

From Waste to Bioethanol

A Feasibility Study on Animal Bedding



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Department of Chemical Engineering
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Preface

This master thesis has been performed at the Department of Chemical Engineering at LTH during spring 2017. The project was a part of the SEGRABIO project.

Firstly, we would like to thank our examiner Ola Wallberg from the Department of Chemical Engineering for proposing this project to us. We would also like to thank our supervisors Borbála Erdei and Mats Galbe, both also from the Department of Chemical Engineering, for providing us with information, advice and support during this process.

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Finally, we would like to thank our families and friends for supporting us and motivating us during tough times.

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Abstract

For the year 2020, the European Union has set common goals to aim for regarding the environment. One of them being to reduce the green house gas (GHG) emissions by 20 percent. The world population is increasing, consequently increasing the demand for energy and food. Since the use of fossil sources is neither sustainable nor environmentally friendly, new techniques of meeting the energy demand need to be found, starting with one of the biggest contributors to GHG – the transportation sector. Alternative fuels such as bioethanol need to substitute today's fossil fuels. Bioethanol in particular can be made from materials such as sugar- or starch-based crops, referred to as first generation (1G) bioethanol. It can also be produced from lignocellulosic substrates, which in this case is referred to as second generation (2G) bioethanol. In case of 2G bioethanol, the sugar components that are necessary for the production of bioethanol are difficult to access. This drawback makes the process of 2G bioethanol production more challenging. However, 2G ethanol does not compete with the food market, *per se*.

The aim of this thesis was to investigate different fermentation configurations using animal bedding as the raw material. Bioethanol from animal bedding, can be referred to as 2G bioethanol. The investigation was conducted experimentally at the Department of Chemical Engineering at LTH.

The animal bedding was collected at a farm in Køge, Denmark. It was once washed with water and later soaked in 0.4 wt-% sulfuric acid. The animal bedding was pretreated in a steam explosion reactor at 190°C for 10 minutes.

Results showed that it is possible to produce bioethanol from animal bedding. When using simultaneous saccharification and fermentation (SSF), an ethanol yield above 0.47 g ethanol/g glucose can be obtained. A slightly higher yield (0.51 g ethanol/g glucose) was achieved when using fed-batch instead of batch. Since it was desired to utilize as much sugar as possible from the animal bedding, simultaneous saccharification and co-fermentation (SSCF) was also tested with the genetically modified yeast strain KE6-12B. No significant amount of ethanol was produced when using SSCF.

Populärvetenskaplig sammanfattning

Från avfall till bioetanol - en undersökning av bioetanolproduktion från djupströ

Under de senaste 50 åren har befolkningen på vår planet fördubblats, vilket har lett till att energiförbrukningen har ökat drastiskt. En av de främsta bovorna är transportsektorn, som dessutom medför stora utsläpp av växthusgaser. Ett sätt att minimera dessa utsläpp är att använda sig av miljövänliga transportmedel så som el- och gasbilar. Dock är körsträckan för dessa fordon oftast kortare, eftersom man måste tanka/ladda oftare än fordon som drivs på fossila bränslen. Ett starkt alternativ för att successivt eftersträva att nyttjandet av miljövänliga bränslemedel är fullständig är användning av bioetanol och biodiesel. Intresset för nya bränslemedel är enormt och i Sverige subventioneras konsumenter till miljövänliga motorfordon av staten.

I denna studie som utförts vid institutionen för kemiteknik vid Lunds Tekniska Högskola har bioetanolproduktionen från djupströ studerats. Djupströ är en blandning av komposterat halm och gödsel som lagrats. I studien undersöktes två olika jäsningsmetoder med två olika jästsorter för att se vilken process som är mest gynnsam vid olika förutsättningar. Den ena jästen kunde bara fermentera glukos (SSF – Simultaneous Saccharification and Fermentation) och den andra var genmodifierad och kunde fermentera både glukos och xylos (SSCF – Simultaneous Saccharification and Co-Fermentation). Vidare studerades även konfigurationer av hur materialet skulle fördelas under jäsningen (sats och satsvis). Odling av den genmodifierade jästen gjordes under examensarbetet för att uppnå tillräcklig mängd till fermenteringen.

Av resultaten framgår det att man kan producera etanol från djupströ. Utbytet är högre vid glukosjäsningen eftersom detta är en mer tålig jäst än den genmodifierade jästen – 0,51 g etanol/g glukos jämfört med 0,09 g etanol/g socker. De bästa resultaten erhöles när satsvis matning användes för både SSF och SSCF. Å andra sidan var utbytet signifikant högre när SSF användes.

Under studien har det påvisats att sammansättningen av djupströ varierar beroende på hur länge det har lagrats. I och med detta innebär det att metoder för förbehandling av materialet inte kan tillämpas på all djupströ. I studien visade det sig att förbehandlingen varit för aggressiv mot materialet. Många inhibitorer bildades som försvårade den fortsatta processen där den genmodifierade jästen användes. Under jästodlingen användes en mindre mängd hydrolysat än önskat för att odla fram tillräckligt hög jästmängd för SSCF. Dessvärre påverkade detta fermenteringen på så sätt att jästen inte klarade av den höga mängden material i fermenteringen.

Idag används djupströ endast som gödningsmedel men med resultat från denna studie framgår det att djupströ är en potentiell råvara för framställning av biobränsle. Många av dagens råvaror som används för framställning av biobränslen härstammar från livsmedelsindustrin. Då djupströ är ett jordbruksavfall kommer detta material inte att konkurrera med framställning av livsmedel.

Lund, Juni 2017

Abbreviations

1G	First generation
2G	Second generation
AIL	Acid insoluble lignin
ASL	Acid soluble lignin
C ₅	Pentose sugar molecules
C ₆	Hexose sugar molecules
EtOH	Ethanol
FPU	Filter paper unit
GHG	Greenhouse gas
H ₂ O	Water
HMF	5-hydroxymethylfurfural
NaCl	Sodium chloride
RPM	Revolutions per minute
SSCF	Simultaneous saccharification and co-fermentation
SSF	Simultaneous saccharification and fermentation
WIS	Water-insoluble solids

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1. Introduction

The range of applications for biomass is large, and it can be utilized in e.g. the biorefinery industry, which includes pulp production, manufacturing of lignosulfonates and other products from biomass. A potential application is production of liquid fuels, and the process of ethanol fermentation of sugars has for a long time been considered one of the most important ones. More specifically, the interest in bioethanol as fuel has grown over the past couple of years. In 2003, the global fuel production of bioethanol was just over 500 PJ, which corresponds to about 23.6 million metric tons of bioethanol (Statens Energimyndighet, 2015). Seven years later in 2010, the production increased to approximately 1750 PJ. (Makkee, *et al.*, 2013)

Essentially, all industrial-scale production of bioethanol is produced from food crops containing starch, where the sugar crops are the most common types of raw material. The bioethanol produced from these types of biomasses are referred to as first generation (1G) bioethanol. There is also second generation (2G) bioethanol that makes use of crop residues. These types of residues consist of mainly cellulose and hemicellulose. Since these materials essentially are considered to be waste, whereas food crops are used for human nutrition, 2G bioethanol can be regarded as favorable over the 1G bioethanol in the sense that it won't compete with food production. (Makkee, *et al.*, 2013)

By producing 2G ethanol with non-food parts, the amount of sustainably produced biofuel is extended. However, the difficulty that comes with 2G bioethanol is the struggle of degrading the raw material due to the useful sugars being inaccessible. This since complex carbohydrates are found in these types of biomass. Essential pretreatment steps are needed to be implemented in order to access the sugars from the lignocellulosic biomass waste. (Makkee, *et al.*, 2013)

Another type of material that can be classified as a raw material for 2G ethanol is animal bedding. The composition of the animal bedding is lignocellulosic biomass together with animal litter. There is potential in utilizing the biomass from the animal bedding to produce sustainable bioethanol. Since there is not currently any other application for the animal bedding other than using it as fertilizer, it is an alternative to other raw materials for 2G bioethanol production.

1.1 Project Background

At Lund University, a project called SEGRABIO has been initiated, where the aim of the project is to produce biofuel from second-grade and low-cost biomass. The requirement for production of renewable fuels, such as bioethanol and biogas is of great interest in Sweden and Denmark, due to the fact that the compounds could replace the fossil sources used in the transportation sector today. Since the method of producing biofuels with the chosen raw material is new and the experiences from the material is limited, a new process has to be developed to achieve cheaper costs. The technology will be focusing on separating the compounds in animal bedding to utilize the manure for biogas production and the biomass for bioethanol production efficiently. (Second Grade Biomass for Biofuels, 2016)

The raw material is projected to be less costly than the currently proposed raw materials, e.g. wheat straw; it is desired to reduce the currently estimated production costs from today's 6 DKK/liter to 4.5 DKK/liter. Furthermore, developing new conditioning and separation

technologies for the mentioned biomass would be important to accomplish the goal. (Second Grade Biomass for Biofuels, 2016)

1.2 Aim and Purpose

The main purpose and aim for the thesis is to study the effect of selected yeast strains on ethanol production from animal bedding. Different yeast strains will be used to observe the impact on pentose (C₅) - and hexose (C₆) fermentation, respectively on the overall ethanol yields. To be able to conclude, some important research questions have been formulated. In this thesis, the main research questions are:

- How great of an impact does simultaneous saccharification and co-fermentation (SSCF) have on the ethanol yield compared to simultaneous saccharification and fermentation (SSF)?
- How great of an impact does fed-batch fermentation have on ethanol yield compared to batch fermentation?
- How great of an impact does pH have on the outcome for the SSCF?

1.3 Outline of Thesis

The outline of this thesis is divided into two parts: one theoretical part (including a literature study) and one experimental part. The theoretical part is presented in section 2. *Literature Review*. In this section, a literature review of the selected raw material and important chemical procedures for the understanding of bioethanol production and this thesis are presented. The experimental part of the thesis is described in section 3. *Material and Method*, and discusses planning of experiments. The results from the experiments, and finally, conclusions and future work are presented in sections 4. *Results and Discussion*, 5. *Conclusion* and 6. *Future Work*.

2. Literature Review

2.1 Biofuel – A Future Investment

Green house gas (GHG) emissions and global energy demand are increasing each year due to the increasing world population, which consequently means that food and energy demands increase. The most energy-intensive sector that releases, and will continue to release high amounts of GHG emission is the transportation sector (Intergovernmental Panel on Climate Change, 2012). This means that GHG emission needs to be efficiently reduced in this area, in order to benefit the environment and to ensure a better future for our planet. Most common GHG are carbon dioxide (CO₂), methane (CH₄) and water vapor (H₂O). (Center for Climate and Energy Solutions, 2017)

The alternative energy source needs to be renewable and sustainable for several reasons. Firstly, to lower GHG emission. Secondly, to ensure that the energy source does not compete with food production and arable land. (Joelsson, 2015)

Transportation fuels produced from biomass are an alternative to fossil fuels. The living plant consumes CO₂ during its life, according to the photosynthesis. However, when using plants for biofuel production, CO₂ is released due to the cellular respiration from the yeast. This is an example of the carbon cycle, which in an ideal case result in a net result of zero CO₂ released. (Erdei, 2013)

Bioethanol, which is ethanol produced from biomass, has shown great potential in replacing fossil fuels to an extent. Today, the raw material used for bioethanol is mostly sugar- or starch based. These raw materials are referred to as first generation (1G) bioethanol (Erdei, 2013). However, they are also used in the food sector, which makes the material questionable for bioethanol production (Sims, *et al.*, 2010). There is also the second generation (2G) bioethanol, which is produced from lignocellulosic material such as agricultural waste. One side effect with 2G bioethanol is the difficulty accessing the sugars in the biomass, making the process more challenging. (Erdei, 2013)

The European Union has a binding legislation to ensure that climate and energy targets are met by the year of 2020. The three key targets are that a cut in GHG emission is cut by 20 percent, that 20 percent of the EU energy is renewable and lastly that there will be a 20 percent improvement in energy efficiency. The national emission reduction targets cover sectors such as housing, agriculture, waste and transport. (European Commission, 2017)

Sweden has set a goal that by the year 2050, a more sustainable and resource-efficient energy supply without generating any net GHG emission needs to be achieved. (SOU, 2013)

2.2 Ethanol Production

Ethanol is a very versatile chemical and can be used in many applications. For example, it can be used directly as a fuel, as an additive in gasoline, or as a chemical that is used as a precursor for many reactions. (Makkee, *et al.*, 2013)

Ethanol can be produced mainly in two different ways. One option is to be produced thermochemically. The process steps then include gasification of the feedstock to produce syngas. This process involves reaction of the material with steam and oxygen, at temperatures above 700°C. The product is a mixture of carbon monoxide, hydrogen, carbon dioxide, methane, and nitrogen. The syngas is later purified to remove tar and ash. (Dwidevi, *et al.*, 2009) This can be performed either by manipulating the gasifier conditions or hot gas cleaning after the gasifier. The hot gas cleaning can be performed using filters, rotating particle separators, or with chemical methods such as thermal or catalytic cracking. Lastly, the ethanol can be produced either through fermentation or catalytic synthesis. (Makkee, *et al.*, 2013)

Another option is to produce ethanol biochemically by using sugar- or starch- containing crops. The main steps include pretreatment, enzymatic hydrolysis, fermentation using yeast or bacteria, and a final product recovery step to utilize sugar molecules and to produce ethanol. Biochemically produced ethanol can be referred to either as first generation or second generation ethanol. As it can be seen in *figure 1*, the feedstocks and the pathways are not the same for the production of 1G- and 2G ethanol. (Dwidevi, *et al.*, 2009)

