Integration of a Hemicelluloses Extraction Step into a Forest Biorefinery for Production of Green Chemicals

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Abstract

Sustainable use of forest and agricultural resources will play an important role for solving urgent global challenges such as the enhanced greenhouse effect and increasing demand for fossil fuels. The development of processes where lignocellulosic biomass can be refined to several different end-products in the same plant, i.e. a biorefinery, will be important in the development towards a more sustainable society where fossil fuels are replaced. To be able to compete with fossil resources, an efficient production of biomass based products is required in order to maximize overall process economics and to minimize negative environmental impact. One solution to increase profitability for forest biomass based plants can be production of value added derivatives produced through fermentation of sugars from hemicelluloses, extracted from lignocellulosic material.

The first part of this thesis investigate the impact of hemicellulose pre-extraction on birch Kraft pulp properties. White liquor and water extractions of hemicelluloses from birch wood chips were performed under conditions compatible with Kraft pulping. The chips from select extractions were subject to subsequent Kraft pulping and the refined pulps were made into hand sheets. Several metrics for hand sheet strength properties were compared with a reference pulp made without an extraction step. This work also includes a demonstration of enzymatic hydrolysis and biological conversion of extracted xylan to succinic acid, a metabolite with the potential of a platform chemical. The study demonstrated that white liquor can be utilized to extract xylan from birch wood chips prior to Kraft cooking without decreasing the pulp yield and paper strength properties, while simultaneously impregnating cooking alkali into the wood chips. Alkaline conditions tested above pH 10 significantly degraded xylan and very low concentrations of xylose were obtained using any of the alkaline extractions. Water extractions resulted in the highest final concentration of xylose, 29.1 g/L; yielding fermentable liquor, but were found to negatively impact some pulp properties including decreases in compression strength, bursting strength, tensile strength and tensile stiffness while exhibiting minimal impact on elongation and slight improvement in tearing strength index. Since hot water extractions gave fermentable liquors, the next study was to integrate the production of green chemicals via hot water hemicellulose extraction of birch wood into a small-scale combined heat and power plant, in this case an externally fired gas turbine. The results show that the extracted wood chips would serve very well as a fuel for combustion and gasification processes due to the relatively high heating value. Most important, the extracted wood chips had low ash content.
and significantly lower concentrations of alkali metals. In addition a fermentable stream with a xylose concentration of 65 g/L was produced.

The second part of this thesis was to optimise the production of the dicarboxylic acid, succinic acid, which can be produced via bioconversion as a renewable building block molecule for production of biodegradable solvents and polyesters. In this study the *E. coli* strain AFP184, which can ferment both five and six carbon sugars with a limited production of other organic acids was used. Earlier work using a high initial sugar concentration resulted in volumetric productivities of almost 3 g/L h, which is above estimated values for economically feasible production, and final succinic acid concentration was around 40 g/L. To further increase succinic acid concentrations, fermentations using NH₄OH, NaOH, KOH, K₂CO₃, and Na₂CO₃ as neutralising agents were performed and compared. It was shown that substantial improvements could be made by using alkali bases to neutralise the fermentations. The highest concentrations and productivities were achieved when Na₂CO₃ was used, 77 g/L and 3 g/L h, respectively. A gradual decrease in succinate productivity was observed during the fermentations, which was shown to be due to succinate accumulation in the broth and not as a result of the addition of neutralising agent or the subsequent increase in osmolarity.
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List of papers

I  Impact of Hemicellulose Pre-Extraction for Bioconversion on Birch Kraft Pulp Properties
Jonas Helmerius, Jonas Vinblad von Walter, Ulrika Rova, Kris A. Berglund and David B. Hodge
Manuscript submitted to Bioresource Technology

II  Integration of a Hemicellulose Extraction Process into a Biomass Based Heat and Power Plant
Joakim Lundgren and Jonas Helmerius
Peer-reviewed full length paper, 2009, 22nd International Conference on Efficiency, Cost, Optimization, Simulation and Environmental Impact of Energy Systems

III  Inhibition of Succinic Acid Production in Metabolically Engineered Escherichia coli by Neutralising Agent, Organic Acids, and Osmolarity
Christian Andersson, Jonas Helmerius, David Hodge, Kris A. Berglund and Ulrika Rova
Biotechnology Progress 2009, 25(1): 116-123
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Introduction

Sustainable use of forest and agricultural resources is essential for solving urgent global challenges such as the enhanced greenhouse effect and the ever increasing demand for fossil fuels. The development of processes where lignocellulosic biomass can be refined to chemicals, fuels, paper and energy in the same plant, i.e. a biorefinery, will be important in the development towards a more sustainable society where fossil fuels are replaced. Although biomass derived chemicals have often have a low raw material cost. Bio based production of chemicals is often set back by a higher production cost compared to petroleum products (1), where the raw material cost is an important factor for the product price. To be able to compete with fossil resources and to minimize negative environmental impact, it is necessary to develop integrated and cost effective processes for conversion of biomass into products based on biomass conversion (2).

Forest biorefinery

Major uses of wood today are paper making, energy generation by burning or combustion, and as building materials (3). Kraft pulp mills are to a significant extent already a biorefinery where energy and pulp fibres are produced from wood and transported out to consumers, mostly paper production. In a combined heat and power (CHP) plant the wood biomass is combusted or burned to generate energy (steam) and power (electricity). The steam and electricity are delivered to other industrial processes or/and district heating. These already established wood biomass logistic capacities give an easy to envision starting point for integration of a process to achieve sugars for bioconversion to value added chemicals and for the development of the next generation of biorefineries.

The carbohydrate portion of lignocellulosic feedstock, where hemicelluloses are on of the major parts, is ideally suited to conversion via biochemical transformations using the catalytic power and specificity of microbes. Many enzymes and metabolic pathways exist for converting carbohydrates to a wide range of metabolites and through metabolic engineering, microbial catalysts can be optimized for the production of transportation fuels, biodegradable polymers, and chemical intermediates (4). Hemicelluloses have a low heating value (13.6 MJ/kg) compared to lignin (27.0 MJ/kg) (5) and one solution to increase profitability for industrial plants currently processing forest biomass, i.e. pulp plants and combined heat and power (CHP) plants, can be an integrated production of value added derivatives through fermentation of hemicelluloses sugars, extracted from lignocellulosic material, Figure 1.
It is important to analyse the effects of the hemicelluloses extraction step on the process into which it is integrated. Different process constraints must be considered such that minimal negative impact is achieved on the final bulk product, for example paper products. It is also important to recognize processes where the integrated hemicelluloses extraction can give a positive impact on the bulk product.

An example of a forest based biorefinery is the integration of a hemicelluloses extraction step prior to the Kraft process (paper I). In a Kraft pulp mill an aqueous caustic (NaOH) and sulphide (Na₂S) solution, called white liquor, is used to cook the wood chips. Lignin and a large fraction of hemicelluloses are dissolved into the liquor, called black liquor, which is burned to generate energy and recover cooking chemicals. Examples of process streams in a pulp mill that can be upgraded and catalytically transformed to high value fuels and chemicals include synthesis gas generated from black liquor gasification or the carbohydrate portion of the biomass. If the hemicelluloses are to be extracted prior to the Kraft process, the following effects must be considered simultaneously: 1) the effect of extraction liquors on the alkali impregnation of wood chips, 2) subsequent cooking requirements and changes in chemical recovery, 3) subsequent recovery and concentration of sugars for fermentations. For example, the paper quality can be affected negatively if the recovery of hemicelluloses is too high since the hemicellulose contributes to the paper strength properties (6). On the other hand, it is
necessary to extract as much hemicelluloses as possible to avoid costly processes to concentrate the hemicelluloses sugars to fermentable feedstock streams.

Another example is integration of a hemicelluloses extraction process into a biomass based heat and power plant (paper II). In an externally fired gas turbine (EFGT) it is of great importance to avoid fouling at the gas side of the heat exchanger surface. Fouling will decrease the heat transfer between gas and air leading to lower electrical efficiency and thereby higher electricity production costs. If advanced gas cleaning equipment must be installed, it may lead to unprofitable plants. The fouling is mainly caused by high concentrations of alkali metals and other ash forming elements in the fuel. It is therefore desirable to generate a cleaner gas, which could be accomplished by using a fuel with low ash forming elements content. Over 70 metals, earth elements and inorganic compounds have been found in wood biomass, with potassium, calcium, magnesium and phosphorous being the major elements in wood. These components are the first ones that can be extracted from wood (3). Through extraction of wood chips it can be possible to extract the ash forming elements and achieve wood chip residues with higher energy content per weight unit compared to fresh wood chips, and at the same time generate a fermentable feedstock stream.

**Hardwood and softwood**

Cellulose and hemicelluloses are the structural carbohydrates in wood that form the supporting structure of the plant cell wall, and between 20–35% of the dry weight of wood is hemicelluloses. Hardwood generally contains more cellulose and hemicelluloses, and less lignin than softwood, the amount of extractives, i.e. resin, is higher in hardwoods, Table 1.

<table>
<thead>
<tr>
<th>Species</th>
<th>Cellulose (%)</th>
<th>Hemicelluloses (%)</th>
<th>Lignin (%)</th>
<th>Extractives (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Softwoods</td>
<td>42</td>
<td>27</td>
<td>28</td>
<td>3</td>
</tr>
<tr>
<td>Birch</td>
<td>45</td>
<td>30</td>
<td>20</td>
<td>5</td>
</tr>
</tbody>
</table>

Hemicelluloses are the matrix substances between cellulose microfibrils, which are the framework of the cell wall. Lignin is the encrusting substance associated with the matrix substances solidifying the cell wall (7). The most striking difference between hardwood, e.g. birch and eucalyptus, and softwood, e.g. pine and spruce, fibres (tracheids for softwood) is that softwood tracheid is significantly longer and thicker than the hardwood fibre, and the structure of hardwood is more complex than softwood (8). Hardwood has different cells for support,
water transport and storage of nutrients, i.e. fibres, vessels and parenchyma cells, and softwood is composed of cells functioning as both support and water transport, mainly tracheids (90-95%) and parenchyma cells. The vessels in hardwood appear in transverse sections in wood as holes, which make hardwood much more porous and more available to chemical treatment than softwood. Softwood are comprised of a limited number and uniform cell types, about 3 axial and 2 radial, whereas hardwood has much greater cell morphology with 5–6 axial and 2 radial cell types. The strongest papers are made of softwood due to its long and strong tracheids while hardwood fibres give a better paper formation. Hardwood fibres are suitable for printing papers as the fibres give a smooth printing surface and high opacity, the ability to prevent the light to passage the paper. To meet both strength and printing properties hardwood and softwood pulps are blended.

**Hardwood and softwood hemicelluloses**

The majority of wood hemicelluloses, which are heteropolysaccharides, have a degree of polymerization up to 200 where the main monomeric pyranose units are hexoses; D-glucose (D-Glc), D-mannose (D-Man), D-galactose (D-Gal), and/or pentoses; D-xylose (D-Xyl), and furanose unit D-arabinose (D-Ara). Other units that occur in small amounts are L-rhamnose, L-fucose, 4-O-methyl-D-glucoronic acid (D-GlcA), D-galacturonic acid and D-glucuronic acid. Glucomannan is the most common hemicelluloses in softwood and hardwood is abundant in glucuronoxylan. Major hemicelluloses in softwoods and hardwoods are presented in Table 2.

**Table 2. Major hemicelluloses in hardwoods and softwoods, table adapted from Pettersen R.C., 1984 (9), and Timell T.E., 1967 (10)**

<table>
<thead>
<tr>
<th>Occurrence</th>
<th>Hemicelluloses</th>
<th>Amount % by Dry Weight</th>
<th>Units</th>
<th>Molar Ratio Approximate Values</th>
<th>Linkage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Softwood</td>
<td>Galactoglucomannan</td>
<td>5–10</td>
<td>β-D-Manp</td>
<td>3</td>
<td>1→4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>β-D-Glc</td>
<td>1</td>
<td>1→4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>α-D-Galp</td>
<td>1</td>
<td>1→6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>O-Acetyl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Softwood</td>
<td>Glucomannan</td>
<td>10–15</td>
<td>β-D-Manp</td>
<td>3-4</td>
<td>1→4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>β-D-Glc</td>
<td>1</td>
<td>1→4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>α-D-Galp</td>
<td>0.1</td>
<td>1→6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>O-Acetyl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Softwood</td>
<td>Arabinoglucuronoxylan</td>
<td>7–15</td>
<td>β-D-Xylp</td>
<td>10</td>
<td>1→4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4-O-Me-α-D-GlcA</td>
<td>2</td>
<td>1→2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>α-L-Araf</td>
<td>1.3</td>
<td>1→3</td>
</tr>
<tr>
<td>Hardwood</td>
<td>Glucuronoxylan</td>
<td>15–35</td>
<td>β-D-Xylp</td>
<td>10</td>
<td>1→4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4-O-Me-α-D-GlcA</td>
<td>1</td>
<td>1→2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>O-Acetyl</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Hardwood</td>
<td>Glucomannan</td>
<td>2–5</td>
<td>β-D-Manp</td>
<td>1-2</td>
<td>1→4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>β-D-Glc</td>
<td>1</td>
<td>1→4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>O-Acetyl</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
Xylan is a heteropolysaccharide having a backbone of \(\beta-1,4\)-linked xylose units with O-acetyl, 4-O-methyl-D-glucoronic acid and arabinofuranosyl substituents. Softwood xylan is mainly arabino-4-O-methyl glucuronoxylan (arabinoglucuronoxylan) with side groups of \(\alpha\)-arabinofuranoside units. The ratio of arabinofuranoside groups to xylose residues is approximately 1:8 and acetyl groups are rarely attached to softwood xylan. Hardwood xylan is mainly \(O\)-acetyl-4-O-methyl glucuronoxylan (glucuronoxylan). Glucuronoxylan is highly acetylated with a ratio acetyl groups at C\(_2\) and C\(_3\) positions to xylose residues of approximately 7:10 (9, 10). The degree of side group substitution of xylan affects the solubility and its ability to bind to cellulose. A high degree of side groups or side chains are more water soluble and bind less tight to cellulose and vice versa.

