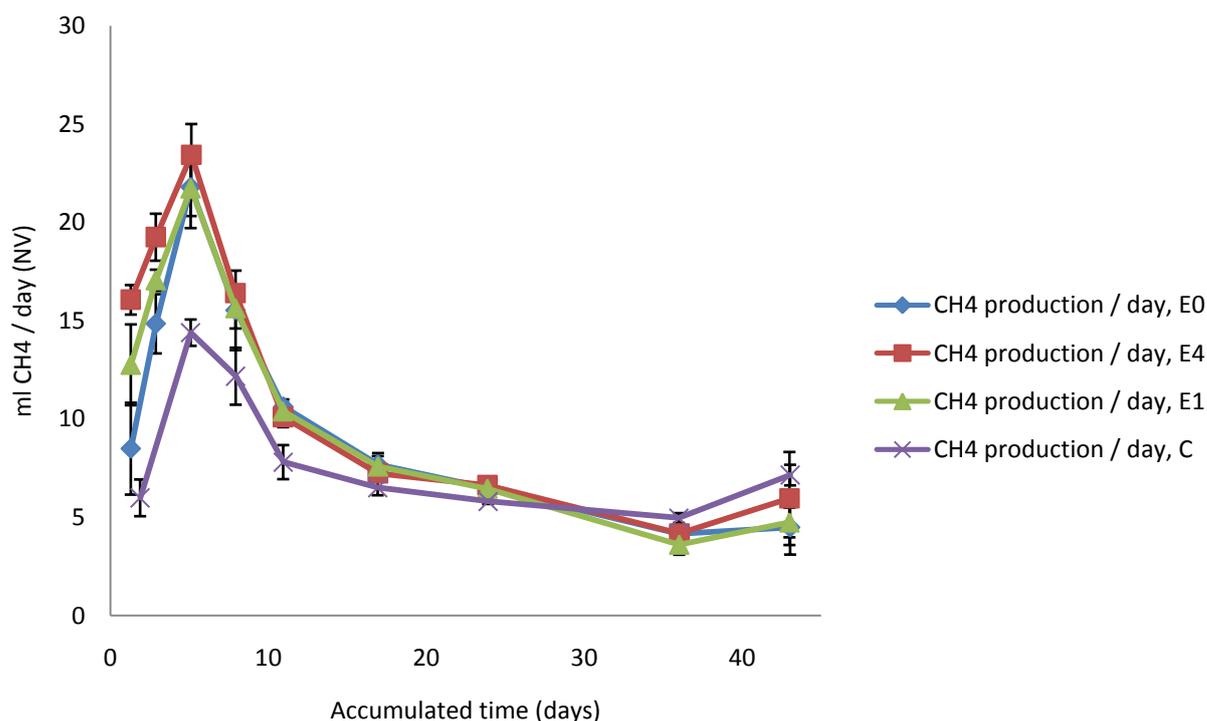


Figure 8 Methane production rates [ml CH₄ (NV) / day] obtained during experiment 2. The series are named by same method as in experiment 1, the number after E indicates the concentration of enzyme added (1X or 4X). The same temperature (55°C) was used for all pre-treatments. C represents the control.

Figure 8 shows that the methane production rates the first 3 days increases with increasing concentration of enzymes. However after 3 days, the untreated (E0) and enzyme treated substrate (E1), at 1X concentration of enzymes, had very similar production rates. However, substrate treated with a high dose of enzyme (4X) gave a significant higher production rate the first 8 days of incubation compared with non treated substrate (E0) and with substrate treated with a lower dose of enzyme (E1; table 4). After 8 days of incubation the methane production rates for the different treatments cannot be separated from each other. The control, without substrate addition, starts to produce more methane, as compared to the enzyme treated material, after ~24 days. A comparison of the two treatments E1 and E0 by student t-test, significance level of $\alpha=0.05$, can be seen in table 4.

Table 4 Results from student t-test, $\alpha=0.05$, with gas production data from experiment 2. Yes or No represents separation possible or not. ^a treatment giving the highest methane production rate if separation is possible.

