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Teknisk och ekonomisk utvärdering av en ny miljövänlig förbehandlingsmetod av biomassaavfall med utspädd salpetersyra

Technical and economical evaluation of a new environmentally friendly pre-treatment method of biomass waste by dilute nitric acid pulping

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Abstract

Bio-refining of renewable resources such as waste biomass into value added products has increased rapidly over the decades. The aim is to find new environmentally friendly yet economically feasible ways of replacing current utilization of non renewable resources. It can be expected that commercially viable next generation bio-ethanol will be produced from lignocellulosic feedstock in the near future.

This master thesis aims at providing a technical and economical evaluation of a recently patented pre-treatment method of biomass wastes which uses dilute nitric acid pulping. Evaluation of producing next generation ethanol from dilute nitric acid pre-treated cellulose rich softwood was performed and sulphite weak acid pre-treated hemi-cellulose rich hardwood Birch pulp was used as a comparison. Experiments were conducted on laboratory scale, using samples from two companies, referred to as Pure Lignin Environmental Technology Ltd (=PLET) (Canada) and SEKAB E-technology (Sweden). The strategy of PLET is currently to find a commercial platform to produce value added products from waste biomass generated by saw mills and pulping industries, while SEKAB E-Technology mainly works with Swedish softwood as a raw material.

The technical part of this Master thesis includes a series of fermentation trials using either SSF (=Simultaneous Saccharification and Fermentation) or SHF (=Separate Hydrolysis and Fermentation). The yields of the enzymatic hydrolysis and subsequent fermentations were estimated with HPLC measurements. The economical part of this master thesis includes the use of Business Model Canvas to define the basic topics and tasks that need to be addressed in the upstart phase of a small consulting bio-tech company.

Summary

Experimental data support the possibility to make lignocellulosic ethanol out of either softwood or hardwood. Ethanol yield from dry material obtained for hardwood birch slurry was 0,43 (g ethanol / g dry raw material). Ethanol yield from dry material obtained for softwood pine washed cellulose was 0,32 g (ethanol / g cellulose). Ethanol yield from dry material obtained for softwood pine unwashed cellulose in the three SHF was 0,48; 0,34 and 0,28 (g ethanol / g cellulose) respectively, while the yield in the two SSF was 0,37 and 0,38 (g ethanol / g cellulose).

The question is if technology provided by PLET can be applied on a commercial, industrial scale, hence the approach is to simulate an industrial full scale process as much as possible in a laboratory environment.

Bio-refining, is at present a rapidly expanding field and it is difficult to tell what will be the next commercially viable process. Therefore, information of what really is cutting edge in the field is essential. In this context, PLET stands out with an interesting new approach to pre-treat waste biomass into value added products, and has therefore received much attention in this master thesis.

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1. Background

This master thesis set out to look at a specific Canadian company in depth (Pure Lignin Environmental Technology Ltd), known as PLET henceforth. PLET has a new approach with a recently patented environmentally friendly technology, which could be extremely lucrative if applied in the right context.

It is desirable to look at the possibility of using the master thesis as a take-off platform to launch a consulting bio-tech company directly after completion. The idea is to accumulate enough knowledge in bio-refining, a relatively new and promising field of science. That is, to study how value added products could be produced from waste biomass.

In addition, economical aspects of such a technical application must be addressed at an early stage of development. This cross-linkage of consideration and understanding between different academic disciplines is necessary to apply as early as possible, in order to save both time and resources. This master thesis aims to consider both the technical issues as well as economical issues.

Last but not least, extensive networking was needed in order to make valuable connections through emails, phone calls, business meetings, conference visits, study visits, scholarship applications, as well as information gathering via consultation by professors. The above mentioned activities have received much attention throughout the project, in addition to experimentation in laboratory. Networking of this kind is absolutely necessary in order to establish a foundation for a future company. However it is difficult to display in an academic report of how much effort that really has been put into this, since it is hard to explain in scientific terminology.

The original plan was to investigate whether the technology of PLET could be used as a commercial platform in Sweden, with laboratory results backing-up a business argumentation. Promotion of the technology could be performed with a mobile factory housed on trucks for demonstration purposes. In this way, a possibility was envisioned to take care of excess saw dust and wood chips along with bark residues and even other products such as black liquor from smaller up to medium sized saw mills and paper pulp industries, by using a somewhat larger version of the already existing mobile demonstration plant of PLET.

2. Introduction

2.1 Master thesis focus

The topic on which this master thesis focuses is next generation lignocellulosic ethanol production with SSF (=Simultaneous Saccharification and Fermentation) and/or SHF (=Separate Hydrolysis and Fermentation), with new enzymes Cellic Ctec2 and modified yeast such as Ethanol Red and a pentose fermenting strain. Raw material used in the experiments was pre-treated biomass samples (pine and birch) supplied by PLET and SEKAB E-Technology, respectively.

2.2 Biomass characterization: Cellulose / Hemi-cellulose / Lignin

2.2.1 Categorization of biomass

Biomass can be divided into five basic categories including virgin wood (= softwood and hardwood); energy crops such as rape seed, agricultural residues such as wheat straw, industrial wastes, such as discarded packaging material, old construction timber and finally food waste such as orange peels [1].

Softwoods are for example gymnosperms like spruce and pine, whereas hardwoods are woody angiosperms such as oak and birch. In addition to hardwood and softwood there are annual plants, also known as herbaceous angiosperms. Fractions of lignin, hemi-cellulose and cellulose vary a lot between different plant species. The general structure of the ligno-cellulosic matrix in common wood is presented below [2].

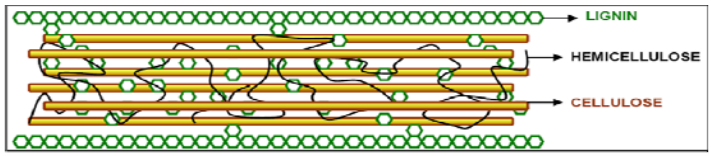

Figure 1. *The lignocellulosic structure in virgin wood displaying the fractions lignin, hemi-cellulose and cellulose.*

2.2.2 Description of cellulose

Cellulose is the main structural component in plants. It consists of unbranched chains of β-D-glucose units closely packed in parallel fiber structures. The CH_2OH- groups are alternating above and under the plane of elongation. Cellulose fibers are usually 2-20 nm in diameter and about 100-40000 nm long. Cellulose generally consists of 2000-14000 residues held flat by hydrogen bonds and is insoluble in water due to its network of hydrophobic ribbons that faces outwards. Structural unit of cellulose is called β-(1→4)-D-glucopyranose [3] [4].

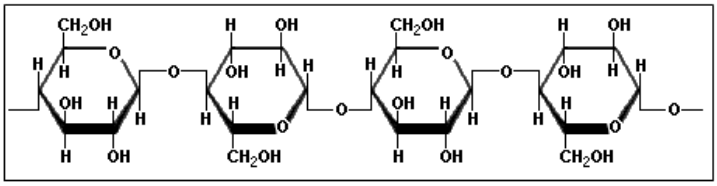

Figure 2. *Example of the chemical structure of cellulose β-(1→4)-D-glucopyranose.*

2.2.3 Description of hemi-cellulose

Hemi-cellulose is branched and built up mainly from D-xylose and other sugars. The content of hemi-cellulose is generally higher in hardwoods compared to softwoods, but it is in both cases a major structural component. Annual plants (=herbaceous angiosperms) have even greater percentage of hemi-cellulose embedded in its structure. One common component in hemi-cellulose is xylan, which contain multiple D-xylose units with β-(1→4)-linkages [4].

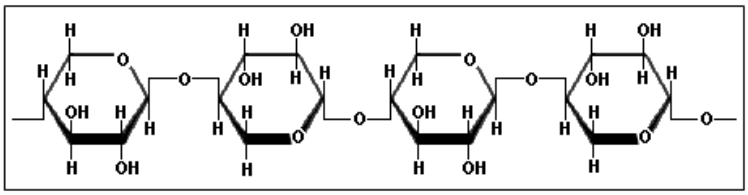

Figure 3. *Example of the chemical structure of hemi-cellulose (Xylan).*

2.2.4 Description of Lignin

Lignin is a main component in vascular plants, such as trees, bushes and grass. Lignin is one of the most abundant organic polymers on Earth, occupying ≈ 30 % of non-fossil organic carbon and constituting from a quarter to a third of the dry mass of wood. A fiber originates from the cambium as a living cell but soon loses its components. These fibers are then developed as a thickened secondary wall, which is made from cellulose, hemi-cellulose and lignin. Cellulose is strong in tension, while lignin is strong in compression. A figurative comparison could be made with reinforced concrete, where steel is the cellulose fibers and the concrete is lignin and hemi-cellulose [5].

The basic structure of lignin is built from three monolignol monomers. These are methoxylated to a varying degree. The three monomers are P-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, incorporated in lignin polymers in the form of phenylpropanoids, also known as guaiacyl (=G), syringyl (=S) and *P*-hydroxyphenyl (=H). All lignins contain small amounts of incomplete or modified monolignols, and other monomers are prominent in non-woody plants [6].

There are three types of plants which are rich in lignocellulosic material; softwood (=gymnosperms), hardwood (=woody angiosperms) and annual plants (herbaceous angiosperms). Gymnosperms have a lignin that consists almost entirely of (G) with small quantities of (H). That of dicotyledonous angiosperms is more often a mixture of (G) and (S), with very little (H). Monocotyledonous lignin is a mixture of all three phenylpropanoids. Many grasses have mostly (G), while some palms have mainly (S) [6].

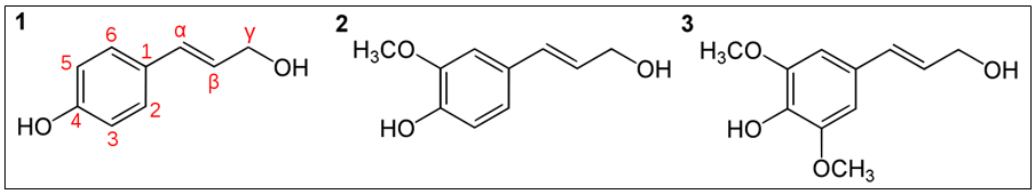

Figure 4. *Chemical structure of the three basic phenyl propanoid monomers which are the building blocks of lignin, also called monolignols.* ***1*** *= Coniferyl alcohol,* ***2*** *= Sinapyl alcohol and* ***3*** *= p-Coumaryl alcohol. Red numbers and greek letters in the figure define carbon positions.*

2.3 Bio-refinery emerging

Today there are great expectations for the benefits that can emerge from biotechnology. Especially, what could be done with the three major components cellulose, hemi-cellulose and lignin, found in abundance in lignocellulosic biomass? Expectations are particularly evident when looking at the world's stock market exchanges, where there are a lot of speculations of how fast future development will be. In some cases, these anticipations could be unrealistic, but are nevertheless inspiring, given the fact that mankind as a whole somehow has to solve the supply of energy in the future, mitigate the greenhouse effect and find a sustainable approach to co-exist with nature and at the same time maintain economic development.

Biotech solutions provide a platform to convert our society's use of non renewable resources, like the use of crude oil, to environmentally friendly utilization of renewable resources. In this context, conversion of waste biomass into cellulosic ethanol is an easily comprehensible and logical step. Currently, waste biomass is abundant and relatively cheap to purchase and has a huge potential as a raw material, suitable for processing into high grade commercial products, given that the constituents can be purified at a low cost and with minor environmental impact. Still there are many problems to be solved in bio-process design and optimization of bio-refining, since it is a relatively new and immature technology. This is, about to change.

2.3.1 Major pre-treatment options in Bio-refining and how they are funded

The general purpose of a pre-treatment of biomass is to expose the embedded cellulose and hemicelluloses from the lignin matrix, thus allowing enzymatic hydrolysis of sugars in the subsequent fermentation. There are several techniques in use to achieve this today [7]. The European Union has allocated resources to support research in strategically important areas. This is mainly done within the framework programs issued by the European commission. There are of course many other research projects within the EU, as well as there are independently funded projects with no governmental or political involvement.

A common approach is to use the SO_2-treatment or dilute acid treatment, which to a large extent hydrolyzes the hemicelluloses, while leaving a large part of the lignin still connected to the cellulose fibers. This technology is performed by SEKAB E-Technology among others. Another method is the steam explosion, which physically disrupts the biomass structure. Lignin remains attached to the fibers. Thirdly, there is the ammonia fiber explosion (AFEX), uses alkaline conditions to allow enzymes access to the cellulose and hemicelluloses. Fourthly, there is the expensive Organosolv approach, which hydrolyzes and removes the lignin and other components from the fibers.

Neither of these methods has yet paved the way for a commercial breakthrough. A method which is today practiced by SEKAB E-Technology and other companies is to pre-treat the cellulose material chemically in order to partially disrupt the structure and subsequently expose it to cellulase enzymes. The advantage with enzymes is that the conversion from cellulose to monomeric sugars is selective, which allows for higher yields. The disadvantage, again, is the cost and probably the enzymatic approach will require on-site production of cheap cellulase enzymes.

2.3.2 The sugar platform

The sugar platform could be defined as a generic value chain including the production of transport bio-fuels such as ethanol and butanol, plastics like PLA (=Poly Lactic Acid), food additives like amino acids and vitamins as well as medical applications like antibiotics and hormones. The refining within the sugar platform uses expertise in both chemistry and biotechnology.

In general terms the sugar platform stands for use of a renewable raw material usually processed from wastes from the paper and pulping industry, which is then refined to a product with higher value. One of the best and well known examples of this is the extraction of xylose from hexose rich pulp at sulphite pulp mills and its refining into xylitol and bio-ethanol.

Wood chips are preferably used as raw material for pulp production while saw dust is incinerated to make bioenergy for other processing or production of district heating. It is likely that the best sugar sources for bio-refining are found among the wet carbohydrate rich by-product waste streams in the conventional paper and pulping industry [8].

2.3.3 Projects in the past

NILE, New Improvements in Lignocellulosic Ethanol, stretched over a period of four years (2005-2009). NILE was supported by the European commission's 6th framework programme. Through this programme resources were allocated to support SEKAB E-technology's research. One of the many topics addressed, was the improvement of enzymatic hydrolysis, since this process step is estimated to contribute to about 30-50% of the cost of the ethanol production process when a lignocellulosic feedstock is used. Another topic addressed is the development of new types of yeast for fermentation of sugars into ethanol [9].

All lignocellulosic material must undergo pre-treatment, if the enzymes are ever going to reach the cellulose fibers and perform their task. The NILE project has addressed several approaches to find suitable pre-treatments. In general terms, a standard pre-treatment include conditioning (size reduction and impregnation) of the raw material followed by a thermo-chemical process to break up the lignocellulosic matrix.

Attention within the NILE project was focused on *Trichoderma reesei*, a fungi known for its cellulase production. *T. reesei* produces nine major enzymes which are utilized in enzymatic hydrolysis of lignocellulosic material. The enzymes *T. reesei* provide could be categorized in three distinct types of activity; two are cellobiohydrolases which liberates cellobiose, five are endoglucanases which attack the cellulose at random points and finally there are two β-glucosidases which splits cellobiose into glucose units.

If the enzymatic hydrolysis is successful, the result is a mixed sugar solution ready to be fermented by yeast into ethanol. Fermenting strains used must possess inherently good tolerance to both high levels of ethanol concentration as well as inhibitors. This has resulted in an improved xylose fermenting capacity as well as a reduced lag phase [9].

2.4 Enzymatic hydrolysis of lignocellulosic material

All enzymatic hydrolysis of lignocellulosic material requires pre-treatment. Enzymes are regarded as a good compliment to dilute acid hydrolysis. In bio-refining, concentrated acid hydrolysis should be contrasted with dilute acid hydrolysis. Dilute acid hydrolysis can be performed with or without enzymes. Without enzymes conditions must be harsh in order to get a good yield. Harsh conditions imply formation of inhibitors and additional losses of sugars due to formation of by-products. With enzymatic treatment the need for harsh pretreatment is reduced. In addition, enzymatic hydrolysis can be performed with lower energy consumption and much reduced environmental impact. Concentrated acid hydrolysis works well technically, but has problems with corrosion on equipment and expensive recycling of chemicals used in the process.

In enzymatic hydrolysis, the yield of pure glucose is high, as is the decreased formation of inhibitory by-products, which is favorable for a subsequent fermentation into ethanol. Cellulases in enzymatic hydrolysis of lignocellulosic material are aws follows; β-1-4-endoglucanases, β-1-4-exoglucanases or cellobiohydrolases and β-glucosidases. Enzymes such as β-1-4-endoglucanases attack regions of low crystallinity, thus creating free chain ends. Then there are β-1-4-exoglucanases or cellobiohydrolases, which removes cellobiose units from the free chain ends. Last but not least, there are the β-glucosidases, which hydrolyzes cellobiose into glucose units [2].