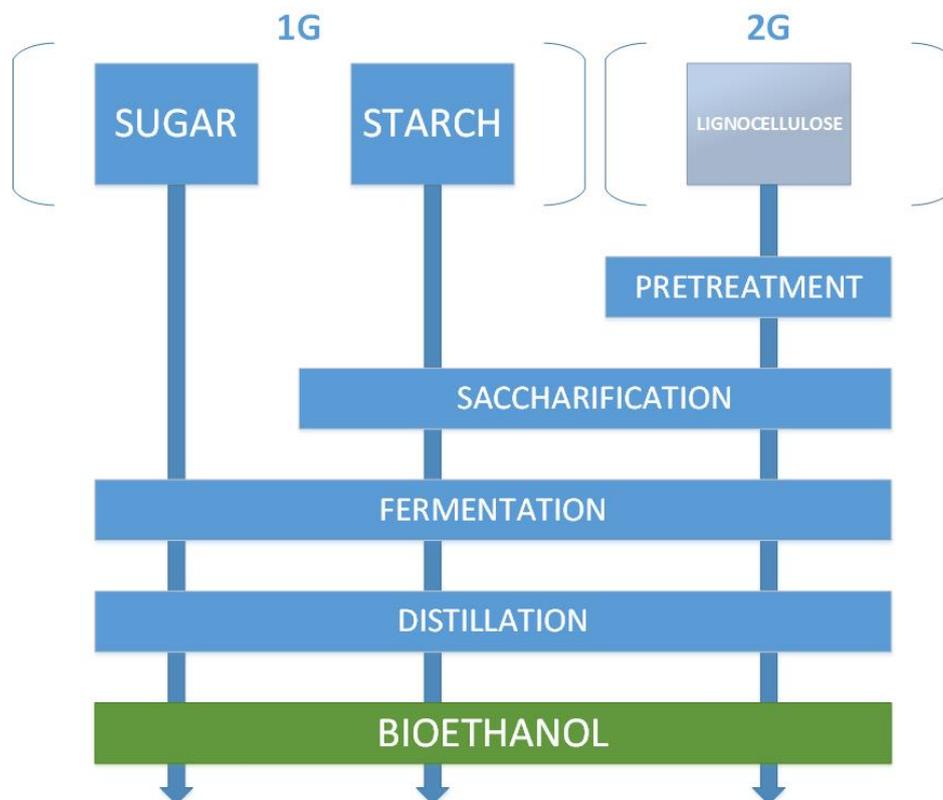


Figure 1. Process pathways for 1G and 2G ethanol production respectively.

2.3 Pretreatment

To enhance the digestibility of lignocellulosic biomass, it is necessary to pretreat the material due to its complex chemical structure (Galbe & Zacchi, 2012) (Hendriks & Zeeman, 2009). For bioethanol production from biomass, pretreatment is necessary since it is desirable to achieve high yield of liberated sugars in the hydrolysis step, and consequently improve the rate of production. In other words, in order for microbial or enzymatic degradation of biomass to occur at a sufficiently high rate, polymeric sugar chains need to be easily accessible. An overview of degradation of biomass is presented in *figure 2*.

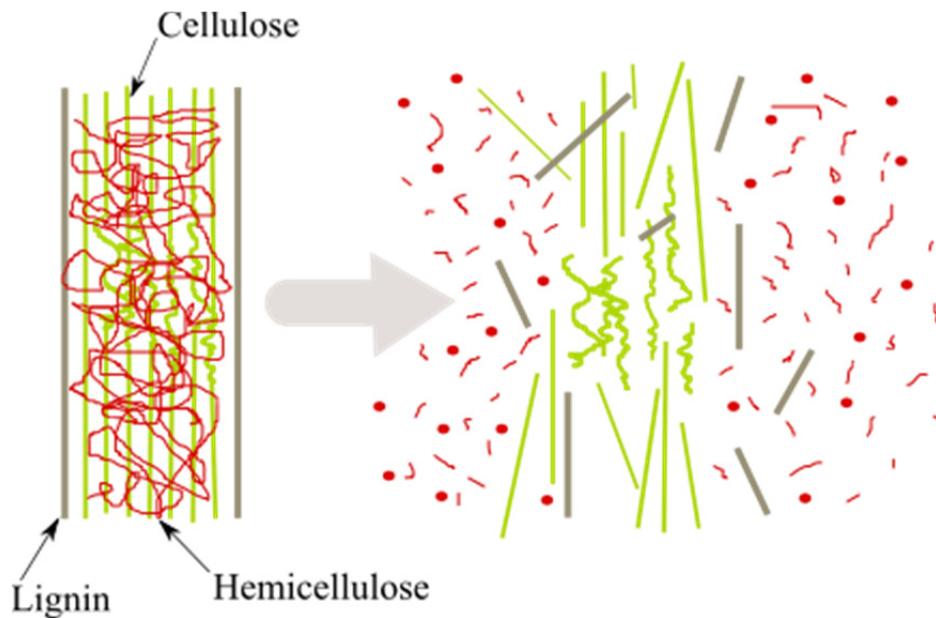


Figure 2. Overview of the degradation of biomass after steam pretreatment. (Bondesson, 2016)

Alternatives for pretreatment methods include chemical-, physical-, and biological pretreatment. The second mentioned type involves size reduction and extrusion by milling or grinding of the biomass. This is often used in combination with chemical pretreatment, and is referred to as physio-chemical (chemical and physical) pretreatment. By milling, the material's fiber structure opens up and increases the surface area. (Angelidaki, *et al.*, 2010) (Galbe & Zacchi, 2012). Different pretreatment alternatives are represented in *figure 3*. (Galbe & Zacchi, 2012)

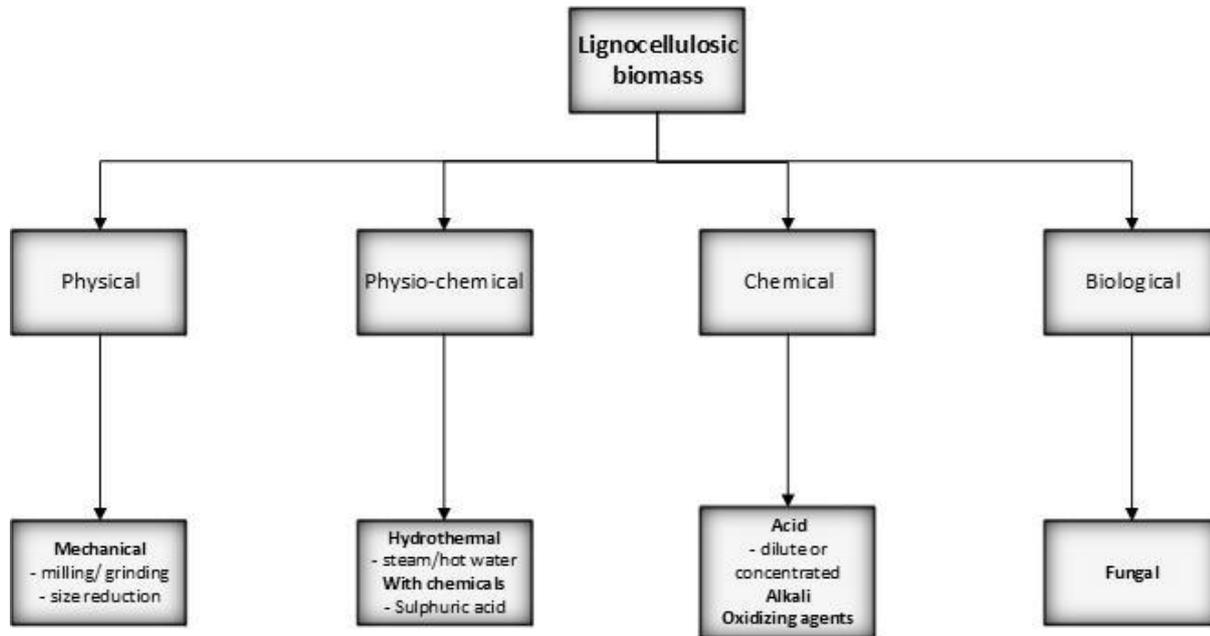


Figure 3. Overview of different pretreatment methods for lignocellulosic biomass.

2.3.1 Steam Explosion

Steam explosion (often called steam pretreatment) is a physio-chemical pretreatment method where the main goal is to open up the fibers in various types of biomass, in order to prepare it for further degradation or treatment (Angelidaki, *et al.*, 2010). During this steam pretreatment, hot steam under high pressure is used. Common temperature ranges are 160-240°C, and pressure ranges are 6-33 bar, depending on the material (Galbe & Zacchi, 2002). The steam penetrates the structure of the biomass, which can lead to some degree of solubilization of the hemicellulose fraction, especially in the presence of an acidic catalyst. When the pressure is released, decompression in the biological material takes place causing rupture and opening of the cellular structure.

The result of the steam pretreatment is a slurry where a liquid phase and a solid phase are present. The liquid phase contains most of the soluble hemicellulosic components, whereas cellulose and lignin can be mainly found in the solid phase (Barrett, *et al.*, 2009).

Steam explosion can be performed with or without a catalyst; either acid or alkaline catalysts can be used. The most commonly used acid catalysts are sulfuric acid (H₂SO₄), and sulfur dioxide (SO₂) (Barrett, *et al.*, 2009). The impregnation of acid catalyst prior to steam explosion has been proved to increase sugar yields. (Ballesteros, *et al.*, 2006) (Galbe, *et al.*, 2008)

One disadvantage with steam pretreatment (and many other) methods is formation of inhibitors. Inhibitors are found in the liquid fraction, due to generation of toxic compounds

derived from sugar degradation. The inhibitors can affect the subsequent hydrolysis and fermentation. (Jonsson, *et al.*, 2013)

2.3.1.1 Inhibitors

During steam pretreatment, sugar degradation products are formed. These products are considered as inhibitors for the upstream processes. However, the inhibitors (or toxic compounds) may be present in the biomass originally, such as acetyl groups and extractives (Bondesson, 2016). The amount of inhibitors increases with increasing parameter values, such as higher temperature, longer residence time and higher acid concentration that would result in a more severe pretreatment. (Abatzoglou, *et al.*, 1992)

The most common inhibitors and toxic compounds are weak acids, phenolic- and furan derivatives. On the other hand, inhibitors formed by degradation of cellulose, hemicellulose and lignin are 5-hydroxymethylfurfural (HMF), furfural, phenolics, and acetic acids. HMF and furfural can later be converted in less toxic forms, e.g. from furfural to furfuryl alcohol. (Bondesson, 2016). The most common inhibitors and toxic compounds are seen in *figure 4*.

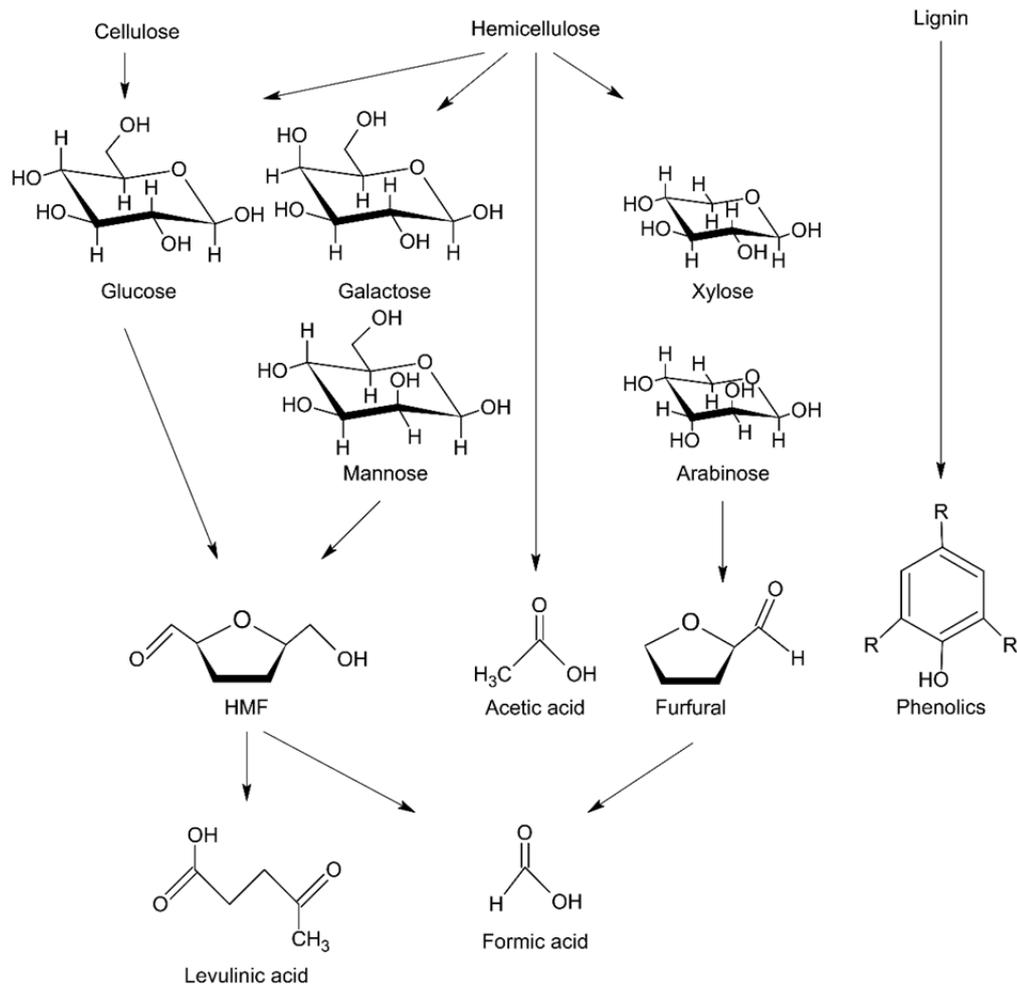
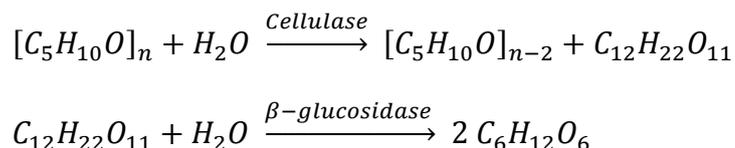


Figure 4. Common inhibitory compounds found in pretreated biomass.