**Extraction of hemicelluloses**

Hemicelluloses extractions have a numerous important operational factors. The chemical alterations and solubility of hemicelluloses, celluloses and lignin depend on the composition of the extraction liquor, temperature and incubation time, which subsequently impact the composition of the extracted liquor and the extracted wood chips. If hemicelluloses are to be extracted from lignocellulosic material for subsequent fermentation it is necessary to leave both cellulose and lignin as essentially un-degraded polymers. The problem in refining lignocellulosic materials is that cellulose, hemicelluloses and lignin, cannot be simultaneously isolated as polymers because the processes used involve the degradation of at least one of the polymers (11). The chemical and thermal stability of hemicelluloses are generally lower than that of cellulose (12). Cellulose is crystalline and has a high degree of polymerisation. The strong and regular interactions between the chains and the organisation of fibrils give cellulose unusual properties among polysaccharides (7, 13), For example, cellulose is totally insoluble in water in spite of all hydroxyl groups present. However, there are a number of suitable solvents for cellulose, ranging from mineral acids to strong alkali, but they might cause hydrolysis and other chemical changes in the cellulose. The cellulose surface is very hydrophilic, e.g. defatted cotton can absorb ten times its own weight of water. The top and bottom of each glucose unit in cellulose is hydrophobic and have a similar size as aromatic rings, which might be important for the interaction of the aromatic polymer lignin with cellulose. Hemicelluloses are amorphous and contain non-glucose units with different ring structures and hydroxyl configurations than the glucose residues. These other sugars units generally have higher reactivity than the glucose residues, which often makes hemicelluloses more selectively removed from lignocellulosic substrates than cellulose (13).
**Lignin-carbohydrate complexes (LCC)**

Results from a number of studies strongly support the existence of covalent linkages between the lignin and wood polysaccharides, which together form lignin-carbohydrate complexes (LCC) (13). The major possible lignin-carbohydrate linkages are benzyl ester, benzyl ether and glycosidic bonds. The hydrolysis behavior of these bonds varies considerably with their chemical structures and the reaction environments. For example, under alkaline conditions the ester type is readily hydrolysed, and the etherified unit is comparatively stable even under alkaline pulping conditions. The hydrolysis of ester bonds is probably the result of saponification of lignin-carbohydrate linkages associated with the 4-O-methylglucuronic acid units of xylan. Presence of ester linkages between lignin and glucuronic acid in glucuronoxylan has been suggested and around one-third of the glucuronic acid present in the LCCs are estimated to be involved in the ester linkages (14). It is also suggested that softwood LCCs consist of linkages between lignin benzyl positions and galactoglucomannan, arabinool-4-O-methylglucuronoxylan and arabinogalactan, and that hardwood LCCs are exclusively linked between lignin and 4-O-methylglucuronoxylan (15). The ether type of linkage can involve all types of polysaccharides in wood, including xylan, galactoglucomannan and cellulose (16-20). The ether linkages are alkali stable and the nature of these linkages has been obtained mostly from analysing the sugar residues following the typical methylation, Smith degradation and acid hydrolysis (21, 22). The glycosidic linkages between lignin and polysaccharides have not been thoroughly investigated and most evidence exists for ether and ester linkages (13, 15).

**Hot alkali and water pretreatment of hardwood**

There are a wide variety of possible approaches for hemicelluloses extraction or pretreatment (23-26) including pretreatments that span the complete range of pH and can use a wide range of inorganic lignin-acting reagents such as sulphur, ammonia, or oxygen. The generation of compounds that are inhibitory to microbial processing such as acetic acid, sugar degradation products, phenolic compounds from partial breakdown of lignin, and the inorganics as sulphur and sodium, in the extracted liquor need to be considered for process integration. High sugar concentrations in the final extracted liquors are essential for an economically feasible conversion of sugar to desired fermentation product. Alkaline or acidic conditions will lead to different mechanisms of degradation during extraction of hemicelluloses from hardwood.

The mechanism behind hardwood hemicelluloses solubilisation using alkaline hydrolysis, is proposed to be saponification of intermolecular ester bonds cross-linking xylan hemicelluloses,
lignin and other hemicelluloses (26). Under conditions of concentrated alkali, polysaccharides undergo degradation reactions that are important during extraction of hemicelluloses, these reactions are also significant reactions at elevated temperatures and pH during pulping and bleaching processes, such as Kraft pulping and alkaline sulphite pulping. The first reaction under alkaline conditions is random cleavage of glycoside linkages along the polysaccharide chain, relatively few breaks decreases the average degree of polymerisation and also reduces fibre strength. The second reaction, the alkaline peeling reaction, cleaves sugar units from the reducing end by breaking of a glycoside adjacent to the end unit containing a carbonyl group. The stopping reaction ends the alkaline peeling reaction by leaving the reducing end of polysaccharide as a carboxylic acid (27). The alkaline peeling reaction degrades glucomannan rapidly under alkaline conditions, while solubilised oligomeric xylan is more stable due to the methyl glucuronic acid side chains. Thus, hot alkali pretreatment can be suitable to extract hemicelluloses from hardwood but not from softwood (28).

High temperature and strong alkaline conditions can form over 100 different compounds through oxidation, fragmentation and dehydration reactions. The presence of oxygen yields products which lead to the formation of a number compounds such as glyceric acid, pyruvic acid, lactic acid, formaldehyde, glyoxal, oxalic acid, acetic acid, formic acid, erythrose, and saccharinic acids. Alkaline dehydration reactions can lead to formation of hydroxymethylfurfural (HMF) and further to levulinic acid (29). Dilute alkaline conditions during pre-treatment of lignocellulosic material can slowly cause C-2 epimerisation, a change in configuration of the second carbon atom, of monosaccharides and the reducing group of polysaccharides, Table 3. Alkali is more effective than acid to catalyze aldose-ketose isomerisation and epimerisation reactions and most of carbohydrates have increasing stability with decreasing pH, with the highest stability at pH 3-4 (29).

Table 3. Products from 5-carbon xylose and 6-carbon glucose treated under acidic and alkali conditions, table adapted from Biermann J.C. 1996, (27)

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Strong OH</th>
<th>Weak OH</th>
<th>Weak H⁺</th>
<th>Strong H⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylose</td>
<td>Acids</td>
<td>C-2 epimers</td>
<td>No reaction</td>
<td>Furfural</td>
</tr>
<tr>
<td>Glucose</td>
<td>Acids</td>
<td>C-2 epimers</td>
<td>No reaction</td>
<td>HMF*</td>
</tr>
</tbody>
</table>

* 5-(hydroxymethyl) furfural
The mechanism behind hot water extraction of hardwood xylan is a self catalytic process, autohydrolysis. This mechanism of hydrolysis lies in cleavage of O-acetyl and uronic acid substitutions that result in the formation of acetic and other organic acids, which makes it possible for further hydrolysis of polysaccharides to oligomers and monomers (30). The hot water extraction can be considered as mild acid hydrolysis. The main degradation pathways of hemicelluloses under acidic conditions liberate xylose, mannose, galactose, glucose, and acetic acid. If the temperature and pressure is too high or incubation time too long during water and acid hemicelluloses extraction, the degradation of xylan can proceed further (31). Degradation of xylose to furfural is possible and degradation of hexose to 5-hydroxymethylfurfural (HMF) also proceeds, Table 3. When furfural and HMF are broken down formic acid is formed, degradation of HMF can also lead to formation of levulinic acid. Partial breakdown of lignin and carbohydrate degradation can generate phenolic compounds.

Hot water pretreatment of wood material is less severe compared to dilute acid or alkali pretreatment, and has been shown to increase accessible surface area, removes hemicelluloses, and alter lignin structure slightly (21). Hot water pre-treatment maintains a liquid phase under pressure, keeping the pH not too low in order to avoid cellulose hydrolysis and sugar degradation reactions (32). Autohydrolysis has a wide range of applications including (11); 1) fractionation or pulping processes, in which there is removal of hemicelluloses with selectivity towards cellulose degradation and splitting the ether bonds of lignin, 2) defibration for fibreboard production, in process using high pressure steam, and 3) as a pretreatment for the enzymatic hydrolysis of cellulose. Autohydrolysis limits corrosion problems and generates no sludge; while capital and operational costs are low (21). Degradation of hardwood hemicelluloses probably occurs at a higher rate during alkali condition than acidic condition, but acidic conditions maintain more of oligomeric xylan and monomeric xylose in extracted liquid.

Alkaline pre-treatment of wood chips can be considered well-integrated with Kraft pulping, since it will lower alkali charge when cooking. It is further established that solubilized polysaccharides are almost completely degraded to saccharinic and hydroxyl acids by the completion of Kraft pulping (30, 33). Alkali treatment at moderate temperatures is an established laboratory method for extracting hemicelluloses (34-36), and is the basis of at least one approach for hemicelluloses extraction from wood prior to pulping (37). In order to extract the hemicelluloses from aspen wood (hardwood) chips, a mild alkaline, low
temperature, pretreatment was applied to the wood chips (37). The extraction was performed at 50°C with 2.08 M NaOH and 90°C with 1.67 M NaOH, liquid to dry wood ratio of 4:1, for 4 hours. The recovery of hemicelluloses was 40-50 kg per ton chips and the yield of pulp after Kraft cooking the extracted residues was the same as for a control cook. The pretreatment of chips allowed shorter cooking time and lower chemical charges. The Kraft pulps obtained from extracted chips had a decrease in tensile strength, around 10%, compared to the control pulp, improved brightness and lower shive content. The yield of hemicelluloses was low, and under the conditions used possible maximum concentration of fermentable sugars in liquor stream was 15 g/kg liquor. When the same liquid to dry wood ratio was used during a hot water extraction it resulted in a liquor containing 35 g/kg fermentable sugars (3) (see below). The effect of hot water extraction of hemicelluloses and recovery is not well documented for combining pulp and paper production with bioconversion of hemicelluloses.

In order to extract sugar maple (hardwood) wood chips (3), hot water extractions were performed isothermally at 160°C for 2 hours with water to solid ratio of 4:1. The heating time to 160°C was 150 minutes. Table 4 shows the main components before and after extraction. It can be observed that most of the cellulose (glucose) and acid insoluble lignin is retained by the residual wood chips and that the hemicelluloses (xylose) part is found in the extraction liquor. If the water to solid ratio is 4:1, a xylose concentration was achieved in final extraction liquor of approximately 25 g/kg liquor, total sugars 35 g/kg liquor. The pH dropped from 6.3 to final 3.5, mostly due to formation of acetic acid. The higher lignin content in residual wood chips indicates a higher heating value per weight in wood chip residues compared to the fresh wood chips. The extracted wood chips were also pulped and the overall yield was low, 40.4%, and 12% lower than a control pulp made of untreated chips. Compared with the alkali extraction above (37), where there was no loss in pulp yield, it can be concluded that hot water extractions affect pulp yield negatively, but the yield of sugars in liquor is much higher.

Table 4. The distribution of carbohydrates, organic acids and lignin before and after the hot water extraction of sugar maple wood chips. Other refers to the sugars that have been converted to compounds that were not identified during analysis. Carbohydrate compositions were measured by sulphuric acid hydrolysis followed by H NMR (3). The total weight in each row is higher than total weight of solids due to conversion from polymeric chains to monomers. Glucose (Glc), Xylose (Xyl), Mannose (Man), Galactose (Gal), Arabinose (Ara), Rhamnose (Rha), Acetic acid (AA), Acid Insoluble Lignin (AIL), Acid Soluble Lignin (ASL).

<table>
<thead>
<tr>
<th>Dry Solids</th>
<th>Glc (g)</th>
<th>Xyl (g)</th>
<th>Man (g)</th>
<th>Gal (g)</th>
<th>Ara (g)</th>
<th>Rha (g)</th>
<th>AA (g)</th>
<th>AIL (g)</th>
<th>ASL (g)</th>
<th>Other (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wood chips: 100 g</td>
<td>45.30</td>
<td>17.52</td>
<td>2.37</td>
<td>0.89</td>
<td>0.66</td>
<td>0.47</td>
<td>3.57</td>
<td>22.30</td>
<td>2.94</td>
<td>12.46</td>
</tr>
<tr>
<td>Extract: 23 g</td>
<td>4.98</td>
<td>10.16</td>
<td>1.04</td>
<td>1.46</td>
<td>0.62</td>
<td>0.92</td>
<td>1.76</td>
<td>1.22</td>
<td>2.05</td>
<td>5.22</td>
</tr>
</tbody>
</table>
Post-hydrolysis of extracted xylan

A secondary hydrolysis, acid or enzymatic, is needed to depolymerise polymeric xylan to xylose, if this is to be used as substrate in fermentation processes. Depending on the process conditions during the secondary hydrolysis, different toxic and inhibitory compounds can be formed in the liquor and degraded xylan that lower the xylose concentration in the final extracted liquor (31, 38). Large quantities of acetic acid can be released when hydrolysing xylan from pretreated hardwood. Even at low concentrations acetic acid can have an inhibitory affect on both microbial growth and product formation, although aerobically, common bacteria and yeast such as *Escherichia coli* and *Saccharomyces cerevisiae* can metabolise acetic acid for growth (39-41). Enzymatic hydrolysis of extracted liquor is much less severe than acid hydrolysis and no toxic or inhibitory compounds can form by sugar degradation, Table 5. The drawbacks for enzymatic hydrolysis are that the yield of monomeric xylose on xylan is less and enzymes are expensive compared with acids used in hydrolysis, also enzymatic hydrolysis takes much longer time. If acid hydrolysis is used, it requires a detoxification step, while enzyme hydrolysis can be performed without detoxification in the same reactor as the fermentation, prior or/and simultaneous to the fermentation.

Table 5. Recovery and relative composition of enzyme and acid hydrolysed oligosaccharide containing hydrolysate obtained from autohydrolysed BSG. The commercial enzyme preparations were diluted in 0.05 M sodium citrate buffer of pH 5.5, and 1 ml of enzyme mixture was added to 25 ml of oligomeric containing liquor. Table adapted form Duarte, L. C., et al, 2004 (42)

<table>
<thead>
<tr>
<th>Enzyme mixture</th>
<th>Glucose (%)</th>
<th>Xylose (%)</th>
<th>Arabinose (%)</th>
<th>Acetic acid (%)</th>
<th>HMF (%)</th>
<th>Furfural (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Celluclast 1.5L</td>
<td>94</td>
<td>63</td>
<td>77</td>
<td>115</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Novozyme 342</td>
<td>52</td>
<td>36</td>
<td>79</td>
<td>92</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Viscozyme L</td>
<td>119</td>
<td>63</td>
<td>74</td>
<td>105</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pentopan 500BG</td>
<td>44</td>
<td>32</td>
<td>77</td>
<td>96</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pulpzyme HC</td>
<td>9</td>
<td>12</td>
<td>52</td>
<td>68</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Multifect Xylanase</td>
<td>9</td>
<td>28</td>
<td>60</td>
<td>86</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Multifect GC</td>
<td>13</td>
<td>46</td>
<td>69</td>
<td>90</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Sulphuric acid hydrolysis is compared with enzyme hydrolysis in Table 5, using different commercial enzyme mixtures. The oligosaccharide containing hydrolysate was obtained through autohydrolysis of starch free Brewery’s spent grain (BSG) (42), and the hydrolysate contained mostly oligomeric arabinoglucuronoxylan which is not readily acetylated.
Catalyst concentration, reaction time and temperature are the most important factors affecting dilute acid hydrolysis. Enzyme hydrolysis is also dependent on factors as pH-regulation, substrate structure and enzymatic activities. The main differences between the enzyme mixtures in Table 5 is the enzyme activity and concentration of each individual enzyme and by mixing different enzyme mixtures it can be possible to reach higher yields of sugars (43). Important enzymes for xylan degradation are endo-1, 4-β-xylanases that attack the main chain, β-xylosidases hydrolysing oligomers to xylose, and also enzymes such as acetyl esterases which release the acetyl group from the pyranose unit, α-Glucuronidases and α-arabinofuranosidases which liberates substituents from the main chain (44).