Cellulases (=enzymes which degrade cellulose), are needed to break down the cellulose fibers into fermentable sugars. This process is called enzymatic hydrolysis and the goal is to obtain as much D-glucose units possible from the pre-treated lignocellulosic material. One of the arguably most cost efficient enzymes available today is the Cellic Ctec 2, supplied by Novozymes. The current commercial variant, Cellic Ctec 2 has already reduced enzyme cost by 50%, to as low as 0,5 US dollar/gallon produced ethanol [10].

Active enzymes in Cellic Ctec 2 are based upon extractions of GH61-proteins and genes expressing them are inserted into the filamentous fungi *Trichoderma reesei*. These glycoside hydrolases (=GH) catalyze the hydrolysis of hemicelluloses and celluloses. The GH61-proteins lack to a large extent measureable hydrolytic activity by themselves, but in the presence of a divalent metal ion the protein loading is significantly reduced. The structure of one highly active GH61- protein has been solved and the results indicate that it is not a glycoside hydrolase. This is because it is devoid of conserved juxtaposed acidic side chains which would otherwise serve as the general proton donors and nucleophile/base in the hydrolytic reaction. Enhancement of cellulase activity by GH61 is not limited to just dilute acid pre-treated biomass but could also be used on steam-exploded biomass as well as on organosolv pulps. Surprisingly, the efficiency of the enzymatic activity does not work better on pure cellulosic substrates, rather is the opposite is favored. An unusual reaction mechanism for the hydrolytic catalysis cannot be ruled out [11].

2.5 Fermentation techniques

Fermentation techniques could be divided into SSF, SHF, SSCF and CBF. SSF stands for Simultaneous Saccharification and Fermentation, whereas SHF is an abbreviation for Separate Hydrolysis and Fermentation. Both techniques allow yeast to utilize hexose fermentation (6-carbon sugar) under anaerobic conditions to produce ethanol. Accumulated ethanol is distilled after completed fermentation. The conventional SHF could be described as follows; firstly there is the initial pretreatment in which the lignocellulosic matrix is broken down, and then follows the enzymatic hydrolysis, which depolymerizes cellulose into glucose, after that the slurry is filtered and the sugar-rich fraction is transferred to the fermentation, where it is fermented into ethanol, normally with *S. cerevisiae* as the fermenting organism. Finally, there is the distillation/dehydration step, in which ethanol is extracted [12].

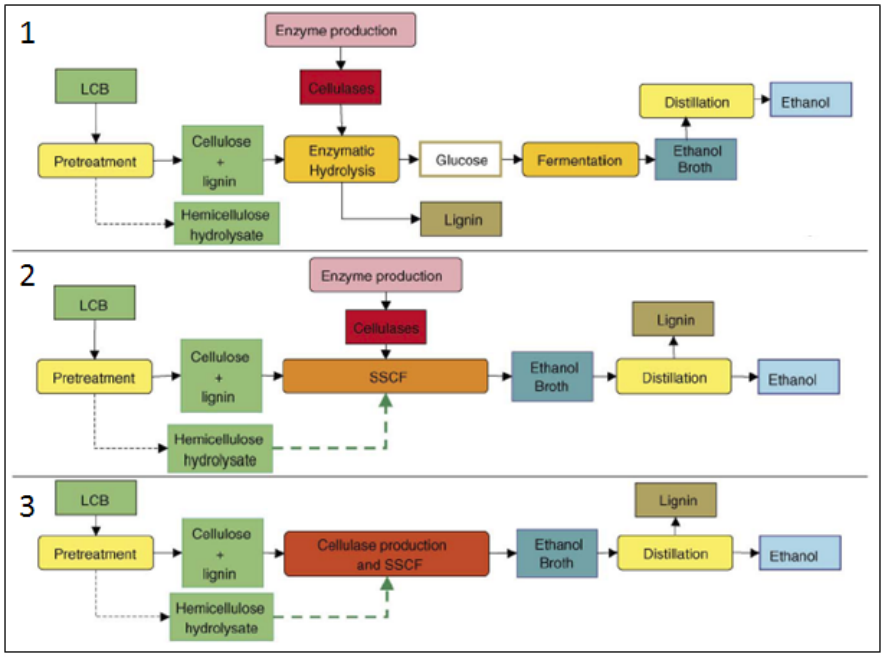

Figure 7. *Flow diagram 1 shows the conventional SHF process for producing ethanol from Lignocellulosic biomass (=LCB); flow diagram 2 shows the Simultaneous Saccharification and Co-Fermentation process (= SSCF) of hexoses and pentoses. And finally flow diagram 3 shows the Consolidated BioProcessing (=CBP), where the enzymes needed for hydrolysis are produced by the fermenting organism* [12].

2.5.1 Advantages/ disadvantages with SSF /SHF

The advantages of SSF compared to SHF are a simpler design, an extra process step is not needed, and there really is no product inhibition. One disadvantage with SSF is that nitrogen in the fermenting culture remains associated with the solid fraction and will thus be incinerated together with the solid residues, which causes formation of NO_x-gases. In SHF, the yeast can instead be re-circulated and the hydrolysis temperature is better optimized. There are also combinations of SSF and SHF available.

2.6 Metabolic pathways in yeast

Sugars such as glucose, fructose, mannose and other sugars are used by the fermenting organism as a carbon and energy source. Ethanol is produced under anaerobic conditions. A high yield of ethanol is the result of a successful fermentation. An important parameter is the amount of accumulated glycerol, which is either produced as the result of high osmotic stress or formed as a side-effect of biomass production.

In addition to the hexose related metabolism presented below in figure 8, fermenting organisms could also acquire pentose fermentation through genetic modification. In this context there are two main pathways to consider. The fungal type pathway uses two enzymes, *xylose reductase* and *xylitol dehydrogenase* with xylitol produced as an intermediate to produce D-xylulose. The inserted genes give the yeast the ability to overcome the problem of an unbalanced co-factor requirement. Both bacterial type pathway and fungal type pathway convert the pentose sugar D-xylose into D-xylulose.

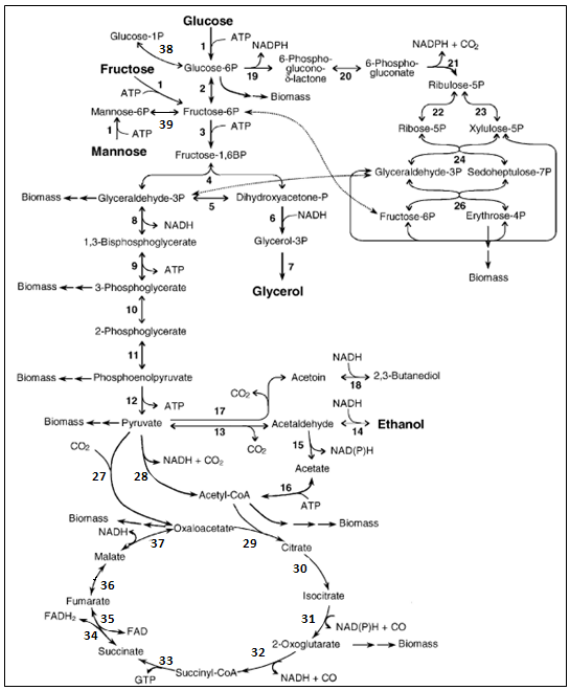

Figure 8. *Metabolic pathways in S. cerevisiae including the glycolysis sugar assimilation during anaerobic fermentation. The osmolyte glycerol is produced along with ethanol during fermentation* [13].

2.7 Optional fermenting organism

Traditional *Saccharomyces cerevisiae* (=Baker´s yeast) is a well characterized robust ethanol tolerant and ethanol producing organism. However, only hexoses are metabolized by the unmodified yeast strains [14].

Furthermore, *Zymomonas mobilis* has a potentially higher productivity of ethanol and lower biomass accumulation compared to *S.cerevisiae* but is more sensitive towards inhibitors. *Z. mobilis* is quite easy to modify genetically, but is currently only used as a hexose fermenting organism [15].

Moreover, there are bacteria such as *Escherichia coli*. *E. coli* is very well characterized indeed, but has in comparison to *S. cerevisiae* faster accumulation of biomass and relatively low ethanol production as well as a much higher sensitivity towards inhibitors [16].

There is also the *Mucor indicus*, which is a filamentous fungus with high tolerance towards ethanol and has a high yield of ethanol versus substrate. It can also metabolize five carbon sugars to some extent. The major drawback is that *M. indicus* is filamentous, which in turn leads to increased viscosity when the filaments are intertwined into a thick matrix [17].

In addition, there is also *Pichia stipidis*. *P. stipidis* is a hexose and pentose fermenting yeast but is bad for industrial purposes. Genes from *P. stipidis* for pentose fermentation has been transferred to Baker´s yeast, thus enabling a new robust strain competence of pentose fermentation [18].

2.8 The yeast strains Ethanol Red and a pentose fermenting strain

In this master thesis, two strains of *S. cerevisiae* were used during experimentation. Firstly, there is the Ethanol Red and secondly there is the pentose fermenting strain.

2.8.1 Ethanol Red

Ethanol Red is a developed strain of *S. cerevisiae* with excellent ethanol tolerance especially developed for the ethanol industry. The strain is however not competent to perform pentose fermentation. Characteristics displayed by the strain are higher cell viability during high gravity fermentation at elevated temperature (35°C), which results in lower cooling costs. Yields of 0,48 g/g ethanol and a final ethanol concentration of 18% v/v have been reported by the supplier Fermentis, a divison of S. I. Lesaffre [19].

2.8.2 The pentose fermenting strain

Recent research has resulted in a strain with genes capable of conversion of practically all hexoses as well as pentoses in biomass into ethanol. This is performed by insertion of bacterial genes for the enzyme *xylose isomerase* into yeast. This bacterial type pathway is then over expressed. The *xylose isomerase* is in addition assisted by another enzyme, *aldose 1-epimerase*, which increases the conversion rate between the xylose anomers β-D-Xylopyranose and α-D-Xylopyranose even further. Furthermore, the non-oxidative part of the pentose phosphate pathway is upregulated by over expressing several other enzymes such as; *xylulokinase*, *ribose-5-phosphate isomerase*, *transaldolase* and *transketolases*. All these enzymes ensure a fast metabolism of D-xylulose towards the glycolytic pathway via the intermediates Glyceraldehyde-3-phosphate and Fructose-6-phosphate [20].

2.9 Scandinavian approaches in Bio-refining

In Scandinavia there is a great demand for utilizing the vast resources of lignocellulosic readily abundant raw material via bio-refining. Currently, three different approaches in bio-refining are successfully emerging. The first is SEKAB E-technology, which is developing a technology that aspires to convert wood chips into ethanol as the main product. Secondly, there is Lignoboost, which tries to complement an existing conventional pulp mill, to extract the lignin from Black liquor. And thirdly, there is Borregaard Lignotech, which focuses on the Lignin-fraction to make various lignosulphonates, in addition to specialty cellulose products.

2.9.1 SEKAB E-technology, Örnsköldsvik, Sweden

At SEKAB E-Technology in Örnsköldsvik, Sweden, technology is developed to produce cellulosic ethanol from both hardwood and softwood. SEKAB´s research is based upon decades of various testing of process parameters, with Etanolpiloten at Örnsköldsvik as the current platform of development. Experience accumulated through Etanolpiloten encompass more than 29000 operative hours, since it was taken into operation in 2005. SEKAB E-Technology has received funding from both the Swedish Energy Agency and the European regional development fund [21].

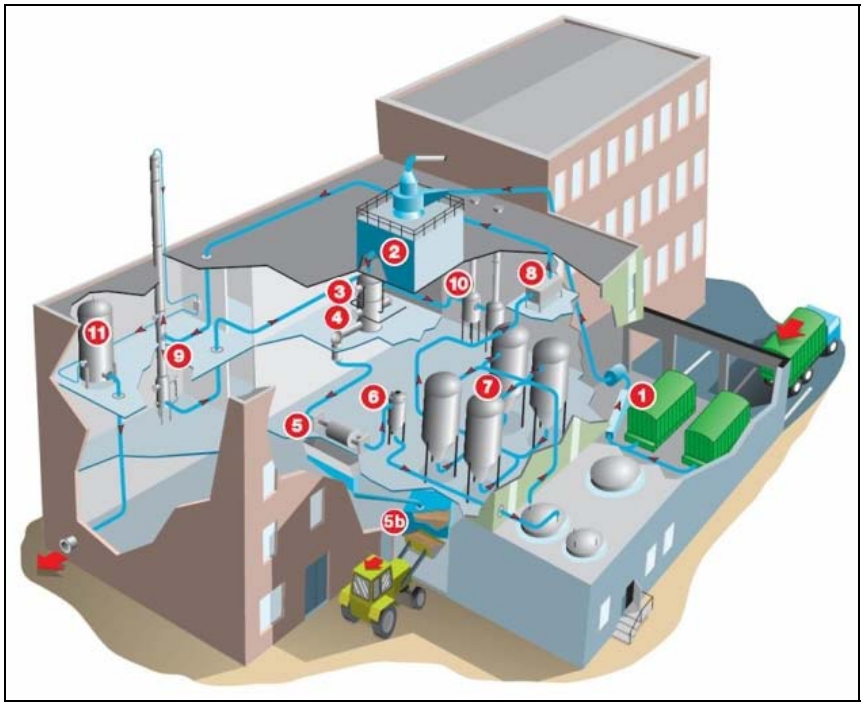

Figure 9. SEKAB´s Etanolpiloten. 1. *Intake, material is screened to remove large particles;* ***2.*** *Steaming, is used to preheat material and to remove air;* ***3.*** *Pre-saccharification, hemicellulose is leached out with acid at 170-200°C;* ***4.*** *The cellulose reactor, where cellulose is decomposed with acid at 200-300°C, followed by a cleavage process into soluble sugars;* ***5.*** *Membrane filter press, the lignin is filtered away. (If SSF is used, this stage could take place after stage 7);* ***5b.*** *The solid lignin, is removed;* ***6.*** *Detoxification, which removes inhibitory substances;* ***7.*** *Fermentation, sugar solution is fed to tanks kept at 35°C, enzymatic hydrolysis can also be carried out at this stage;* ***8.*** *Yeast separator, reuse of yeast from mash;* ***9.*** *Distillation, the distilled ethanol vapour is collected at the top of the distillation column, while stillage at the bottom of the column is discharged;* ***10.*** *Evaporation, after ethanol has been extracted, attention is given the stillage, which contain both liquid and solid fractions. These are concentrated through evaporation and then incinerated to produce heat. Process water streams undergo biological wastewater treatment before discharged;* ***11.*** *Product tank, ethanol is kept in large tanks before transport* [22].

2.9.2 LignoBoost at Bäckhammars Bruk, Sweden

At Bäckhammars Bruk, Sweden, currently operated by Innventia, the Lignoboost demo plant demonstrates a value added product biorefinery of black liquor into high purity lignin to be used as fuel additive in a lime kiln or to be sold for other applications. Since most pulp mills in Sweden are fairly similar to the facility in Bäckhammar it is appropriate to see how a bio-refining approach can complement an already established industry [23].

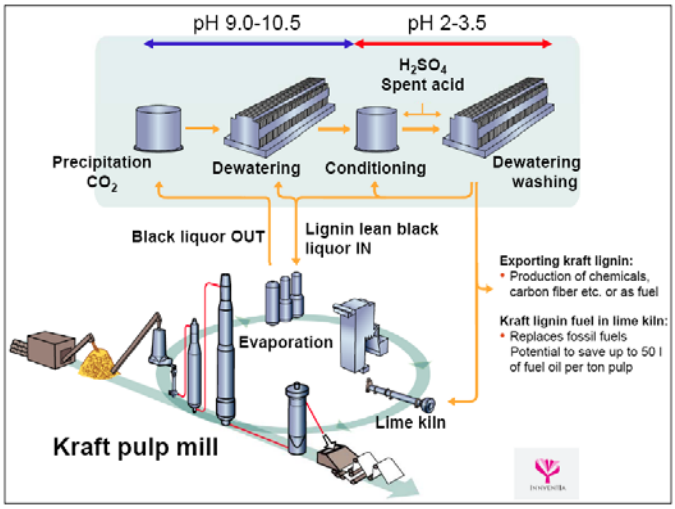

Figure 10. *LignoBoost process added to a conventional Kraft Pulp Mill at Bäckhammars Bruk, Sweden* [24].