2.4 Enzymatic Hydrolysis

After pretreating biomass, there is still a great number of undigested polysaccharides that need to be decomposed to monomeric sugars before fermentation. This is achieved by using different enzymes in a process step called enzymatic hydrolysis.

The enzyme group cellulase splits cellulose into oligosaccharides and the enzyme β -glucosidase fulfills the decomposition by hydrolyzing the oligosaccharides into monomeric sugars. The hydrolysis is performed when water and appropriate enzymes are added to the polysaccharide. (Galbe & Zacchi, 2002)



However, β -glucosidase is product inhibited, thus, when too much glucose is formed, the glucose formation will be affected negatively. (Galbe & Zacchi, 2002)

Due to the complex chemical composition of lignocellulosic material, several enzymes are required to degrade the polymeric sugar molecules. On the other hand, enzymatic hydrolysis is a relatively expensive process method. Thus, acidic hydrolysis can be an alternative due to its low price and higher effectiveness; however, the corrosive action of the acid may cause equipment to be prohibitively high. In addition, formation of inhibitors or toxic substances are also a problem (Arifin, *et al.*, 2012)

2.5 Fermentation

Using yeast or bacteria, ethanol can be fermented from sugars found in the liquid phase from hydrolyzed biomass. When lignocellulosic material is hydrolyzed, the hydrolysate contains pentose (C_5) - and hexose (C_6) sugars, as well as lignin and- lignin derivatives; in addition, also inhibitory compounds. Glucose (C_6) and xylose (C_5) are the main sugars fermented from the hydrolysate.

For fermentation of C_6 sugars, a common type of ethanol producing yeast, *Saccharomyces cerevisiae* (*S. cerevisiae*) can be utilized, whereas a genetically modified yeast is required for combined C_5 and C_6 sugar fermentation, for example the yeast strain *S. cerevisiae* KE6-12B. (Erdei, 2013) (Bondesson, 2016)

S. cerevisiae can be used for both starch- and lignocellulosic-based biomass, since it has a high tolerance to ethanol and many by-products that can be formed after pretreatment of lignocellulosic material. Besides these qualities, a suitable yeast should also have tolerance to low pH. By reducing the pH, the risk of contamination decreases. (Gírio, *et al.*, 2010) For fermentation with lignocellulosic material, the optimal operating temperature is approximately 30°C and a favorable pH is between 4.5 and 5.5. (Erdei, 2013)

The maximum ethanol yield that can be achieved from fermentation is based on glucose to ethanol conversion. The chemical reaction for glucose to ethanol is as followed:



The maximum theoretical ethanol yield, Y_T , that can be produced from glucose is determined from the following equation:

$$Y_T = \frac{2 \cdot 46.07}{180.16} = 0.51 \frac{g \text{ EtOH}}{g \text{ glucose}}$$

2.5.1 Simultaneous Saccharification and Fermentation

SSF is a method where fermentation and enzymatic hydrolysis is performed in the same bioreactor. Thus, derived sugars and bioethanol are formed simultaneously. When yeast is in the presence of cellulolytic enzymes, the accumulation of inhibitors within the reactor decreases, which *increases* the saccharification rates and the yields. Infections are also less likely to happen from undesired microorganisms when ethanol is present. Moreover, the production costs decrease when both reactions occur in the same reactor. (Ferreira, *et al.*, 2010) On the other hand, mixing the lignin together with the yeast makes recycling of yeast difficult. The optimal temperature for the yeast and the enzymes differs, which consequently means that the temperature will be a compromise during fermentation that might not be optimal either for the yeast or the enzymes. (Bura, *et al.*, 2007) The operating temperature is therefore between 35 and 37°C in SSF (Erdei, 2013).

Furthermore, if choosing a process configuration like SSF, with compromised operating temperature, at low pH, the tolerance is favorable for the microorganism used. The reason is that a low pH minimizes the risk for contamination. In general, there is not a single microorganism that fulfills all requirements. (Erdei, 2013)

When fermenting both pentose and hexose sugars, the process is called SSCF. (Erdei, 2013) In this configuration, a more sensitive yeast can be used that is able to ferment both glucose and xylose. However, in some cases, a low concentration of glucose is needed for an efficient xylose fermentation. Furthermore, the advantages and disadvantages with SSCF are similar to those for SSF. (Meinander, *et al.*, 1999)

2.5.2 Feeding Mode

There are different methods for feeding the bioreactors with material. The most common operations are batch-, fed-batch- and continuous feeding. The first two are used to handle low-volume and high value products, for instance amino acids and antibiotics. (Lim & Shin, 2013)

In batch operations, all of the material and necessary medium components are added initially at batch start. Using batch procedures, it is difficult to control the concentrations of the substances in the fermenter. On the other hand, the concentrations are allowed to vary since living cells are working and producing. This definition of batch operation can also be questioned, since in some cases, oxygen and/or nutrients can sometimes be added during the fermentation. (Lim & Shin, 2013)

A fed-batch procedure is a method where material is added sequentially in a controlled manner, via one or several separate streams. Addition of nutrients and materials are set and fed at certain times during the fermentation. By controlling the frequency of feeds or concentration of each feed, a better control can be achieved of the growth rate of the microorganisms and the concentration of the biomass in the reactor. By using a fed-batch bioreactor, a reduction of inhibition can occur, since the concentrations of toxic or inhibiting compounds are lower. (Lim & Shin, 2013)

When the bioreactor is fed with the necessary compounds, media or material continuously, it is referred to as a continuous bioreactor. The amount of feed that is put into the reactor should correspond to the amount of material removed from the bioreactor. This type of process is quite sensitive to contamination and biomass washouts. Continuous operation is more common in conventional chemical industries than in biotechnology industry. (Lim & Shin, 2013)

2.6 Ethanol Recovery

After ethanol fermentation, the concentration of the product is low and by-products have to be removed, thus, product recovery is needed. This step involves solid-liquid separation and distillation. The distillation includes both strippers and rectification columns. The first column separates the ethanol from the non-volatile and solid compounds, as well as removes water to increase the ethanol concentration. The ethanol is later concentrated closed to the azeotropic point in the rectification column. The ethanol recovery has a considerable effect on the overall production costs. (Galbe, *et al.*, 2011)

2.7 Lignocellulosic Feedstock

An interesting type of biological raw material is one that is considered a waste product from agriculture and forestry industry. For instance, it can be animal bedding or crop residues. These types of material can be interesting since there is an abundance of them, which makes them a less expensive raw material to use in biochemical processes and industries. (Mubeen & Saleem Khan, 2012)

It has to be pointed out that when waste material can be used in some way, e.g. as raw material in a process; thus, they can be considered to have a higher value, from an economical point of view than before. Even though animal bedding and crop residues both are waste products that can be used to produce 2G ethanol, they are treated as two different types of material. Animal bedding is currently regarded as a waste only, while straw has a value.

2.7.1 Biomass Composition

The chemical composition of biomass mainly comprises hemicellulose, cellulose and lignin, together with other components such as ash and extractives. The cell wall of a plant consists of cellulose, which is mainly made up of linear chains of β -1, 4-linked glucose units. The repeating unit is the disaccharide cellobiose, which is a dimer of glucose. The chemical structure of cellulose can be seen in *figure 5*. Cellulose is stable and orderly in its structure, due to hydrogen bonds found between the linear chains. However, the structure makes cellulose insoluble in most solvents. (Makkee, *et al.*, 2013)

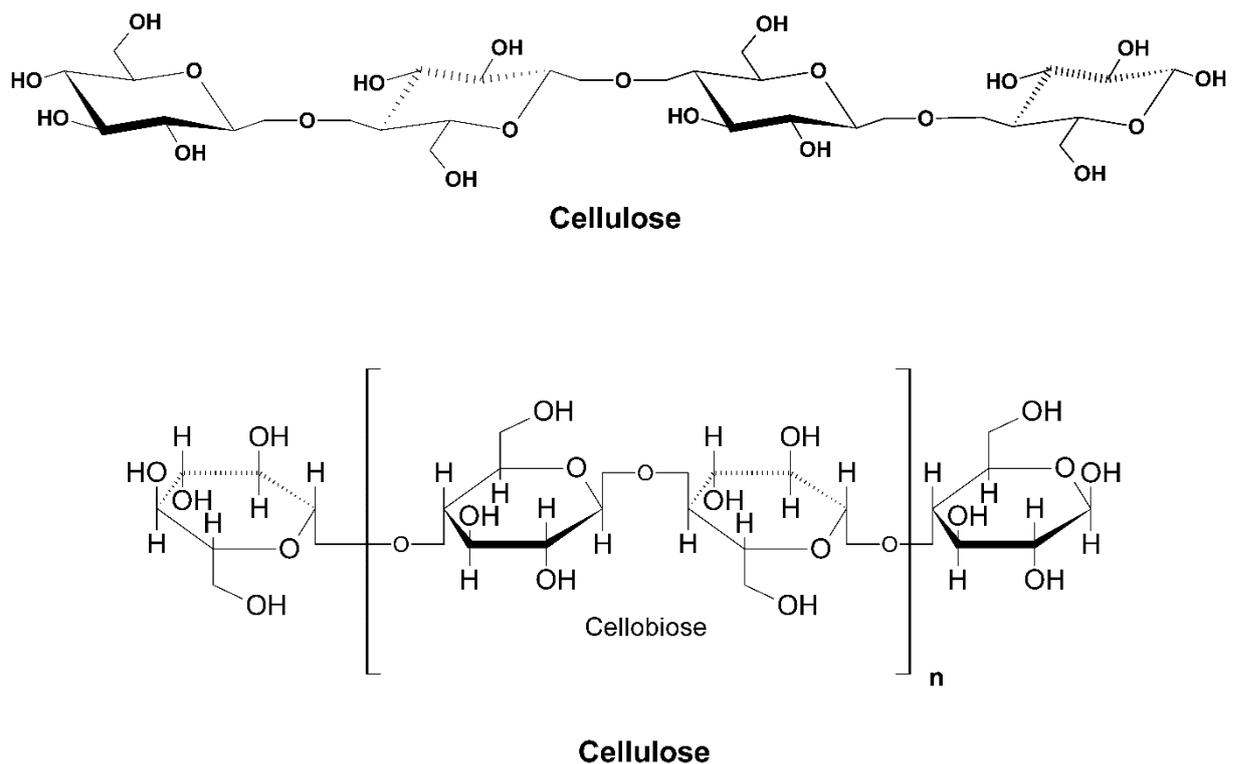


Figure 5. Cellulose consists of polymeric of cellobiose ($n=2500-5000$).

Hemicellulose consists of highly branched heterogeneous polysaccharides. It consists of both C_5 and C_6 sugars. Even though the chemical composition of hemicellulose varies depending on the origin of the biomass, the most common component in all agricultural residue is xylan, followed by arabinan and mannan. Figure 6 shows the chemical composition of xylan. (Makkee, *et al.*, 2013)

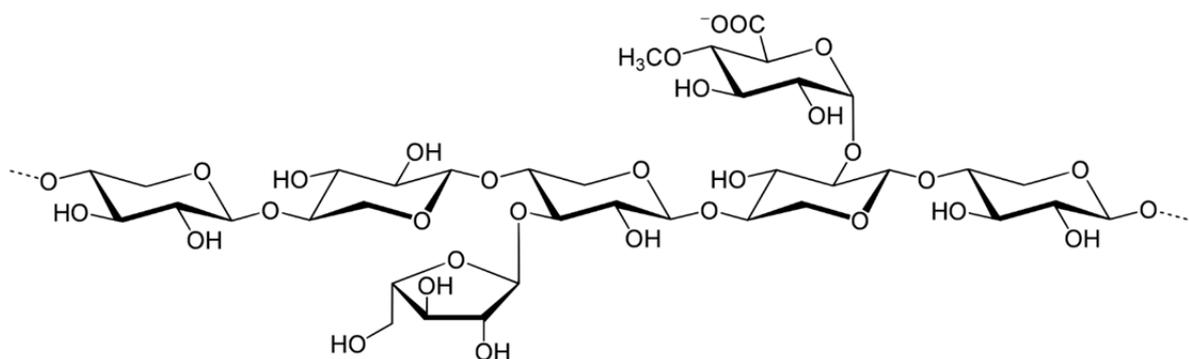


Figure 6. Chemical composition of xylan, which is a typical component in hemicellulose. (Wikimedia, 2015)

Lignin can be found between cellulose and hemicellulose in plant cell walls. Its chemical structure, see figure 7, is highly amorphous and consists of a three-dimensional polyphenolic compound. Together with cellulose, lignin is the main component, which makes the structure of the plant strong and rigid. In comparison with cellulose and hemicellulose, lignin has properties that make the compound resistant to enzymatic- or chemical degradation due to its structure. (Makkee, *et al.*, 2013)