Incubation time (days)	E4 versus E1	^a Highest rate	E1 versus E0	^a Highest rate
1.5	Yes	E4	Yes	E4
2.5	Yes	E4	Yes	E4
5	Yes	E4	Yes	E4
8	Yes	E4	Yes	E4
11	No		No	
17	No		No	
24	No		No	
36	Yes	E4	No	
43	No		No	

Treatments E1 and E0 can be separated at a 99 % significance level until point 2.9 days. After that the treatments cannot be separated, with one exception; at point 36 days, where E0 has a significant higher rate, on a 95% significance level. All treatments are separated from the control at a significance level of at least 97.5 %. The methane production rate in the controls is lower than obtained from bottles with substrate during the first 24 days of incubation. After that the control starts to produce more methane than E0, E1 and E4.

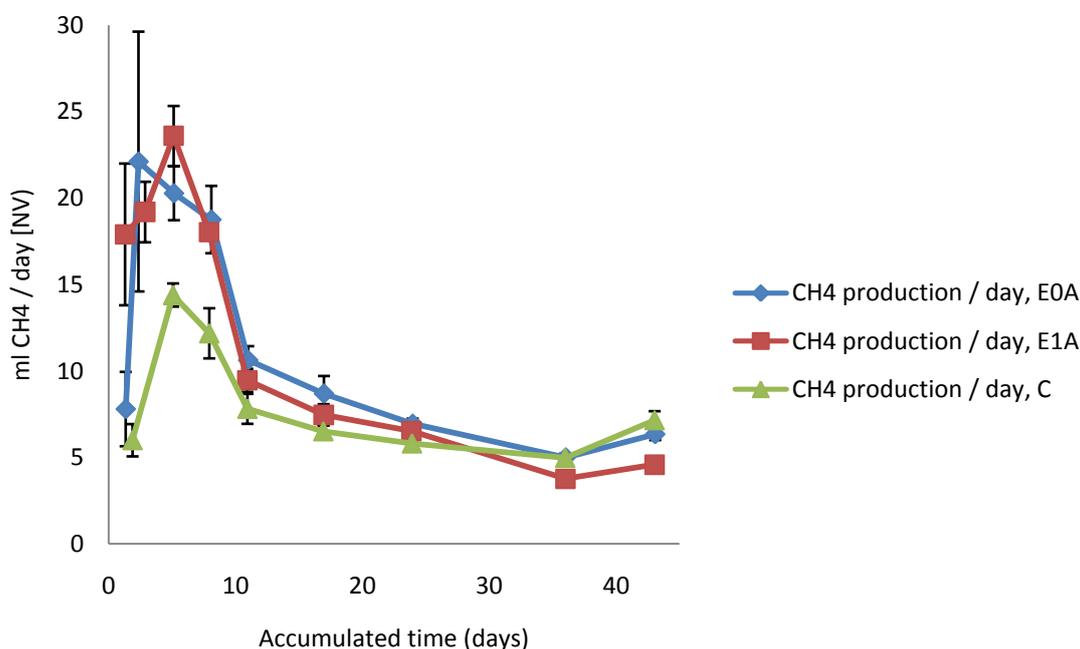


Figure 9 Methane production rates [ml CH₄ (NV) / day] obtained during experiment 2. E and number represents the concentration of enzyme added (1X or 4X). The same temperature (55°C) was used for all pre-treatments. A represents alkaline peroxide pre-treatment. For example, E1A is alkaline peroxide pre-treatment followed by an enzymatic pre-treatment, enzyme concentration 1X.

At the first measuring point after 1.5 days of incubation there is a big difference in methane production rates, with the enzyme pre-treated substrate (E1A) giving the highest production rates (figure 9). After this point in time the methane production rate

from the non- enzymatically treated material (E0A) increases in methane production rate and after 5 days the same production rates as E1A was reached, with the exception of the measuring point after 2.5 days of incubation. The high value and variation at 2.5 days of incubation seen for E0A is caused by an uneven start of the methane production between parallel bottles. At that point one bottle had started to produce methane with a slightly higher rate than the others. This bottle had a lower production rate at the next measuring point. Also, the control, with only inoculum gave a high background production of methane. After 23 days, the control even starts to produce more methane than treatments E1A and E0A.

A comparison of the treatments E0A and E1A, by student t-test, significance level of $\alpha=0.05$, can be seen in table 5. At measuring point 1.5 days after start the treatments can be separated at a 99.9 % significance level. Both series can be separated at significance level 95 % from the control until 24 days after start. After 24 days series E1A shows a significant lower production rate compared to the control and E0A. E0A cannot be separated from the control at a 90 % significance level at point 36 and 43 days.