2.9.3 Borregaard Lignotech at Sarpsborg, Norway

At Borregaard Lignotech, Norway, a new pilot plant is planned to demonstrate the potential of the newly patented BALI-process. The BALI-process is a sulphite based pre-treatment. Borregaard Industries claims to be a leading supplier of speciality cellulose as well as the global leader in high performance lignin derived chemicals. Further, Borregaard is the only producer of the flavour sweetener vanillin ($C_8H_8O_3$) from lignocellulosic raw material. Lignocellulosic bio-ethanol has been produced by Borregaard since 1938, and current production is approximately 20000 m^3 annually. Borregaard LignoTech lists the most popular industrial applications and functional uses for their lignosulphonate as follows; Binding Agent, Emulsion Stabilizers, Dispersing Agent, Extrusion Aids, Dust Suppressant, Retarders, Crystal Growth Modifier and Rheology Control [25].

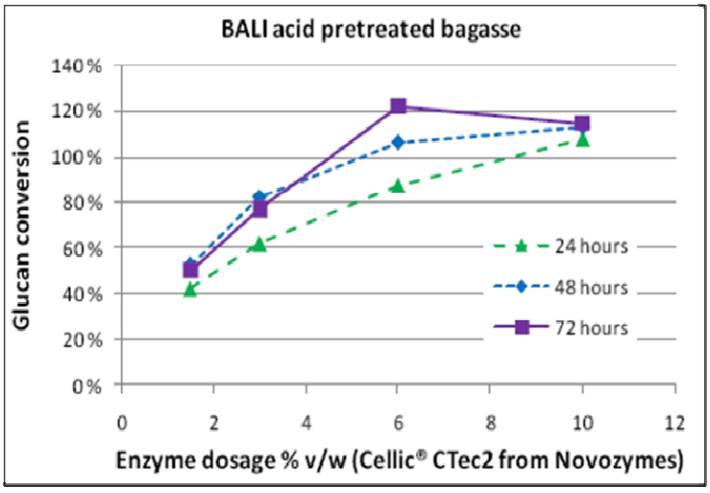

Figure 11. *The effectiveness of the enzyme Cellic Ctec2 in the BALI-process. Operation parameters were 6,3 % glucan loading, dry solids were 9,2%, Temperature was 50°C and the flasks was kept at 200 rpm in a shaking incubator. 50 mM Sodium citrate buffered around pH 5, Total reaction mass was 50 g in 100 mL flasks, and 0,01 % NaN_3 was added for microbial control. Enzyme producer Novozymes comment the results above as follows; "This is probably among the best 10-15 % of all results we have seen"* [25].

3. The Concept

The concept in this master thesis is to combine the cheap environmentally friendly pre-treatment of biomass performed by PLET, with enzymatic treatment and fermenting organisms in SSF or SHF on the cellulose and sweet liquor fraction, in order to produce a competitive next generation lignocellulosic ethanol.

3.1.1 A description of PLET and the patented process.

At present, PLET is an independent Canadian "Family and Friends"-sized business, and is thus considered as small company. The organization consists of the owners, consulting representatives and business relations managers. Current operations are carried out at a small demonstration plant in Canada. The company has been registered for about 6-7 years.

PLET is currently in the difficult process of leaping from lab and demo scale into full industrial scale. PLET is by far not unique; there are thousands of other recently started companies all over the world facing the very same challenges. What really catches attention is the way, in which PLET approaches one of the most difficult tasks in bio-refining. PLET provides a novel and innovative way of solving one of the toughest problems in bio-refinery. That is, the separation, fragmentation and purification of the complex biomass, into three high grade commercial products, with less generation of unwanted by-products, while also keeping the costs at a minimum [26].

Seen in figure 12, is a PFD (=Process flow diagram) of the patented technology from PLET. In brief words the process is described as follows. First the biomass is soaked in dilute nitric acid and then churned through an auger mechanism into a bioreactor where the nitric acid is evaporated and re-circulated. The cooked mesh is then transferred to a digester where the cellulose pulp is separated with alkaline treatment whereas the black liquor undergoes precipitation to separate the lignin as a dry product. The remaining sweet liquor could then undergo fermentation by Torula yeast (=*Candida utilis*) into unicellular protein [27].

The catalytic reactor process (=CRP), is performed by PLET in a continuous and batch system, in which lignocellulosic material (=wood chips, saw dust or other waste biomass) undergo acid catalyzed hydrolysis by dilute nitric acid. The impregnated wood chips are partially depolymerised from the lignin matrix when heated in the low pressure catalytic reactor. After that, follows distillation, condensation and almost complete recovery of dilute nitric acid. After the CRP, cellulose pulp is separated from the black liquor by an alkaline solution (=NaOH). The obtained black liquor is pumped to a separation tank to precipitate lignin and sweet liquor through filtration. The final step is to dry the lignin, while the sweet liquor is fermented by Torula yeast (=*Candida utilis*) into unicellular protein [26]. It is interesting to see if the process Company X provides, also could offer the solution to make next generation of cellulosic ethanol from waste biomass.

The lignin, extracted from the process is given extra attention according to PLET, since it has such high purity. Thus, it would be a potential candidate for a lot of new and interesting applications. PLET´s lignin is stated to have unique properties, which include high molecular weight and water solubility, to mention but a few. This extraction is possible thanks to the mild CRP, which uses low pressure, low temperature and not so harsh chemicals. While the lignin must be regarded as the main product, the cellulose fraction extracted and separated through the process is considered to be of standard commercial grade cellulose pulp.

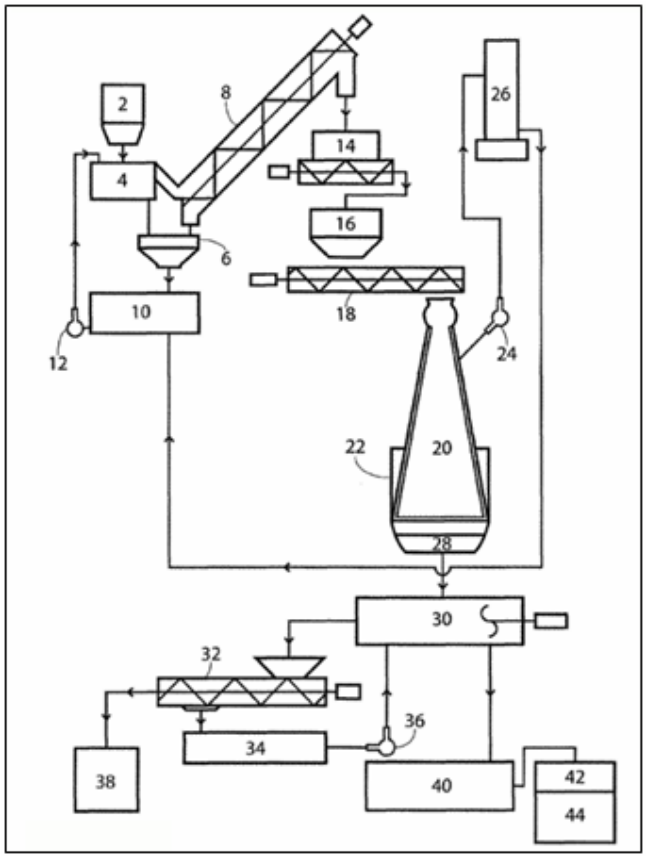

Figure 12. *A PFD (=Process flow diagram) of PLET patented technology, which incorporates a continuous and batch system to treat biomass in a closed loop. Raw material (=wood chips) are feed from a storage [2] over to impregnation chambers [4] along with dilute nitric acid from a solution tank [10]. After a soaking time the contents are transferred to a heated, low pressure reactor [20] by auger mechanisms [8]. After this the mesh [28] is treated with alkaline solution in a digester [30]. Evaporated impregnate and nitric acid and/or ammonium hydroxide is recovered via an absorption tower [26] and recycled back to the solution. The mesh is first heated then cooled in the alkaline treatment to separate the cellulose pulp [38] from black liquor. The black liquor is transferred to a lignin tank [40] in which the lignin is cooled and precipitated through filtration [42]. The lignin is dried and the remaining sweet liquor is ready for fermentation [44] into unicellular protein by Torula yeast. The unicellular protein could then be marketed as nutritious yeast. Depending of the intended results, the extracted fractions of cellulose, lignin and sweet liquor require extensive washing* [27].

According to statements given at PLET´s homepage; "For every 2,2 tons input of wood chips, the process produces 1,00 ton of cellulose, 0,42 tons of lignin and 0,78 tons of sweet liquor. After fermentation the sweet liquor is converted to 0,25 tons of protein." The prospect is to see if the cellulose and sweet liquor fractions could be used to cellulosic ethanol [26].

3.1.2 Sweet liquor, the by-product of dilute nitric acid pulping

The sweet liquor extracted from dilute nitric acid pulping contain more or less all the other chemical compounds not separated as pure cellulose and pure lignin from the original raw material. One way of making something valuable out of this diversified and complex mixture is to let Torula yeast (*Candida utilis*) grow on it in order to produce unicellular protein for animal food supplements. This sturdy robust yeast metabolizes almost everything of the otherwise toxic and inhibitory chemical compounds found in the extracted brown coloured sweet liquor.

Torula yeast has excellent nutritional properties, since it has such high nucleotides content, in particular RNA (ribonucleic acid). This makes Torula a good source for pet foods, especially for cats, but it is also frequently used as feed supplement to livestock in general. Furthermore, the Torula has a clean flavor profile, compared to the sometimes bitter taste of ordinary Yeast extracts from Baker´s yeast (=*Saccharomyces cerevisiae*), which makes torula useful as a flavor enhancer in animal food [28].

3.1.3 Advantages of the PLET concept

There are a number of arguments for choosing the PLET approach to process waste biomass. First of all, the starting raw material could be wet, since water takes part in the soaking pre-treatment with dilute nitric acid in the acid catalysed hydrolysis. It is generally regarded as bad water economy to dilute the raw material in conventional pre-treatment, but a wet starting material is actually preferable in the CRP-process. It is basically a fairly simple hydrolysis technology, which is performed at a low temperature and pressure along with a low input of energy to run the process. Only dilute acids and bases are used, thereby reducing raw material costs and unnecessary degradation of final products. In addition, usage of dilute acids and bases reduces the wear and tear on equipment used. The dilute nitric acid catalyst is recovered in a closed loop, which makes the processing almost pollution free.

Furthermore, the CRP generates commercial grade products such as high yield of α-Cellulose, native unique Klason lignin along with sweet liquor appropriate for a unicellular protein via fermentation of Torula yeast (=*Candida utilis*). The international EPA (EPA=Environmental Protection Agency) carbon dioxide pollution credit system ensures extra revenues, for implementation of a factory using CRP. Last but not least, CRP is flexible, since it could use any vegetation and/or waste biomass [27].

3.1.4 Disadvantages of the PLET concept

However, there are also some disadvantages that need to be addressed regarding the CRP. This is especially relevant when dealing with a new technology, which has not been put into full scale production yet. It is hard to compare the actual advantages of dilute nitric acid pulping to conventional pulping, since it has not been done before, at least not in the way PLET does it. Today there is no data apart from PLET´s own research supporting the approach to use nitric acid in pulping instead of sulphuric acid or other methods to extract high purity lignin and celluloses.

CRP offers another way of processing a lot of different raw materials, leading to three main products, each with their own specific value, all depending on oil price and commercial availability on a global market. So, the size and design of a future full scale processing unit must be made both according to customer specifications, and expected demands of the global market [27].

4. Technical and economical aspects of lignocellulosic ethanol

The technical and economical aspects of lignocellulosic ethanol could be described with the commercial flowsheeting programme Aspen Plus. The cost of major process equipment could be estimated by Icarus Process Evaluator (=IPE) from Aspen technology. Another alternative is to use computer software like Superpro for the technical layout of a process design and use Capcost to estimate the investments necessary if designing a large scale process plant. In this way it is possible to estimate the flow rate economy, its composition and energy flows for all processing streams.

A satisfactory concentration of fermentable sugars would be around 80 g/L or higher to support an industrial implementation, in addition the biomass yield during cultivation should be 0,5 g/g fermentable sugars. Ethanol must be concentrated in a distillation step consisting of stripper columns and a rectification column. The degree of distillation depends on how much ethanol could be concentrated during fermentation. It is generally regarded as "bad water economy" to add water during the processing since it has to be removed in the distillation step. In general, SSF:s has a better "water economy" than SHF:s [29]. It is however impossible to get accurate figures of what everything would actually cost, since prices of both equipment, raw materials, chemicals, utilities and other cost varies a lot depending on what amounts of quantities that are processed and current world market prices on bulk volumes [29].

Raw material		
Salix	555	SEK/dry metric ton
Corn stover	497	SEK/dry metric ton
Spruce	528	SEK/dry metric ton
Co-product income		
Solid fuel	185	SEK/MWh
CO_2	0.03	SEK/kg
Chemicals		
SO_2	1.5	SEK/kg
NaOH (50%)	1.5	SEK/kg
NH_3 (25%)[a]	2.0	SEK/kg
H_3PO_4 (50%)[a]	5.0	SEK/kg
Defoamer	20.0	SEK/kg
$(NH4)_2PO_4$[b]	1.5	SEK/kg
$MgSO_4 \cdot 7H_2O$[b]	4.4	SEK/kg
Molasses	1.0	SEK/kg
Enzymes	19.0	SEK/10^6 FPU
Utilities		
Electricity	300	SEK/MWh
Cooling water	0.14	SEK/m^3
Process water	1.4	SEK/m^3
Other costs		
Labour	600 000	SEK/employee/year
Insurance	1	% of fixed capital per year
Maintenance	2	% of fixed capital per year

[a] Chemical used in wastewater treatment.
[b] Chemical used as nutrient in SSF.

Figure 13. *Economic evaluation of three different feedstocks of lignocellulosic material, (Salix = Hardwood, Corn stover = biomass waste (herbaceous angiosperms) and Spruce = Softwood) as well as chemicals chemicals and other requirements needed, done by Department of Chemical engineering at Lund University. SEK is the currency in Sweden (1 SEK ≈ 0,09 Euro). Calculated example is taken from a potential not yet constructed processing plant of 200000 tonnes dry weight annually. The raw material is steam pretreated with SO_2. Note the high enzyme cost in the calculation, which is substantially reduced today [29].*

The process economy benefits from larger scale, but it is not certain that it is economically feasible. Although abundant, biomass requires storage when transported; in addition it is not a homogenous material, which makes it harder to accumulate sufficient amounts of raw material to the production plant. Raw material cost contributes most to the overall production cost, while the ethanol yield is the most important parameter for reducing the cost of ethanol production [29].

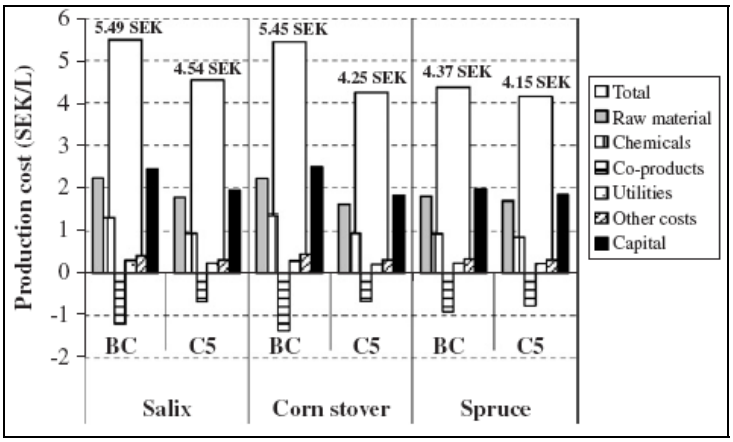

Figure 14. *Production cost calculation for the three cases (Salix = Hardwood, Corn Stover = biomass waste (herbaceous angiosperms) and Spruce = Softwood) done by Department of Chemical engineering at Lund University. All data refers to the same calculated example given in figure 17. BC refers to the base case while C5 refers to the pentose fermenting cases [29].*

The commercial viability of next generation lignocellulosic ethanol depends on; cost, availability and quality of the feedstock, pre-treatment technical process parameters and enzyme loading [30]. Finally, the product revenue could vary a lot due to world market demand. Still, the main indicator of commercial price of any product is the peak oil index.