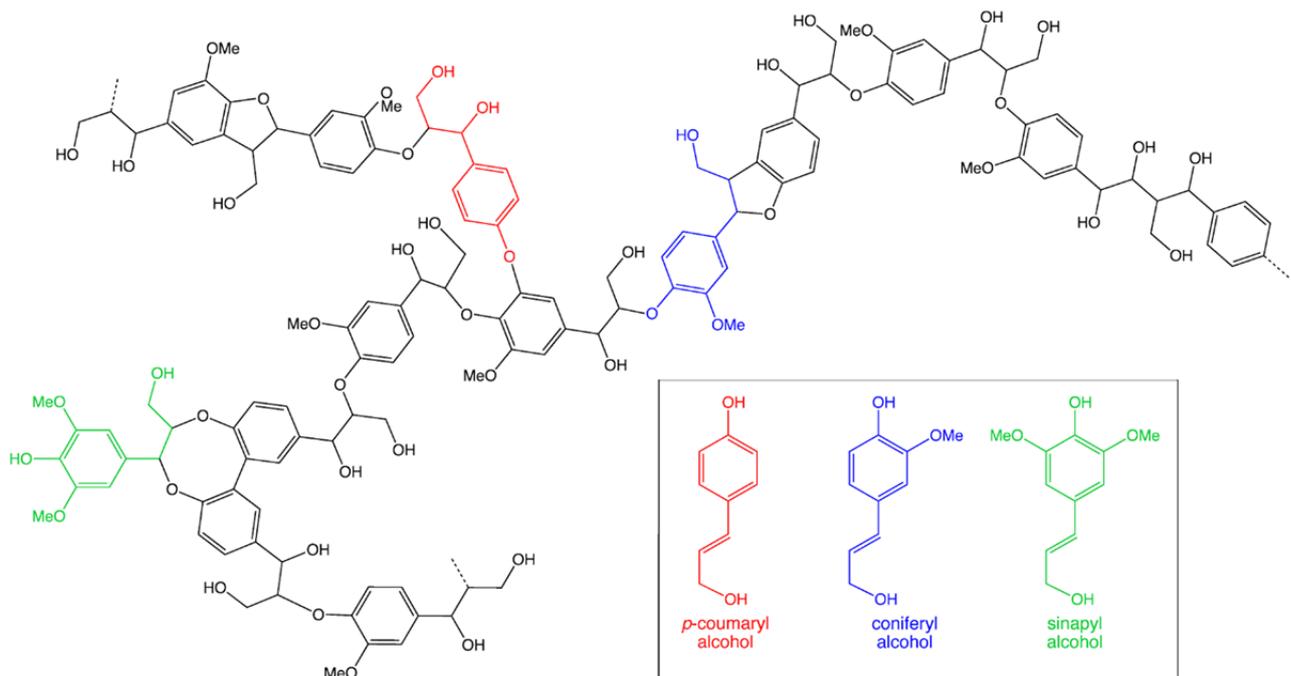


Figure 7. A possible chemical structure of lignin. The three imbedded units in the structure are formed from their precursors: *p*-coumaryl, coniferyl and sinapyl alcohol. (Wikimedia, 2014)

2.7.2 Animal Bedding

Animal bedding covers the floors in the litter areas of barns where already in the barn some composting occurs (Jordbruksverket, 1992). The material is regarded to be cheap; it is used in 8 percent of Sweden's barns to give cattle and pigs a more natural environment, which the animals can imitate (Karlsson & Salomon, 2002). Instead of removing the animal bedding, i.e. manure and straw and replacing it with fresh straw, the fresh straw is added to a new layer, leading to storage of the animal bedding. However, if further treatment of the animal bedding is desired, the manure has to be removed to facilitate the degradation of the carbohydrates. (Elumalai, *et al.*, 2014)

The straw consists of carbohydrates, such as, cellulose, hemicellulose and lignin. Cellulose mainly consists of glucose, while hemicellulose mainly consists of xylose. The chemical structures of monomeric sugars present in cellulose and hemicellulose are shown in *figure 8*. (Erdei, 2013)

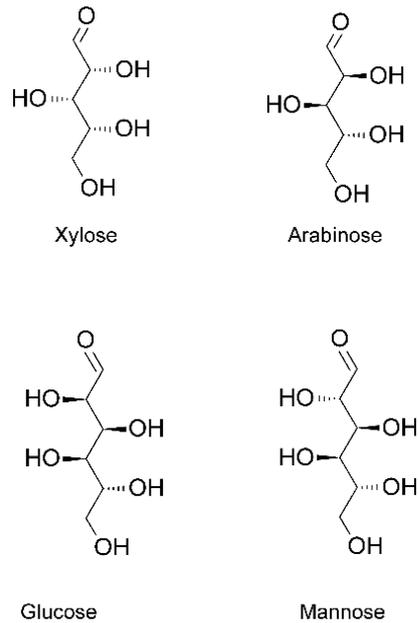


Figure 8. Monomeric sugar components found in cellulose and hemicellulose. Xylose and arabinose are found in hemicellulose, while glucose and mannose are mainly found in cellulose.

The dry matter often comprises more than 25 percent of animal bedding. However, the composition of the material varies depending on the type of bedding (short-cut straw, peat or wood chips), and on the duration of storage (the time the manure has lied in the pile). In this project, animal bedding containing wheat straw is considered. In general, fresh animal bedding that has not been stored has a dry-matter content between 18 and 26 percent, whereas stored animal bedding has a dry-matter content between 28 and 37 percent. Thus, the stored material has a lower heterogeneity than the fresh. Furthermore, the resistance to decomposition is lower for stored animal bedding, making the material easier to process. (Karlsson & Salomon, 2002)

2.8 SEGRABIO: Proposed Process

According to the proposed process by SEGRABIO, animal bedding is used as a feedstock for the production of biofuel. The animal bedding needs first to undergo feedstock conditioning to separate the manure from the lignocellulosic biomass. Feedstock conditioning is followed by separation of the wet fraction and fibrous fraction, in order to utilize as much material as possible. The wet fraction is then treated in anaerobic digestion to potentially produce biogas, while the dry fraction is subjected to hydrolysis and fermentation to produce bioethanol. The overall overview of the proposed process can be seen in figure 9. (Second Grade Biomass for Biofuels, 2016)

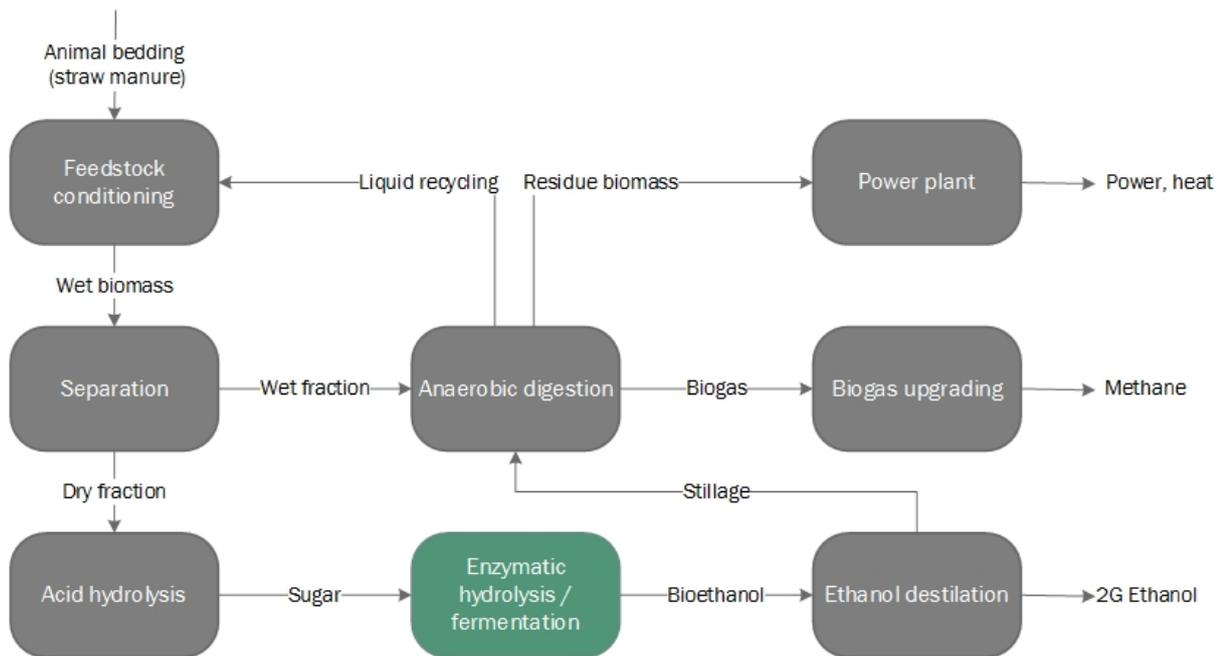


Figure 9. Block diagram of the proposed process. Adapted from (Second Grade Biomass for Biofuels, 2016)

3 Material and Method

3.1 Animal Bedding

The animal bedding was provided by a Danish farm close to the city of Køge, Denmark. The animal bedding had been stored for a couple of weeks inside the cattle stable before the collection. Later, the bedding was transported to the company TK Energy, also located in Køge. At their facility, the material was washed using a concrete mixer for one minute. The slurry was then sieved and pressed at approximately 16 bar to remove all of the excess water.

A total amount of 32.4 kg of animal bedding was washed with 80 kg of water. As a result, 14 kg of washed animal bedding was brought back to the faculty of LTH for further experimental testing. Samples of the original animal bedding (before washing) were collected, as well as samples from the pressed liquid. The animal bedding before and after washing can be seen in *figure 11*.

3.2 Pretreatment

The animal bedding was treated a second time by soaking the material in dilute 0.4 wt-% sulfuric acid solution (20 g liquid/g dry animal bedding) for a total of one hour. After the impregnation, the material was pressed in a 5 L filter press (Fischer Maschinenfabrik, GbmH, Germany) at 16 bar to remove access liquid.

The pretreatment step was performed at 190°C for 10 minutes per run using a steam-explosion reactor (Bondesson, 2016), see *figure 10*. After the pretreatment of all washed animal bedding, a total of 26.5 kg of steam exploded material was obtained. The pretreated material can also be seen in *figure 11*.



Figure 10. Steam explosion reactor in the Apparatus hall at the Department of Chemical Engineering (LTH).



Figure 11. Left picture: Original (raw) animal bedding. Middle picture: Once washed animal bedding. Right picture: Pretreated animal bedding.

After the steam pretreatment, a portion of the material (100 g) was filtered to obtain a liquid fraction and a solid fraction for further composition analysis. Furthermore, the same amount of both washed and soaked material were separated for composition analysis.

3.3 Yeast Preparation

3.3.1 Yeast Cultivation for SSCF

The genetically modified yeast strain *S. cerevisiae* KE6-12B (Taurus Energy AB, Lund, Sweden) (Bondesson, 2016) was cultivated before the SSCF. The cultivation was divided into two parts: pre-cultivation and cultivation, leading to a total cultivation residence time of 96 h.

Preparation of various solutions was performed prior to pre-cultivation and cultivation. The concentrations of the media are presented in *table 1*. Both the vitamin solution and the metal solution were previously prepared. In appendix I, a detailed recipe for the trace metal solution and the media for the vitamin solution are presented.

Table 1. Conditions for the media for the entire cultivation.

Material	Concentration (g/L)
<i>Salt solution</i>	
(NH ₄) ₂ SO ₄	100
KH ₂ PO ₄	60
MgSO ₄ x7H ₂ O	10
Glucose	300
Xylose	300
Cas-aminoacids	100
Vitamin solution	1000
Metal solution	1000

For the pre-cultivation, two kinds of solutions were prepared. 12 flasks of each solution were needed to obtain about 4 g of dry weight yeast. For the solution prepared in 100 mL pressure-proof flasks, 8 mL of salt solution and 20 µL of metal solution were used. Pressure-proof flasks were used since a small volume of liquid was prepared. For the solution prepared in

500-mL Erlenmeyer flasks, 15 mL salt solution, 34.5 mL distilled water and 300 µL metal solution were added.

The flasks, together with required material, such as pipettes and measuring cylinders, were autoclaved. After the material had cooled down, it was important to work in sterile conditions.

The solution from one pressure- proof flask was added in one Falcon tube, together with 1 mL glucose, 0.5 mL cas-amino acid, 10 µL ml vitamin solution and 50 µL yeast. In total, 12 Falcon tubes were prepared to be used for the pre-cultivation. The incubation time was 24 h and the incubation temperature was 30°C.

After 24 h the pre-cultivation was stopped. 15 mL of hydrolysate (pH 5), 30 mL distilled water, 150 µL vitamin solution, 15 mL glucose and 30 mL xylose solution, and the solution from one of the pre-cultivated Falcon tubes were added to the Erlenmeyer flasks. The cultivation time was 72 h, at the same temperature as previously used.

After 72 h, the cultivation was stopped and the yeast cells were centrifuged using a Jouan C4-12 centrifuge (St Herblain, France) with appurtenant flasks at 5000 rpm for 15 minutes to remove access liquid. Later, the cells were washed with NaCl solution (9 g/L) and re-centrifuged to obtain the yeast cells. Lastly, a small portion of the yeast was used for DM determination.

3.3.2 Yeast Preparation for SSF

For hexose fermentation, 4 g/L of *S. cerevisiae* (Ethanol Red, Lesaffre, Marcq- en- Baroeul, France) was used in each fermentation batch. The yeast had a DM content of 94%. The yeast had to be activated with warm distilled water approximately 15 minutes prior to usage.

3.4 Enzymes

For all fermentation experiments, an amount of the enzyme *Cellic CTec2* (Novozymes, Bagsvaerd, Denmark) corresponding to 15 FPU/g WIS was used. The enzyme solution was diluted twice before being added to the fermentation. The dilution was necessary since the enzyme solution was viscous. The diluted solution was added when the fermenter had reached 35°C.