Table 5 Results from student t-test, $\alpha=0.05$, with gas production data from experiment 2. Yes or No represents separation possible or not. ^atreatment giving the highest methane production rate if separation is possible.

Incubation time (days)	E1A versus E0A	^aHighest rate
1.5	Yes	E1A
2.5	No	
5	Yes	E1A
8	No	
11	No	
17	No	
24	No	
36	Yes	E0A
43	Yes	E0A

The specific gas potential (total accumulated methane from substrate minus total accumulated methane from the control per g added VS) was calculated after 24 days of incubation (figure 10). These calculations show that the treatments E1A, E0A and E4 gave a higher methane production than E1 and E0. To be able to separate the series from each other a t-test was performed. The test showed that treatments E4, E0A and E1A cannot be separated at a 95% confidence level but they can be separated from E1 and E0 on a 97.5% confidence level. Apparently, the alkaline peroxide treatment significantly improved the gas potential, as did the highest dose of enzymes (E4). The specific gas potential obtained from material treatment with a low dose of enzyme (E1) and from non-treated material (E0) are not separated, illustrating that the concentration of enzymes were critical for the results.

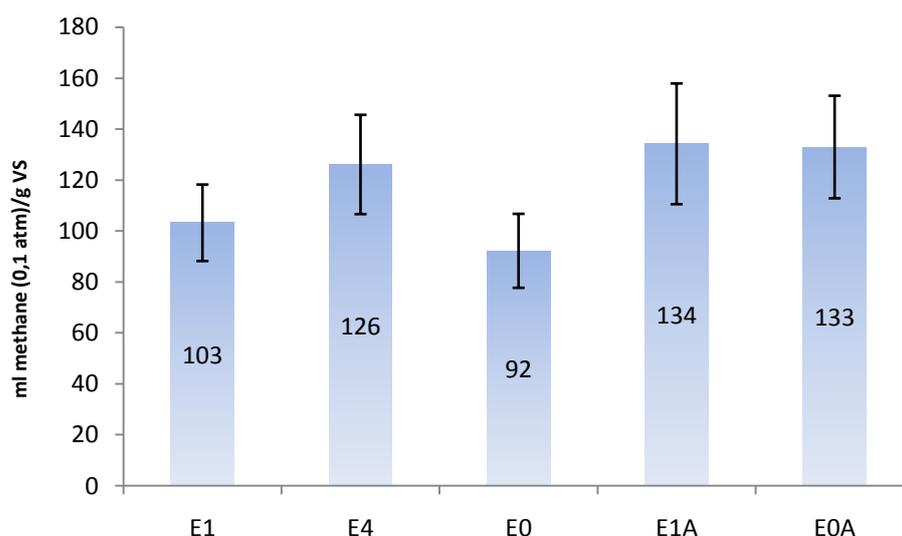


Figure 10 Methane potentials [ml methane (NV) / g VS], obtained after different pre-treatment regimes as performed in experiment 2. E and number represents the concentration of enzyme added (1X or 4X). The same temperature (55°C) was used for all pre-treatments. A represents alkaline peroxide pre-treatment. For example, E1A is alkaline peroxide pre-treatment followed by an enzymatic pre-treatment, enzyme concentration 1X.

To clearly illustrate the effects, the results from the different treatments and their methane production rates as presented as the quota of two methane production rates (table 6). Calculating the quota makes it clear that an enzymatic pre-treatment with the low dose of enzyme increased the methane production during the first 2.5 days of incubation. A higher dose of enzymes gave, compared to a lower dose, or no dose, a higher methane production, which also lasted for a longer period of time. When using the alkaline peroxide pre-treatment, with a preceding enzymatic pre-treatment, a high increase in methane production could be seen in an early stage of incubation. However, after 8 days of incubation a lower gas production for E1A as compared to E0A was obtained

Table 6 Quotas of mean methane production rates obtained from different pre-treatment regimes on wheat straw

Incubation time	E1/E0	E4/E0	E4/E1	E1A/E0A
1.5	1.50	1.89	1.26	2.30
2.5	1.15	1.30	1.13	0.87
5	1.00	1.08	1.08	1.16
8	1.01	1.06	1.05	0.96
11	0.98	0.95	0.97	0.89
17	0.99	0.94	0.95	0.86
24	1.00	1.02	1.03	0.94
36	0.87	1.00	1.16	0.75
43	1.06	1.33	1.26	0.72

Calculations of theoretical energy in totally produced methane per g added VS shows that the use of wheat as a substrate for biogas production in this study results in more than two times the amount of methane compared to the use of wheat straw (table 7). When using wheat straw and boss as a substrate the highest energy yield was obtained when the material was subjected solely to a pre-treatment with alkaline peroxide.