In a study from 2003, the commercial prospects of lignocellulosic ethanol were evaluated by a comparison of the two major process configurations, SSF and SHF. The study was based upon softwood spruce. Ethanol production costs for the SSF and SHF were 4.81 SEK/L and 5.32 SEK/L or 0.57 USD/L and 0.63 USD/L, respectively. (1 USD = 8.5 SEK, in this study). SSF has lower production cost since the ethanol yield is higher than in SHF, the major drawback with SSF is the recirculation of yeast. The SSF could be improved by higher substrate loading and recirculation of process streams. If these arrangements were to be implemented, then their cumulative effect would result in a production cost of 3.58 SEK/L (0.42 USD/L) Seen in retrospect the decrease in production cost of lignocellulosic ethanol has been substantial over the last few years [31].

This study on the technical and economical aspects on lignocellulosic bio-refining into ethanol was however written before the pilot plant was inaugurated and the promised performance eventually turned out to be exaggerated. Despite the meager outcome of the pilot plant it is still interesting to analyze the approach. There are not that many studies that discuss both the technical and economical aspects at such early stage.

4.1 Business model canvas and the three phases envisioned

There are several ways available for formulating a business plan for a future company. This master thesis will use the simple business model canvas together with a timeline defined in phases, the most crucial topics and tasks that need to be addressed in the process of making a successful introduction of a small consulting bio-tech company. Some of the topics addressed in the business model canvas are obvious, but must nevertheless be clearly defined [32].

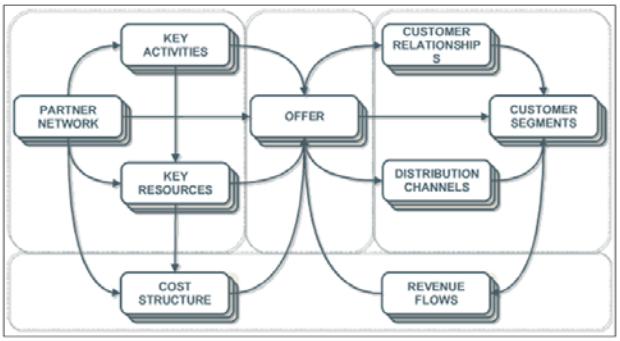

Figure 15. *Layout of the business model canvas, in which topic and tasks are defined and addressed with post-it tags on a large paper during a brainstorming session [32].*

4.1.1 Partner network, key activities and key resources

Partner Network, consists of PLET, SEKAB E-Technology, Högskolan i Borås, ESPIRA Inkubator, ALMI, Drivhuset, Innovationsbron, Innventia, Kommerskollegium, Svebio, Vinnova, Skogssällskapet Förvaltning AB. Affärsänglar (=Venture capitalists), Crowd funding [33] and/or Equity Crowd funding. And there are also the suppliers of enzymes (Novozymes) and yeast strains. *Key activities* would be marketing research of PLET products as well as displaying PLET technology at fairs, conferences and business meetings. Offer technical support for buyers and design applications of PLET process. Bio-tech research performed at Högskolan i Borås, thereby optimizing process parameters. *Key Resources* could be process data, support, licenses supplied by PLET, Högskolan i Borås. As well as experiments performed during master thesis and knowledge of how to handle equipment. A business network needs to be established and raw materials need to be assured. Other resources could be waste biomass produced at saw mills and paper pulping industries. Also there is a need to prepare proper conference material to support a business argumentation and put the technology in a current context as a cutting edge bio-refining alternative. Besides this, there may be necessary to specifically design computer software applications. Finally, there is also a possibility to utilize private capital and/or scholarship funding.

Customer relationships is to actively consult saw mills and paper pulp industries so that biomass waste could be turned into ”Value added products” on site, or collecting waste and take it to a future facility for processing. To consult industries involved in agricultural wastes thereby taking care of wastes like corn stovers, sugar cane bagasse, wheat straw et c, and textile lump of various origin (=cellulose rich substrates). Also, to consult and supply the chemical industry with high grade products from a demonstration facility for further synthesis. *Distribution channels* must transport raw material and processed products to and from facilities. This could be organized with trucks, trains or ships. Information, marketing and monetary transactions would be facilitated via internet. *Customer segments* include potential customers which would be saw mills and pulping industries.

The offer is to provide a non specific high grade separation of waste biomass into cellulose, lignin and sweet liquor, through an environmentally friendly pre-treatment step with dilute nitric acid pulping followed by enzymatic treatment and conversion into ”Value added products”. The commercial products are cellulose, lignin and sweet liquor or hemi-cellulose.

Revenue flows should come from consulting fees during the design/construction phase. Distribution of licenses; sold to partners using equipment designed by PLET. Revenues could also come from support agreements and equipment operations on site. Of course the major part of revenues would come in to the company when products such as specialty cellulose, ethanol, pure lignin, and unicellular protein for animal food are sold. *The cost structure* is important to consider. First of all a pilot plant must be purchased for demonstration purposes. License agreements must be made with PLET and an enzyme supplier. Other costs would encompass promotion, advertisements, business meetings, conference visits etc. The construction costs of a pilot plant and production plant are large and must be calculated with an estimated payback time. Equipment maintenance and estimated depreciation must be assessed. Administration, training of workers, salaries and social fees, taxes, permits etc. Design of future automation of processes at full scale production plant via PLC (=Programming Logical Control). Operation costs; transportation of raw material. Energy needed to run the processes. Chemicals; HNO_3, H_2O, NH_4OH, H_2SO_4, and HCl. Biological laboratory needed to cultivate large volumes of both enzymes and fermenting organisms like yeasts under licenses.

4.1.2 Economics of a future production plant

A production plant designed for processing 56 tons of wood chips per day of wood chips would generate annual revenues of ≈ 7500000 US dollars. The annual profit would be ≈ 2500000 US dollars, and capital cost (equipments) ≈ 5000000 US dollars, while the payback time on investment would be 2-3 years. The products are estimated to have the following values; 25 tons/day of pure Cellulose with a market value of 400-500 US dollars/ton, 10 tons/day of pure Lignin with a market value of 1000-1200 US dollars/ton and finally 21 tons/day of sweet liquor with a market value of 50-100 US dollars/ton [21].

4.1.3 Envisioned phases

Phase 1. First a master thesis of 30 ECTS must be written, in which samples from PLET pilot plant is analyzed in laboratory at Högskolan I Borås. Meanwhile a market analysis is performed, involving visits to conferences and business meetings. The prospect must be discussed with agencies and authorities and experienced people, as well as future investors such as Nyföretagarcentrum Sjuhärad, Banks etc. Time envisioned is six months. **Phase 2.** Involve the formation of a share holding company including study visits to PLET. There would be a need to purchase or lend/lease of 1st pilot plant. Personnel need to be hired to do tasks. A future factory would be designed in computer software such as Superpro or ASPEN Plus along with an economical calculation of estimated costs and incomes using CAPCOST. Marketing could be done on exhibitions, fairs and other events. Time estimated for these tasks are one year. **Phase 3.** The actual construction and supervision of a factory for production of lignocellulosic products must be done. The factory envisioned would employ about 20 persons. Supply lines need to be administrated, as well as even larger full scale production plants must be designed. A network needs to be established. And production needs to be optimized in order to reduce costs. If these tasks are successfully executed the company would expand to other sites in Scandinavia. Expected time for this would be at least ten years.

4.2 Technical and Customer oriented parameters to consider

There are many ways to estimate what is important to consider before launching a project in bio-refining. Below are two examples given of how these parameters/factors could be addressed and formulated as questions, which a future entrepreneur in bio-refining must be prepared to answer satisfactory. The technical and customer oriented parameters are to be considered. In addition, these parameters must be ranked and put into an international context.

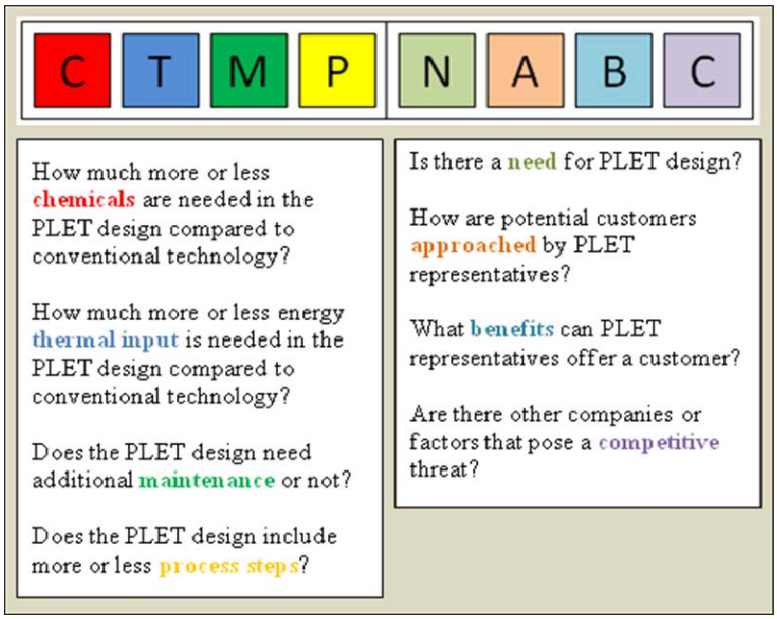

Figure 16. *Technical parameters are C = Chemical, T = Thermal, M = Maintenance and P = Process. Customer oriented parameters are N = Need, A = Approach, B = Benefit, C = Competition* [34].

Parameters to consider could be ranked after importance according to Lena Dahlman at Svebio.se. Starting with; economic framework, feedstock availability, financial markets and investment capital from banks, authorities and legislation, energy markets, process energy requirements, market saturation level, political stability and public acceptance [35].

5. Methods and materials

This section includes a description of the raw material, equipment used for experiments, as well as a more detailed description of the six conducted experiments.

5.1 Raw material

In experiment 1, dilute SO_2- pretreated birch slurry from SEKAB E-Technology was used. Samples from SEKAB E-Technology serves in this master thesis as a comparison since the dilute SO_2- pretreated birch slurry process is well documented and relatively close to commercial application. The approach during this project is to determine whether the presumably simpler process suggested by PLET is preferable compared to SEKAB E-Technology´s processing of biomass, due to lower cost and potentially environmental impact.

In experiments 2 to 6, samples from the pilot plant of PLET were used. Dilute nitric acid pretreated samples supplied by PLET were taken from different stages of the whole process. Within the scope of this project the washed cellulose fraction (WC), the unwashed cellulose fraction (UWC) and the sweet liquor (SW) were used for analysis and trials, while only minor attention was given the main product pure lignin. According to PLET, the lignin produced already is a commercial grade product. Hence focus was concentrated on determining if pretreatment suggested by PLET is sufficient to produce the next generation of lignocellulosic ethanol from softwood.

5.2 Equipment used during experiments

5.2.1 Biostat B-Plus fermentor

A Biostat B-Plus fermentor was used for SSF and SHF experiments. In addition, experiments performed in the Biostat B-plus fermentor was complemented with shake flask fermentations.

Parameters applied in experiments with Biostat B-Plus were as follows. The *pH*, was kept within an interval of 5,0 to 5,5 by addition of 1 M sulfuric acid (H_2SO_4) and/or 1 M sodium hydroxide (NaOH). The *temperature* was set to 50 °C to increase enzymatic activity in SHF, while lowered to 35 °C, during the subsequent fermentation. In SSF experiments, the temperature was set to 35 °C throughout the whole process. A *stirring speed* of 1200-1500 rpm was initially used in order to maintain satisfactory mixing (rpm = rounds per minute). However, the stirring speed was lowered to 400-500 rpm as soon as viscosity decreased sufficiently during the enzymatic hydrolysis. In shake flask experiments the stirring speed was set to 120 rpm.

Figure 17. *The Fermentor Biostat B-Plus, linked to a computer controlled interface for automatic monitoring and adjustment of stirring rate, temperature, pH and O_2-supply* [36].

5.2.2 HPLC-analysis

HPLC, high performance liquid chromatography, was used to determine concentrations of sugars and accumulated ethanol in collected samples. Computer software supporting HPLC analysis was EmpowerPro version 6.2. Two columns were used in experiments; the H-column (Aminex® HPX-87 H, 300 mm*7,8 mm) and the P-column (Aminex® HPX-87 P, 300 mm*7,8 mm) supplied from BioRad [37]. For the P-column samples, removal of sulphate was required before run in the HPLC. This was done by addition of $Ba(NO_3)_2$ to each sample, enabling easy separation of insoluble $BaSO_{4(s)}$.

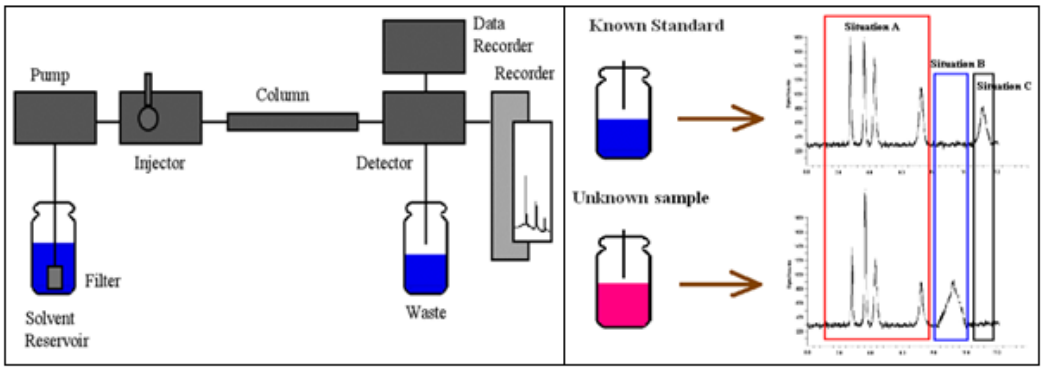

Figure 18. *Simplified structure of the HLPC equipment and how HPLC chromatograms could be interpreted. HPLC compares the retention time for samples and standards. Corresponding peaks are found (situation A), which means that there are the equivalent chemicals in both standard and sample. Concentration of chemicals in the sample equals to the concentration of chemicals in Standard *(peak area in sample/ peak area in standard). In situation B, there is a peak in the sample, but no peak at the same fraction in standard, which means that the sample contains a certain chemical not present in standard. In situation C, there is a peak in the standard, but no peak at the same fraction in sample, which means that the standard contains a certain chemical not present in the sample* [38].

5.2.3 Dry weight measurements

Dry weight measurements were determined by sampling of 5 ml hydrolysis or fermentation broth. Samples were transferred to glass tubes and centrifuged at 5000 rpm for 5 minutes and then washed with deionized water and dried in an oven overnight at 105 °C. The weight was measured and compared to the weight of the empty (dry) glass tube [39].

5.2.4 Enzyme kit

Glucose enzymatic kit uses the measurable UV-absorbance of NADH at 340 nm as a measurement of glucose content in a solution. The enzyme hexokinase converts glucose into Glucose-6-Phosphate assisted by ATP. Then the enzyme G6PDH catalyzes the reaction of Glucose-6-Phosphate and NAD^+ into 6-Phosphogluconate and the optically active NADH. The NADH contributes to an increase of absorbance measured by spectrophotometer at wavelength 340 nm, which on a molar basis is equivalent to the initially present glucose molecule [40]. Cell density was in some applications estimated with a spectrophotometer by measuring the optical density (OD) at 610 nm. Absorbance is compared to a standard from dry weight measurements and then recalculated to concentration in g/L [41].

5.3 Summary of conducted experiments

In Table 1, there is a brief description of the six different experiments performed. In the first five experiments, extracted samples were collected for HPLC analysis. These extracted samples were complemented with corresponding dry weight measurements in order to determine ethanol yield of processed raw material. Experiment 6 was a simple lignin dry weight / polycondensation estimation which purpose only was to analyze general characteristics.

Table 1. *Showing the different experiments performed in laboratory.*

	Raw material	***Procedure***	***Enzymatic treatment***	***Fermenting organism***
Experiment 1	*Birch slurry, (SEKAB E-technology)*	*SSF*	*Cellic Ctec2*	*Pentose fermenting strain*
Experiment 2	*Washed cellulose (WC), (PLET)*	*SHF*	*Cellic Ctec2*	*Ethanol Red*
Experiment 3	*Unwashed cellulose (UWC), (PLET)*	*SSF/SHF*	*Cellic Ctec2*	*Ethanol Red*
Experiment 4	*Sweet liquor (SL), (PLET)*	*SSF*	*Cellic Ctec2*	*Pentose fermenting strain*
Experiment 5	*Sweet liquor (SL), (PLET)*	*SSF*	*Cellic Ctec2*	*Pentose fermenting strain*
Experiment 6	*Pure lignin, (PLET)*	*Dry weight, Polycondensation*	-	-

A general outline of the mass flow modes in SHF and SSF are difficult to explain unless a graphical presentation is used, which preferably can be seen in figure 19 and 20.