3.5 Fermentation

The batch set-up is presented in section 3.5.1. *Batch*, while the fed-batch is presented in section 3.5.2 *Fed-Batch*.

3.5.1 Batch

SSF and SSCF were performed in 2 L fermenter reactors (Infors AG, Bottmingen, Switzerland), see *figure 12*. A total weight of 1 kg of total material weight was used.



Figure 12. Bioreactor at the Department of Chemical Engineering (LTH).

The pretreated material had a WIS-content of 12.9%. This was adjusted by dilution with deionized water to achieve 10% WIS during fermentation. Later, the fermenters and all material that was needed for SSF were sterilized using an autoclave.

After sterilization, the equipment was cooled and then prepared for the start-up of the fermentation. When the fermenter was prepared, the reactor parameters were set and the fermentation could begin. The reactor temperature was set to 35°C and the slurry was adjusted to have a pH of 5. The pH was adjusted automatically using 10 vol-% sodium hydroxide. The experiment was performed during 168 h and samples were taken at various times during the fermentation. During the first day, samples were taken four times. From day 2 to day 5, samples were taken twice a day, and from day 6 to day 8, they were only taken once a day. The exact amounts of each material used during the batch runs are presented in *table 2*. After 168 h, the last sample was taken and the remaining material in the fermenter was recovered and weighed.

Table 2. Amount of each material used for one batch fermenter, with a total material weight of 1000 g.

Substance	Amount	Unit
Slurry	775	g
Yeast Suspension	33.3 ^a	g
Enzyme Solution	13.9	g
(NH ₄) ₂ HPO ₄ ^b	5.0	mL
Vitahop	0.1	mL
H ₂ O ^c	172.5	g

^a 33.3 g corresponds to 4 g dry weight

^b Concentration of 100 g/L

^c 50 g of the water is put in the reactor during autoclaving, together with the ammonium phosphate. 13.9 g water is used for enzyme dilution and 20 g water for activation of Ethanol red yeast. Rest (88 g) is used during startup of the fermentation. Observe, 108 g of water is used during start-up for fed-batch feedings.

3.5.2 Fed-Batch

For fed-batch fermentation, the material was divided into three parts and fed at three different times: 0 h, 24 h and 28 h, as presented in *table 3*. For SSCF A, slurry at pH 5 was fed each of the three times. For SSCF B, hydrolysate, also at pH 5, was fed once followed by feeding pressed fiber twice. The pressed fiber had a WIS content of 37.0%. The rest of the procedure is described in section 3.5.1. Samples were taken one hour before and after each feeding on day two.

Table 3. Amount of material that has to be fed at a specific time for different fed-batch fermentation types. For SSCF A, only slurry was fed into the reactor. For SSCF B, hydrolysate and fiber were fed separately into the reactor.

Fermentation type	SSF	SSCF A	SSCF B
Time (h)	Amount of material (g)		
0	388	388	505
24	194	194	135
28	194	194	135

3.6 Sample Analysis

Composition analysis of all the types of material e.g. raw animal bedding, washed animal bedding, soaked animal bedding and pretreated animal bedding, were analyzed according to National Renewable Energy Laboratory (NREL) standard methods (National Renewable Energy Laboratory, 2008).

Analysis of ethanol and degradation products received from the SSF and SSCF, was performed with an HPLC. Samples were filtrated using a filter with a 0.20 µm pore diameter. The samples were also diluted with an appropriate dilution factor, prior to analysis. The HPLC consisted of a Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) and used a 5 mM H₂SO₄ solution as eluent. The operation conditions during the run were: temperature at 50°C and flow rate of 0.5 mL/min.

3.7 Yield Calculations

The ethanol yield from the ethanol production was based on the amount of available sugar in the process. For SSF, the ethanol yield was based on glucose, and for SSCF, both glucose and xylose were taken into consideration. The amounts of available sugars are determined from the amount of available sugars in the slurry, both in the fibrous and in the liquid fraction, at the start of the fermentation.

The ethanol yield, Y_{EtOH} , was calculated as followed:

$$\frac{g EtOH_{end}}{g sugar_{start}} = Y_{EtOH}$$

$g EtOH_{end}$ is gram ethanol at the end of the fermentation. It is calculated as followed:

$$C_e \cdot V = g EtOH_{end}$$

Where C_e is the concentration of ethanol at the end of the fermentation and V is the total liquid volume in the reactor at the start of the fermentation.

$g Sugar_{start}$ is gram sugar in the material at the start of the fermentation. In SSF, glucose is the sugar component taken into consideration, while for SSCF both glucose and xylose are taken into consideration. $g Sugar_{start}$ is calculated as followed:

$$m \cdot (1 - WIS) \cdot [C_s] = g Sugar_{solid\ fraction}$$

$$\frac{m \cdot WIS \cdot X_s}{\rho} = g Sugar_{liquid\ fraction}$$

$$g Sugar_{solid\ fraction} + g Sugar_{liquid\ fraction} = g Sugar_{start}$$

Where m is the amount of material put in the reactor, WIS is the WIS content for the material, $[C_s]$ is the concentration of a specific sugar component (glucose or xylose) at the start of the fermentation and ρ is the density of the liquid fraction of the material. X_s is the molar fraction of the sugar component. It is calculated by multiplying the concentration of sugar with 1.11 and 1.13 for glucose and xylose, respectively.

It is to be noticed that by using the liquid volume at the beginning, it is assumed that the WIS content of the slurry is constant throughout the fermentation. Using this method, the calculated ethanol yield will be considered a minimum value.

4 Results and Discussion

4.1 Fractionation

From a total of 32.4 kg of animal bedding, a total weight of 12.0 kg soaked straw was obtained after the washing and soaking steps. The dry weight content from the animal bedding compared to the content in the soaked straw is 7.6 kg and 4.1 kg, respectively. The loss in dry weight is reasonable considering that the material was handled manually, and contained manure that was washed of. An overview of how the animal bedding has been fractionated is presented in *figures 13, 14 and 15*.

The washing strategy was considered successful. A significant amount of manure was removed after the first washing. A low amount of dry weight was found in the pressed soaking liquid. This is also seen in *figure 13*.

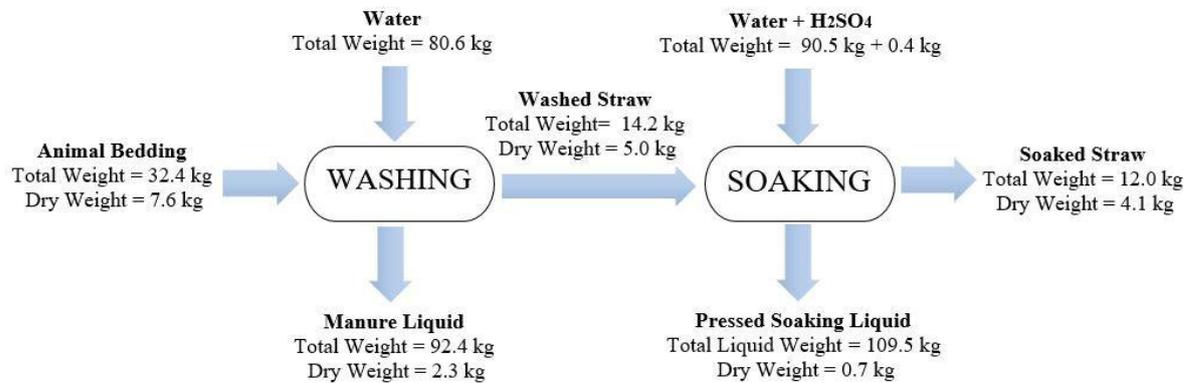


Figure 13. Fractionation diagram over washed and soaked animal bedding.

After soaking the washed straw, steam pretreatment of the material was performed. From this step, a total amount of 26.5 kg of pretreated slurry was obtained, having a dry weight of 4.3 kg. A slight increase in dry weight was observed after the pretreatment. However, this increase is not significant.

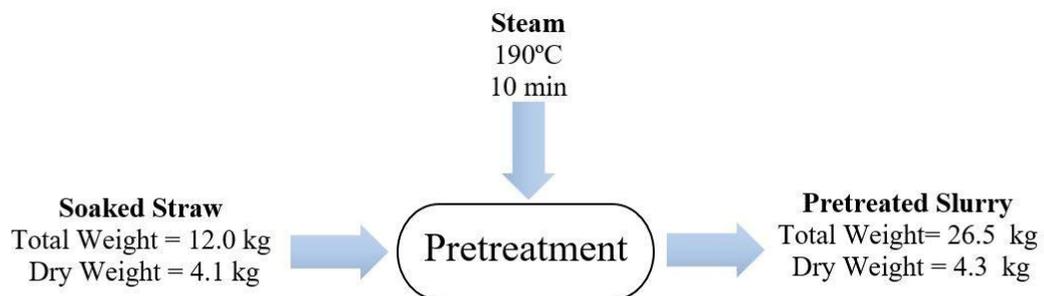


Figure 14. Fractionation diagram over pretreated animal bedding.

The results in *figure 15* show that a low amount of ethanol was produced during the study. However, if the experiment would have been run under optimal conditions for fermentation, higher ethanol yields would have been obtained. In this study, twice as much material was used for SSCF studies than SSF. Further, if only SSF would have been used with the available material, approximately 0.9 kg of ethanol could have been produced.

During the fermentation, approximately 4 kg of input has been lost. Reasons for this can be: sample taking, loss of material during weighing, and spillage.

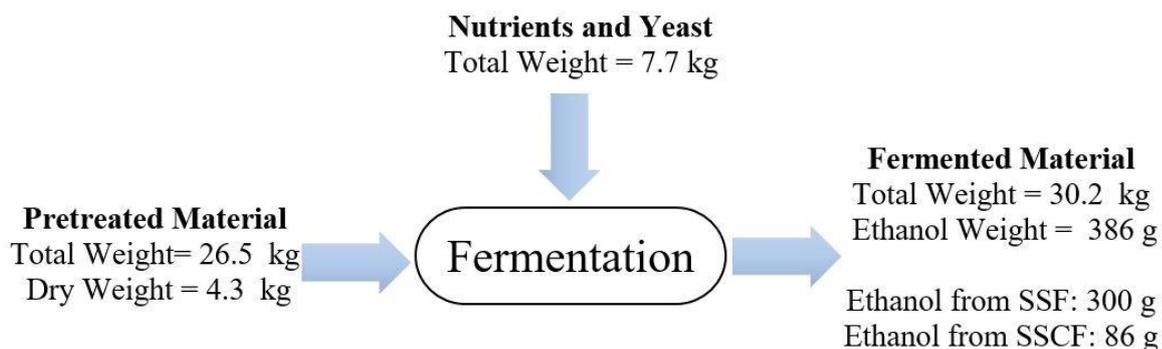


Figure 15. Fractionation diagram over the fermentation. The amount of ethanol produced from the fermentation is based on the yields from the experiments, presented in section 4.3.3.

4.2 Composition Analysis

The compositional analysis of the washed animal bedding showed that the major sugar components in the straw were glucan, followed by xylan. The washed animal bedding in this study has a composition that resembles raw dry wheat straw; they are less similar to washed animal bedding from another study, see *table 4*.

This may be because the studied animal bedding was stored for a shorter time than what the washed animal bedding from a previous study was. In particular, that material had been stored for a longer period of time, which may have resulted in degradation of sugar components.

The ash content is higher in animal bedding than in raw dry wheat straw, which can be a result of a “dirtier” raw material.

Table 4. Composition of the studied washed animal bedding compared to animal bedding and wheat straw from other studies. (Sancho Sebastián, 2017)¹ (Erdei, et al., 2010)²

Components	Washed Animal Bedding	Washed Animal Bedding ¹	Raw Dry Wheat Straw ²
Solid fraction (% of WIS)			
Xylan	21.7 ± 1.0	16.6 ± 2.1	22.2 ± 0.3
Arabinan	0.8 ± 0.1	3.2 ± 0.1	4.7 ± 0.1
Glucan	36.7 ± 2.1	24.6 ± 3.5	38.8 ± 0.5
Mannan	1.5 ± 0.0	3.7 ± 0.2	1.7 ± 0.2
Galactan	BDL ^a	3.2 ± 0.1	2.7 ± 0.1
Total solids	35.5 ± 1.0	-	-
AIL	23.7 ± 1.3	24.8 ± 1.8 ^b	16.1 ± 0.1
ASL	1.1 ± 0.0	-	2.4 ± 0.0
H ₂ O Extractives	10.0 ± 0.3	-	-
EtOH Extractives	2.9 ± 0.4	-	-
Total Extractives	12.9 ± 0.7	13.6 ± 0.0	-
Ash	8.6 ± 1.7	12.0 ± 0.6	5.8 ± 0.1

^aBDL = Below detection limit

^bThe result refers to the total lignin amount in the dry matter.

After soaking, the xylan concentration slightly increased, see *table 5*. This is an effect of a successful soaking, since hemicellulose mainly degrades in acidic conditions. An increase of the glucan concentration indicates that also some cellulose has been degraded.

There is a significant difference in ash content between washed, and soaked materials, see *tables 4 and 5*. Since the ash content decreases after soaking, it can be assumed that the material has become cleaner.

Table 5. Composition of the straw in soaked animal bedding.