Table 5 Theoretical energy in totally produced methane/ g VS from substrates subjected to different pre-treatment regimes

Treatments	Total energy in produced methane (kJoule / g added VS)	Substrate used
E1(37)	10.7	Wheat
E0(37)	11.2	Wheat
E1(55)	3.7	Wheat straw and boss
E0(55)	3.9	Wheat straw and boss
E0A	4.8	Wheat straw and boss
E1A	3.9	Wheat straw and boss
E0	3.3	Wheat straw and boss

No accumulation of VFA occurred in any of the batch bottles in experiment 2 and the concentrations of total analyzed acids were below 0.6 g / l.

4.4 CONTROL OF THE EFFECT OF ENZYME ADDITION DIRECTLY TO THE BIOGASPROCESS

In the experiments, in which different dosage of enzyme was added directly to the biogas process, no differences in biogas or methane production rates could be seen, independent of dosage. Also no differences in total biogas or methane production could be seen (figure 11). The treatments are named as before, the number after E indicates the added enzymes concentrations. Control is equal to E0, no addition of enzymes.

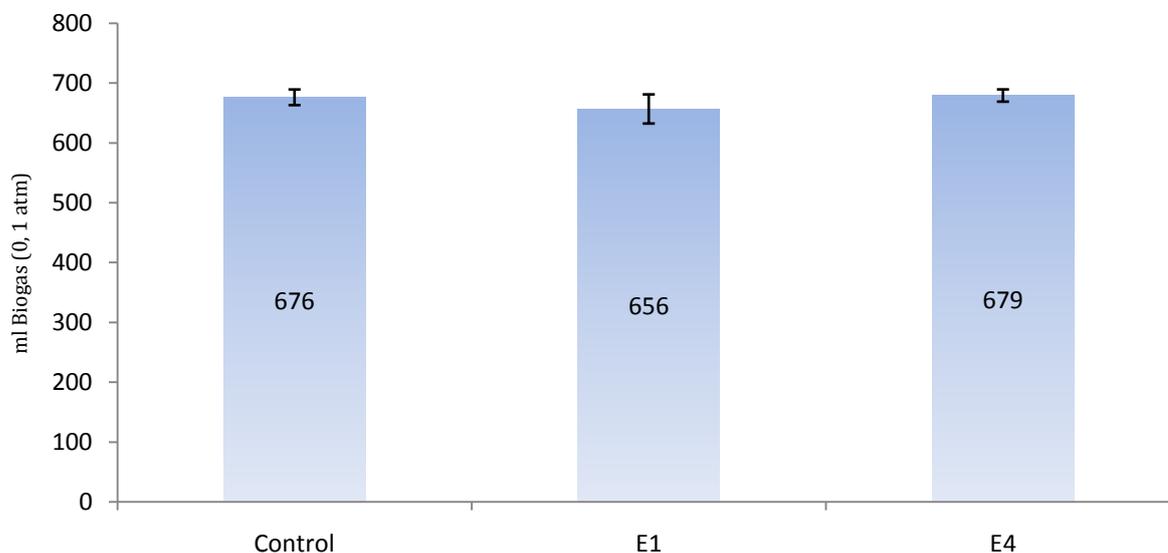


Figure 11 Total accumulated biogas [ml (NV)] after addition of enzymes directly to batch cultures (experiment 3). C is control without addition of enzyme.

No accumulation of volatile fatty acids occurred during the experiment and the concentration of total amount of acids, over the whole experiment period, were below 0.5 g / l.

4.5 MNP COUNTING OF SULPHATE REDUCING BACTERIA

The amount of sulphate reducing bacteria, both acetate and H₂/CO₂ consuming species, was determined to be 10⁶ bacteria / ml inoculums.