**Fermentation**

Many organic acids can be produced through biocatalytic processes since they are intermediates or/and products of the cellular metabolism. Figure 2 shows the glycolysis and mixed acid fermentation in *Escherichia coli*, starting with glucose as a substrate. To increase the production of a metabolite, an organism can be metabolic engineered where the characteristics of one or several genes are altered to direct the carbon flow towards the wanted end product. The organism can also be designed to tolerate different toxic and inhibitory compounds and genetically engineered to utilise substrates not normally utilised by the organism.

![Figure 2. Schematic sketch of glycolysis and the mixed acid fermentation in *Escherichia coli*, starting with glucose](image_url)
The extracted liquor from wood biomass and the post-hydrolysis of polysaccharides must comply with the fermentation demands, such as high substrate (sugar) concentration and with a minimal concentration of inhibitory and toxic compounds. This is important so that the screening and optimisation of the fermentation towards high productivity and high final concentration of biofuels and chemicals can be achieved.

**Production of carboxylic acids through fermentation**

Citric, acetic and lactic acid are the most significant carboxylic acids with large existing markets, which can be produced through fermentation processes (45-47). Today citric acid is primarily produced biochemically by aerobic cultivation of *Aspergillus niger*, the applications includes food and beverage acidulates, and pharmaceuticals. Lactic acid is produced biochemically by anaerobic cultivation of *Bacillus* and *Lactobacillus*, and polylactic acid is used in the production of biodegradable polymers. Acetic acid is today mostly produced in the petrochemical industry by carbonylation of methanol for vinyl acetate used for the production of polymers and solvents. Acetic acid is also produced in a much smaller scale biochemically by aerobic cultivation of *Acetobacter* and anaerobic by *Clostridium* for producing vinegar.

**Succinic acid**

One of the top twelve building block chemicals produced from biomass is succinic acid according to a report from the U.S. Department of Energy (48, 49). Building block chemicals are molecules with multiple functional groups that possess the potential to be transformed into new families of useful molecules. Figure 3 show feed stocks for producing succinic acid and derivates produced from succinic acid. Succinic acid, a dicarboxylic acid, is today mainly produced from butane through maleic anhydride in petrochemical processes (50), and is mainly used as a surfactant, detergent extender and foaming agent. If succinic acid is produced using biomass instead of petrochemicals as raw material the feed stocks are renewable and the production is not contributing to the accumulation of greenhouse gases. Chemicals based on benzene and other intermediate petrochemicals can be substituted with succinic acid for production of biodegradable polymers and solvents. Other chemicals that can be produced from succinic acid are food ingredients, fuel additives and plant growth stimulants (50).
Succinic acid production through fermentation has been demonstrated with a number of different organisms (51-57) where one of the most studied is *Anaerobiospirillum succiniciproducens* (58, 59). Table 6 present some of the organisms and their volumetric productivity, final concentrations and yield. *A. succiniciproducens* and *M. succiniciproducens* has been demonstrated to ferment glucose and xylose/glucose, respectively, derived from hardwood hydrolysates (58, 60). The fermentations using *A. succinogenes* were performed in vial flasks or one litre reactors and have not been repeated in larger scale (61-63), and only with reagent grade glucose added to media. Succinic acid concentrations above 80 g/L have been achieved in fermentations using *A. succinogenes*, fermentation neutralised with MgCO₃. Using neutralising agents NH₄OH or sodium alkali, the succinic acid concentration reached up to around 60 g/L.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Yield [g(SA)/g(Sugar)]</th>
<th>Productivity [g/L h]</th>
<th>Concentration [g (SA)/L]</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anaerobiospirillum succiniciproducens</em>.</td>
<td>0.88</td>
<td>10.0</td>
<td>30.0-35.0</td>
<td>(58, 59)</td>
</tr>
<tr>
<td>Strict anaerobe</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Mannheimia succiniciproducens</em>.</td>
<td>0.70</td>
<td>3.9</td>
<td>50.0</td>
<td>(53, 64, 65)</td>
</tr>
<tr>
<td>Facultative anaerobe</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Actinobacillus succinogenes</em>.</td>
<td>0.85</td>
<td>No data</td>
<td>105.8</td>
<td>(61-63)</td>
</tr>
<tr>
<td>Facultative anaerobe</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 3.** Succinic acid feed stocks and products
The facultative anaerobe *E. coli* is known to produce a mixture of organic acids and ethanol under anaerobic conditions (66), Figure 2. The fermentation typically yields 0.8 moles ethanol, 1.2 moles formic acid, 0.1-0.2 moles lactic acid, and 0.3-0.4 moles succinic acid per mole glucose consumed. In the 1990s US Department of Energy initiated the Alternative Feedstock Program (AFP) with the aim to metabolically engineer *E. coli* strains to increase succinic acid production, and promising mutants developed by the program were AFP111 and AFP184 (52, 67, 68). AFP111 is a spontaneous mutant and the mutations resulted in increased succinic acid yield of 1 mole succinic acid per mole glucose. AFP184 is metabolically engineered by deliberately insert three mutations, that were spontaneous in AFP111, into the wild type *E. coli* strain C600 (ATCC 23724), which can ferment both 5- and 6-carbon sugars and have strong growth characteristics (67). Fermentations performed with *E. coli* strain AFP111 and AFP184 are called dual-phase fermentations, one relative shorter aerobic growth phase to desired cell density and then transition to an anaerobic succinic acid production phase were CO₂ is consumed. Final succinic acid concentrations above 60 g/L using NH₄OH or sodium alkali as neutralising agent has been reported using metabolic engineered *E. coli* strains (69-71).

The increased interests for biodegradable polymers give succinic acid production the opportunity to be an economical driving force for a current biomass processing plant. However, costs of manufacturing succinic acid are affected by raw material costs, utilisation, productivity and yield, and recovery methods. To develop a bio based industrial production there are three main issues. The first and most important factor is the volumetric productivity (paper III), to make succinic acid production economically feasible it will be necessary to achieve productivities above 2.5 g/L h (48). Second is that a low cost medium must be used and third, the organism used must be able to utilise a wide range of sugar feedstock and produce succinic acid in high yields.
Present investigation

The development of processes where lignocellulosic biomass can be refined to several different end-products in the same plant, i.e. a biorefinery, will be important in the development towards a more sustainable society where fossil fuels are replaced. In this present study the integration of hemicelluloses extraction step into two different processes were investigated, the Kraft process and a combined heat and power plant (CHP). The extracted mono- and oligosaccharide containing liquor and extracted hardwood chips were evaluated in both cases. Also in order to improve the economical viability of bioconversion of sugars for succinic acid production the final succinic acid concentration must be increased and the volumetric productivity should be maintained at elevated values, above 2.5 g/L h, for an extended period of time. Following approaches were applied in present study:

- The combination of hemicelluloses extraction with chemical pulping processes is one approach to generate a sugar feedstock amenable to biochemical transformation to fuels and chemicals. White liquor and water extractions of hemicelluloses from birch wood chips were performed under conditions compatible with Kraft pulping. The chips from select extractions were subject to subsequent Kraft pulping and the refined pulps were made into hand sheets. Several metrics for hand sheet strength properties were compared with a reference pulp made without an extraction step.

- The idea to integrate the production of green chemicals via hot water hemicelluloses extraction of birch wood into a small-scale combined heat and power plant. The fresh wood chips, extraction residues and extraction liquor were analysed in order to evaluate how the extraction residues and final extraction liquor will serve as a fuel for combustion and/or gasification, and as a fermentable xylose stream, respectively. A techno-economically successful concept could provide the option to turn a small- to medium scale CHP plant into a small- to medium scale biorefinery.

- Optimising the production of succinic acid via bioconversion as a renewable building block molecule for production of biodegradable solvents and polymers. In this study the *E. coli* strain AFP184, which can ferment both five and six carbon sugars with a limited production of other organic acids was used. Earlier work using a high initial sugar concentration resulted in volumetric productivities of almost 3
g/L h, which is above estimated values for economically feasible production, and final succinic acid concentration was around 40 g/L. To further increase succinic acid concentrations, fermentations using different neutralising agents were evaluated.
Paper I

This study presents the combination of birch wood (*Betula pendula*) hemicelluloses extraction with Kraft pulping to generate a sugar feedstock amenable for bioconversion to fuels and chemicals. In the Kraft process a large fraction of the hemicelluloses, together with the lignin, are lost to the black liquor stream during cooking. The black liquor is evaporated and further burnt in the recovery boiler to recover cooking chemicals (NaOH and Na2S) and to produce the heat and power requirements for the mill. Most of the hemicelluloses dissolved from wood chips during cooking are completely degraded to hydroxy and saccharinic acids due to the severe alkaline conditions (30, 33). The hemicelluloses that are retained in wood chips affect the pulp properties and subsequently also the paper quality, for example the paper strength properties (6). Considering that hemicelluloses has a low heating value (13.6 MJ/kg) compared to lignin (27.0 MJ/kg) (5), recovery of hemicelluloses at an early stage of the Kraft process followed by bioconversion into value added products such as ethanol and succinic acid might provide a more diverse product portfolio with potentially improved economics. By extracting the wood chips with white liquor alkali, low effective alkali charge, or water prior cooking it can be possible to provide a fermentable sugar feedstock and in the case of using white liquor at the same time impregnate the wood chips and subsequently lower the alkali charge during cooking (37).

In order to assess the potential of using the xylose contained in hardwood 4-O-methyl glucuronoxylan (xylan) as a feedstock for bioconversion and the effect of its removal on pulp properties, a number of trials were performed to extract xylan prior to pulping. This screening was used to identify conditions fulfilling equipment and process integration constraints, such as yield, final pH, and concentration of total xylan. The xylan was measured as xylose. At a liquid to dry wood ratio of 3:1, the extraction times ranged between 20-90 minutes, temperatures 130°C-160°C, effective alkali charges 0%-7%. The chips from select extractions were pulped and refined and made into handsheets. Metrics for handsheet structural, strength, stiffness, surface and optical properties were compared with a reference pulp made without a preceding extraction step. This work also includes a demonstration of enzymatic hydrolysis and biological conversion of extracted xylan to succinic acid, a metabolite with a potential use as a platform chemical.
The screening of the xylan extraction showed that the glucan (measured as glucose) concentration in the final extraction liquors did not exceed 1.1 g/L in any extractions performed, indicating that most of the cellulose was retained in wood chips. The xylan+xylose concentration ranged from 0.1 g/L to 29.1 g/L. Water extractions at 150°C and 160°C, hold times 60-90 minutes resulted in the highest final xylose concentrations in the liquor. The highest final xylose concentration, 29.1 g/L, was at the expense of high material losses, 76.9% mass yield after extraction. Increasing the effective alkali (EA) charge using white liquor resulted in a decreased yield, less material for pulp production, and decreased final xylose concentration, less substrate for fermentation process. Decreased EA charge, increased temperature and longer reaction time increased the final xylose concentration in extracted liquor. Only the hot water and 3% EA extractions showed increasing final xylose concentrations with increasing total solids extracted. Four of the white liquor alkali extractions using EA charge of 3% reached final xylose concentrations above 2.9 g/L in liquor, with the highest concentration of 5.8 g/L, corresponding to approximately 8.7% of the xylan content of the birch wood. Two of these extractions had acceptable material losses, above 91% of material left for pulping. The neutralisation of hydroxide ions by the acetic acid liberated from the xylan during extraction, birch wood xylan is highly acetylated, decreased the final pH with increasing reaction severity, (temperature and time). The highest EA charge of 7% resulted in higher amounts of residual alkali in extracted liquor and at 130°C, 20 minutes the final pH was 11.92, slightly below the final pH of an ordinary cook.

The final concentration of xylose was not promising in most of the alkali extractions performed using white liquor and from the screening, several conditions resulting in higher final xylose concentrations at high wood yields were selected for further investigations. To compare different pulps obtained at different conditions, the same target extent of delignification had to be reached, in this case a K-number of 16-18. The H-factor is widely used for characterising delignification during alkaline pulping and collapses time and temperature into a single reaction ordinate to predict delignification. H-factors for the subsequent pulping conditions for extracted materials were estimated based on the knowledge of the trends for yield and K-number to reach the targeted K-number. The selected extractions conditions were repeated in order to perform Kraft pulping on the extracted chips. The new final xylose concentration and yields are presented in Table 7, together with pulping data; the new extraction data were comparable with the original screening data.
Table 7. Properties for the cooking and resulting K-number, total yield, and also recovered xylose from the xylan extractions performed prior to cooking. Calculated K-numbers in parentheses.

<table>
<thead>
<tr>
<th>Cond.</th>
<th>Extraction</th>
<th>Cond.</th>
<th>Pulping</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time (min)</td>
<td>Temp (°C)</td>
<td>Yield (%)</td>
</tr>
<tr>
<td>Ref.</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>90</td>
<td>150</td>
<td>92</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>150</td>
<td>96</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>160</td>
<td>91</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>150</td>
<td>92</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>160</td>
<td>94</td>
</tr>
</tbody>
</table>

The properties of handsheets from the reference pulp and all the extracted pulps were normalised in terms of the freeness (°MSR) and significant differences in pulp properties between extraction conditions were obvious for beating energy, brightness, compression strength index, tensile strength index and tensile stiffness index.

The reference material had the highest freeness with increased refining (beating energy), together with material obtained using condition 2 and 4. The other hemicelluloses extracted materials did not have the same increase in freeness with increased refining, indicating losses of hemicelluloses and less internal fibrillation with increased refining. Pulps from water extracted material condition 2 and alkali extracted material condition 4 were the most similar in terms of properties to the reference pulp, for all paper tests performed. Water extracted material condition 1 had the highest value of tearing strength index, which is interesting since it had the highest concentration of xylose, 11.7 g/L, in extracted liquor, Table 7. The lowest value of brightness at all freeness levels had the reference sample even though it had the highest K-number. Condensation of lignin at low residual alkali in the hemicelluloses extraction might contribute to a darker sheet or alternatively may be due to less dense material absorbing more light. The water extracted materials 1 and 2 had higher elongation properties and lower values of compression strength index compared to the reference. More flexible fibers increase the bonding that increases the resistance against compression. Elongation is the opposite of compression and elongation increases with increasing shrinkage and higher moisture content. Less flexible fibers contains maybe more moisture since it avoids brakeage from the capillary forces upon drying, and this could maybe explain the higher elongation among the water extracted materials. Fibre flexibility and strength are the most significant factors affecting the tensile strength. Less shrinkage, more flexible fibers, increased the tensile strength index for the reference and condition 4. Also, the reference pulp and pulps from condition 2 and 4 had more flexible fibres with more bonding between fibres giving higher values of tensile energy absorption index compared to the other pulps. The tensile stiffness properties indicated that the
pulps from water extracted birch wood chips can give unsatisfactory bending stiffness due to very low tensile stiffness. Bursting strength index and tensile strength index showed almost the same result, reference pulp and pulp from condition 4 are similar in properties due to more bonding between fibres.