Soaked Animal Bedding WIS	
Components	Percentage of DM
Xylan	24.5 ± 1.2
Arabinan	0.6 ± 0.0
Glucan	41.4 ± 1.8
Mannan	1.5 ± 0.0
Galactan	BDL
Total solids	33.1 ± 4.5
AIL	23.1 ± 0.2
ASL	1.1 ± 0.0
H ₂ O Extractives	2.9 ± 0.2
EtOH Extractives	3.4 ± 0.2
Ash	2.7 ± 0.5

Previous studies on animal bedding showed that when soaking the material in 0.2 wt-% sulfuric acid, the pretreatment was less successful compared with when regular wheat straw was pretreated utilizing the same conditions. The previous results were not satisfying; so, in this study the material was soaked in 0.4 wt-% sulfuric acid. The results from pretreatment of each type of material are presented in *table 6*.

Table 6. Composition of studied pretreated animal bedding compared to animal bedding and wheat straw from other studies. The studied animal bedding was soaked in 0.4 wt-% H_2SO_4 . The compared animal bedding and wheat straw were soaked at 0.2 wt-% H_2SO_4 . (Sanchis Sebasti a, 2017)¹ (Erdei, et al., 2013)²

Material	Studied Pretreated Animal Bedding	Pretreated Animal Bedding ¹	Pretreated Wheat Straw ²
<i>WIS (%)</i>	12.9	8.5	12.4
Solid fraction (% of WIS)			
Arabinan	BDL	1.0 ± 0.0	-
Glucan	46.5 ± 4.6	41.2 ± 1.5	51.4
Xylan	1.5 ± 0.2	8.2 ± 0.3	5.6
Mannan	1.4 ± 0.1	2.7 ± 0.1	-
Galactan	BDL	0.6 ± 0.0	-
AIL	38.3 ± 0.1	37.4 ± 0.1 ^a	29.2
ASL	1.0 ± 0.0	n.d ^b	2.0
Ash	4.2 ± 0.1	n.d	5.0
Liquid fraction (g/L)			
<i>Monomers</i>			
Glucose	2.1 ± 0.1	0.2 ± 0.0	4.0
Xylose	13.5 ± 0.1	3.7 ± 0.0	30.0
Galactose	0.9 ± 0.1	0.3 ± 0.0	-
Arabinose	1.8 ± 0.1	0.8 ± 0.0	6.7
Mannose	0.4 ± 0.0	0.5 ± 0.0	-
<i>Oligomers</i>			
Glucose	2.2 ± 0.2	1.6 ± 0.0	3.9
Xylose	5.9 ± 0.7	13.7 ± 0.3	9.2
Galactose	0.2 ± 0.0	0.7 ± 0.0	-
Arabinose	0.4 ± 0.0	1.2 ± 0.0	-
Mannose	0.6 ± 0.0	1.3 ± 0.0	-
<i>By-products</i>			
Furfural	4.8 ± 0.4	0.5	2.8
HMF	0.5 ± 0.1	0.1	0.3
Acetic acid	3.6 ± 0.1	n.d	3.6
Formic acid	BDL	n.d	0.7

^a The result refers to the total lignin amount in the dry matter.

^b n.d = not detected

Observations, see *table 6*, from the studied material show that the xylan fraction in the fiber has almost completely degraded. The concentration of monomeric xylose is 13.5 g/L in the liquid fraction, which indicates that the pretreatment was successful. However, comparing to regular pretreated wheat straw, the pretreatment is still less effective.

Since the pretreatment was more severe, it resulted in higher amounts of by-products. These by-products could prevent a successful fermentation.

All analyses were performed with small amounts of material (0.3-2.0 g), which can however, be seen as a source of error.

4.3 Ethanol Production

The results from the SSF and the SSCF are presented as figures and tables in this section. It can be seen that there are dips present in the graphs for all components at a specific time. This can be a result from error in analysis, dilution, or evaluation of the chromatograms. Moreover, when fed-batch is used, dips of glucose and xylose concentration should occur due to feeding of material. Lastly, increased glucose concentration indicates that saccharification occurs.

4.3.1 SSF

Duplicates of the batch SSF were performed. In both cases, ethanol was produced and low amount of the inhibitor lactic acid was available, see *figure 16*. However, in batch SSF 1, the fermentation started earlier, resulting in a longer steady state phase.

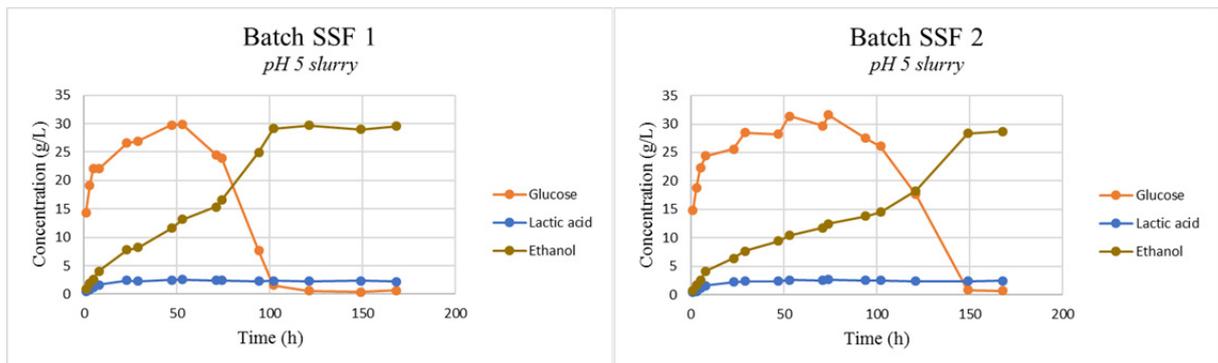


Figure 16. Concentration profiles for duplicates of SSF performed with the yeast *S. Cerevisiae*. Left graph represents batch-feeding no.1 of slurry at pH 5, while right graph represents batch-feeding no.2 of slurry at pH 5.

Duplicates of the fed-batch SSF were also performed. Ethanol was also produced with this process concept, and low amount of lactic acid was available, see *figure 17*. The duplicate followed the same trend and only differed slightly in concentration.

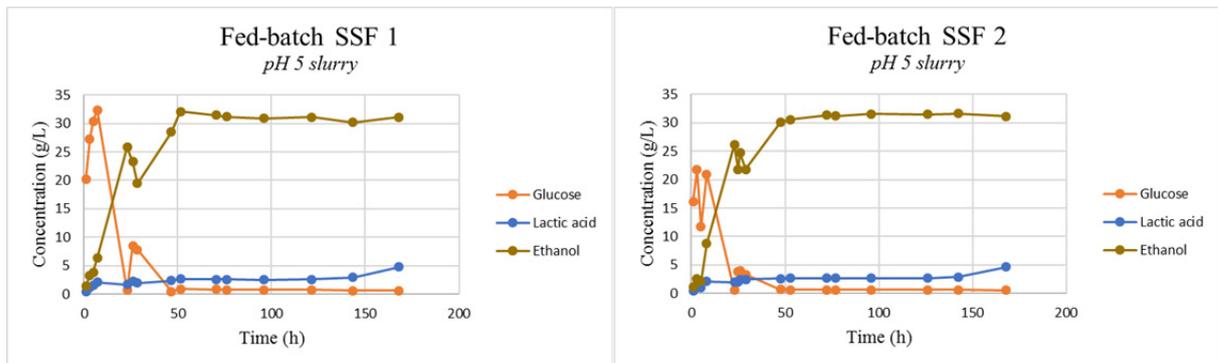


Figure 17. Concentration profiles for duplicates of SSF performed with the yeast *S. Cerevisiae*. Left graph represents fed-batch feeding no. 1 of slurry at pH 5, while right graph represents fed-batch feeding no. 2 of slurry at pH 5.

The ethanol production rates were higher in the beginning of the fermentations when fed-batch was used compared to batch. During the first 50 hours of the batch process, only saccharification occurred, which lead to an increase of glucose concentration. This might be a result of the yeast having a difficulty adapting to its environment.

Another observation is that steady state for ethanol production was obtained at an earlier stage for fed-batch when compared to batch. This occurred after approximately 50 hours, while the ethanol production for batch at this time had not yet reached steady-state. Low amounts of

lactic acids were detected in the both process operations, indicating that ethanol production can occur under these conditions.

4.3.2 SSCF

Duplicates of the batch SSCF was performed. The results show that no significant amount of ethanol was produced when the pH was set to 5, see *figure 18*. However, hydrolysis did occur in both cases, since there is an increase of glucose concentration.

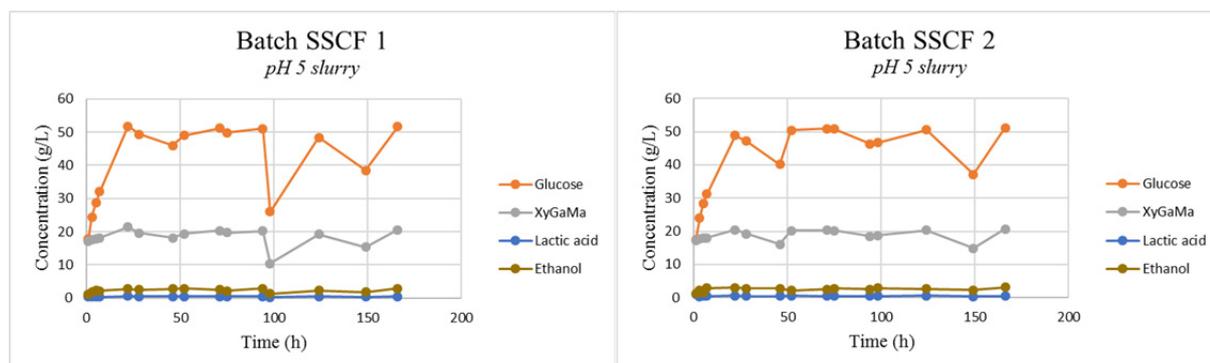


Figure 18. Concentration profiles for duplicated of SSCF performed with the genetically modified yeast strain KE6-12B. Left graph represents batch-feeding no.1 of slurry at pH 5. Right graph represents batch-feeding no.2 of slurry at pH 5.

Fed-batch SSCF experiments were also performed with xylose fermenting yeast. Similar results were obtained in fed-batch SSCF, in both cases, no significant amount of ethanol was produced, see *figure 19*. However, hydrolysis occurred also in these cases. The significant increase in glucose for the separate hydrolysate and fiber feeding is because the fiber contains a high amount of glucose, see *table 6*.

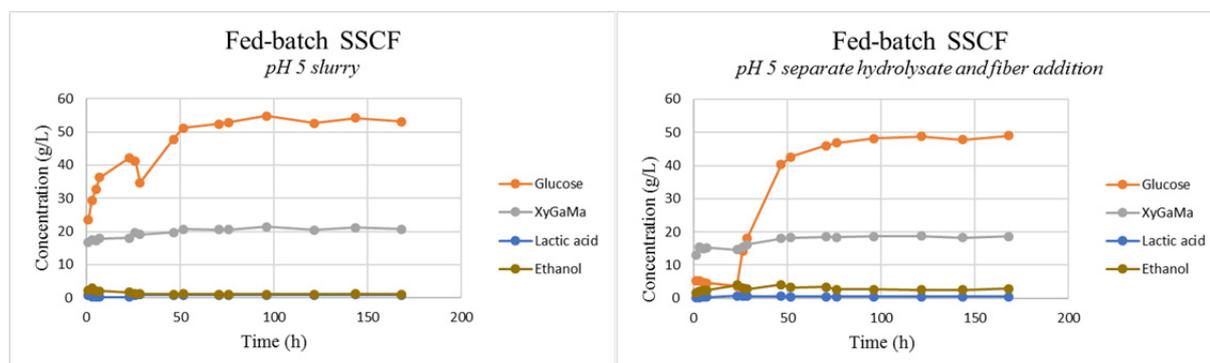


Figure 19. Concentration profiles for SSCF performed with the genetically modified yeast strain KE6-12B. Left graph represents a fed-batch feeding of slurry at pH 5. Right graph represents a fed-batch feeding where hydrolysate at pH 5 was fed, followed by two additions of fiber.

It is observed from the composition analysis that there are high amounts of inhibitors in the material. This is also noticed in *figure 20*. Further, observations from figures 18 and 19 show that xylose was not consumed in neither of the SSCF. Thus, it can be concluded that the yeast has had difficulty to adjust to the environment. In order to decrease the toxicity level for the yeast, a pH adjustment from pH 5 to 5.5 was made.