5 DISCUSSION

5.1 EFFECT OF ENZYMATIC- AND ALKALINE PEROXIDE TREATMENT

The reason for why not an effect of the enzymatic treatment of wheat and distillers waste liquid could be seen, may be that the cellulose concentration in wheat is relatively low (12.5 %) and/or that the effect of the enzymatic pre-treatment is “hidden” in the background deviation (2-4%). Cellulose is naturally degraded by the microorganisms in an anaerobic biogas reactor why it is possible that the effect of an enzymatic pre-treatment on the final methane production is limited. The fact that the differences in methane production were bigger in experiment 2, with a more cellulose rich material, (wheat straw and boss) indicates that this was the case. Wheat contains too little cellulose for an enzymatic treatment to be useful.

When a more cellulose rich material, such as wheat straw and boss, was used as raw material an increase in methane production rate at an initial stage after the enzymatic pre-treatment was seen. This increase of methane production rate at an early stage is of importance in a continuous biogas process. In a continuous biogas process the initial rate of degradation is important as there is both a continuous flow in and out of the process. If the substrate is slowly degraded the result is a low biogas production and a low total degradation of the substrate. Big losses of non degraded substrate may also result in a high biogas production in the post storage of the biogas sludge. Thus, a fast hydrolysis of the substrate results in a low level of non-degraded substrate going out from the continuous biogas reactor, if the process is stable and no fatty acids is accumulated. To evaluate the importance of higher methane production rates, caused by enzymatic pre-treatment, for a continuous biogas process, calculations of total yields were performed. The calculation was based on experimental data from treatment E4 and E0 and with theoretical retention times of 20 days and 30 days. For a continuous biogas plant feed with enzymatic pre-treated wheat straw and with retention 20 days the increase in methane yield would be 11 %, as compared to non treated wheat straw. If the retention time were increased to 30 days the importance of the enzyme treatment is lowered and the increase in methane yield would then only be 4.5 %.

A high dose of enzymes resulted in a higher increase of methane production rates at an early stage of degradation compared to a lower dose. Furthermore, a higher dose also resulted in an effect that lasted for a longer period of time. This increase in methane potential, from wheat straw (figure 9), shows a potential of obtaining an increased biogas yield not only in a continuous process but also in a batch process.

The alkaline peroxide pre-treatment resulted in a higher initial effect of the enzymatic – pre-treatment, as compared to alkaline treatment of not pre-treated wheat straw. Apparently the chemical treatment made the cellulose structure more accessible to the enzymatic treatment. However, the alkaline peroxide treatment by it self also resulted in a high methane yield. Likely this was a consequence of the alkaline peroxide treatment making the cellulose more available also for the enzymes naturally occurring in the biogas process. Interestingly, the alkaline peroxide treated material (E0A) had a higher methane production rate than the material subjected to a preceding enzyme treatment (E1A) after 10 days of incubation. This higher methane production in E0A compared to E1A may be caused by an increase of internal extracellular enzymes due to an adaption

of the enzyme producing microorganism to the substrate available. This result shows the importance of optimizing the process to the substrate used.

No effect could be seen by an addition of enzymes straight into the inoculums. This indicates that the enzymes used cannot be active in these environments, or has a very low activity level compared to the enzymes naturally occurring. It also shows that the enzymes do not have an effect on biogas production due to their own degradation.

5.2 INOCULUM

Inoculum used for the batch experiments were taken from Norrköping biogas plant at several different occasions. To avoid high background production of gas from the degradation of endogenous material, the inoculum was incubated at 37°C for 4-7 days before start of the batch experiments. But even these pre-caution action wasn't always enough as a very high background production of gas occurred during experiment 2 and 3 (the same batch of inoculums were used in these experiments). The background of methane production in these experiments corresponded to a load of 7 g VS of wheat / l. The reason for this high background production of methane may be that the feeding rate was changed in the Norrköping biogas plant just before start of experiment 2 and 3. Apparently, one could not assume that inoculum, taken from the same biogas plant at two different occasions, always are similar. To avoid big differences in background methane production during batch experiment it is recommended to check VS and DS of every inoculum to be used. A high background gas production of from the inoculum makes it more difficult to evaluate and calculate the gas production from the specific substrate added. However, because a control with only inoculum was started in every batch experiment the background deviation could be determined and different treatments was thus possible to compare. When using the control for comparable analysis it is also important to take into consideration that the inoculum in the batch series is not a control which tells us the true story about the background of biogas production. The biogas process is a complex process which changes over time due to physical and biological changes and it is therefore important to consider that substrate initially in the inoculum may be degraded faster/slower in presence of additional substrate as compared in the control with only inoculum. Possible explanations for such a difference are that; 1) the microorganisms prefers an easily degradable substrate and thus degrade this material before attacking the more complex organic structures presence in the inoculum. If so, the batches with added substrate will initially produce gas only from the added material and not from the endogenous material present in the inoculum. 2) The lack of easily degraded substrates in the inoculum enhances the microorganism production of extra cellular enzymes. Thus the control will have a higher gas production rate from endogenous material as compared to inoculum with externally added substrate.