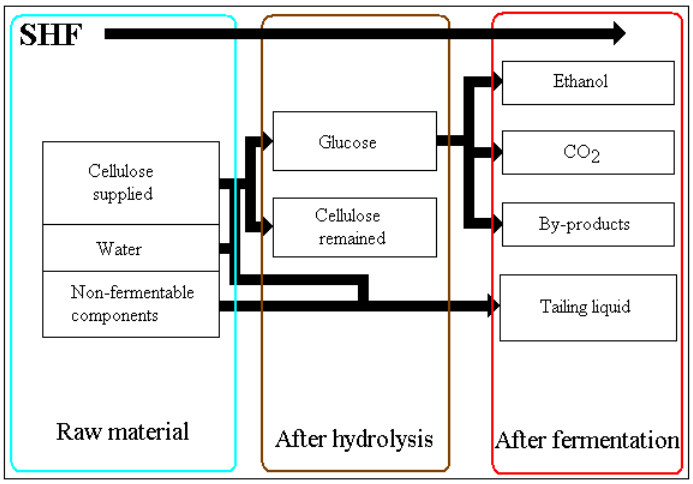

Figure 19. *SHF Mass flow mode*

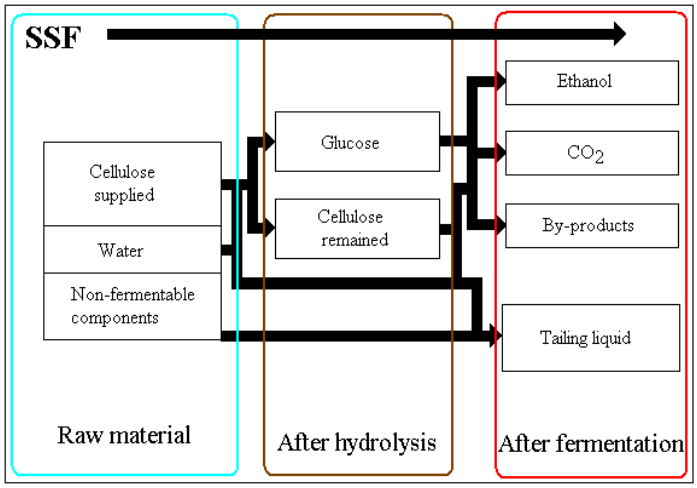

Figure 20. *SSF Mass flow mode*

5.4 Experiment 1

Firstly, 2000 mL glucose-xylose containing liquid medium (100 g glucose, 100 g xylose, 5,0 g peptone, 5,0 g yeast extract) was prepared. The pentose fermenting yeast was inoculated into a 100 mL medium containing 5,0 g glucose, 5,0 g xylose, 2,0 g yeast extract and 2,0 g peptone. At t = 0, the 4 mL of yeast suspension was added to medium. At t = 0 hours, the first addition of birch slurry 300 g was mixed with 20 mL 1 M NaOH in order not to expose the yeast culture to a pH shock. The pH was adjusted to 5,5. The total weight of the first addition of birch slurry to Biostat B-Plus was 321 g. Simultaneously at t = 0 hours, 20 mL Cellic Ctec2 enzyme was added. A second addition of (321 g) was prepared and added to the Biostat B-Plus at t = 8 hours. After 48 hours of cultivation, the volume was adjusted to 700 ml. At the time t = 53,5 hours the third birch slurry addition (321 g) was added. When the last sample was collected, fermentation stopped. All samples were centrifuged for supernatant extraction at (10000 rpm, 5 minutes) followed by storage in freezer until HPLC. Samples were extracted at t = 0; 2; 26; 53; 72; 80; 96 and 100 hours. See layout of experiment in figure 21.

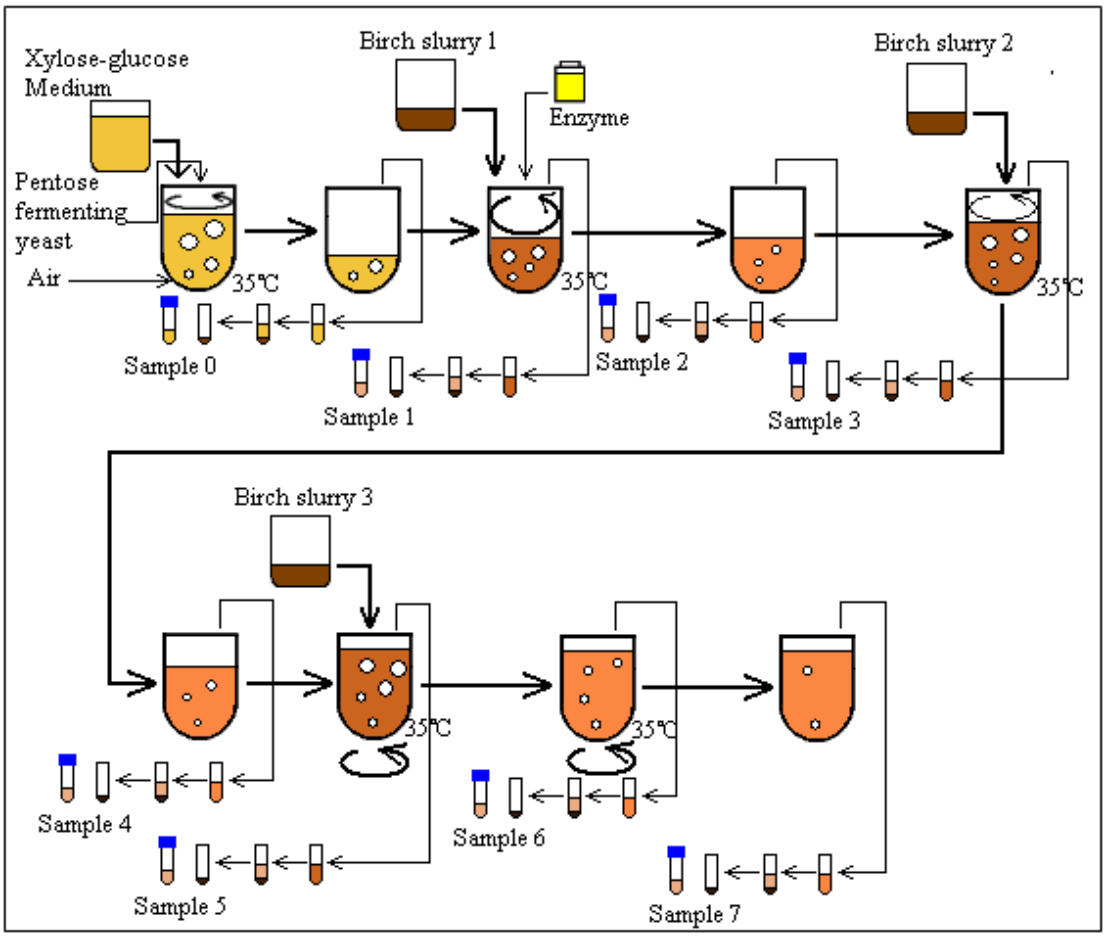

Figure 21. *The performed birch slurry fermentation. Three additions of birch slurry were added to the pentose fermenting yeast medium. Samples were extracted throughout the hydrolysis and fermentation process for HPLC and dry weight estimation.*

5.5 Experiment 2

From a wet yellow brown mesh of washed cellulose fibers 75 g of floccules were well mixed with 300 mL milliQ-water. Then pH was adjusted to 5,5 with 1 M sulfuric acid (1M). Slurry was after that transferred to Biostat B-Plus, which was autoclaved at 121 °C for 20 min, and cooled to 50 °C. The pH of the slurry was readjusted to 5,5. Water was added to the slurry until the viscosity was manageable. At t = 0 hours, 7 mL of enzyme Cellic Ctec2 was added and left for hydrolysis for 24 hours at 50°C. A second addition of 75 g slurry in 300 mL water was prepared and added to Biostat B-Plus at 25 hours. The hydrolysis continued until 49 hours until the third addition of slurry was made. The hydrolysis continued until 72 hours after which the Biostat B-Plus was cooled down to 35°C. At t = 95 hours, 10 g of dry Ethanol Red yeast was added to the Biostat B-Plus for fermention. The fermentation was stopped at the time t = 100 hours. Samples were extracted at t = 24; 48; 72 and 100 hours. See layout of experiment in figure 22.

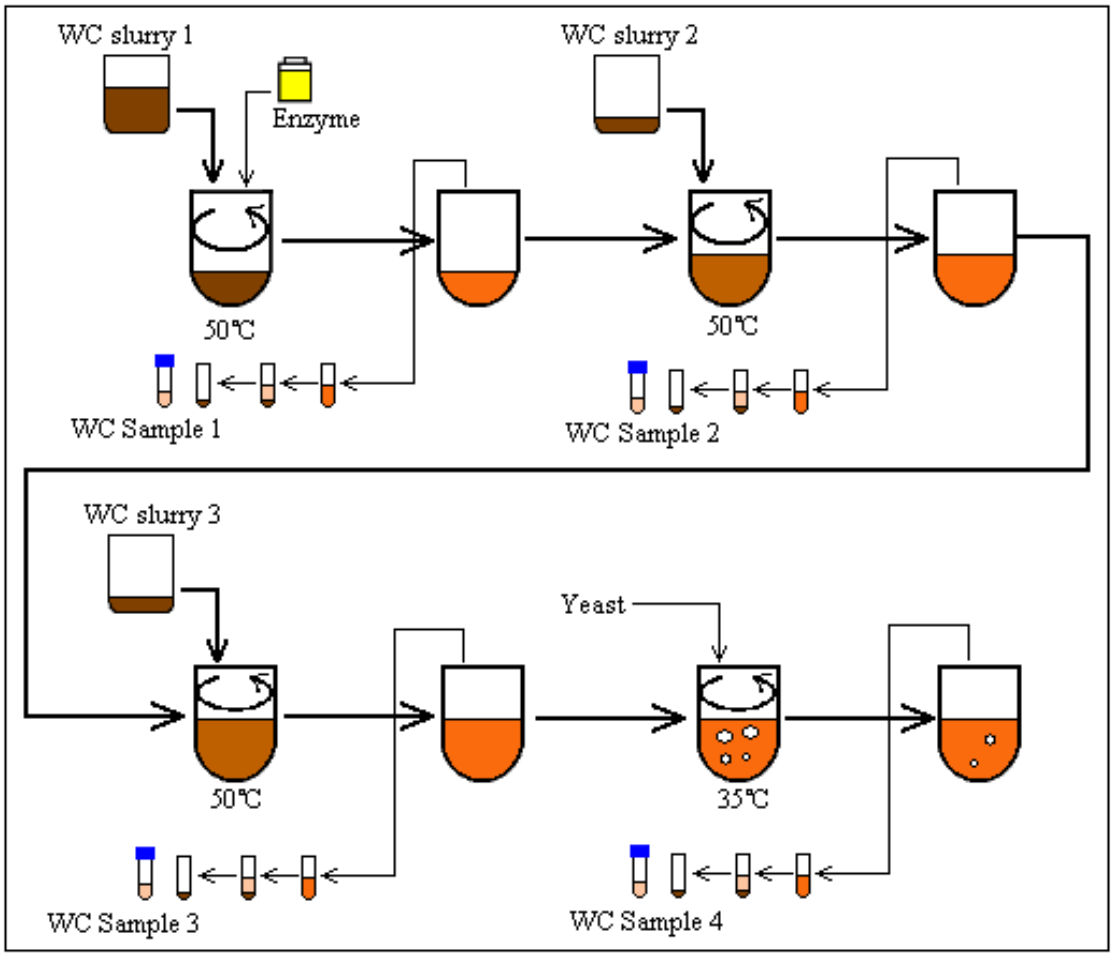

Figure 22. *Washed cellulose (WC) fermentation. Three batches of WC slurry were mixed with enzyme and hydrolyzed at 50°C. Yeast was added to hydrolyzed medium and fermentation was performed at 35°C. Samples were collected during hydrolysis and fermentation process.*

5.6 Experiment 3

From a compact cake of unwashed cellulose, 150 g was churned into smaller pieces and mixed in 300 mL milliQ-water. Then, pH was adjusted to 5,5 with 1 M sulphuric acid. Thus was the slurry prepared. Slurry was then transferred to the Biostat B-Plus fermentor and autoclaved at 121°C for 20 minutes, then cooled to 50 °C. The pH was readjusted to 5,5 and sufficient milliQ-water was added to Biostat B-Plus in order to enable stirring to a total volume of 1540 mL. Suspended solids (SS) concentration was 86,0 g/L. Then, 25 mL Cellic Ctec2 enzymes was added at t = 0 hours, which resulted in an enzyme concentration of 1,6 % starting hydrolysis. At 24 hours 150 mL of the slurry was extracted from the Biostat B-Plus to start fermentation in shake flasks A and B each given 75 mL of the slurry along with 10 g dry Ethanol Red yeast, labeled as 1FA and 1FB (1FA= first fermentation flask A). Flasks were left for 24 hours fermentation at 35 °C. At 48 hours, a second shake flask fermentation was started in the same way. Meanwhile, the hydrolysis continued in Biostat B-Plus. A second batch of slurry, 150 g unwashed cellulose, was prepared and added at 25 hours. A third batch of slurry was prepared in the same way and added at t = 49 hours. After extractions for a third shake flask fermentation at 71 hours, the Biostat B-Plus temperature was cooled to 35 °C. At 71 hours, 10 g dry Ethanol Red yeast, 10 mL Enzyme Cellic Ctec2 and 2 g ammoniumsulphate $(NH_4)_2SO_4$ was added to the Biostat B-Plus to start a fermentation, which ended at 120 hours. In parallel with the fermentation in Biostat B-Plus an additional shake flask fermentation was started. Samples were extracted at t = 0; 24; 25; 48; 49; 72; 95 and 120 hours. See layout of experiment in figure 23.

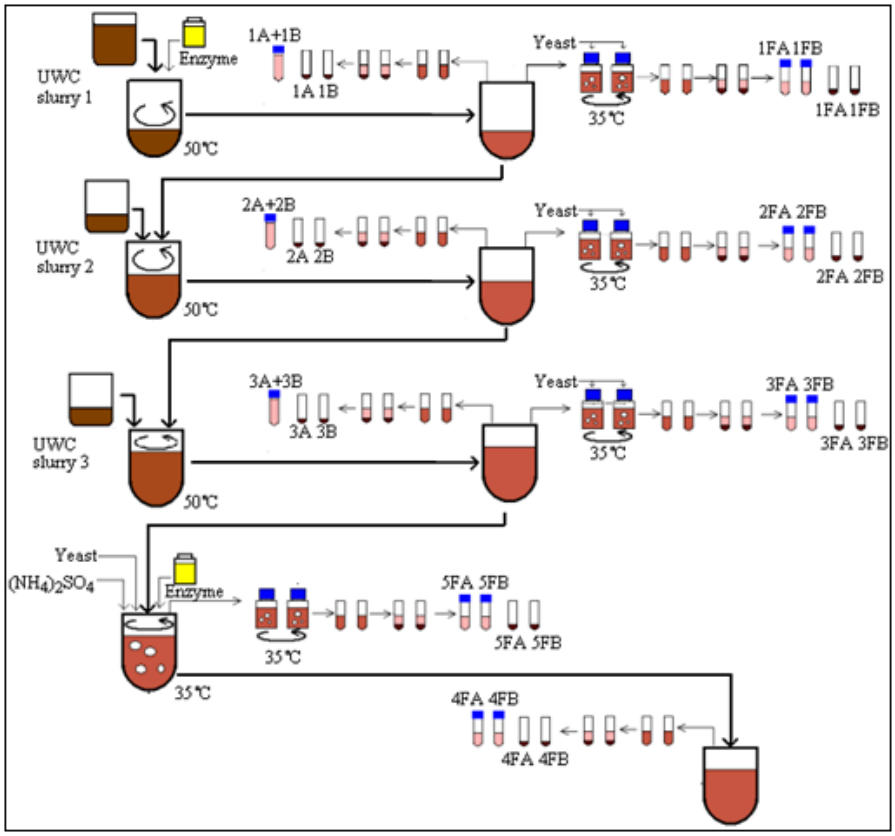

Figure 23. *The unwashed cellulose fermentation. Three batches of UWC slurry were mixed with enzyme and hydrolyzed. Five series of hydrolyzed slurry were collected and added yeast to ferment. Samples were collected during hydrolysis and fermentation process.*

5.7 Experiment 4

The sweet liquor is a dark brown liquid. The pH of sweet liquor is 3,2. Experiment 4 was a fermentation of differently diluted sweet liquor, fermented by pentose fermenting yeast. The experiment was duplicated in five dilution sets (50 ml/0ml = no dilution, 40 ml/10 ml, 30 ml/20 ml, 20 ml/30 ml, 10 ml/40 ml) all adjusted to pH= 5,0. The pH was adjusted to 5,0 with 1 M NaOH before addition of 2 g $(NH_4)_2SO_4$, 2 g KH_2PO_4 and 8 ml pentose fermenting yeast suspension to each bottle. The fermentation was performed at 35 °C, and 100 rpm for 72 hours. Samples were collected at 0 and 72 hours. See layout of experiment in figure 24.