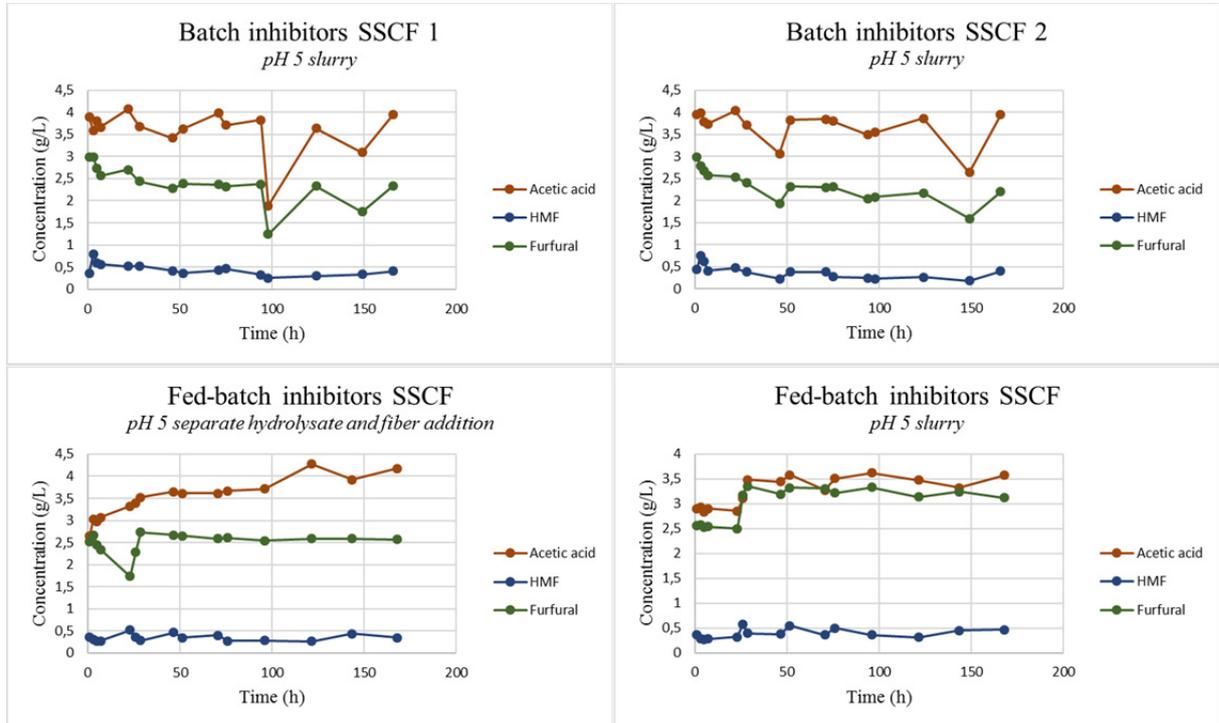


Figure 20. Concentration profiles for by-products formed during SSCF performed with the genetically modified yeast strain KE6-12B. Upper left graph represents by-products from batch feeding no.1 of slurry at pH 5. Upper right graph represents by-products from batch feeding no.2 of slurry at pH 5. Lower left graph represents by-products from fed-batch feeding where hydrolysate at pH 5 was fed, followed by two feedings with fiber. Lower right graph represents by-products from fed-batch feeding of slurry at pH 5.

After increasing the pH to 5.5, it was observed that a low amount of ethanol was produced for the two cases of fed-batch, see *figure 21*. However, the amount of ethanol is slightly higher than when using pH 5. There is no consumption of xylose but the saccharification was successfully performed also in this case.

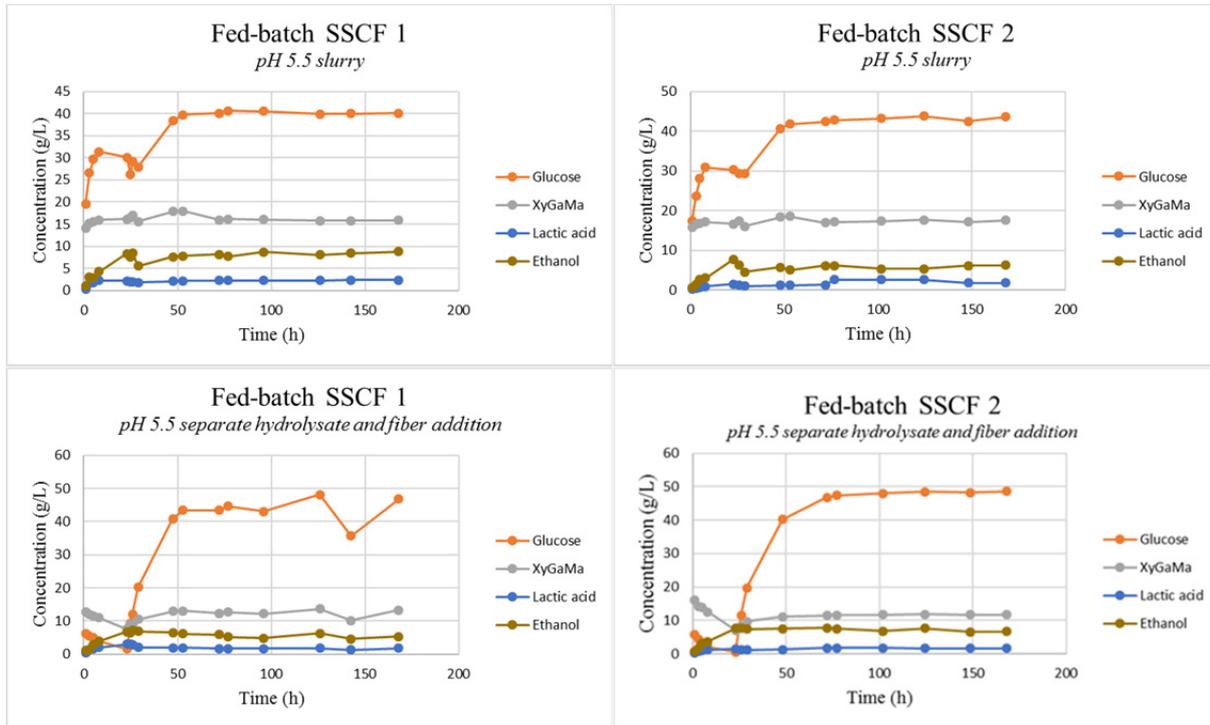


Figure 21. Concentration profiles for SSCF performed with the genetically modified yeast strain KE6-12B. Upper left graph represents a fed-batch feeding no. 1 of slurry at pH 5.5. Upper right graph represents a fed-batch feeding no. 2 of slurry at pH 5.5 Lower left graph represents a fed-batch feeding no. 1 where hydrolysate at pH 5.5 was fed, followed by two feedings with fiber. Lower right graph represents a fed-batch feeding no. 1 where hydrolysate at pH 5.5 was fed, followed by two feedings with fiber.

The concentration of inhibitors has changed when increasing the pH, see *figure 22*. It is noticed that the amount of acetic acid increases slightly, while the concentration of furfural decreases during the SSCF with pH 5.5. The increase of acetic acid, compared to earlier study with pH 5, indicates that the yeast was active, but did not have the optimal conditions to produce ethanol instead. Furthermore, the increase of acetic acid during the SSCF is due to addition of the material.

The decrease of the other inhibitors is due to the yeast activity. Since the inhibitor concentrations were high and HMF does not disappear completely, the yeast could not manage to assimilate all the inhibitors to be able to start producing ethanol.

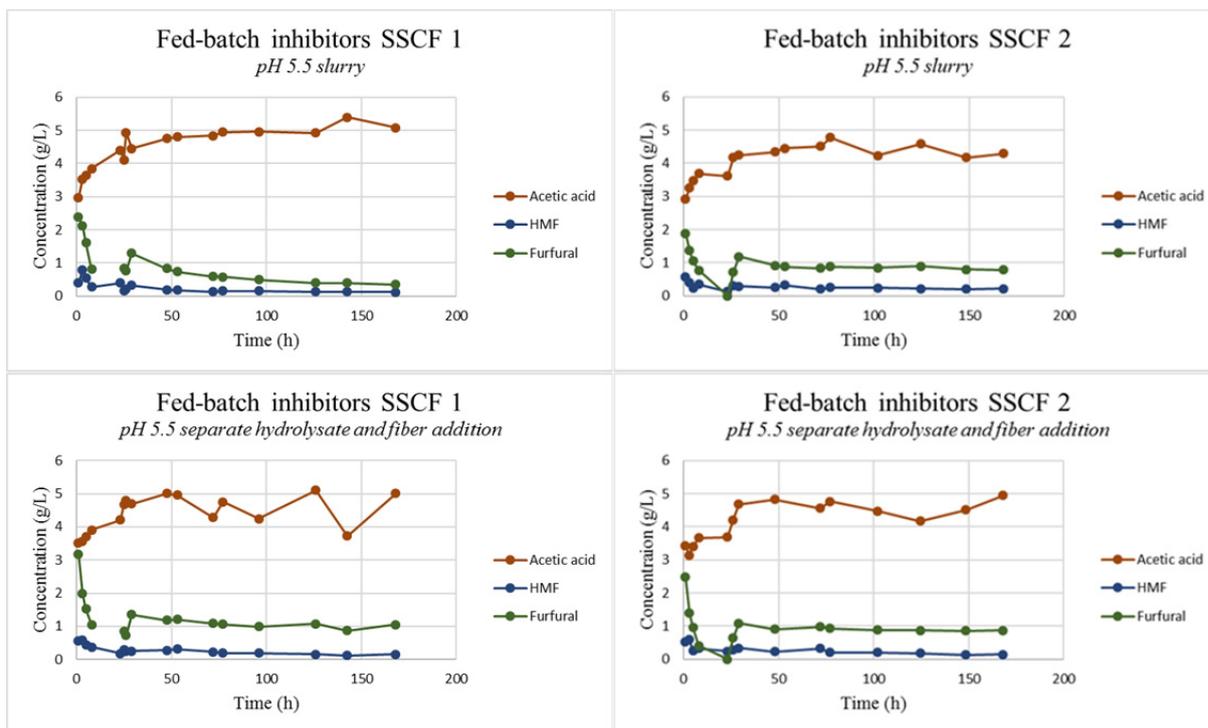


Figure 22. Concentration profiles for by-products formed during SSCF performed with the genetically modified yeast strain KE6-12B. Upper left graph represents by-products from fed-batch feeding no.1 of slurry at pH 5.5. Upper right graph represents by-products from fed-batch feeding no.2 of slurry at pH 5.5. Lower left graph represents by-products from fed-batch feeding no.1 where hydrolysate at pH 5.5 was fed, followed by two feedings with fiber. Lower right graph represents by-products from fed-batch feeding no.2 where hydrolysate at pH 5.5 was fed, followed by two feedings with fiber.

4.3.3 Ethanol Yields

Ethanol yields from SSF are presented in *figure 23*. Overall, high yields of ethanol were obtained for both processes, indicating that it was possible to produce ethanol under these conditions.

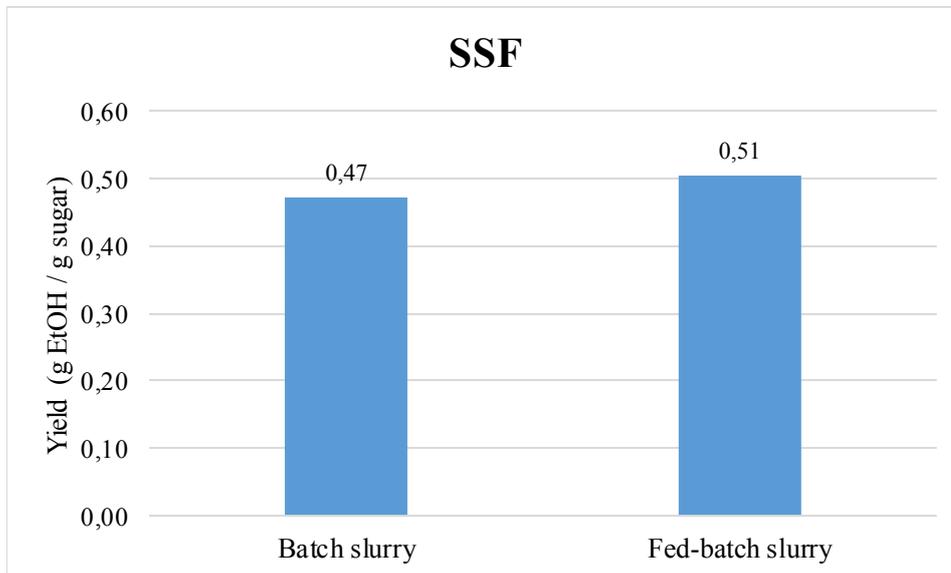


Figure 23. The average yield from SSF when using duplicates of batch and fed-batch, respectively. The standard deviations for the duplicates were 0.01 g EtOH/ g sugar for batch slurry and 0.00 g EtOH/ g sugar for fed-batch slurry.

In *figure 24*, ethanol yields from SSCF are presented. It is clearly seen that the SSCF at pH 5.5 is preferable for ethanol production. Nevertheless, the yields are low, which once again indicates that the yeast did not have a favorable environment.

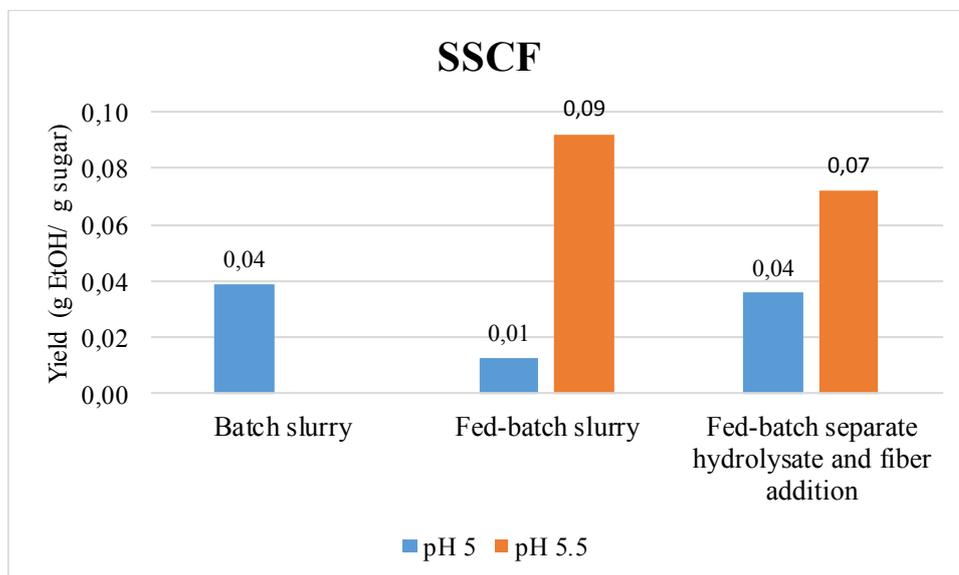


Figure 24. The average yield from SSCF when using duplicates of batch and fed-batch, respectively. Single samples were taken for fed-batches run at pH 5. The standard deviations for the duplicates were 0.00 g EtOH/ g sugar for batch slurry, 0.02 g EtOH/ g sugar for fed-batch slurry at pH 5.5 and 0.01 g EtOH/ g sugar for fed-batch separate hydrolysate and fiber addition at pH 5.5.