In these experiments a low specific gas production was seen as compared to a previous investigation using the same anaerobic digestion batch method but with a different inoculum (Moglia, 2007). The specific gas production for wheat was approximate 25% lower in the batch experiment using inoculum from Norrköping (table 8) as compared to when the inoculum was taken from Västerås biogas plant. In experiment 1, in this study, an energy yield of approximately 11 kJoule per gram added VS were obtained and in the previous experiment the energy yield was approximately 14.5 kJoule per gram added VS. Apparently there is a difference in the total biogas yield depending on the inoculums used.

Table 6 Specific methane production from wheat in experiment 1, in this study, and from a previous investigation with inoculum from Västerås biogas plant (Moglia, 2007).

Origin of inoculums:	Methane potential of wheat [ml CH₄ (NV) / day]
Norrköping	305 ±13
Västerås	400±23 (Autumn wheat) 404±78 (Spring wheat)

One big difference between the inoculum from Västerås- and Norrköping is the sulphate concentration in ingoing substrate. Distiller's waste liquid has high levels of sulphate, due to the use of sulphuric acid in the pre-treatment of wheat and as a pH regulator in the ethanol production process. The silage and household waste used as a substrate in Västerås is not as high in sulphate.

In a biogas process rich in sulphate, sulphate reducing bacteria can compete with the methane producing microorganisms for hydrogen and acetate and as the former have a comparably more efficient metabolism they generally outcompetes the methane producing microorganisms (Gerardi, 2003). The concentration of sulphate is very important and first at an acetate-to-sulphate ratio less than 2 the sulphate reducing bacteria can outcompete the methane producing organisms (Gerardi, 2003). Results from the MNP counting of sulphate reducing bacteria showed that a significant amount (106 /ml inoculums) of sulphate reducing bacteria were present in the inoculums from Norrköping. Furthermore hydrogen sulphide, the product of sulphate reduction, has an inhibitory effect on methane producing organism and also on acetate forming bacteria. At low concentrations of sulphide the effect on acid-forming bacteria is less than that on the methane producing bacteria, which may lead to a accumulation of VFA (Gerardi, 2003). Furthermore many metal sulphides are insoluble and high concentrations of sulphide may thus lead to a shortage of micronutrients available for microorganisms (Edmond-Jacques N. 1986). High contents of sulphate may therefore lead to low methane yields and may also be the reason for the comparably low methane yield in experiments presented in this thesis. The obtained methane production potential in the experiments might therefore be a little bit underestimated. However a comparison between treatments is still relevant and the results are likely not affected of this lower gas production.

There have been some theories that the problem of sulphate containing substrates might be overcome by an inhibition of sulphate reducing bacteria by a single or continuous dosing of molybdate (Ranade et. al, 1999)). However investigations have also shown that continuous use of molybden also disturb and inhibit the methane forming microorganism. Therefore molybden cannot be seen as the solution to be used in a continuous anaerobic reactor run on sulphate rich material (Ranade et. al., 1999). The methane producing microorganism seems not to be able to acclimatise to an addition of MoO⁴⁻, like in the case of high ammonia concentrations (Hasnain and Anderson, 2005). Another problem of using molybden as an inhibitor is the risk of accumulating molybden on agricultural land when using biogas residues as fertilizers (Ranade et. al., 1999).