To demonstrate the feasibility of at least one of the extraction approaches, water extraction, with regard to biological process integration, enzymatic hydrolysis of the xylan was performed together with fermentation of the xylose to succinic acid by a metabolically engineered *E. coli*. Enzymatic hydrolysis using commercial enzyme mixtures Pulpzyme HC and Celluclast 1.5 from Novozymes®, Denmark, showed that the enzymatic de-polymerisation of the acetylated xylan is a feasible approach since the yield of monomeric xylose was around 75%. The subsequent dual-phase fermentation of xylose to succinate showed that during the aerobic growth phase both acetate and xylose were consumed as substrates for cell growth, while under the anaerobic phase the remaining xylose was converted into succinate at a yield of approximately 0.73 g succinate/g xylose. Since the acetate can be utilised as a carbon source for growth during the aerobic phase the first fermentation phase will act as a detoxification step for the subsequent anaerobic succinic acid product phase.

There are a number of specific criteria for effective process integration of a hemicelluloses extraction step prior to Kraft pulping, providing sugar for bioconversion to value added chemicals. One is that the extraction cause minimal impact on the overall process and the quality of the resulting pulp. Second, it is important to obtain high final hemicelluloses sugar concentrations in the extracted liquor while minimising the loss in pulp yield due to the equipment requirements both for downstream bioconversion and the pulping process. Third, the pH after extraction must not be too low that uneven impregnation of the wood chips by alkali occurs. Finally, the generation of compounds that is inhibitory to microbial processing in the extracted liquor need to be considered for process integration if the same liquor is to be used during the biological conversion. Approaching with the results achieved all of these criteria are discussed in Paper I.
This study presents the idea to integrate the production of green chemicals via hot water hemicelluloses extraction, autohydrolysis, of birch wood (hardwood) into a small-scale combined heat and power (CHP) plant, in this case an externally fired gas turbine (EFGT) (72, 73). Birch wood hemicelluloses constitute mainly of glucuronoxylan (xylan), around 25-30% of dry wood content, which is highly acetylated, acetic acid/xylose unit ratio of approximately 7:10 (9, 10). In an EFGT it is of great importance to avoid fouling, caused by alkali metals and other ash forming elements in the fuel, at the gas side of the heat exchanger surface. Fouling will decrease heat transfer between gas and air leading to lower electrical efficiency. By extracting the fresh wood chips before combustion it can be possible to extract the ash forming elements together with parts of the hemicelluloses and provide (3):

- Cleaner wood chip residues with higher energy content per weight unit compared to fresh wood chips
- A fermentable sugar stream, amenable for bioconversion to green chemicals using microbial catalysts

In order to evaluate how the extraction residues and final extraction liquor will serve as a fuel for combustion and/or gasification, and as a fermentable xylose stream, respectively, water extractions were performed in rotating autoclave cylinders for 90 minutes isothermally. The cylinders were heated to target temperatures between 160-180°C at a rate of approximately 1.6°C/min. Liquid to dry wood ratio (L/DW) was between 1.44 and 2.37. In order to investigate how the chemical properties and characteristics of the wood chips changed after hot water extractions, the extracted residues and the fresh wood chips were evaluated. The free liquor was collected after extraction and analysed for its contents of xylose, glucose, acetic acid, hydroxymethylfurfural (HMF) and furfural before and after acid hydrolysis (4% H₂SO₄, 60 minutes at 121°C).

The carbohydrate and lignin content of feedstock and extracted residues after approximately 23% of dry material was water extracted (160-165°C) from wood chips indicated; (i) Most of the cellulose remained intact, due to high glucose content in the residues. (ii) Most of the acid insoluble lignin (AIL) was retained in the residues while a part of the acid soluble lignin (ASL) was extracted. (iii) Xylose and acetic acid concentration were much lower in the residues and it was possible to extract a large part of the xylan.
The analysis (% wt dry, ash free) and heating values of the birch wood chips and extracted residues showed that the heating values of the residues were higher; calorimetric 19.54 MJ/kg and the lower heating value 18.71 MJ/kg (dry, ash free), compared to fresh chips, 18.09 MJ/kg and 17.26 MJ/kg respectively. The ash content was significantly lower in residues, 0.1% compared to 0.4% in fresh chips, which means that the ash forming elements have been extracted from chips. For example, 86% of the potassium, more than 80% of the phosphorus and the magnesium, and more than 70% of the calcium and the mangan have been extracted from wood chips. Only iron increased slightly, probably due to contamination from the steel autoclave cylinders.

The yield of xylose after extraction and secondary hydrolysis was at an average of 58.4% of the total content in dry wood for extractions performed at 160-165°C, and 35.6% at 170-180°C. The extraction liquor from eight different extractions had xylose concentrations, after acid hydrolysis (4% H₂SO₄, 121°C for 60 min), between 40.9 g/L and 69.4 g/L. Extractions performed at 170-180°C gave final xylose concentrations between 40.9 g/L and 47.3 g/L, and extractions performed at 160-165°C gave xylose concentrations between 62.0 g/L and 69.4 g/L. After extraction, without any secondary hydrolysis performed, the analysis showed a much higher xylose concentration in liquor for extractions performed at 170-180°C than at 160-165°C; 34.2-39.5 g/L and 18.3-26.0 g/L, respectively.

Also a correlation between final pH and extraction temperatures was found, the final pH was between 2.9 and 3.0 for extractions at 170-180°C and between 3.1 and 3.2 for extractions performed at 160-165°C. A lower final pH together with higher acetic acid and xylose concentrations after extraction, without any secondary hydrolysis performed, indicates that the degradation of xylan was at a higher rate during extractions performed at 170-180°C. The higher xylose concentration after secondary acid hydrolysis for extractions performed at 160-165°C compared to 170-180°C also indicates that the degradation is at a high rate at 170-180°C, causing additional degradation of xylose during the secondary hydrolysis. The maximum concentrations of furfural and HMF were obtained after extractions performed at 170-180°C, 2.3 g/L and 2.2 g/L, respectively. No HMF was detected after secondary hydrolysis in any extracted liquors and the highest furfural concentration was 2.3 g/L, also from an extraction performed in the 170-180°C interval. This indicates further degradation of HMF and furfural during secondary hydrolysis, and that the degradation of sugars was at a higher rate using extraction temperature 170-180°C. The L/DW ratio influences the final
concentrations of different compounds and final pH in liquor. A too low L/DW ratio lowers pH, caused by high acetic acid formation, which causes degradation of xylose in a high rate. The acetic acid/xylose ratios after extraction and secondary hydrolysis were at an average of 0.86 for extractions performed at 160-165°C, and 1.95 at 170-180°C, indicating losses of xylose, due to degradation, at the higher temperature range.

The considerably lower ash content in hot water extracted residues, and higher heating values compared to fresh birch wood chips is promising. The use of extracted wood chips in an EFGT will probably lower the fouling significantly and give a higher efficiency, without any costly gas cleaning equipment. The extracted residue will serve as a better fuel than the fresh wood chips and give the techno-economical opportunity to turn a CHP plant into a biorefinery. The hot water extraction of birch wood chips can be performed so that the extraction liquor can serve as a fermentable feedstock stream with the 5-carbon sugar xylose as main substrate for microbial growth and product formation. Most carbohydrates have increasing stability with decreasing pH, with the highest stability at pH 3-4. The combination of pH below 3 and the higher temperatures during hot water extractions, or the higher temperature forming acetic acid to levels that will lower pH below 3, is probably too severe and will give great losses of xylose and subsequent less substrate for microbial growth and product formation. The acetic acid/xylose ratio indicates how much xylan hemicelluloses that are extracted and also how much xylose that is further degraded. The lower temperature range during hot water extractions performed in this study gave a much higher final xylose concentration in the extraction liquor than if a higher temperature range was used. The extraction of ash forming elements is probably not affected by the differences in extraction temperatures used in this study, because these elements will leave the wood chips first during extraction and the extraction can be optimised towards high final xylose concentration in extraction liquor.
This study presents the optimisation of the volumetric productivity and final succinic acid concentration by using different neutralising agent during dual-phase fermentations using a metabolic engineered *E. coli*. The facultative *E. coli* strain (AFP184) used is genetically engineered to utilise both 5- and 6-carbon sugars during growth and mixed acid fermentation, and the carbon flow during mixed acid fermentation is directed towards succinic acid as product. In earlier work a decrease in succinic acid production was observed when the fermentations accumulated organic acids (74). The fermentations in the earlier investigation were performed at regulated pH of 6.6-6.7 and since organic acids have a pKₐ about 4 the produced organic acids were dissociated. The cytoplasmic membrane should be relatively impermeable to the acid anions and the protons (75, 76). However, other studies indicate that acid anions can travel over the membrane (77), and the effect would be accumulation of the anions in the cytoplasm. The metabolic effects of succinate anions on anaerobe succinic acid production is unknown and the neutralising agent used in the earlier investigation, NH₄OH is known to cause growth inhibition in *E. coli* at concentrations above 3 g/L (78, 79). In the earlier investigations the highest anaerobic volumetric productivity was approximately 2.9 g/L h after 22 hours total fermentation time using NH₄OH as neutralising agent and glucose as substrate. The highest final succinic acid concentration was approximately 40.6 g/L. In this work fermentations with the bases NH₄OH, KOH, K₂CO₃, NaOH or Na₂CO₃ were conducted and further evaluated. The substrate used was glucose.

The use of alkali carbonates Na₂CO₃ and K₂CO₃ for neutralisation showed higher volumetric productivities after 20 hours total fermentation time, 2.95 g/L h and 3.02 g/L h respectively, than the alkali hydroxides NaOH and KOH, 2.47 g/L h and 2.62 g/L h respectively. The increased productivity is probably caused by an increased availability of hydrogen carbonate. The enzyme PEP-carboxylase catalyses the carboxylation of PEP to oxaloacetic acid using HCO₃⁻ as a substrate for the reaction (80), Figure 2. The higher productivity using CO₃ bases for neutralisation indicates that the medium is not saturated by the sparged CO₂. The highest final succinic acid concentration, 77 g/L, was achieved when Na₂CO₃ was used as base. Using NaOH resulted in 69 g/L, K₂CO₃ in 64 g/L, KOH in 61 g/L and NH₄OH in 43 g/L.
Productivities per viable cell provide other information than volumetric productivity; volumetric productivity gives the total amount of succinate produced per volume and time unit and there is no information regarding the state of the cells. The productivity per viable cell reveals the production capacity of each viable cell, thus indicate if the cells are inhibited or not. In general the productivities per viable cell were initially high but decreased after approximately 20 hours of total fermentation time. Using NH$_4$OH as base completely stopped the succinate productivity after 32 hours, whereas using the other bases showed gradually decreasing productivities during the remaining anaerobic phase. During the first 20 hours of the anaerobic production phase the viability of the cultures decreased significant but the remaining time of fermentations showed only a small decrease in viability. Using K$_2$CO$_3$ and Na$_2$CO$_3$ as pH regulators resulted in higher cell viability during the anaerobic phase compared to using KOH and NaOH.

Also in this study fermentations were conducted in which 150 ml of either a 140 g/L succinic acid solution or a sodium phosphate buffer (pH 6.6) were added gradually during the anaerobic phase. The amount of succinic acid produced when the buffer was added was significantly higher than when succinic acid solution was added. The viable cell concentration was not negatively affected in the fermentations conducted, but the externally added succinic acid resulted in a decreased anaerobic productivity per viable cell when succinic acid concentration increased. The osmolarity of the medium appeared to have only marginal effect on succinate productivity and that was also further shown by the results from fermentations with added osmoprotectant glycine betaine. Addition of osmoprotectants should improve succinate production if the reduced productivity was caused by increased osmolarity (81). It has been shown that increased intracellular concentrations of the osmolytes trehalose in ethanologenic E. coli did not improve growth in the presence of formate, lactate or acetate (82). Thus, the decrease in productivity was probably due to organic acid toxicity and not caused by osmotic stress. In order to further improve productivity the product acids in media must be separated from the cells.
Conclusions

It was concluded in Paper I that it is possible using white liquor to extract xylan from birch wood chips prior to Kraft cooking without decreasing the pulp yield and paper strength properties while simultaneously achieving an impregnation of alkali into the wood chips. However, by using these conditions, it is not possible to attain a liquor containing xylan at industrially attractive concentrations for microbial conversion. By using hot water extractions of xylan prior to Kraft cooking it was possible to obtain liquor with higher titres and minimal degradation of xylan at the expense of decreases in compression strength, tensile strength, tensile stiffness and burst strength. The hot water extraction decreases the pH to acidic conditions which will lead to increased alkali charge during cooking and it might be a problem to obtain a homogenous cook due to presence of pH gradients in chip pores.

In Paper II it was concluded that hot water extractions of birch wood chips will integrate well with a combined heat and power (CHP) plant. The hot water extracted residues had a considerably lower ash content and higher heating values compared to fresh birch wood chips. The use of extracted wood chips in an externally fired gas turbine will probably significantly lower the fouling caused by the ash forming elements and give a higher efficiency. The extracted residue will serve as a better fuel than the fresh wood chips and give the techno-economical opportunity to turn a CHP plant into a biorefinery. The extraction of ash forming elements is probably not affected by the differences in extraction temperatures used in this study, because these elements will leave the wood chips first during extraction and the extraction can be optimised towards high final xylose concentration in extraction liquor.

In Paper I it was demonstrated that the water extraction integrates well with downstream biological processing in that there are few additional process requirements for the subsequent bioconversion of the extracted liquor, although some detoxification may be necessary. In Paper II it was shown that hot water extractions can result in liquors containing xylose at industrially attractive concentrations for bioconversion to value added chemicals. This can be contrasted with the alkali extraction processes performed in Paper I, which integrate well with existing Kraft pulping, but integrate poorly with the downstream biological processing in that some xylan is degraded, recovered xylan requires significant concentration, and potentially sodium from the white liquor might need to be removed since it can be inhibitory or toxic to biological conversion and/or product recovery. Overall, this implies that water extraction
yielding high concentrations of oligomeric xylan and monomeric xylose might be a more promising solution for integration into a CHP plant than integrated into a Kraft mill, if not decreases in pulp strength properties can be accepted.