A summary of the ethanol yields can be seen in *table 7*. It is observed that SSF is preferable to use instead of SSCF under the studied conditions. However, the results from SSF are remarkably high, especially for fed-batch, since almost 100% of the theoretical maximum ethanol yield is achieved. When working in a laboratory environment, these results are not common.

Furthermore, using fed-batch results in higher yields than using batch.

Table 7. Summary of calculated ethanol yield¹ based on available sugar in each batch and theoretical ethanol yield² referred to as the percentage of the theoretical maximum yield, also based on available sugar in each batch.

Batch type	Specifications		Yield (g EtOH/ g sugar)¹	Yield (%)²
	pH	Feeding material		
Batch SSF	5	Slurry	0.47 ± 0.01	92.6 ± 1.9
Fed-batch SSF	5	Slurry	0.51 ± 0.00	99.0 ± 0.1
Batch SSCF	5	Slurry	0.04 ± 0.00	0.08 ± 0.5
Fed-batch SSCF ^a	5	Slurry	0.01	2.5
Fed-batch SSCF ^a	5	Hydrolysate and fiber	0.04	7.0
Fed-batch SSCF	5.5	Slurry	0.09 ± 0.02	18.0 ± 4.3
Fed-batch SSCF	5.5	Hydrolysate and fiber	0.07 ± 0.01	14.2 ± 2.3

^a Single samples were taken.

5 Conclusion

In this thesis a successful fermentation study was performed; where it was shown that it is possible to produce bioethanol from animal bedding. The results can be seen as a starting foundation for further studies for the SEGRABIO project. Moreover, several conclusions have been made and are presented below.

Initially, fractionation of the animal bedding was overviewed, in order to follow the distribution of the material. The results showed that there are no significant material losses from collection to fermentation and it is possible to fractionate the material in this manner.

The composition analysis for the studied material showed that animal bedding is similar to wheat straw. However, the composition of the material varies depending on the storage time, since sugar decomposition occurs. Furthermore, the analysis also showed that the pretreatment of the animal bedding was too severe, since inhibitors were formed at relatively high concentrations.

For the SSF, ethanol was successfully produced when the two types of process concepts were used. However, a slightly higher yield of ethanol was obtained when using fed-batch instead of batch configuration. This is reasonable, since fed-batch feeding allows the yeast to better adapt itself to the environment. Comparing to theoretical yields, the obtained yields were very satisfying. Moreover, low concentrations of inhibitors were observed in the two cases.

There was a difficulty cultivating the yeast, since the hydrolysate was too toxic for it. Hence, the yeast could only be cultivated with a smaller amount of hydrolysate than it ordinarily would have been. However, the successful cultivation did not result in a successful SSCF, since low yields of ethanol were obtained. Increasing the pH lead to a higher ethanol yield, but still no significant increase. On the other hand, the concentration of by-products increased, indicating that the yeast worked properly.

Conclusively, this study shows that SSF is better than SSCF and that fed-batch is a better process option than batch. However, further studies are needed to ensure that the obtained results are valid. It is important to investigate the reasons for the less successful utilization of pentose-fermenting yeast in comparison with what can be obtained using fresh wheat straw.

6 Future Work

In this study, different fermentation configurations were investigated. The results showed that the conventional SSF method gave promising results. However, when using the genetically modified yeast strain KE6-12B for the SSCF, low ethanol yields were obtained. Thus, further studies need to be performed in order to see if the process can be feasible.

One of several reasons to why SSCF did not work is that the pretreated slurry was highly inhibitory. This may be due to the pretreatment that was considered to be too severe for the used animal bedding compared to other studies on animal bedding. Therefore, it is recommended that further studies on a suitable pretreatment method need to be investigated. Moreover, further studies on animal bedding need to be designed according to the need to find a representative composition for the material. It was shown that the raw materials are different in composition depending on storage time.

In order to use the yeast strain for SSCF, cultivation is needed to be performed. When cultivating, hydrolysate was used so that the yeast could adapt to the environment in the fermentation. In this study, a low amount of hydrolysate was used, since it contained compounds that killed the yeast. It can be of interest to further investigate the yeast cultivation procedure to increase the chance of the yeast surviving the fermentation.

Parameters such as pH and WIS can be studied to see if there is any major difference in the result outcome. It was seen that the pH affects the yeast's ability to ferment. However, only one WIS-content during the fermentation was studied. Nevertheless, it is an interesting parameter to study, since it affects the amount of ethanol that can be produced.

7 Bibliography

- Abatzoglou, N., Chornet, E., Belkacemi, K. & Overend, R. P., 1992. Phenomenological kinetics of complex systems: The development of a generalized severity parameter and its application to lignocellulosics fractionation. *Chemical Engineering Science*, 47(5), pp. 1109-1122.
- Alvira, P., Ballesteros, M., Negro, M. J. & Tomás-Pejó, E., 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresource Technology*, 101(13), pp. 4851-4861.
- Angelidaki, I., Karakashev, D. & Talebnia, F., 2010. Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation. *Bioresource Technology*, 101(13), pp. 4744-4753.
- Arifin, Z. et al., 2012. *Production of Biocellulosic Ethanol from Wheat Straw*. [Online] Available at: <http://repository.um.edu.my/34946/1/Production%20of%20Biocellulosic%20ethanol%20from%20wheat%20straw.pdf>. [Accessed 13 February 2017].
- Ballesteros, I. et al., 2006. Ethanol production from steam-explosion pretreated wheat straw. *Applied Biochemistry Biotechnology*, 129(132), pp. 496-508.
- Barrett, D. M., Delwiche, M. J., Kumar, P. & Stroeve, P., 2009. *Methods for Pretreatment of Lignocellulosic Biomass*. [Online] Available at: <http://ucanr.edu/datastoreFiles/234-1388.pdf> [Accessed 22 February 2017].
- Bondesson, P.-M., 2016. Ethanol Production. In: *Evaluation of Pretreatment and Process Configurations for Combined Ethanol and Biogas Production from Lignocellulosic Biomass*. Lund: Media-Tryck, Lund University, pp. 13-17.
- Bura, R., Saddler, J., Zacchi, G. & Öhgren, K., 2007. A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover. *Process Biochemistry*, 42(5), pp. 834-839.
- Center for Climate and Energy Solutions, 2017. *Main Greenhouse gases*. [Online] Available at: <https://www.c2es.org/facts-figures/main-ghgs> [Accessed 09 05 2017].
- Dwidevi, P., Alavalapati, J. R. & Lal, P., 2009. Cellulosic ethanol production in the United States: Conversion technologies, current production status, economics, and emerging developments. *Energy for Sustainable Development*, 13(3), pp. 174-182.
- Elumalai, S., Markley, J. L., Roa-Espinosa, A. & Runge, T. M., 2014. *Combined sodium hydroxide and ammonium hydroxide pretreatment of post-biogas digestion dairy manure fiber for cost effective cellulosic bioethanol production*. [Online] Available at: <https://sustainablechemicalprocesses.springeropen.com/articles/10.1186/2043-7129-2-12> [Accessed 13 February 2017].
- Erdei, B., 2013. *Development of integrated cellulose- and starch- based ethanol production and process design for improved xylose conversion*. Lund: Media-Tryck, Lund University.
- Erdei, B. et al., 2010. Ethanol production from mixtures of wheat straw and wheat meal. *Biotechnology for Biofuels*, 3(16).
- Erdei, B., Frankó, B., Galbe, M. & Zacchi, G., 2013. Glucose and xylose co-fermentation of pretreated wheat straw using mutant of *S. cerevisiae* TMB3400. *Journal of Biotechnology*, Volume 164, pp. 50-58.

- European Commission, 2017. *Climate Action*. [Online] Available at: https://ec.europa.eu/clima/policies/strategies/2020_en [Accessed 09 05 2017].
- Ferreira, V., de Oliveria Faber, M., Pereira Jr., N. & da Silva Mesquita, S., 2010. Simultaneous saccharification and fermentation process of different cellulosic substrates using a recombinant *Saccharomyces cerevisiae* harbouring the β -glucosidase gene. *Electronic Journal of Biotechnology*, 13(2).
- Galbe, M., Guido, Z., Jakobsson, E. L. & Linde, M., 2008. Steam pretreatment of dilute H₂SO₄ - impregnated wheat straw and SSF with low yeast and enzyme loadings for bioethanol production. *Biomass and Energy*, 32(4), pp. 326-332.
- Galbe, M., Wallberg, O. & Zacchi, G., 2011. Techno-economic aspects of ethanol production from lignocellulosic agricultural crops and residues. In: *Comprehensive Biotechnology*. Burlington: Academic Press, pp. 615-628.
- Galbe, M. & Zacchi, G., 2002. A review of the production of ethanol from softwood. *Applied Microbiology and Biotechnology*, 59(6), pp. 618-628.
- Galbe, M. & Zacchi, G., 2012. Pretreatment: The key to efficient utilization of lignocellulosic materias. *Biomass and Bioenergy*, Volume 46, pp. 70-78.
- Gírio, F. et al., 2010. Hemicelluloses for fuel ethanol: A review. *Bioresource Technology*, 101(13), pp. 4775-4800.
- Hendriks, A. & Zeeman, G., 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource Technology*, 100(1), pp. 10-18.
- Intergovernmental Panel on Climate Change, 2012. *Renewable Energy Sources and Climate Change Mitigation*, Cambridge, United Kingdom and New York, USA: Special Report of the Intergovernmental Panel on Climate Change.
- Joelsson, E., 2015. *Techno-economic evaluation of integrated lignocellulosic ethanol production*. Lund: Media Tryck, Lund Univeristy.
- Jonsson, L., Alriksson, B. & Nilvebrant, N.-O., 2013. Bioconversion of lignocellulose: inhibitors and detoxification. *Biotechnol. Biofuels*, Volume 6, p. 16.
- Jordbruksverket, 1992. *Lagring av stadsgödsel i stuka direkt på mark*, Jönköping: Jordbruksverket.
- Karlsson, S. & Salomon, E., 2002. *Djupströgödsel till vårsäd*, s.l.: JTI - Institutet för jordbruks- och miljöteknik.
- Lim, H. C. & Shin, H. S., 2013. Introduction to Fed-Batch Cultures. In: *Fed-Batch Cultures: Principles and Applications of Semi-batch Bioreactors*. New York, USA: Cambridge University Press, pp. 1-6.
- Makkee, M., Moulijn, J. A. & van Diepen, A., 2013. *Chemical process technology*. 2 ed. Chichester: John Wiley & Sons Inc.
- Mubeen, U. & Saleem Khan, T., 2012. Wheat Straw: A Pragmatic Overview. *Current Research Journal of Biological Sciences*, 4(6), pp. 673-675.
- National Renewable Energy Laboratory, 2008. *Biomass Compositional Analysis Laboratory Procedures*. [Online] Available at: <https://www.nrel.gov/bioenergy/biomass-compositional-analysis.html> [Accessed 28 February 2017].

Sanchis Sebastián, M., 2017. *An experimental study on producing ethanol from animal bedding*. Lund: Media-Tryck, Lund University.

Second Grade Biomass for Biofuels (2016) SEGRABIO.

Sims, R., Mabee, W., Saddler, J. & Taylor, M., 2010. An overview of second generation biofuel technologies. *Bioresource Technology*, Volume 101, pp. 1570-1580.

SOU, 2013. *Fossilfrihet på väg- Del 1 - Betänkande av Utredningen om fossilfri fordonstrafik*, Stockholm: 2013:84.

Statens Energimyndighet, 2015. *Energiläget 2015*, Bromma: Arkitektkopia.

8 Appendix

Appendix I – Metal Solution and Vitamin Solution

For preparation of 100 mL of trace element solution (Metal Solution), the components and amounts can be found in *table A*. Components required for the preparation of the vitamin solution can be seen in *table B*. Note that the vitamin solution was prepared prior to start of the experiments and that no description of the preparation for the solution is presented.

Table A. The dilution factor and amounts of each component needed for preparation of the metal solution.

Dilution factor	1000
Component	Amount needed (g)
EDTA(disodium)	1.5
ZnSO ₄ ·7H ₂ O	0.45
MnCl ₂ ·2H ₂ O	0.1
CoCl ₂ ·6H ₂ O	0.03
CuSO ₄ ·5H ₂ O	0.03
Na ₂ MoO ₄ ·H ₂ O	0.04
CaCl ₂ ·2H ₂ O	0.45
FeSO ₄ ·7H ₂ O	0.3
H ₃ BO ₃	0.1
KI	0.01

Firstly, EDTA is dissolved in 50 mL water. Following, each new component is added separately. For each new addition, the pH needs to be adjusted to 6.0. When the final component is added, the pH is adjusted to 4.0 and the volume is adjusted to 100 mL with distilled water. Afterwards, the solution is sterile filtered and stored in 50 mL tubes in the refrigerator.

Table B. The dilution factor and amounts of each component needed for preparation of the vitamin solution.

Dilution factor	1000
Component	Concentration (g/L)
Biotin	0.05
Ca-pantothenate	1
Nicotinic acid	1
Myo-inositol	25
Thiamin.HCl	1
Pyridoxine.HCl	1
P-aminobenzoic acid	0.2
Riboflavin	0.2
Folic acid	0.002