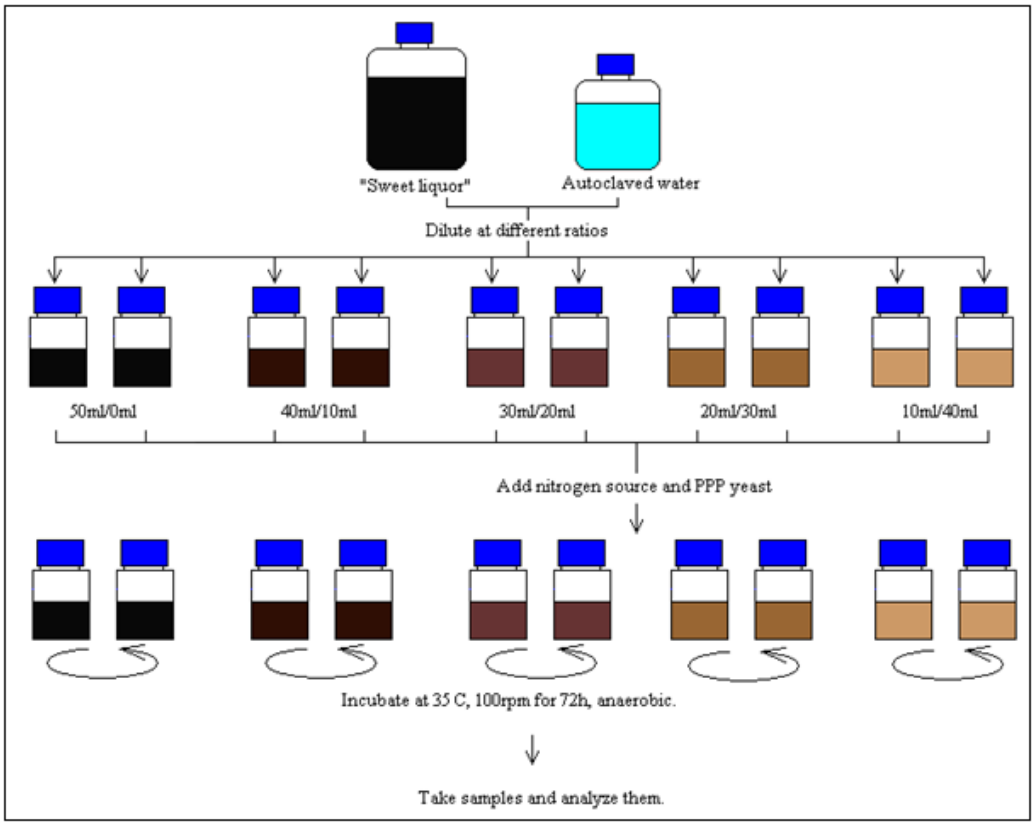

Figure 24. *A layout of experiment 4. Five sets of media were prepared from sweet liquor and autoclaved water at different dilutions. All series of media were mixed with an additional nitrogen source and pentose fermenting yeast as fermenting organism. Samples were taken after fermentation and were analyzed.*

5.8 Experiment 5

Experiment 5 was a fermentation of various concentrations of added yeast extract and cell concentration. Yeast extract levels were 0 g/L, 1,0 g/L, 5,0 g/L while pentose fermenting yeast cell levels were 0,5 g/L, 1,0 g/L, 1,5 g/L. The pH was adjusted to 5,0 with 1 M NaOH before addition of 2 g KH_2PO_4 and specific amount of pentose fermenting yeast cells and yeast extract according to the presentation seen in figure 25. The fermentation carries out for 72 hours at 35 ℃, 100 rpm. Samples were collected at t = 0 and 72 hours.

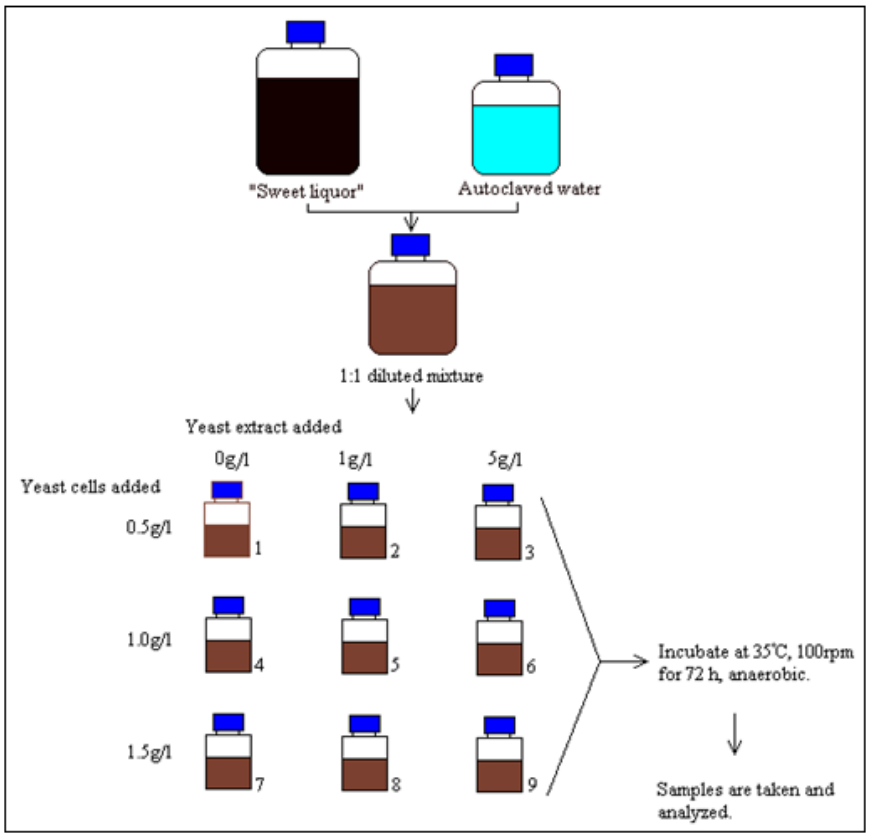

Figure 25. *Seen above is the experimental layout of experiment 5. A medium was prepared from sweet liquor and autoclaved water. This medium was separated into 9 bottles and yeast extract and different amounts of yeast cells were added. Samples were extracted and analyzed after 72 hours of fermentation.*

5.9 Experiment 6

In order to determine whether the lignin from Company X can be used as raw material for polyphenolic resin production or not, a polycondensation experiment was performed by complete mixing of 10 g lignin with 0,2 g $NaOH_{(s)}$ and 10 g furfural $(OC_4H_3CHO)_{(l)}$. The mixture was then incubated at 150 °C for 20 minutes, after which it was cooled to 20°C. The polycondensation was complemented with dry weight estimation [42].

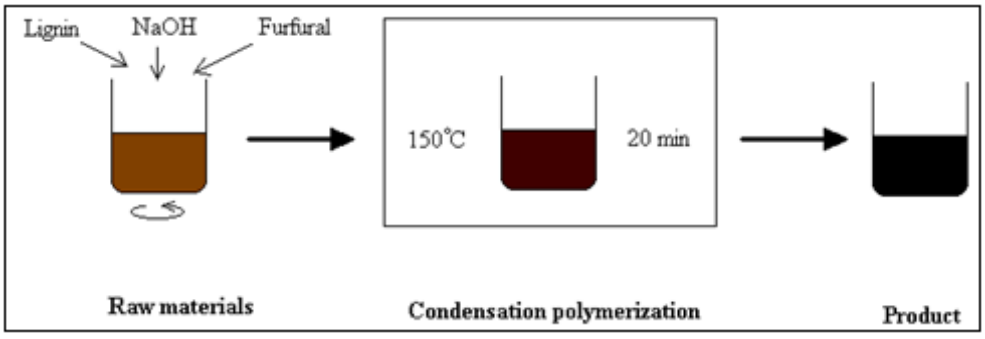

Figure 26. *A graphic presentation of the lignin polycondensation experiment.*

6. Results and Discussion

In this section, results from the six different experiments are displayed and discussed. Examples of calculations of dry weight, yield, cell density and glucose concentration are also included. All raw materials in lignocellulosic ethanol production can be classified into two components: fermentable and non-fermentable sugars derived from celluloses and hemi-celluloses, plus all other still remaining structural wood components. The fermentable part is a complex mixture, where cellulose can be considered as the major raw material component. In order to simplify the theoretical yield calculation it was assumed that the washed cellulose of PLET sample contained 100 % pure cellulose, which is a simplification.

The hardwood birch slurry with hexoses derived from cellulose and pentoses derived from hemi-cellulose from SEKAB E-Technology, in contrast to the pure cellulose from PLET, which has a simplified mass flow mode as follows.

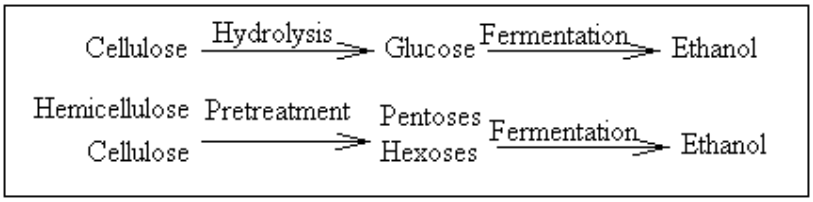

Figure 27. *The two steps, hydrolysis and fermentation in a SSF and/or SHF.*

Formulas used to calculate yields stoichiometrically was [m = n * *M*], [n = c * v] and [c_1*v_1 = c_2* v_2], where m = mass in gram, n = number of mol, *M* = molar mass in g/mol, c = concentration in g/dm^3 and v = volume in dm^3 or L. Dry weight percentage is expressed as the cellulose supplied plus non fermentable dry matter divided by the cellulose supplied plus non fermentable matter plus water. So, the dry initial raw material could be considered as cellulose supplied plus non fermentable matter.

6.1 Yield calculations (theoretical versus actual yield)

Reaction 1 is cellulose into ethanol stoichiometrically balanced net reaction, whereas reaction 2 and 3 show simplified balanced reactions of hemicelluloses into ethanol under aerobic and anaerobic conditions respectively if no consideration is taken to biomass formation.

Reaction 1: $(C_6H_{10}O_5)_n + n\ H_2O \rightarrow n\ C_6H_{12}O_6 \rightarrow 2*n\ C_2H_5OH + 2*n\ CO_2$
Reaction 2: $(C_5H_8O_4)_n + n\ H_2O \rightarrow n\ C_5H_{10}O_5 + 0{,}5*n\ O_2 \rightarrow 1{,}5*n\ C_2H_5OH + 2*n\ CO_2$ [43]
Reaction 3: $(C_5H_8O_4)_n + n\ H_2O \rightarrow n\ C_5H_{10}O_5 \rightarrow 1{,}67*n\ C_2H_5OH + 1{,}67*n\ CO_2$ [43]

Table 2. *Showing the molar weights of molecules that take part in hydrolysis and fermentation.*

$M_{(Cellulose\ unit)}$ = 162 g/mol	$M_{(Water)}$ = 18 g/mol	$M_{(D\text{-}Glucose)}$ = 180 g/mol
$M_{(Hemicellulose\ unit)}$ = 132 g/mol	$M_{(D\text{-}Xylose)}$ = 150 g/mol	$M_{(Ethanol)}$ = 46 g/mol
$M_{(Carbondioxide)}$ = 44 g/mol	$M_{(Oxygen)}$ = 32 g/mol	

In a SHF both the hydrolysis and fermentation must be considered as two steps;

Theoretical hydrolysis yield = 180 / 162 = 1,11 (g glucose / g cellulose)
Theoretical fermentation yield = (2 * 46) / 180 = 0,51 (g ethanol / g glucose)
Theoretical total yield = (2 * 46) / 162 = 0,57 (g ethanol / g cellulose)

Hydrolysis yield = Glucose / Cellulose supplied
Fermentation yield = Ethanol produced / Glucose
Actual yield = Ethanol produced / Cellulose supplied
Percentage yield = Actual yield / Theoretical yield

Whereas theoretical yield calculations are simplified in a SSF, where the total reaction is considered as;

Theoretical total yield = 2*46 /162 = 0,57 (g ethanol / g cellulose)
Actual yield = Ethanol produced / Cellulose supplied
Percentage yield = Actual yield / Theoretical yield

6.2 Experiment 1: Birch Slurry SSF

6.2.1 Results of Experiment 1

The birch slurry is a red-brown pasty substance with a pH at 2,5. The birch slurry SSF involved three sequential additions (total amount added 900 g) of birch slurry to a pentose fermenting yeast medium along with 20 mL Cellic Ctec2 enzymes. In the dry solid measurement the cellulose fraction is marginal compared to free sugars. The total SS content, also known as the dry solid concentration was 185,8 g/L.

In all calculations presented below, the density ratio is approximated as 1 g = 1 mL. In the 700 mL start-culture there were substantial amounts of glucose, xylose and ethanol which cannot be neglected. These amounts have to be subtracted from the actual SSF performed;

Glucose: 0,6 g/L* 0,7 L = 0,4 g
Xylose: 27,2 g/L * 0,7 L = 19,0 g
Ethanol: 7,1 g/L* 0,7 L = 5,0 g

When the actual SSF was initiated to Biostat B-Plus, there were three separate additions of 300 g slurries plus 21 g NaOH, with dry solids content of 16 %. It is assumed that the solid fraction of the slurry contained 50 % cellulose. Consequently, the Biostat B-Plus received;

Dry solid weight: 3 * 300 * 0,16 = 144 g
Dry solid concentration: 144 / 1,6 L = 90 g/L
Glucose: (3,8 g/L * (0,7 L + 0,321 L) - 0,4 g) * 3 = 9,3 g
Xylose: (36,7 g/L * (0,7 L + 0,321 L) - 19,0 g) * 3 = 44,4 g
Cellulose: 144 g * 0,50 = 72 g

According to HPLC data the final solution contained;

Glucose: 8,0 g/L * 1,6 L = 12,8 g
Xylose: 10,8 g/L * 1,6 L = 17,3 g
Ethanol: 44,4 g/L * 1,6 L = 71,0 g

Yield calculation of the ethanol produced and the productivity of birch slurry SSF requires that the amounts added from pre-culturing is subtracted. The total volume of the pre-culture plus the added slurry during the experiment was considered as 1,6 L, which includes an approximation for the volume lost during sampling. The theoretical fermentation yield of 0,51 g ethanol /g glucose cannot be assumed, at best a 90 % of theoretical yield could be expected as described below;

Ethanol from pre-culture: (0,4 g + 19,0 g) * 0,51 g/g * 0,90 = 8,9 g
Ethanol from slurry: 71,0 g - 8,9 g = 62,1 g
Actual total yield: 62,1g / 144 g = 0,43 g ethanol / g dry raw material
Ethanol from free sugar: (0,4 g + 19,0 g + 9,3 g + 44,4 g) * 0,51 g/g * 0,90 = 33,6 g
Ethanol from cellulose: 71,0 g - 33,6 g = 37,4 g
Cellulose consumed: 37,4 g / (0,9 * 0,57 g/g) = 72,9 g
Productivity: 62,1 g / (73 h * 1,6 L) = 0,53 g/h*L

Produced ethanol concentration: 37,4 / 1,6 = 23,4 g/L

Table 3. *The concentration of dry material and ethanol for the birch slurry SSF. Assuming that the ethanol production was based on complete consumption of free sugars (with 90 % of 51 % yield = 46%).*

	Dry raw material	Produced Ethanol	90 % of theoretical yield of ethanol	Free sugars added (xylose + glucose)	Free sugars remained (xylose + glucose)	Consumed cellulose
Concentration	185,8 g/L	23,4 g/L	75,5 g/L	40,5 g/L	18,8 g/L	82,5 g/L

Amount of free sugars added to the system was 36,7 g/L xylose + 3,8 g/L glucose, including pentoses and hexoses. The produced ethanol was 44,4 g/L at 73 hours when the experiment was ended. Assuming that the ethanol production was based on complete consumption of free sugars (with 90 % of 51 % yield = 46 %). Since there is 23,4 g/L produced ethanol in the end sample then the initial raw material must have contained at least 82,5 g/L of cellulose. The actual total yield is calculated in "g ethanol / g dry raw material". Then the actual total yield is 0,43 g ethanol / g dry raw material.