**Paper III** demonstrated that it was possible to achieve an almost 100% increase in final succinic acid concentration using Na₂CO₃ as neutralising agent compared with fermentations neutralised with NH₄OH. It was also demonstrated that the duration of high volumetric productivity of succinic acid could be increased by changing neutralising agent. The decrease in productivity and limited final titres could be attributed to accumulation of organic acids or neutralising agent in the fermentation broth resulting in inhibition rather than osmolarity as the primary reason for reduced productivity and limited final titres. This finding also points out the importance in avoiding accumulation of inhibitory and toxic compounds and elements during the xylan extractions that are discussed in **Paper I** and **Paper II**.
References


Paper I
Impact of Hemicellulose Pre-Extraction for Bioconversion on Birch Kraft Pulp Properties

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Abstract
The combination of hemicellulose extraction with chemical pulping processes is one approach to generate a sugar feedstock amenable to biochemical transformation to fuels and chemicals. Extractions of hemicellulose from silver birch (Betula pendula) wood chips using either water or Kraft white liquor (NaOH, Na₂S, and Na₂CO₃) were performed under conditions compatible with Kraft pulping, using times ranging between 20-90 minutes, temperatures of 130°C-160°C, and effective alkali (EA) charges of 0%-7%. The chips from select extractions were subjected to subsequent Kraft pulping and the refined pulps were made into handsheets. Several metrics for handsheet strength properties were compared with a reference pulp made without an extraction step. This study demonstrated that white liquor can be utilized to extract xylan from birch wood chips prior to Kraft cooking without decreasing the pulp yield and paper strength properties, while simultaneously impregnating cooking alkali into the wood chips. However, for the alkaline conditions tested extractions above pH 10 resulted in low concentrations of xylan. Water extractions resulted in the highest final concentrations of xylan; yielding liquor without the presence of toxic or inhibitory inorganics and minimal soluble aromatics that we demonstrate can be successfully enzymatically hydrolyzed to monomeric xylose and fermented to succinic acid. However, water extractions were found to negatively impact some pulp properties including decreases in compression strength, bursting strength, tensile strength, and tensile stiffness while exhibiting minimal impact on elongation and slight improvement in tearing strength index.

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Introduction

Chemical pulp mills can be considered as examples of existing chemical/thermochemical biorefineries that utilize technology developed over the last century to chemically fractionate and convert woody biomass into products including pulp, cellulose derivatives, extractives such as tall oil, as well as a number of minor products, while the unused biomass fractions, including lignin and hemicellulose solubilized during pulping, are thermochemically converted to heat and power. Currently, North American and European pulp mills are in a period of decreased profitability due to a number of contributing factors including increased competition and higher raw material costs resulting in significant industry consolidation. One solution to this problem is the diversification of products from the mills, that include directing the hemicelluloses and lignin solubilized during chemical pulping towards the generation of green fuels and chemicals (Huang et al., 2010; Stuart, 2006; van Heiningen, 2006).

Much of the recent focus on bioenergy has been on liquid transportation fuels with a particular focus on ethanol from lignocellulosics. Considering that a chemical pulp can be produced with a value in the range of $600/ton, this would require that the ethanol generated from the same carbohydrates contained in this pulp to be valued at greater than $4/gal to be economically competitive. Rather than focus on the cellulose fraction of woody biomass that currently has a higher value for its fiber applications, approaches for fuels and chemicals from woody biomass should be directed towards the hemicellulose and lignin fractions that are not utilized in pulp fibers.

In general, several broad strategies can be identified to address the utilization of the lignin and hemicellulose fraction in chemical pulp mills. One approach includes gasification of
the black liquor after pulping which allows for recovery of the organic components of the biomass in a chemically reduced form that can be catalytically upgraded to renewable fuels and chemicals. Alternatively, these biopolymers can be utilized with more of their original structure features intact by recovery from liquid phase streams either from the black liquor after chemical pulping or with a dedicated extraction stage prior to pulping.

These approaches that target hemicellulose and lignin recovery are based on the chemistry of chemical pulping which has the overall effect of increasing lignin and hemicellulose solubility by depolymerization and/or chemical modifications. These methods all require the utilization of soluble lignins and hemicelluloses from a liquor containing a complex mixture of solubilized organics as well as the inorganics used in the process which can present process challenges. Specifically for hemicellulose extraction, there are a wide variety of available chemical pretreatments (Yoon et al., 2008; Al-Dajani and Tschirner, 2008; Kenealy et al., 2006; Mosier et al., 2005; Sun and Cheng, 2002) that span the complete range of pH and can use a wide range of reagents such as alkali, sulfur, ammonia, or oxygen. These pretreatments all share common features with chemical pulping that include chemically modifying and solubilizing a portion of the hemicellulose and lignin.

There are unique constraints placed on the strategy available to recover or utilize the solubilized biopolymers depending on the chemical pulping process employed. The chemistry of sulfite pulping allows a number of uses from chemicals in its black liquor. During sulfite pulping lignin is solubilized, while under the slightly acidic conditions hemicellulose sugars are hydrolyzed to lower molecular weight oligomeric and monomeric sugars. Ethanol production from sulfite black liquors from the 6-carbon sugars of softwood glucomannans and galactoglucomannans was once more ubiquitous (Helle et al., 2004). Due to the gradual phase-
out of sulfite mills in favor of Kraft process, only a few sulfite mills remain that continue to practice ethanol fermentation of hemicellulose monosaccharides. The Kraft process is the dominant chemical pulping process due to the highly efficient chemical recovery, reduced effluent treatment and emissions, and the process robustness to produce a quality pulp from a variety of feedstocks. In the Kraft process, in addition to lignin a large fraction of the hemicellulose is lost to the black liquor stream during the cooking procedure (Bhaskaran and von Koeppen, 1970). These modified, solubilized biopolymers in the black liquor stream are typically concentrated and combusted to produce the heat and power requirements for the mill while the pulping chemicals (NaOH and NaS) are regenerated and reused. Considering that polysaccharides such as hemicelluloses have a low heating value (13.6 MJ/kg) compared with lignin (27.0 MJ/kg) (van Heiningen, 2006), recovery of hemicelluloses at an early stage of the Kraft process followed by biochemical conversion into value-added products such as ethanol or succinate (Werpy et al., 2004; 2006) might provide a mill with a more diverse product portfolio with potentially improved economics. Using the catalytic power and specificity of enzymes and the coupled enzymatic networks within cellular metabolic pathways the carbohydrate portion of lignocellulose has the potential to be biochemically upgraded. Through metabolic engineering, microbial catalysts can be further optimized for the production of transportation fuels, biodegradable polymers, and chemical intermediates (Willke and Vorlop, 2001).

Hardwoods contain a higher fraction of total carbohydrate fraction (holocellulose), a lower lignin content than softwoods, as well as a more open vascular structure, all of which render hardwoods more amenable to chemical pretreatment (Polizeli et al., 2005). Silver birch (Betula pendula) is the hardwood species with the most intensive industrial utilization in northern Europe and its hemicellulose consists of primarily 4-O-methyl glucuronoxylan (xylan),
which can contribute to more than 20% of the total dry weight of wood. The alkaline peeling reaction degrades glucomannan (the dominant softwood hemicellulose) rapidly under alkaline conditions, while de-acetylated, solubilized oligomeric xylan is more stable due to the 4-O-methylglucuronic acid side chains. Thus, alkali pretreatment should be more suitable for hemicellulose extraction from hardwoods rather than softwoods (Simonson, 1965). Hot water pretreatment of woody biomass solubilizes hemicellulose primarily as oligomers. Hot water pretreatment is a self-catalytic process and the mechanism of hydrolysis is based on the cleavage of acetyl and uronic acid ether substitutions that result from the formation of acetic and other organic acids, with further hydrolysis of polysaccharides to oligomers and monomers possible (Niemelä and Alén, 1999).

Treatment with alkali at moderate temperatures is a well-established laboratory method for extracting hemicelluloses from plant cell walls (Dashek et al., 1997; Vuorinen and Alén, 1998; Ebringová and Heinze, 2000) and is the basis for at least one approach for hemicellulose extraction from wood prior to pulping (Al-Dajani and Tschirner, 2008). The effectiveness of moderate temperature alkali extraction is limited for the larger particle sizes that are utilized in pulping, however. It is well-established that solubilized polysaccharides are completely degraded to saccharinic and hydroxy acids by the completion of Kraft pulping (Bhaskaran and von Koeppen, 1970; Niemelä and Alén, 1998). Data from Axelsson et al. (1962) for soda pulping of *B. pendula* at 18% EA suggests that 30% of the xylan are solubilized early in the cook at a number average degree of polymerizations (DPn) in range of 130-180 and at concentrations of up to 20 g/L before degradation. These results are encouraging, and suggest that a significant portion of the xylan can be recovered from pulping liquors during the early stages of pulping or for example with a modified liquor impregnation stage.
A critical process integration challenge for biological conversion of polysaccharides in alkaline pulping liquors is the high concentration of both toxic organics (acetate and degradation products of polysaccharides and lignin) and inorganics (from the pulping chemicals). One approach to overcome this challenge is by precipitation of polymeric xylan since these high-DP, deacetylated hemicelluloses are significantly less soluble at non-alkaline pH or in other solvents. Examples of how this might translate into a process includes precipitation of hemicelluloses (N’Diaye et al., 1996) with alcohols (e.g. 2-propanol, methanol, or ethanol) which is a standard laboratory method for hemicellulose recovery (Puls et al., 2006; Glassner et al., 2000) or alternatively by acidification (e.g. acetic acid, H₂SO₄, or CO₂). This approach would achieve two technology breakthroughs simultaneously. The first is that this separates the hemicellulose from the liquor so that microbial toxicity to organics and inorganics in the black liquor is overcome. The second is that recovered hemicellulose oligomers can be concentrated to very high levels for a subsequent enzymatic hydrolysis and microbial utilization which can result in high product titers.

Alkali extraction of hemicelluloses from wood chips can be considered as well-integrated with an existing alkaline process such as Kraft pulping, since it will lower the alkali charge when cooking, although the effectiveness of hemicellulose extraction and recovery is not well-documented for combining pulp production with bioconversion of hemicellulose. Hot water extraction of hemicelluloses does not present the same integration challenges for the biological integration, however, as this work will investigate, may present process integration and product quality challenges for chemical pulping. Examples of how these approaches might integrate with an alkaline pulping process are given in Figure 1.
Specifically, the present work proposes to evaluate the implications of birch hemicellulose extraction prior to Kraft pulping using both hot water and Kraft white liquor alkali extractions on the final pulp quality (as determined by a number of metrics for handsheet properties) as well as characterize the potential for xylan recovery from these extraction liquors and demonstrate a enzymatic hydrolysis and biological conversion of extracted xylan to succinate, a metabolite with the potential of a platform chemical.

Materials and Methods

Hemicellulose Extraction

Xylan extractions were performed in autoclave cylinders (3 L) fastened on a rotating wheel inside a glycol bath set at the target temperature. Silver birch (Betula pendula) grown in northern Sweden was chipped to dimensions not exceeding 45 mm x 8 mm x 8 mm and were obtained from Smurfit-Kappa Kraftliner AB (Piteå, Sweden). These were found to have a composition of 42.5% glucan, 23.6% xylan, 6.3% acid soluble lignin, 14.3% acid insoluble lignin, 5.0% extractives, and 7.0% acetate, using the methods of Sluiter et al. (2008a). Autoclave cylinders were weighed and filled with wood chips (100 g based on dry wood) and steamed at 110°C for 10 minutes, then weighed again to measure water content inside the autoclave. After steaming, make up water plus white liquor with a composition according to Table 1 or water alone were added to achieve a liquid to wood (L/W) ratio of 3:1. The extraction temperatures were 130°C, 140°C, 150°C and 160°C with hold times of 20, 30, 60 and 90 minutes after the set temperature was reached. After extraction the chips were thoroughly washed to remove soluble solids. Total pulp mass yield, total solids in the extracted liquid, and final liquor pH were
measured. The carbohydrate oligomer, monomer, and acetate composition of the extracted liquor were determined as described by Sluiter et al. (2008b) using an HPLC (Perkin Elmer) equipped with refractive index detection and an Aminex HPX-87H column (BioRad) using a mobile phase of 0.005 M H₂SO₄ in water at a flowrate of 0.6 mL/min, a column temperature of 65°C, and an injection volume of 20 μL.

**Kraft Cooking**

For five selected conditions, the hemicellulose extractions were performed in the same way as described above, with the difference that no washing was performed and a larger total mass was used (330 g of dry solids). The extracted liquid inside the autoclave cylinders was removed and weighed. White liquor and water were prepared and added to the autoclave cylinders, corresponding to an L/W ratio of 3.5:1 and yielding a total final EA of 21%. Reference cooking without xylan extraction was also performed with a 21% EA charge following steaming at 110°C for 10 minutes before insertion into the glycol bath. The glycol bath was heated to 85°C, and the autoclave cylinders were put inside. The heating procedure increased the temperature with 1°C/minute to 120°C. This temperature was constant for 30 minutes and then further increased to 160°C with 1°C/minute. The total heating procedure took 100 minutes. When the desired H-factor was reached, the cylinders were removed and cooled rapidly in a water bath. The black liquor was removed and the cooked chips were then washed.

**H-Factor vs. Kappa Number Correlation**

In order to compare different xylan extractions followed by Kraft pulping, a basis of consistent comparison in terms of delignification or Kappa number (K-number) must be used,
and therefore a correlation in terms of cooking extent using an H-factor was developed. For this, a range of 16-18 in K-number was targeted for eight reference cooking trials with H-factors ranging from 300 and 600. Overall, the conditions for pre-Kraft pulping extractions chosen as a guideline consisted of one batch each of water-extracted chips or 3% EA-extracted chips at a 92% yield, H-factor of 300, three batches of water-extracted chips at 95% yield (H-factor 200, 350 and 500), and a reference curve for Kraft pulping without pre-extraction. For the 95% water extraction and Kraft pulping reference curve, the data were fitted with exponential curves to yield a correlation between the K-number and the H-factor as to determine the required cooking time at the specified temperature. Based on the two water and alkali extractions at different yields, a similar curve was estimated so that all HC-extracted samples could be pulped to a consistent K-number irrespective of yield. K-number was evaluated using standard methods (ISO 302:2004).

Refining and Preparation of Cooked Material

The resulting pulp was refined twice at low consistency in a laboratory disc refiner (PFI mill) using 0.3 mm and 0.1 mm disc gaps for the respective passes. The wet pulp was centrifuged for 10 minutes, and then the cake was put into a large blender to homogenize the samples. After 5 minutes the pulp was collected and weighed. The dry content was also measured to determine the yield. Samples for K-number analysis were prepared.

Sheet Formation and Paper Testing

All methods used follow the standard protocols of the International Organization for Standardization (ISO) and the Scandinavian Pulp, Paper and Board Testing Committee (SCAN-
To analyze paper qualities, dilute pulp samples were refined to varying degrees and formed into handsheets (ISO 5264-2 and ISO 5269-1). Drainability or freeness of the pulps were determined (ISO 5267-1) in Modified Shopper-Riegler degrees (°MSR). Handsheets were reduced in size using a circular cutter, with a diameter of 20 cm, and the weight was recorded. Other standard analyses that were performed on the handsheets included thickness and bulk density (ISO 5270), air permeance (SCAN-P 85:02), Bendtsen roughness (ISO 8791-2), brightness (ISO 2470), tear strength (ISO 1974), short span compression test or SCT (ISO 9895), tensile testing: tensile strength index, elongation, tensile energy absorbance index, tensile stiffness index (ISO 1924-2), and bursting strength (SCAN-P 24:99).