5.3 WHEAT STRAW AS A SUBSTRATE

Wheat straw has, by JTI (Swedish institute of Agricultural and Environmental Engineering), been estimated to have a biogas potential corresponding to approx. 7 TWh. However wheat straw has due to high transport costs and low biogas yields also been ruled out as a substrate for the biogas production by SGC (Swedish centre of gas technology AB) and SOU (The Swedish government's official investigations). On the other hand SGC also states that wheat straw should be taken into account during comparisons with other bio energy production methods using wheat straw. (Nordberg U, 2007). Wheat straw can also be interesting as carbon source in a co-digestion system with a high nitrogen containing substrates (Nordberg A., 1997). The results from this thesis show that if the biogas plant in Norrköping would use alkaline pre-treated wheat straw as a substrate instead of untreated wheat the biogas production would almost be halved.

If the methane potential value obtained in the experiments for wheat straw is adjusted with an increase of 25 %, due to low methane yields (chapter 5.2 INOCULUM), the methane potential of untreated wheat straw would be 0.115 l/g VS, and for the alkaline treatment (E0A) the potential would be 0.17 l / g VS. This is theoretically comparable to 4.1 kJoule/g VS and 6 kJoule/g VS, respectively. When burning wheat the effective heating value is 16.9 kJoule / g DS (Bernesson and Nilsson, 2005).

5.4 METHOD DEVELOPMENT

During testing of different experimental set-up it became clear that the different pre-treatments of the substrates should be performed directly in the batch bottles. This conclusion was draw as a consequence of difficulties, due to inhomogeneous substrates, in transferring equal amount of treated material to all bottles. Consequently the pre-treatment should not be performed in a big batch and later be divided, but directly into smaller bottles, later used in the batch experiment. In this thesis, the smallest bottles possible to use without obtaining big variations, due to small errors in added enzymes and substrate, had a volume of 309 ml.

Another important factor for the outcome of the experiment is the degree of fractionation of the substrate. In order to minimize variations in gas production, the substrate to be used in batch experiment has to be as homogenous as possible. Therefore, during co-digestion experiments the material should be weighed and added one by one. Mixing followed by weighing can also result in big variations. An example on the importance of a homogenous substrate can be seen in table 9. The table shows a big difference in CV value (standard deviation in % of mean value) of the biogas production for two experiments with differences in substrate handling, A and B. In experiment A, grinded wheat mixed with hacked and grinded wheat straw and boss were weighted into the batch bottles, no separation by particle size was made. Experiment A was not included in the results presented in this thesis due to the very high deviations of biogas production, making it impossible to compare the effect of different pre-treatments. In experiment B a wheat straw and boss were mixed and filtered. The fractions that passed by the 0.5 mm filter, but did not pass through the 0.1 mm filter, were used. The wheat straw and boss were assumed to be close in composition and therefore weighted in together, no addition of wheat were made in experiment B. It should also be mentioned that during experiment B the background deviation from the control were 0.015-0.02. No significant background deviation was measured during experiment A.

Table 7 A comparisons of CV values for methane production rates? between two experiments with differences in handling of substrate. For both experiment A and B four treatments were started, with identical pre-treatment procedure. The CV values were calculated for five parallels of every treatment. In experiment A grinded wheat, wheat straw and boss of different fraction sizes was mixed and used as a substrate In experiment B grinded wheat straw and boss were filtered through filters and one fraction with a particle size of X were chosen for the experiment.

Experiment A	Experiment B
0.27	0.03
0.12	0.04
0.10	0.03
0.16	0.03

To be able to statistically compare treatments and to rule out measurements which are obviously wrong, maybe due to leakage of gas and/or mistakes during measurements, at least five parallel batch bottles should be started. The variation is smaller when using only inoculum, and no substrate are added. Therefore three parallels are sufficient for such controls.