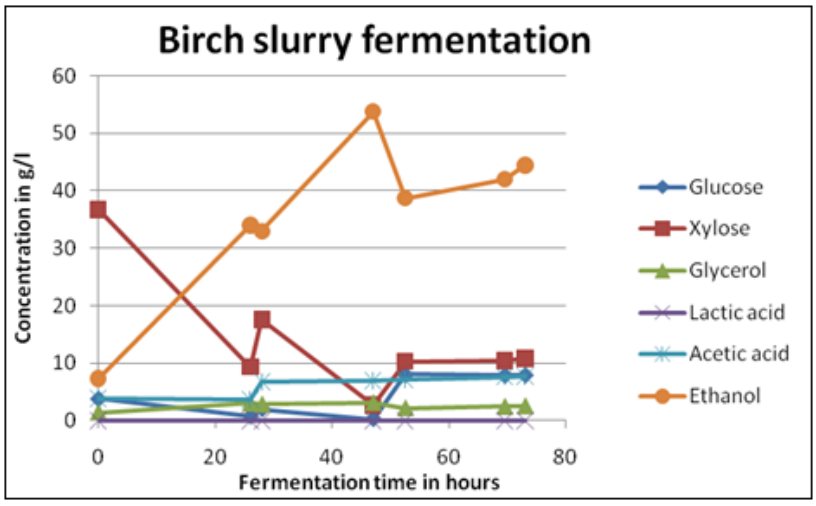

Figure 28. *Shows the HPLC-data from experiment 1. SSF on SEKAB E-Technology´s birch slurry. Y-axis display concentration given in (g/L) and x-axis display time given in hours.*

6.2.2 Discussion of Experiment 1

Experiment 1 involved a SSF with birch slurry provided by SEKAB E-Technology. Slurry was enzymatically treated with 20 mL of the enzyme Cellic Ctec2 and fermented with a pentose fermenting yeast. This experiment is considered successful since the yield of ethanol was high. Seen in retrospect, more samples should have been taken during the duration of experiment in order to achieve a curve with better precision. However, every time a sample is collected, the anaerobic conditions are disturbed, so sample extraction must always be kept at a minimum, while precision of actual concentrations must still be distinguishable.

Ethanol concentration was high once fermentation was established with values close to 40 g/L and increasing throughout the continuation of experiment. According to the three consecutive additions of slurry, the fermentation can be divided into 3 phases. In the first phase, glucose concentration decreased to 3,0 g/L, xylose concentration decreased to 27,4 g/L, whereas ethanol concentration increased to 26,6 g/L, according to the measurements. In the second phase, the glucose concentration decreased to 1,7 g/L and the xylose concentration decreased to 14,9 g/L, whereas ethanol concentration increased to 20,8 g/L. In the third phase, glucose concentration decreased to 0,06 g/L, xylose concentration increased to 0,48 g/L, ethanol concentration increased to 5,7 g/L.

In experiment 1, both xylose and glucose are utilized by pentose fermenting yeast, resulting in ethanol production. Ethanol concentration increases, as concentration of glucose and xylose decreases. The (xylose and glucose) / ethanol yield is during a short period higher than the theoretical value. Seen in figure 28 at t = 53,5 hours. Explanation for this is that there was other fermentable raw material still remaining in slurry from previous slurry additions, which is hydrolyzed during fermentation, thereby adding cumulatively to the total amount of fermentable sugars during the process. Comparing the three phases, the fermentation rate decreased during the whole process. It can be seen in data as the activity of yeast cells decreased. When the third phase was entered, cells entered a stationary stage resulting in an increase of ethanol concentration. The ethanol concentration increased moderately while (xylose and glucose) concentration levels remained stable.

6.3 Experiment 2: Washed cellulose (WC) SHF

6.3.1 Results of Experiment 2

The washed cellulose (=WC) samples from PLET, is a floccules wet matter, which contains 69,4 % water. The raw material was cellulose which had been washed three times according to PLET, and the dry mass was thus considered as 100 % pure cellulose. Three consecutive additions of WC slurry with 30,6 % dry mass content was added to the Biostat B-Plus during hydrolysis performed at 50 °C. The load of cellulose (= suspended solids) were 95,9 g/L. Temperature was decreased to 35 °C, and yeast was added at t = 72 hours at a concentration of 12,5 g/L. The total mass transferred to Biostat B-Plus was;

Cellulose: 75 g * 3 * 0,306 = 68,9 g

To simplify calculations the total volume occupied in Biostat B-Plus during the experiment was approximated as 0,75 L despite the fact that substantial volume was extracted during sampling. This effect was however counteracted by almost equal volume metric additions of NaOH-solution for pH-adjustment during both hydrolysis and fermentation. According to HPLC data the glucose concentration was 68,4 g/L.

Total volume occupied: 0,75 L
Glucose: 68,4 g/L * 0,75 L = 51,3 g
Actual hydrolysis yield = 51,3 g / 68,9 g = 0,74 g glucose / g cellulose

The fermentation yield, amount of cellulose consumed and productivity was calculated according to HPLC data, which stated that the ethanol concentration after fermentation was;

Ethanol concentration after fermentation: 30,5 g / L* 0,75 L = 22,9 g
Actual fermentation yield = 22,9 g / 51,3 g = 0,45 g ethanol / g glucose
Actual total yield = 22,9 g / 68,9 g = 0,33 g ethanol / g cellulose
Cellulose consumed = 22,9 g / (0,57 g/g * 0,9) = 44,6 g
Productivity = 22,9 g / (100 h * 0,75 L) = 0,31 g/h*L

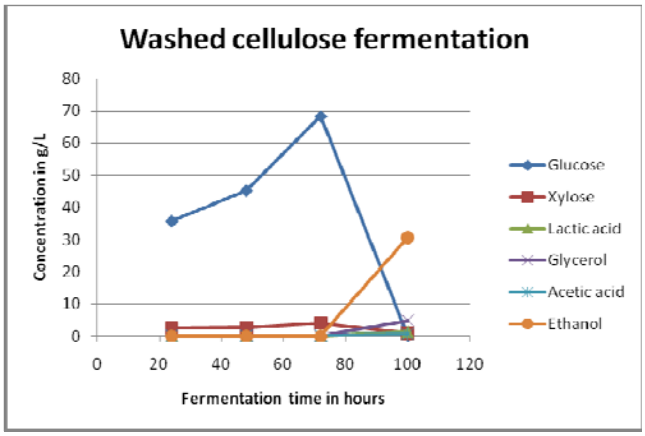

Figure 29. *HPLC data for washed cellulose. Y-axis display concentration given in (g/L) and X-axis display time given in hours.*

Table 4. *The concentration of the dry material, hydrolysis and ethanol.*

	Cellulose before hydrolysis	Glucose after hydrolysis	Ethanol after fermentation
Concentration	95,9 g/L	68,4 g/L	30,5 g/L

Table 5. *Cellulose concentration was based on dry weight measurements, glucose concentration and ethanol concentration were based on HPLC data.*

Actual hydrolysis yield	0,74 g glucose / g cellulose
Actual fermentation yield	0,45 g ethanol / g glucose
Actual total yield	0,33 g ethanol / g cellulose
Productivity (fermentation)	1,09 g/h*L
Productivity (total process)	0,31 g/h*L

6.3.2 Discussion of Experiment 2

Experiment 2 of washed cellulose is considered as a good experiment with ambiguous results since the real peak ethanol concentration cannot be determined. Still, this is a valuable result indeed, showing that the input of yeast and enzymes on pretreated biomass, can produce 0,3 tons of ethanol per ton cellulose. This yield can be converted to 99,2 US gallons per ton. It is quite high compared with USDA economically achievable yield which is in the range of 65-75 US gallons per ton [44].

However, this must also be seen in the context of how much water that was used to wash the cellulose. According to PLET the washed cellulose was washed three times with water. The productivity achieved was 1,09 g ethanol per liter per Biostat B-Plus fermentor volume and 28 hours during fermentation.

It can only be speculated that cells are starving at the end of fermentation due to difficulty to find sufficient levels of glucose as the experiment progressed. All glucose is consumed at 100 hours and ethanol concentration reached 30 g/L, which is a proof of a successful fermentation. When starving, during the last hours of fermentation the yeast cells are hence forced to consume the previously produced ethanol as an energy and carbon source in the presence of oxygen. Fully anaerobic conditions could not be performed in this experiment.

If this argumentation is correct then the obvious conclusion would be that peak ethanol concentration would be substantially above 30 g/L somewhere in the time interval between 80-90 hours. In the end, it is perhaps better to argue that there is insufficient data to say anything about peak ethanol concentration. More samples should definitely have been taken in the time interval between 72 hours to 100 hours.

6.4 Experiment 3: Unwashed cellulose SSF+SHF

6.4.1 Results of Experiment 3

Unwashed cellulose samples were extracted from a hard compact cake, which contained substantial amounts of salt. When re-suspended, the pH was 11,6, which required adjustment with H_2SO_4 to a pH of 5,5. Three batches of UWC slurry were mixed with a single addition of 25 mL Cellic Ctec2 enzyme and then hydrolyzed in the Biostat B-Plus.

Five series of hydrolyzed slurries were collected at different times to start fermentation by 10 g dry Ethanol Red yeast in shake flasks. In addition, a mixed SHF/SSF was performed with 10 g dry Ethanol Red yeast in the Biostat B-Plus in parallel with another SHF/SSF simultaneously performed in shake flask with addition of 10 g dry Ethanol Red yeast and 10 mL Cellic Ctec2 enzymes. The HPLC data and hydrolysis data are displayed in figures 30-31 and tables 6-9 show calculated yields.

In the first SHF, labeled as SHF 1 in table 6 and 7 below, the dry solid content of the unwashed cellulose sample was 88,2 %, which was re-suspended in 1,54 L. The produced glucose accumulated during the first hydrolysis was 116,4 g.

Dry raw material: 150 g * 0,882 = 132,3 g
Volume: 1,54 L
Accumulated glucose: 75,6 g/L * 1,54 L = 116,4 g
Hydrolysis yield = 116,4 g / 132,3 g = 0,88 g glucose / g dry raw material

The following 24 hour fermentation of SHF 1 in shake flasks resulted in an ethanol concentration of 41,3 g/L. The extra high yield reflects the fact that the enzyme Cellic Ctec2 still was active during fermentation. Assuming a 90 % conversion of the theoretical value for cellulose into ethanol, an estimation of how much cellulose that actually was consumed can be drawn. The fermentation yield, total yield, cellulose consumed and total productivity are calculated as follows;

Accumulated ethanol: 41,3 g/L
Fermentation yield = 41,3 g/L / 75,6 g/L = 0,55g ethanol / g dry raw material
Total yield = 0,88 g/g * 0,55 g/g = 0,48 g ethanol / g dry raw material
Cellulose consumed = (41,3 g/L * 0,1 L) / (0,57 g/g * 0,9) = 8,05 g
Productivity (fermentation) = (41,3g/L *0,1 L) / (24 h * 0,1 L) = 1,72 g/h*L
Productivity (total) = (41,3g/L *0,1 L) / (48 h * 0,1 L) = 0,86 g/h*L

The other SHF:s, SHF 1 and SHF 2 was calculated in the same way as for SHF 1, with consideration taken to the extracted volumes from Biostat B-Plus and the corresponding time. Results are presented in table 6 and 7. For the combined SSF 1 and SSF 2 fermentation, only the total yield could be calculated. Since volumes of the previous SHF:s were extracted, these must considered when calculating the actual concentration present within the Biostat B-Plus. The reached ethanol concentration is very high due to the long hydrolysis time. Data is summarized below in table 8 and 9. For the combined SSF1 performed in Biostat B-Plus; the dry raw material, volume compensation, ethanol produced, yields, cellulose consumed and productivity are calculated as follows;

Dry raw material: 365,9 g * (2,54 L - 0,15 L - 0,1 L) / 2,54 L = 329,9 g
Volume: 2,54 L - 0,15 L - 0,1 L + 0,01 L + 0,05 L = 2,35 L
Ethanol produced: 53,8 g/L * 2,35 L = 126,4 g
Total yield = 126,4 g / 329,9 g = 0,38 g ethanol / g dry raw material
Cellulose consumed = 126,4g / (0,57 g/g * 0,9) = 246,4 g
Productivity = 126,4 g / (144 h * 2,35 L) = 0,37 g/h*L

And for the combined SSF 2 performed in shake flask; the dry raw material, volume compensation, ethanol produced, yields, cellulose consumed and productivity are calculated as follows;

Dry raw material: 329,9 g * (0,1 L / 2,35 L) = 14,04 g
Volume: 0,1 L
Ethanol produced: 52,5 g/L * 0,1 L = 5,25 g
Total yield = 5,25 g / 14,04 g = 0,37 g ethanol / g dry raw material
Cellulose consumed = 5,25 g / (0,57 g/g * 0,9) = 10,23 g
Productivity = 5,25 g / (144 h * 0,1 L) = 0,36 g/h*L

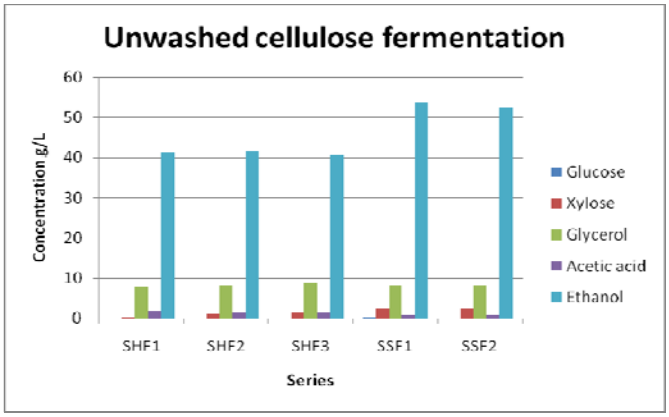

Figure 30. *HPLC-data presented in a graph for unwashed cellulose fermentation. For the SHF:s the hydrolysis continued in the Biostat B-Plus using 10 mL enzyme Cellic Ctec2, and each subsequent fermentation was performed for 24 hours, and for the combined SSF:s the time was 48 hours. In each shake flask fermentation was 1 g of Ethanol Red yeast added.*

Table 6. *Dry mass concentration during hydrolysis exceeded theoretical yield due to the fact that previously added dry material contributes cumulatively so that more sugars is produced.*

	Cellulose added before hydrolysis	Glucose after hydrolysis	Ethanol after fermentation
SHF 1	86,0 g/L	75,6 g/L	41,3 g/L
SHF 2	122,6 g/L	72,2 g/L	41,7 g/L
SHF 3	144,2 g/L	81,7 g/L	40,4 g/L

Table 7. *The SHF yield data for experiment 3.*

	SHF 1	SHF 2	SHF 3
Hydrolysis yield	0,88 g glucose / g cellulose	0,60 g glucose / g cellulose	0,57 g glucose / g cellulose
Fermentation yield	0,55 g ethanol / g glucose	0,58 g ethanol / g glucose	0,49 g ethanol / g glucose
Total yield	0,48 g ethanol / g cellulose	0,35 g ethanol / g cellulose	0,28 g ethanol / g cellulose
Productivity (fermentation)	1,72 g/h*L	1,74 g/h*L	1,68 g/h*L
Productivity (total)	0,86 g/h*L	0,58 g/h*L	0,28 g/h*L

Table 8. *The combined SSF 1and SSF 2, concentration and yield data for experiment 3.*

	Combined SSF 1	Combined SSF 2
Cellulose added before SSF	144,2 g/L	144,2 g/L
Final ethanol concentration	53,8 g/L	52,5 g/L
Actual total yield	0,38 g ethanol / g cellulose	0,37 g ethanol / g cellulose
Productivity	0,37 g/h*L	0,36 g/h*L

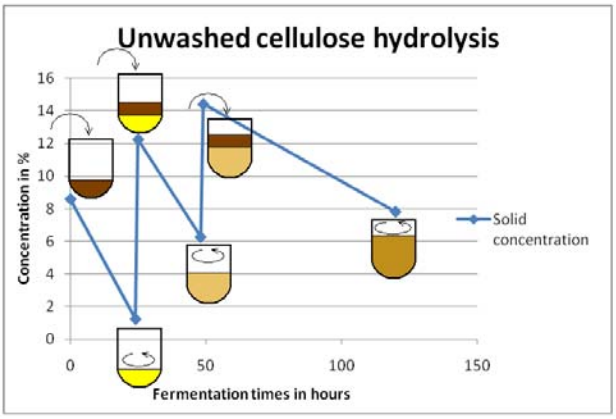

Figure 31. *Hydrolysis curve of unwashed cellulose. After each slurry addition, the solid concentration increases due to the extra addition of solids, then it decreases due to ongoing hydrolysis.*

6.4.2 Discussion of Experiment 3

The decreasing hydrolysis rate during experiment can be explained with a decrease of the enzymatic activity. The SSF 1 and SSF 2 both had higher total yield than SHF 3, since an extra addition of 10 mL Cellic Ctec2 enzymes were added to the Biostat B-Plus, when these were initiated. In addition, the combined SSF1 and SSF2 both had access to hydrolyzed material not yet converted by the enzyme Cellic Ctec2. In laboratory experiments, enzyme cost are often neglected, but in industrial production, the cost of enzymes must be considered. Higher yield versus lower cost is always important to consider if the laboratory experiments ever will find an industrial platform.