**Enzymatic Hydrolysis and Fermentation**

Enzymatic hydrolysis of water-extracted xylan was performed using the commercial enzyme preparations Pulpzyme HC (alkaline endoxylanase) at the high enzyme loading of 7.8 mg protein/g xylan (as assayed by the Bradford method) and Celluclast 1.5L (both from Novozymes A/S, Bagsværd, Denmark) at 22.5 mg protein/g xylan. The inclusion of the Celluclast 1.5L was due to the presence of its minor activities for acetylxylan esterase, α-glucuronidase, endoxylanase, and β-xylosidase (Niels Erik Krebs Lange, Novozymes, personal communication). Birch xylan hydrolyzates were prepared by water hydrolysis as described previously at 160 °C for 90 minutes only with a L/W of 2.1:1 resulting in a hydrolyzate containing 53.2 g/L of total hemicellulose sugars (as xylose) of which 29.4% was monomeric xylose and the remainder was polymeric xylan. Hydrolyzates were treated with 0.05 g/mL commercial activated carbon (ColorSorb G5, Jacobi Carbons, Kalmar, Sweden) according to Hodge et al. (2008) and subsequently 520 mL of detoxified hydrolyzate were filter-sterilized
(0.45µm pore size) and added to a sterile 1.0 L working volume bioreactor (Applikon, Schiedam, The Netherlands). The pH was next adjusted to 5.5 with 12% (v/v) NH₄OH and the enzymes were added and saccharification was allowed to proceed with periodic sampling. At the completion of the enzymatic saccharification of the xylan, an additional 50 mL containing both inoculum (metabolically engineered succinate fermentor *Escherichia coli* AFP 184) and fermentation media (based on final reactor concentrations of K₂HPO₄, 1.4 g/L; KH₂PO₄, 0.6 g/L; (NH₄)₂SO₄, 3.3 g/L; MgSO₄ × 7H₂O, 0.4 g/L; 15 g/L corn steep liquor from Sigma-Aldrich) were added to the reactor with succinate fermentation and quantification performed as described previously (Hodge et al., 2008).

**Results**

**Xylan Extraction**

In order to assess the potential of using the xylose contained in hardwood 4-O-methyl glucuronoxylan (xylan) as a feedstock for bioconversion to fuels or chemicals and the effect of its removal on pulp properties, a number of trials were performed to extract xylan prior to pulping. This screening was used to identify conditions fulfilling equipment and process integration constraints, such as yield, final pH, and concentration of total xylan. Following this, xylan-extracted chips were subjected to a Kraft cook to the desired K-number and pulp quality testing was performed.

Table 2 presents the results of the extractions in terms of yield, final pH, and xylan concentration (determined as xylose). Glucan concentration in the extracted liquids did not exceeded 1.14 g/L in any extractions performed (data not shown), and the xylan + xylose concentration ranged from 0.09 g/L to 29.10 g/L. Increasing the effective alkali (EA) charge
using white liquor resulted in a decreased yield. Using 3% EA resulted in mass yields of between 89% and 96%. The yield using 5% EA was between 84% and 95%, and the yield further decreased to 80% - 88% when using 7% EA. Water xylan extractions at 150°C and 160°C using 60 and 90 minutes extraction time resulted in the highest final concentrations of xylan. The highest xylan concentration obtained in this set of studies was 29.1 g/L at the cost of high material losses (76.9% mass yield after xylan extraction). When using alkali extraction, the final xylan concentrations in the extracted liquid increased with decreased EA charge, increased temperature and longer reaction time. During the extractions using alkali, the neutralization of hydroxide ions by acetic acid liberated from the xylan decreased the final pH with increasing reaction severity (temperature and time). The higher charge of alkali, 7% EA, resulted in higher amounts of residual alkali in the extracted liquid. At 130°C and 20 minutes the final pH was 11.92, slightly below the final pH of an ordinary cook. The final pH range at 3% EA was 5.0 - 9.5 compared to 8.0 - 11.0 for 5% EA or 9.0 - 12.0 for 7% EA. The final concentration of xylan was not promising in most of the alkali xylan extractions performed. Only four of the xylan extractions performed using white liquor (WL) and 3% EA reached final xylan concentrations above 3 g/L in the extracted liquor (Table 2) with the highest concentration of 5.79 g/L corresponding to 8.7% of the xylan content of the original wood.

From this screening, several conditions resulting in higher xylan concentrations at high wood yields were selected for further investigations and are highlighted in Table 2. Xylan extractions at 130°C and 140°C, with reaction times, 20 and 30 minutes, were excluded from further investigation due to low final xylan concentrations. Extractions using EA charges of 5% and 7% were also excluded since the yield was too low in combination with a low final xylan concentration in the extracted liquid. Figure 2 plots the results of recovered xylan, pH, and
solubilized acetate in the extraction liquors as a function of the total solids extracted. These data show clear trends which are independent of the extraction temperature and time. For example, only the hot water and 3% EA extractions show increasing xylan concentrations with increasing total solids extraction (Fig. 2A), while the final pH (Fig. 2B) for 3% EA shows the characteristics of a titration curve, indicating the neutralization of base by the release of acetate, uronic acids, and the carboxylic acid degradation products of carbohydrates. The acetate solubilization (Fig. 2C) is significantly higher in the alkali-extracted liquors than for the water-extractions presumably since the acetate-glycoside ether bond is more alkali-labile.

**H-factor vs. Kappa Number Correlation**

To be able to compare different pulps obtained at different conditions, the same target extent of delignification (measured as K-number) had to be reached, in this case 16-18. This was done using the H-factor for predicting the extent of pulping. The H-factor is widely used for characterizing delignification during alkaline pulping and collapses time and temperature into a single “reaction ordinate” to predict delignification. This considers that the rate of delignification is first order and therefore proportional to time and an Arrhenius rate constant (proportional to the exponential of the inverse T) for a single raw material at constant EA and sulfidities as originally derived by Vroom (1957). This parameter is comparable to the combined severity factor (CSF), which roughly estimates the effectiveness of dilute acid pretreatments with a single parameter that includes temperature, time, and pH (Schell et al., 2003; Söderström et al., 2003).

The correlations used to predict K-number based on the H-factor are presented in Figure 3 which were based on the Kraft pulping of water-extracted chips at 95% yield and the reference
Kraft pulping with no extraction. The empirical correlation $K = 1678.38H^{0.7713}$, for water extraction at yield 95% was used as the guideline kinetic ordinate for the water extracted materials at yields 92% and 96%. The correlation $K = 2196.38H^{0.7652}$ developed from reference pulping of unextracted wood (i.e. yield 100%) was the guideline for the alkali extracted materials at yields 91%, 92% and 94%. H-factors for the subsequent Kraft pulping were estimated based on knowledge of the trends for yield and K-number to reach the targeted K-number. While this is not a detailed kinetic analysis, as shown in Table 3, this approach was at predicting final K-numbers from pulping of extracted material.

**Pulp Properties**

Xylan extractions were repeated in order to perform Kraft pulping on the extracted chips. The new final xylan concentrations and yields are presented in Table 3 which are comparable with the original screening data (Table 2). The subsequent pulping conditions based on the correlations developed from Figure 3 and results are also given in Table 3. These were all performed to give both a total EA charge of 21% and yield a final K-number in the range of 16-18. A reference pulp for these conditions without any extraction was also performed as a basis for comparison of pulp properties. The predicted K-number is given in parenthesis next to the measured K-number in Table 3 and shows that the correlation is valid.

The properties of handsheets from all the extracted pulps and the reference pulp normalized in terms of the freeness (°MSR) are plotted in Figure 4. From this figure, the significant differences in pulp properties between extraction conditions are obvious for subplots 4A, 4D, 4J, 4I, 4K, corresponding to beating energy, brightness, compression strength index, tensile strength index, and tensile stiffness index.
As plotted in Figure 4A, the reference material had the highest freeness with increased refining (beating energy). The material obtained using condition 2 and 4, were close to the reference at all revolutions. The other xylan-extracted samples did not have the same increase in freeness, indicating losses of hemicelluloses and less internal fibrillation with increased refining. Table 3 shows that other factors also influence the result in freeness since the drainability should increase with increasing yield after cooking, indicating that more refining is needed for samples 3, 5 and also 1 to reach the same value of freeness as the reference. The density is dependent on the amount of fines, and extent of fibrillation. The reference sample had the highest density (Fig. 4B), together with samples using conditions 2, 4 and 1. Air permeance (Fig. 4C) is closely connected to the density since denser sheets have higher air resistance. The materials that had the highest density also had the lowest value of air permeance.

Brightness decreased with increased freeness (Fig. 4D). The reference sheets had lower values at all revolutions even though it had the highest K-number 17.9 (Table 3). The differences in the xylan-extracted samples cannot be explained by differences in K-number. At higher refining energy the brightness decreased with increased freeness. For the Bendtsen roughness, more flexibility and fines create a smoother surface, allowing less air through the surface structure, and this property (Fig. 4E) is strongly correlated with the air permeance (Fig. 4C).

Figure 4F shows that the tearing strength index increased more in the xylan-extracted materials than the reference material with increased refining. Reference sample and condition 4 had lower values of tearing index, indicating more flexible fibers. Tearing index increased with increased extraction time for the water extracted samples. Increased temperature increased the
tear index for the alkali xylan-extracted materials and shorter extraction times at the same
temperature also increased tear index.

The reference pulp had the highest values of compression (Fig. 4G), even though the
compression for sheets obtained under condition 4 increased rapidly and has almost the same
value at maximum freeness and refining. The water extracted materials had lower values of
compression than the alkali extracted materials, and the compression strength index decreased
with increasing extraction time for the water extracted samples. The elongation does not show
significant differences between conditions (Fig. 4H).

At all pulp freeness values, the reference material had highest tensile strength index (Fig.
4I). At higher values of freeness the extracted materials at conditions 2 and 4 increased more in
tensile strength index than the reference. Tensile strength index decreased with increasing
extraction time in the water extracted samples. A shorter extraction time at the same temperature
decreased the tensile strength index for alkali-extracted samples. Condition 4 is slightly lower in
tensile strength index then the reference. Figure 4J shows the tensile energy absorption index
and how it changed with freeness. The reference, conditions 2, and 4 have the highest energy
absorption at the same freeness, indicating more flexible fibers. Tensile energy absorption index
decreased with increasing extraction time in the water xylan-extracted samples. Increased
extraction temperature decreased the tensile energy absorption index at the same freeness for the
alkali-extracted materials and a shorter extraction time at the same temperature decreased also
the tensile energy absorption index. Figure 4K shows that the tensile stiffness is higher for
condition 4 than the reference sample. Tensile stiffness index decreased with increasing
extraction time in the water-extracted samples. Increased temperature decreased the tensile
stiffness index for the alkali-extracted samples and a shorter extraction time at the same
temperature also decreased the tensile stiffness index. The burst strength is highest in the reference material and condition 4 (Fig. 4L). This is due to more bonding between the fibers. Water-extracted materials have lower values of burst strength than alkali-extracted materials. Burst strength index decreased with increasing extraction time in the water-extracted samples.

Pulps from conditions 2 (water-extracted) and 4 (alkali-extracted) are the most similar in terms of the properties discussed so far to the reference sample. Water extracted material using condition 1 (Table 3) had the highest value of tearing strength index, which is interesting since it had the highest final concentration of xylan, 11.75 g/L in extracted liquid. The reference sample had the highest K-number, 17.9 (Table 3), but the lowest value of brightness at all freeness levels (Fig. 4D). Condensation of lignin at low residual alkali in the xylan extraction might contribute to a darker sheet or alternatively may be due to less dense material absorbing more light. Compression (Fig. 4G) is the opposite of elongation (Fig. 4H), and more flexible fibers increases the bonding that increases the resistance against compression. Elongation increases with increasing shrinkage and higher moisture content also increases the elongation properties. Less flexible fibers contains perhaps more moisture since it avoids breakage from the capillary forces upon drying. This could explain why the water-extracted materials from conditions 1 and 2 have slightly better elongation properties compared to the reference. The tensile strength index increases with less shrinkage, and the higher values from the reference sample and 4 are due to more flexible fibers (Fig. 4I). Fiber flexibility and strength are the most significant factors when it comes to the tensile strength. The reference and pulps from condition 4 have more flexible fibers, with more bonding giving more energy absorption (Fig. 4J). The tensile stiffness (Fig. 4K) indicates that water-extracted birch wood chips can give unsatisfactory bending stiffness due to low tensile stiffness. Bursting strength index (Fig. 4K) showed almost the same result as
tensile strength index, sample 4 and reference sample are similar in properties due to more bonding between the fibers. Alkali-extracted sample (condition 4) and reference sample showed similar properties for all paper tests performed.

**Enzymatic Hydrolysis and Fermentation**

Enzymatic hydrolysis and fermentation of the xylan to succinic acid by a metabolically engineered *E. coli* was performed to demonstrate the feasibility of at least one of the extraction approaches with regard to biological process integration. The enzymatic hydrolysis of water-extracted xylan to xylose is presented in Figure 5A which shows near saccharification of polymeric, partially acetylated 4-O-methylglucuronoxylan to monomeric xylose at a yield of 75% monomers with an unoptimized enzyme cocktail (Sørensen et al., 2007), demonstrating that enzymatic depolymerization is a feasible approach. This same enzyme-treated hydrolyzate was further subjected to fermentation to succinate by a metabolically engineered *E. coli* (Figure 5B) which shows that during the aerobic growth phase (left of dashed line) both acetate and xylose are consumed as substrates for cell growth, while under anaerobic conditions (right of the dashed line) the remaining xylose is completely converted to succinate at a yield of approximately 0.73 g succinate/g xylose. The findings that acetate can be utilized as a carbon source is significant in that the aerobic growth phase acts as a detoxification for the acetate. Some detoxification of the water-extracted xylan was necessary to remove toxic soluble aromatics from the hydrolyzate, however, toxicity is not a significant concern for this process configuration. Fermentation of alkali-extracted, ethanol precipitated xylan was not performed due to the large volumes of hydrolyzate required to perform this fermentation in a 1 L reactor.
Discussion

Hemicellulose extraction prior to pulping in a Kraft mill functions effectively as a pretreatment of the wood chips. As an example, acidic hemicellulose extractions prior to alkaline pulping are feasibly performed commercially in mills producing viscose (dissolving) pulps used for cellulose derivatives, often as an acid sulfite stage. The ideal xylan extraction step should result in high sugar concentrations at high pulp yields, while causing minimal interference with the subsequent pulping process. High sugar concentrations in the final extracted liquors are essential for an economically feasible conversion of sugar to a desired fermentation product. There are a number of daunting challenges or process constraints for integrating biological catalysis of carbohydrates into alkaline chemical pulping. These are derived both from the challenges to the chemical pulping and biological conversion.