6 CONCLUSIONS

- Enzymatic pre-treatment of ingoing substrate can enhance the biogas yield in a biogas process.
- Enzymatic pre-treatment with a relatively high dose of enzyme can increase the total methane production potential of a cellulose rich substrate. However, an enzyme dose considered as optimal for treatment of corn did not give an increase of methane production potential when used for wheat straw. More research is needed, including for example testing of different enzyme mixtures, to be able to say anything general about the use of enzymes for hydrolysis of lignocellulosic biomasses to be used in biogas processes.
- A pre-treatment aiming at destroying the fibre structure and making the cellulose more available for enzymatic degradation enhanced the biogas potential of wheat straw more than an enzymatic pre-treatment solely. Furthermore, the treatment also enhanced the initial biogas production after a preceding enzymatic treatment. However, the effect from this preceding enzymatic pre-treatment did not last for long and did not give an increase in the overall methane production potential of wheat straw. These results indicate a big potential for structure breaking treatments when lignocellulosic biomasses is used as a substrate for biogas production.
- The natural ability of degrading cellulose is relatively high in a biogas process

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8 APPENDIXES

8.1 BATCH BOTTLE – AND GAS SAMPLING BOTTLES VOLUME

All bottle volumes were measured; initially the empty bottles were weighted together with the rubber plug used to seal the bottles. The bottles were then filled with 25°C water and sealed with the rubber plug. The bottles were filled by using two syringes, one for injecting the water and one who were put through the plug in order to release possible surplus of water injected. By doing this, air bubbles could be eliminated. The bottles were dried on the outside and weighed again. The volume was calculated from the water density at 25°C and 1 atm.

Table 8 Determination of batch bottle volume (309 ml)

Number of bottles measured:	8
Mean:	309.4 ml
Std:	1.4 ml
CV:	0.005

Table 9 Gas sampling bottles volume

Number of bottles measured:	10
Mean:	12.10
Std:	0.14
CV:	0.01

8.2 EXCEL DATA SHEET

A	B	C	D	E	F	G	H	I	J	K	L
Atm. Tryck (mbar):	1013	Vikt ymp:	130								
Start:	2007-10-15 00:13	Temp:	37								
Flaska nr:	Datum	Dagar:	Timmar:	Min	CO2 (%)	CH4 (%)	Tryck (mbar)	Kommentar	Akkumulerad tid (dagar)	N2 (%)	Summa:
118	2007-10-16	1	20	0	16	16,4	626		1,3	0,65	97,8
133	2007-10-18	3	10	0	20	19,1	522		2,9	0,43	82,2
168	2007-10-20	5	15	0	24	49,7	643		5,1	0,26	100,1
202	2007-10-23	8	11	10	26	54,8	619		7,9	0,16	97,2
223	2007-10-26	11	11	10	28	55,5	419		10,9	0,12	95,1
280	2007-11-01	17	11	40	28,5	60,9	504		16,9	0,08	97,1
295	2007-11-08	24	10	0	31	59,8	524		23,9	0,05	95,9
295	2007-11-20	36	13	30	29	59,8	507	Fel i gas provtagning?	36,0	0,03	92,2
325	2007-11-27	43	13	0	32	56,4	366		43,0	0,02	90,9
						0,0			0,0	0,00	0,0
						0,0			0,0	0,00	0,0
						0,0			0,0	0,00	0,0
						0,0			0,0	0,00	0,0
						0,0			0,0	0,00	0,0
						0,0			0,0	0,00	0,0
						0,0			0,0	0,00	0,0
						0,0			0,0	0,00	0,0
						0,0			0,0	0,00	0,0
Tryck faktor	Volym vid fatm.	Volym Gas	Norm Vol. (NV)	IV CH4	Ack. Biogas	Ack. CH4	Ack. CH4 / g VS	0			
1,62	159,4	257,9	86,7	14,2	86,7	14,2	11,8	0,0	7,132282986	11,8	
1,52	159,4	241,5	72,3	13,8	159,1	28,0	1,0	0,0	27,41029688	1,0	
1,63	159,4	260,6	89,1	44,3	248,2	72,3	30,7	0,0	53,84229861	30,7	
1,61	159,4	256,8	85,8	47,0	333,9	119,3	57,6	0,0			
1,41	159,4	225,3	58,1	32,2	392,0	151,5	63,5	0,0			
1,50	159,4	238,7	69,8	42,5	461,8	194,0	58,4	0,0			
1,52	159,4	241,8	72,6	43,4	534,4	237,4	76,4	0,0			
1,50	159,4	239,2	70,3	42,0	604,7	279,4	136,4	0,0			
1,36	159,39	216,97	50,71	28,62	655,40	308,0	227,6	0			
1,00	159,39	0,00	0,00	0,00	0,00	0,0	0,0		227,5887021		

Figure 8 A example of data sheet for one batch bottle, all indexes is in Swedish.