The most interesting results are found in the SHF, where the actual ethanol yield range between 0,48 - 0,28 g ethanol / g cellulose. This provides a strong argument for a future production of lignocellulosic ethanol from unwashed cellulose from the simple pre-treatment developed by PLET. The higher yields (0,37 - 0,38 g ethanol / g cellulose) accumulated in the combined SSF:s are also inspiring, but are somewhat inconclusive since it is hard to rule out the cumulative effect of the ongoing hydrolysis.

Experiment 3 has undoubtedly provided this master thesis with the best experimental results. It is, however, also the hardest one to interpret since it was a combined SSF and SHF experiment. The ethanol yield was very high, close to the theoretical yield. It is preferable to use unwashed cellulose in industrial ethanol production to reduce the use of water, which eventually will have to be removed in distillation at great cost.

The Biostat B-Plus was used as a separate hydrolysis reactor from which batches were taken every day to start fermentation in shake flasks. Only one end sample was taken from the fermentations in shake flasks to prove the production of ethanol. More samples should have been collected during experimentation. As everything turned out, there was still substantial ethanol content in all shake flasks, thereby proving that pre-treated unwashed cellulose from PLET is excellent for making industrial ethanol. For the combined SSF:s results, the Biostat B-Plus was used as a standard SSF bioreactor loaded with enzymes in large excess to see if complete conversion of cellulose into glucose could generate a much higher ethanol yield compared to the previous separate fermentation performed in shake flasks. The main reason for this approach is to see if the increased ethanol yield could compensate the extra use of enzymes.

6.5 Experiment 4: Sweet liquor (10 flasks)

6.5.1 Results of Experiment 4

The HPLC-data indicate no sign of successful fermentation in experiment 4. Five sets of media were prepared from sweet liquor and autoclaved water at different dilutions. All series of media were mixed with an additional nitrogen source and pentose fermenting yeast as fermenting organism. The initial sugar concentration was however not determined.

Table 9. *HPLC data of experiment 4.*

Samples	10:40 ml	20:30 ml	30:20 ml	40:10 ml	50:0 ml
Xylose (g/L)	1,79	2,40	3,24	3,89	3,76
Lactic acid (g/L)	0	0	0	2,24	3,16
Glycerol (g/L)	0	0	0	0,29	0,30
Acetic acid (g/L)	0,29	0,81	0,91	0,71	0.68
Ethanol (g/L)	3,60	1,85	3,35	3.40	3,18

6.5.2 Discussion of Experiment 4

Experiment 4 from PLET samples is considered as a failed experiment since the sweet liquor did not contain sufficient fermentable sugars, which made fermentation unfeasible. The initial sugar concentration was not determined. HPLC data shows only the presence of contaminations and a large percentage of smaller hydrocarbons. The low concentration of ethanol is most probably coming from the transferred pre-cultured yeast cell medium, thus would have nothing to do with fermentation of sweet liquor.

6.6 Experiment 5: Sweet liquor (9 bottles)

6.6.1 Result of Experiment 5

A medium was prepared from sweet liquor and autoclaved water. This medium was separated into 9 bottles and yeast extract and different amounts of yeast cells were added. Samples were extracted and analyzed after 72 hours of fermentation. No sign of successful fermentation according to HPLC data.

Table 10. *HPLC data of experiment 5. Where A, B and C refers to the initial cell concentration respectively concentration 0,5 g/L, 1,0 g/L and 1,5 g/L. The numbers 1,4 and 7 refer to bottle with no yeast extract added while 2,5 and 8 refer to addition of 1,0 g/L of yeast extract and finally 3, 6 and 9 are bottles with 5,0 g/L addition of yeast extract.*

Samples	A1	A2	A3	B4	B5	B6	C7	C8	C9
Lactic acid (g/L)	3,66	4,03	3,89	3,82	3,70	3,81	3,65	3,77	3,78
Acetic acid (g/L)	0,57	0,75	0,62	0,52	0,51	0,58	0,46	0,49	0,50

6.6.2 Discussion of Experiment 5

Experiment 5 from PLET samples is considered as a more or less failed experiment since the sweet liquor did not contain sufficient fermentable sugars, which made fermentation unfeasible. HPLC data shows only the presence of contaminations and a large percentage of small hydrocarbons.

6.7 Experiment 6: Lignin (Dry weight / Polycondensation)

6.7.1 Results of Experiment 6

A polycondensation experiment was performed by complete mixing of 10 g lignin with 0,2 g $NaOH_{(s)}$ and 10 g furfural $(OC_4H_3CHO)_{(l)}$. The mixture was then incubated at 150 °C for 20 minutes, after which it was cooled to 20°C. The polycondensation was complemented with dry weight estimation. The pure lignin from PLET has a dry solid percentage of 63,8 %. When subjected to a polycondensation the result was a hydrophobically insoluble, brittle, porous, tarnish black cake.

6.7.2 Discussion of Experiment 6

The pure lignin of PLET looks like small brownish granules. These granules are hydrophilic and have rosin like melting and freezing behavior, mostly amorphous. Phenol formaldehyde resins (PF) include synthetic thermosetting resins such as obtained by the reaction of phenols with formaldehyde and similar chemicals such as furfural. Lignin monomers have phenolic hydroxyl group. Lignin can be used as a phenol in polycondensation reaction.

Experiment 6 was only a short study of general properties. Since this master thesis did not get access to necessary analyzing equipment it was impossible to conduct any scientific analysis better than analysis already performed by PLET. It is however a bit disturbing that the product of the polycondensation was so brittle. That is, if the pure lignin ever will be applied as a binder or adhesive in paint of glues. Heating as well as pressure applied in experiment was substantially lower than what is normally applied in industry for adhesives production. Probably the preferred adhesive characteristics will be achieved under those conditions.

7. Conclusions.

7.1 Conclusions of results discussed in general

Best ethanol yield was obtained in the experiments with the raw material of unwashed cellulose, followed by less yield of accumulated ethanol from washed cellulose samples and birch slurry samples.

The stickiness of slurry can be a certain problem in experiments. High solid concentration slurry was hard to stir, as the result, faster stirring speed and extra addition of water must be used on slurries before hydrolysis.

In experiment 1, the ethanol yield from dry material obtained for hardwood birch slurry was 0,23 g ethanol / g dry raw material. Whereas in experiment 2, the ethanol yield from dry material obtained for softwood pine washed cellulose was 0,32 g ethanol / g cellulose. And in experiment 3, the ethanol yield from dry material obtained for softwood pine unwashed cellulose in the three SHF was; 0,48, 0,34 and 0,28 (g ethanol / g cellulose) respectively, while the yield was 0,37 and 0,38 g ethanol / g cellulose in the two combined SSF:s. It is interesting that the unwashed cellulose in experiment 3 achieve a slightly higher ethanol concentration compared to the washed cellulose in experiment 2.

Main reason for the increased ethanol yield in experiment 3 compared to experiment 2, is a more complete hydrolysis due to higher enzymatic loading, and that the enzyme Cellic Ctec2 perform better on not absolutely pure cellulose [10]. Decrease of ethanol yield during the three SHF:s in experiment 3 are most probably caused by diminished enzymatic activity, due to product inhibition (glucose concentration is high) and increasing inaccessibility of the substrate in a larger volume.

The fermentation strains, Ethanol Red and the pentose fermenting yeast are both especially developed for the purpose of ethanol production. However, the pentose phosphate pathway is not relevant in the metabolism of Ethanol red strains, thus the pentoses present are only occupying space as not fermentable sugars, whereas in pentose fermenting yeast these pentoses are contributing to increase ethanol yield. This effect is reduced by the fact that softwood only contains a smaller percentage of hemi-cellulose, while a fermenting strain utilizing pentose phosphate pathway is absolutely necessary when using hardwood birch as a substrate. When comparing the accumulated ethanol yield from either fermenting organism, this is important to consider.

There is, however, some general differences that need to be considered between the samples of the two companies, SEKAB E-Technology and PLET. Evidently, SEKAB E-Technology has had time to optimize process parameters of their pre-treatment over several years with a lot of manpower, money and advanced equipment, while PLET have had to make the best of what they have had at their disposal. In addition, there are a lot of other companies that uses dilute SO_2 as a pre-treatment, while no other currently known company uses dilute nitric acid as pre-treatment of lignocellulosic material, at least not in the way PLET does it. In this context SEKAB E-technology is clearly favoured.

Birch slurry of SEKAB E-Technology is of hardwood origin, while the Gorman pine samples of PLET comes from cellulose rich softwood. The hardwood birch slurry contains more inhibitors regarding fermentation compared to the softwood pine. In addition, the washed

cellulose samples from PLET are referred to as pure cellulose, which substantially increases the potential ethanol yield, if put into a SSF and/or SHF. Thus have hemi-cellulose dominated hardwoods both benefits and backdraughts for industrial potential, compared to cellulose dominating softwoods.

Furthermore, fermenting organisms applied in SSF:s on pre-treated samples are different, pentose fermenting yeast for hemi-cellulose rich samples (pentose sugars generating) from SEKAB E-Technology, and Ethanol Red which was applied on cellulose rich (hexose sugars generating) samples from PLET. Hexoses are clearly favoured compared to pentoses, since they are more easily metabolized by already established and preferred metabolic pathways in yeast. The metabolic pentose phosphate pathway is generally not expressed under normal fermentation conditions, whereas the glucose pathway is utilized by yeast as long as there is any glucose still present in media, which further delays the metabolic fermentation of pentoses into ethanol.

Purpose of pre-treatment is to break up the lignocellulosic matrix. The approach is to separate all three major components; cellulose, hemi-cellulose and lignin. It is apparent that the yield of fermentable sugars would be greater if a larger proportion of separated cellulose fraction can be recovered after a successful pre-treatment. Although, SEKAB E-Technology and PLET both use dilute acids in pre-treatment to avoid unnecessary fractional loss of desirable products, the process of PLET must be considered favoured because of milder process conditions, (= lower temperature and low pressure).

The question is, if the pre-treatment performed by PLET really break up the lignocellulosic matrix to a sufficient degree, without producing unwanted inhibitors like furfurals, HMF (= 5-Hydroxymethylfurfural), levulinic acid (= 4-oxopentanoic acid), and excessive loss of the expected products. The answer is that ethanol could be produced at high yield and low cost from the pre-treated cellulose fraction, when enzymes such as Cellic Ctec2 and microorganisms such as Ethanol Red are incorporated in the process.

Moreover, the enzymatic treatment with Cellic Ctec2 efficiency could also vary between the two pre-treatment technologies, since residues of either sulphur or nitrates or other inhibitors could interfere and decrease enzymatic activity, but this is just speculation. More experiments need to be performed to fully evaluate minimum enzyme loading contra substrate loading, to optimize the whole process if the concept will ever see an industrial application.

The Biostat B-Plus fermentor used in SSF and SHF experiments does not fully provide anaerobic conditions, which further decreases the yield of ethanol when yeast metabolize previously produced ethanol during the latter phase of fermentation. Samples extracted were frozen and thawed one time too much before analyzed in HPLC, due to rescheduled planning, which also could have generated minor losses of evaporated ethanol.

As has been shown by the so called “failed” experiments 4 and 5, on sweet liquor from PLET it is clear that this fraction probably contain the majority of all other constituents found in wood, including inhibitors such as HMF (= 5-Hydroxymethylfurfural), Levulinic acid (= 4-oxopentanoic acid) and salts. It would be interesting to apply Torula yeast, as suggested by PLET, on this fraction to see if a commercial grade unicellular protein actually can be produced instead.

7.2 Conclusions on the commercial potential

Before doing anything, the commercial potential of the concept have to be thoroughly evaluated and specified in a time schedule. Activities such as networking, mutual partnership agreements, licence agreements, patent rights, complementary research and process optimization, logistics, funding and production plant design, according to appropriate specifications must be organized within the project in order to ensure success.

At least 40 million SEK is needed as a minimum starting capital to be able to construct a small commercially viable pilot plant, according to PLET [26]. This investment would cover the basic requirements and is scalable. The construction could preferably be done in Sweden in the vicinity of an existing saw mill or paper pulp industry or elsewhere, where a steady supply of cheap raw material could be assured. Size of the operation envisioned, depends greatly on location and adjacent businesses. Large pulp mills in the range of 400-500 MWh would be preferred, the largest pulp mills in Canada process 5000 ton per day which is equivalent to a capacity of 1 GW wood per day (GW = giga watt) [45], but the concept of the technology of PLET must first prove its value in a much smaller scale. An initial investment of about 30 - 40 million SEK is thus reasonable in the first phase, and could later on be scaled up by adding multiple production lines [26].

Despite the simple design of CRP compared to conventional technology, it is hard to explain to investors and venture capitalist the whole concept. This is a general problem for the concept of bio-refining as a whole and not just for the concept of PLET, compared to the already established oil refinery industry, where practically all by-products generated by its refining processes have already found appropriate commercialization.

According to PLET there are some problems related to unwanted nitration on the aromatic ring of the main product Pure Lignin. This could actually be an advantage, given the possibility that a future not yet known application would be found. Nitration on aromatic rings are for example found in explosives such as trinitrotoluen (=TNT) [46]. Perhaps there is a commercial potential to produce new explosives from the pure lignin of PLET as well, or just to use the pure lignin as a raw material for TNT-production.

Unfortunately, because of the nitration of the lignin, it would not be recommended to use it as a combustible fuel, due to environmental regulations regarding emissions of nitrous gases. This is a major problem, considering that the lignin is regarded as a source of energy when incinerated in a heat and power plant. And this means that some commercial use of the lignin must be found. One such possibility is to use it as an additive in paint and other adhesive applications, where the pure lignin would perform well, thanks to its water solubility and high molecular weight.

Currently, it is extremely hard to find investors in Sweden because of general financial instability on the global markets. Especially while networking it was experienced that the patent issues were extremely important since the technology is not of Swedish origin. Very few Swedish investors appreciate and recognize an already approved patent as an attractive investment possibility unless it is domestic. A world patent such as the one PLET possesses would cost several hundred thousand SEK according to the Swedish PRV (= Patent och registreringsverket) [47], and it took PLET several years of processing before it was approved. Despite promises of an agreement to utilize a patented technology under licence, it is really hard to attract the interest from venture capitalist and other agencies.

The promise of a substantial capital investment inserted into the business is not a powerful argument for co-investors either. A small business such as PLET has not the sufficient support of a large organization. Neither does a small consulting business such as the one envisioned here in Sweden. It is important to understand that the initial investment must come from many different sources. Required funding, must be granted by multiple sources, such as national and international research programmes, scholarships, bank loans, own capital, venture capitalist, business institutes, crowd funding initiatives et c.

But more importantly, there is an urgent need for connections and business network, in order to find the appropriate representatives who have the business expertise and know how to set up business arrangements. It is evident that a "snowball effect" will spring into action once a major investor is found, but that is far from an eventual completion of a project.

It is by far too soon to discard the simple, environmentally friendly low cost pre-treatment method of PLET as a plausible alternative to produce next generation of lignocellulosic ethanol. More studies on samples derived from the pilot plant in Canada must be done as soon as possible. Positive results from these future studies would eventually lead to the construction of a larger demonstration plant, which would be used for even further analysis and optimization of process parameters.

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