For this process, there are a number of specific criteria for effective process integration that can be identified. One is that this extraction cause minimal impact on the overall process and the quality of the resulting pulp. Second, it is important to obtain high final hemicellulose sugar concentrations in the extracted liquor while minimizing the loss in pulp yield due to the equipment requirements both for downstream bioconversion of the hemicellulose sugars and the pulping process. Third, the pH after extraction must not be too low that uneven impregnation of the chips by alkali occurs. Fourth, the generation of compounds that are inhibitory to microbial processing such as acetic acid, sugar degradation products, soluble aromatics and lignin degradation products, and the inorganics (sulfur and sodium) in the extracted liquor need to be considered for process integration if the same liquid phase is to be used during the biological conversion.
Irrespective of the extraction method proposed, process changes and equipment requirements need to be considered. For example, the effect of extraction liquors on the alkali impregnation of chips, subsequent cooking requirements, changes in chemical recovery, as well as subsequent recovery and concentration of xylan for fermentations, must all be considered in tandem. Both hot water and acid extraction affect the alkali charge during cooking and therefore demand more extensive process changes in the Kraft pulp mill. Acidic pH will affect the penetration of white liquor into chips, resulting in an uneven cook since acidic groups must undergo neutralization and consume base. At low pH dissolved lignin condenses creating problems during both cooking and bleaching. If the liquor used for extractions have approximately the same composition as the liquor used in the cooking process, then the extraction step can act as an impregnation step and decrease the effective alkali (EA) requirements during the subsequent Kraft cook. The composition of the extracted liquor and desired fermentation product yield a number of different process solutions. Low concentrations of xylan in the extracted liquor increase the costs due to equipment requirements for concentrating xylan in the liquor by either evaporation or ultrafiltration (Kenealy et al., 2006).

For the utilization of alkali-extracted xylan, proposed approaches include xylan precipitation by either solvent addition or acidification as is currently practiced in processes recovering lignin from alkaline black liquors. For example if ethanol is one of the fermentation products, a fraction of the ethanol product could be used to precipitate xylan from the extracted liquor (Figure 1B), simultaneously separating hemicellulose sugars from the toxic components of the liquor, with the potential for alcohol recovery when the liquor passed through the black liquor evaporator chain.
Water extractions conducted in this study with temperature and reaction times not exceeding 160°C and 90 minutes, occurred at mildly acidic conditions caused by liberation of acetic acid from the hemicellulose. Under these conditions, further hydrolysis of hemicelluloses resulted in primarily oligomeric, partially acetylated xylan. These relatively mild extraction conditions largely prevent the degradation of xylose by dehydration to furfural and subsequently formic acid (Palmqvist and Hahn-Hägerdal, 2000). Both water and alkali extraction resulted in a maximum glucose concentrations no greater than 1.14 g/L, indicating minimal cellulose degradation to monomers during extraction, although some acid hydrolysis of cellulose may result in decreases in cellulose DP, contributing to the fiber strength losses.

The correlation between xylan recovered (Fig. 2A) and final pH (Fig. 2B) is very clear in that as pH drops from 10 to less than 6, the corresponding xylan recovery increases with the same trend as is the case for water extractions. Furthermore, it should be noted that this begins to increase the xylan concentrations to values high enough to warrant further studies. Alkali extractions under more severe extraction conditions potentially extract more xylan than is quantified, but due to degradation to saccharinic and lower molecular weight carboxylic acids the final xylan concentrations were low. If yield and final xylan concentration are compared between the different white liquor extractions (Table 2), the degradation of xylan apparently increased with increased EA charge, temperature, and time.

The treatment of fibers for pulp production through refining is very important and results in fibrillation, hydration, and fines. More flexible fibers create more crossing-linking or hydrogen bonding potential between polysaccharide chains, increasing the paper strength. Hemicelluloses located at the surface are another factor that increases paper strength (Fellers and Norman, 1996), especially tensile strength and burst strength and contribute to swelling, internal
fibrillation, which increases flexibility and contact area between the fibers. The hemicellulose content can also affect tearing strength since it contributes to more bonding between the fibers and increases flexibility of the paper sheet. This is because hemicellulose polymers are more amorphous and have a higher surface area to weight ratio than either cellulose microfibrils or plant cell wall surfaces (fibers), and as such promote fiber-fiber bonding (Karlsson, 2006). During alkali pulping, solubilized hemicelluloses re-precipitate as the pH drops and are re-deposited on fibers (Hannuksela and Holmbom, 2002) such that removal of these hemicelluloses will clearly impact subsequent fiber properties.

Under alkaline conditions the acetyl group in hardwood xylan is quickly saponified (Fig. 2C) while the 4-O-methyl glucuronic acid group at C-2 position stabilizes the xylan chain against the alkaline peeling reaction. With increasing temperature and pH this stabilizing group is removed allowing the peeling reaction to continue (Simonson, 1965) and degradation of sugars occurs. During mild acid hydrolysis, as occurs during hot water pretreatment, Figure 2C shows significantly less cleavage of acetyl groups. It has been demonstrated previously that more than half of the acetyl groups can remain bound to the xylan backbone after steam pretreatment of birch (Ebringová and Heinze, 2000). The action of acetyl esterases liberating acetate from acetylated xylan is demonstrated in Figure 5A, which shows a rapid initial increase in the acetate concentration. The total monomeric xylose liberated is approximately 75% of the theoretical maximum (53.2 g/L xylose for this case) indicating the cooperative activity of β-xylosidase and α-glucuronidase. Unpublished data from our laboratory indicates that hydrolysis of deactylated 4-O-methylglucuronoxylan by only endoxylanase results in incomplete hydrolysis to low DP xylan oligomer fragments.
Conclusions

In this study, it was demonstrated that it is possible to use white liquor to extract xylan from birch wood chips prior to Kraft pulping without decreasing the pulp yield and paper strength properties while simultaneously achieving an impregnation of alkali into the wood chips. However, for the conditions tested, it is not possible to attain a liquor containing xylan at industrially attractive concentrations for recovery or microbial conversion. By using hot water extractions of xylan prior to Kraft cooking it was possible to obtain a liquor with higher xylan titers and minimal degradation of xylan at the expense of decreases in compression strength, tensile strength, tensile stiffness and burst strength. Due to the presence of pH gradients in chip pores, obtaining a homogenous cook may be problematic. One important feature of the water extraction is that this integrates well with downstream biological processing in that there are few additional process requirements for the subsequent bioconversion of the extracted liquor (although some detoxification may be necessary) as demonstrated by the enzymatic hydrolysis and succinate fermentation. This can be contrasted with alkali extraction processes, which integrate well with existing Kraft pulping, but integrate poorly with the downstream biological processing in that some xylan is degraded and that recovered xylan requires removal from the liquor since potentially sodium is inhibitory or toxic to biological conversion and/or product recovery. Overall, this implies that water extraction yielding high concentrations of xylan might be a more promising solution, if decreases in pulp strength properties can be accepted.
Acknowledgements

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Abbreviations

DPn  Number average degree of polymerization

DW  Dry wood

EA  Effective Alkali (g alkali as Na₂O / g dry wood)

HC  Hemicellulose

L/S  Liquid-solid separation

L/W  Liquor to wood ratio used during pulping

WL  White liquor

References


Table 1. Composition of white liquor (WL) and green liquor (GL) and different loads on dry wood (DW).

Table 2. Results of screening xylan extractions where xylose concentrations presented are after a secondary hydrolysis of the extracted liquid. The conditions chosen for further study are highlighted; effective alkali (EA), white liquor (WL), not determined (n.d.).

Table 3. Properties for the cooking and resulting K-number, yield, and also recovered xylan from the xylan extractions performed prior to cooking. Calculated K-numbers in parentheses.
**Figure 1.** Examples of process designs with either hot water (A) or white liquor (B) extraction of xylan prior to Kraft pulping.

**Figure 2.** Relation between total solids extracted and recovered xylan (A), final pH (B), and acetate solubilized (C) for water and white liquor (WL) extractions.

**Figure 3.** Correlation between H-factor and final K-number.

**Figure 4.** Handsheet properties as a function of freeness.

**Figure 5.** Enzymatic hydrolysis of hot water extracted xylan (A) and its fermentation to succinate by metabolically engineered *E. coli* AFP 184 (B)
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<th>WL 5% EA (g/100 g, DW)</th>
<th>WL 7% EA (g/100 g, DW)</th>
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Figure 2
Figure 3

$y = 2196.4x^{-0.765}$
$R^2 = 0.8936$

$y = 1678.4x^{-0.771}$
$R^2 = 0.9878$

- Water Extraction, 95% Yield, Reference Curve
- Kraft Pulping Reference Curve
- Water Extraction, 92% Yield
- Alkali Extraction, 92% Yield
Figure 4

The image contains a series of graphs showing the relationship between various properties of paper or other materials and their corresponding values. Each graph represents different conditions or treatments, indicated by various symbols and colors. The properties measured include:

- Brightness (% ISO)
- Air Permeance (ml/min)
- Density (kg/m³)
- Freeness (°MSR)
- Tensile energy abs. index
- Tearing strength index (Nm²/kg)
- Elongation (%)
- Compression strength index (Nm/g)
- Bursting strength index (MN/kg)
- Tensile strength index (kNm/kg)
- Bendtsen roughness (ml/min)
- Tensile stiffness index (MNm/kg)
- Beating Energy (Revolutions)

Each graph compares these properties under different conditions, allowing for a visual analysis of how these factors interact and change under various treatments.
Figure 5

(a) Aerobic cell growth
(b) Anaerobic succinate fermentation

- Glucose
- Xylose
- Acetate
- Biomass
- Xylose
- Acetate
- Succinate
- Biomass
Paper II
INTEGRATION OF A HEMICELLULOSE EXTRACTION PROCESS INTO A BIOMASS BASED HEAT AND POWER PLANT

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Abstract. The development of processes where lignocellulosic biomass can be refined to several different end-products in the same plant, i.e. a biorefinery, will be important in the development towards a more sustainable society where fossil fuels are replaced. This paper presents the idea to integrate the production of green chemicals via hot water hemicellulose extraction of birch wood (hardwood) into a small-scale combined heat and power plant (CHP), in this case an externally fired gas turbine. A techno-economically successful concept could provide the option to turn a small- to medium scale CHP plant into a small- to medium scale biorefinery. The results show that the extracted wood-chips would serve very well as a fuel for combustion and gasification processes due to the relatively high heating value, low ash content and significantly lower concentrations of alkali metals. Under the assumed economic conditions, electricity can be produced to a cost in the range of €85.6 to €196.2 per MWhel and a fermentable feedstock stream with a xylose concentration of 65 g/L to a cost in between €0.44 to €4.15 per kg xylose depending on plant size and number of annual operational hours.

Keywords: biomass, hemicellulose extraction, CHP, biorefinery

1. INTRODUCTION

Sustainable use of forest- and agricultural resources will play an important role in help solving urgent global challenges such as the enhanced green house effect and the ever increasing demand for fossil fuels. The development of processes where lignocellulosic biomass can be refined to several different end-products in the same plant, i.e. a biorefinery, will be important in the development towards a more sustainable society where fossil fuels are replaced. To be able to compete with fossil resources, an efficient production of biomass based products is necessary to maximize overall process economics and to minimize negative environmental impact (René van Ree, 2007).

Currently, very large biorefinery plants are required in order to reach favourable economy-of-scale effects and consequently reasonable production costs. Some of the larger first-generation biofuel plants require in the vicinity of 3,000 tonnes per day of feedstock (such as plants that produce ethanol from corn). The next-generation facilities are envisioned that would call for 6,000 tonnes per day or more of feedstock (such as gasification/Fischer-Tropsch facilities that will convert wood to synthetic diesel). To enable the expansion of biofuel production in such facilities, as well as provide for associated distribution requirements, it is clear that substantial infrastructure planning and development will be needed. It is also important to situate the plant so that the residual heat can be sold as district heating or process heat (Leduc et al., 2009). On the countryside, it may be difficult to find a heat demand matching such a large amount of residual heat generated from large scale production plants. For these reasons, it would be desirable to put efforts in developing small scale biorefinery plants that can be located near the raw material resource and at the same time as competitive production costs are reached. Such plants are requested for example by the National Science Foundation in USA (2008).

The combination of hemiceluloses extraction with combined small-scale heat and power production (CHP) could be one way to generate a sugar feedstock amenable to biochemical conversion to fuels and chemical intermediates, such as ethanol, butanol, succinic acid and lactic acid (Hahn-Hägerdal et al., 2007; Werpy et al., 2004, 2006) at the same time as heat and power is generated. Hemicelluloses have a low heating value (13.6 MJ/kg) compared to lignin (27.0 MJ/kg) (Van Heiningen, 2006), and therefore recovery of hemicelluloses from lignocellulosic material prior heat and power production followed by biochemical conversion into value-added products might offer a better process economy.

This paper presents the idea to integrate the production of green chemicals via hot water hemicellulose extraction of birch wood (hardwood) into a small-scale combined heat and power plant, in this case an externally fired gas turbine. If the concept turns out to be techno-economically viable, it provides the option to turn a small- to medium scale biomass heating- or CHP plant into a biorefinery.

1.1. Hot-water extraction of hemicellulose

The carbohydrate portion of lignocellulosic feedstock is ideally suited for conversion via biochemical transformations due to their crucial role in cellular metabolism. Many enzymes and metabolic pathways exist in which carbohydrates can be converted to a wide range of metabolities. Through metabolic engineering, microbial catalysts can be optimized for the production of transportation fuels, biodegradable polymers and chemical intermediates (Willke and Vorlop, 2001).
Hardwood hemicelluloses consist of mainly glucuronoxylan, while galactoglucomannan is the major part of softwood hemicelluloses. Glucuronoxylan in hardwood contribute with 15-30% of the total dry weight and softwood contains 7-12% arabinoglucuronoxylan (Polizeli et al., 2005). Hot water pre-treatment of wood materials is less severe compared to acid or alkali pre-treatment. Hot water pre-treatment of wood increases the accessible surface area, removes hemicelluloses and alters the lignin structure to a minor degree. Diluted acid treatment has similar effects on wood, but alters the lignin structure to a significant extent (Mosier et al., 2005). Hot water pre-treatment is a self catalytic process and the mechanism of hydrolysis lies in cleavage of O-acetyl and uronic acid substitutions that result in the formation of acetic and other organic acids, with further hydrolysis of polysaccharides to oligomers and monomers possible (Nikitin, 1962). If hemicelluloses are to be extracted from wood, it is important to obtain high final sugar concentration in extracted liquid that can be utilized by organisms in fermentation processes. However, the generation of compounds that are inhibitory to microbial processing such as acetic acid, hydroxymethylfurfural (HMF) and furfural need to be considered in the extracted liquid (Palmqvist and Hahn-Hägerdal, 2000).

The residue from the extraction process consists of intact “washed” wood chips, which may be suitable as a fuel for thermochemical conversion process, such as combustion and gasification for CHP production.

1.2. Externally fired gas turbine (EFGT)

A promising concept for small- to medium scale biomass based CHP are externally fired gas turbines (EFGT) (Kautz and Hansen, 2007; Kjellström, 2007). The technology may be considered to be under development, but the company Talbott’s Biomass Energy LTD already provides a commercial module producing 100 kWel.

In an EFGT plant, a conventional atmospheric combustion chamber can be used to heat up compressed ambient air using the combustion gases via a heat exchanger. The hot air, heated to 800-900°C is expanded in the turbine generating electricity via a power generator. The turbine exhaust, i.e. pure air, may be used as pre-heated combustion air in the furnace and the excess heat in the flue gases can be used to generate district heating or process heat.

It is of great importance to avoid fouling at the gas side of the heat exchanger surface, which would decrease the heat transfer between gas and air leading to lower electrical efficiency and thereby higher electricity production costs. If advanced gas cleaning equipment must be installed, it may lead to unprofitable plants. The fouling is mainly caused by high concentrations of alkali metals in the fuel. It is therefore desirable to generate a cleaner gas, which could be accomplished by using a fuel with low alkali content.

1.3. Objectives

The overall objectives of this paper are to present and describe the idea to integrate the production of green chemicals via hot water hemicellulose extraction of birch wood (hardwood) into an externally fired gas turbine plant and to make an introductory techno-economic evaluation of the integrated process. Specific objectives are

- To investigate how the chemical properties and characteristics of the wood-chips changes after the hot water extraction process. This is done in order to evaluate how the extraction residue will serve as a fuel for combustion and/or gasification processes.
- To carry out hot-water extraction lab-scale experiments using birch wood chips (Betula pendula) from northern Sweden to get a fermentable feedstock mainly containing the 5-carbon sugar xylose at a high concentration. Analysis of the free liquor will provide data of the composition of liquor from the extractions including resulting concentration of xylose, acetic acid, HMF and furfural.
- To find an appropriate extraction temperature range to obtain an as high yield of xylose as possible.
- To make estimations of the costs for electricity production as well as of the fermentable feedstock stream with high xylose concentration for plant sizes in the range of 200-800 kWel. Parts of the results from the extraction experiments will serve as input data for the economic evaluation of the extraction process.

2. DESCRIPTION OF THE INTEGRATED PROCESS

The idea is to integrate a process for hot water extraction of birch-wood chips (hardwood) into a heat- and power production plant consisting of an externally fired gas turbine. Figure 1 shows a preliminary schematic layout of the proposed process.

The processes within the dotted areas are considered in this study meaning that heat, electricity and a fermentable feedstock stream are produced. The acetic acid may be separated from the stream prior the fermentation and partly used in upstream hydrolysis and partly as a final product. A variety of products such as succinic acid, butanol, ethanol etc, may be produced via fermentation and required downstream processes.
Figure 1. Schematic process layout of the integrated plant. The processes within the dotted lines are considered in this paper.

The extraction process is at this stage assumed to be performed batch wise in a pressurized vessel with a slowly rotating mixer. The wood-chips are first extracted during 90 minutes at a temperature in the range of 160°C-180°C. Then the free liquor is separated from the wood chips and the temperature in the vessel decreased to 121°C, which takes approximately 30 minutes. Secondary hydrolysis is performed during 60 minutes at 121°C. To process one batch takes around three hours excluding water filling. It is further assumed that the hydrolysis can be carried out in the same pressurised vessel.

The wood-chips after the extraction contain water up to around 70% wt. This water contains a high sugar concentration, which is desirable to recover for hydrolysis. Therefore, the extracted wood-chips will be mechanically pressed to squeeze out the remaining liquor. The extracted, pressed wood-chips is stored in a container and self-dried until fed into the combustion chamber by screw conveyors.

The excess heat from the exhaust gases may be used for district heating production, heating of the extraction process or for other downstream processes such as distillation. In this paper, only the district heating option is considered. The exhaust temperature will vary depending on a number of parameters.

3. METHODS AND INPUT DATA

3.1 Chemical analysis of feedstock and extraction residue

The dry solid content of the birch wood chips was measured by conventional methods. The composition of the raw birch wood chips was determined by the method suggested by Sluiter et al. (2008a). The elementary analysis of the extraction residue as well as the birch wood-chips was carried out by an accredited laboratory (ALS Scandinavia AB).

3.2 Extraction experiments in lab-scale

The birch wood chips were sieved with the acceptable fraction less than 45 mm length and width, less than 8 mm thick and greater than 7 mm in length and width. Hot-water extractions of the wood-chips were performed in rotating autoclave cylinders for 90 minutes isothermally at a pressure of approximately 7 bar. Heating to target temperatures, 160-180°C, was carried out at a rate of approximately 1.6 °C/min. A weighed amount of water was added to each autoclave cylinder filled with a weighed amount of chips. The cylinders were sealed and put in the rotating heating device and heated to the target temperature. After 90 minutes at steady-state each cylinder was cooled to approximately 60°C and the free liquid was separated from the wood-chips. The liquid was collected for subsequent hydrolysis and analysis, and the chips were put in a bucket of water overnight. The day after, the water was separated from the chips, which were left to dry in ambient temperature. The water/wood ratio was based on dry wood, liquid/dry wood ratio (L/DW).
The final pH was measured by conventional methods. The acetate and xylan (xylose) concentrations in the extraction liquors were determined by HPLC analysis (RI detection, Aminex HPX-87H column at 65°C with 0.005 M H₂SO₄ at 0.6 mL/min flowrate) after dilute acid sulphuric acid hydrolysis at 121°C for 1 hour to hydrolyze oligomeric xylan (Sluiter et al., 2008b).

3.3 Economic evaluation

Based on the elementary composition of the extracted wood-chips, an assumed fuel water content of 35% (as received) and an excess air factor of 1.5, the actual specific combustion air flow (lₑ) and resulting exhaust gas mass flow per mass unit of fuel (gₑ) were estimated via stoechiometric calculations.

Gas turbine cycle calculations based on the input data presented in Tab. 1 were carried out in order to estimate the required air mass flow through the turbine for different power outputs. The inlet air conditions were assumed to be 288 K and 1.013 bar. A mechanical efficiency was assumed to 98%.

Table 1. Assumed gas turbine cycle data

<table>
<thead>
<tr>
<th>Compressor pressure ratio</th>
<th>4.5</th>
<th>Combustion efficiency (ηₜ)</th>
<th>0.90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turbine inlet temperature</td>
<td>1223 K</td>
<td>Heat exchanger efficiency (ηₚₖₑ)</td>
<td>0.80</td>
</tr>
<tr>
<td>Isentropic compr. efficiency</td>
<td>0.768</td>
<td>Pressure loss heat-exchanger air side</td>
<td>2% of compressor delivery pressure</td>
</tr>
<tr>
<td>Isentropic turbine efficiency</td>
<td>0.826</td>
<td>Pressure loss heat-exchanger gas side</td>
<td>0.04 bar</td>
</tr>
</tbody>
</table>


The required air mass flow (mₐ) through the turbine was calculated from the desired power output and the difference between the resulting specific turbine work and compressor work. The required thermal input was calculated according to Eq. (1).

\[ Pₐ = \frac{mₐ \cdot cₐ \cdot ΔT}{ηₚₖₑ} \]  

where \( cₐ \) is the specific heat for air (assumed constant, 1.005 kJ/kg K), \( ΔT \) is the air temperature difference between compressor outlet and turbine inlet. The fuel mass flow to the combustion chamber was calculated according to Eq. (2).

\[ mₕₑ = \frac{Pₚ}{ηₜ \left( Hᵢ + lₑ hₑ - gₑ hₑ \right)} \]

where \( Pₚ \) is the required thermal input (kW), \( Hᵢ \) is the lower heating value of the fuel (as received), \( hₑ \) is the enthalpy of the turbine outlet air which is supplied to the combustion chamber and \( hₑ₁ \) is the enthalpy of the exhaust gas leaving the air/gas heat exchanger.

The required mass flow of fuel gives the average daily demand of extracted wood-chips. Based on the assumption that three batches of extractions are carried out daily, the required volume of the pressurised extraction vessel and equipment costs for the extraction process can be calculated via the method suggested by Sinnott (1998).

Table 2 summarises the general conditions assumed in the economic evaluation.

Table 2. Assumed general economics

| Interest rate | 5% | Additional cost for CHP | 2.2 €/MWhₑ₁ₑ₂ |
| Economic lifetime | 20 years | Fuel cost | 15 €/MWhₑ₁ₑ₂ |
| Technical lifetime | 20 years | Operational hours | Variable |
| Annuity factor | 0.0802 | Labor | 30000 €/year |
| O&M factor | 2% of capital | Plant overhead | 15000 €/year |


The size of a small-scale CHP plants are generally determined by the heat demand, meaning that the electricity producing part can be seen as an additional investment to conventional district heating plant. In this study, the cost of electricity (COE) and a cost of heat (COH) were therefore separated.

As there are only a few commercial plants in operation, it is difficult to get accurate investment figures as a function of plant size. Therefore, as a reference case, the initial investment of a 100 kWₑ₁ module from Talbott’s Biomass Energy...
LTD was used (Gard, 2008). Kjellström (2007) suggests to calculate the specific investment of the heat producing part (€ per kWheat) according to Eq. (3). That amount was subtracted from the total investment to get the specific investment of the electricity producing part. Thereafter, it is assumed that the specific electricity investment follows the same economy-of-scale effects as the heating part, i.e. the same power exponent as in Eq. (3).

$$I_{heat} = 3384 \cdot P_{heat}^{-0.262}$$  \hspace{1cm} (3)

The annual fuel cost was also separated into a heat and electricity part, where the latter was calculated as the difference between the fuel cost for CHP production and the cost if only heat would be produced.

The cost of the production of the fermentable feedstock stream mainly includes the capital cost for the extraction process and operation and maintenance. However, extraction experiments have shown that the dry mass output of extracted wood-chips is 77% of the dry mass input of the birch wood-chips. Therefore, 23% extra mass of dry birch-wood chips must be fed into the extraction plant to fulfill the fuel demand of the CHP process. The cost for that extra fuel is put on the cost of the production of the fermentable feedstock stream.

4. RESULTS

4.1. Chemical analysis of feedstock and extraction residue

The compositions of birch wood from northern Sweden and the extracted wood chips are presented in Fig. 2.

![Figure 2. Compositions of birch wood (left) and extracted wood-chips (right)](image)

In the fresh birch wood, the total lignin content, acid insoluble lignin (AIL) and acid soluble lignin (ASL) were found to represent approximately 20.6%. The hemicelluloses part, mostly xylose and acetic acid, was 29.3% and the cellulose content, mostly glucose, was 42.5%. The composition of the extracted wood chips showed higher lignin content, higher glucose content together with lower acetic acid content and lower xylose content compared to the fresh birch wood. This indicates that the major part of the hemicellulose was extracted with low final glucose concentration in the extracted liquor and that the cellulose remained mostly intact. It also indicates that most of the lignin stayed in extracted wood-chips.

Table 3 shows the heating values and the ultimate analysis of the birch wood chips used in the extraction experiments as well as of the extracted wood-chips.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Birch wood-chips</th>
<th>Extracted wood-chips</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heating value, calorimetric (MJ/kg)</td>
<td>18.09</td>
<td>19.54</td>
</tr>
<tr>
<td>Lower heating value (MJ/kg, dry ash free)</td>
<td>17.26</td>
<td>18.71</td>
</tr>
<tr>
<td>Volatiles (%wt, dry basis)</td>
<td>84.4</td>
<td>83.4</td>
</tr>
<tr>
<td>Ash content (%wt, dry basis)</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>Carbon</td>
<td>49.6</td>
<td>51.0</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>6.3</td>
<td>6.5</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>&lt;0.1</td>
<td>0.25</td>
</tr>
<tr>
<td>Sulphur</td>
<td>0.005</td>
<td>0.006</td>
</tr>
<tr>
<td>Oxygen</td>
<td>43.6</td>
<td>42.2</td>
</tr>
<tr>
<td>Chlorine</td>
<td>0.02</td>
<td>0.03</td>
</tr>
</tbody>
</table>
Table 3 shows that the heating values of the extracted wood-chips are higher than for the birch-wood chips, due to the increased lignin concentration and the lower ash content. The considerably lower ash content means that ash forming elements have left the fuel during the extraction process. Figure 3 shows the results from analysis of the ash forming elements in the original birch wood-chips and the extracted wood-chips.

Figure 3: Ash forming elements in the original birch wood-chips and the extracted wood-chips. (Note the different scale in the figures)

As shown in Fig 3, all the main alkali metals have decreased significantly after extraction. For example, 86% of the Potassium, more than 80% of the Phosphorus and Magnesium and more than 70% of the Calcium and the Mangan have left the wood-chips during the extraction process. Only Iron has increased slightly, most probably due to contamination of stainless steel from the autoclave cylinders.

4.2. Hemicellulose extraction experiments

The glucose concentration in the extracted liquors did not exceed 4.5 g/L in any hot-water extractions performed, indicating that the cellulose remained mostly intact after the extraction. Table 4 shows the xylose and acetic acid concentrations in extracted liquors, the water/dry wood ratio (L/DW) in the extraction experiments and the final pH. The hemicelloses in birch also contain low concentrations of other sugars than xylose, such as mannose, which are not shown in the table. The amount of extracted material from the wood chips was approximately 23% (HWE2 and HWE5). Previous, not yet published studies have shown approximately same result. The share of extracted xylose after hydrolysis was at an average of 58.4% of the total content in dry wood in the experiments HWE1-HWE5 and 35.6% in HWE6-HWE8.

Table 4. Results of the hot-water extraction experiments

<table>
<thead>
<tr>
<th>Name</th>
<th>L/DW (g/g)</th>
<th>pH (final)</th>
<th>Xylose (g/L)</th>
<th>Acetic acid (g/L)</th>
<th>After Hydrolysis (4% H₂SO₄)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HWE1</td>
<td>2.06</td>
<td>3.20</td>
<td>18.3</td>
<td>10.9</td>
<td>68.0 20.3</td>
</tr>
<tr>
<td>HWE2</td>
<td>1.90</td>
<td>3.17</td>
<td>26.0</td>
<td>14.2</td>
<td>68.3 25.2</td>
</tr>
<tr>
<td>HWE3</td>
<td>1.84</td>
<td>3.15</td>
<td>22.0</td>
<td>12.4</td>
<td>69.4 24.9</td>
</tr>
<tr>
<td>HWE4</td>
<td>2.34</td>
<td>3.10</td>
<td>21.1</td>
<td>9.8</td>
<td>63.0 21.3</td>
</tr>
<tr>
<td>HWE5</td>
<td>2.37</td>
<td>3.09</td>
<td>25.3</td>
<td>10.9</td>
<td>62.0 21.8</td>
</tr>
<tr>
<td>HWE6</td>
<td>2.36</td>
<td>2.99</td>
<td>37.7</td>
<td>18.7</td>
<td>47.3 24.8</td>
</tr>
<tr>
<td>HWE7</td>
<td>1.44</td>
<td>2.90</td>
<td>39.5</td>
<td>32.6</td>
<td>41.9 40.3</td>
</tr>
<tr>
<td>HWE8</td>
<td>1.87</td>
<td>2.92</td>
<td>34.2</td>
<td>28.8</td>
<td>40.9 34.9</td>
</tr>
</tbody>
</table>

The maximum concentrations of furfural and HMF were obtained after extraction HWE8, 2.31 g/L and 2.22 g/L, respectively. No HMF was detected after hydrolysis in any extracted liquors, probably due to degradation. The highest concentration of furfural after hydrolysis was in HWE7, 2.30 g/L. The trend was that HWE1-HWE5 had lower concentrations of furfural and HMF than in HWE6-HWE8, probably due to the lower extraction temperature used in extraction HWE1-HWE5. This is illustrated in Figure 4, which shows the extraction temperature levels in the experiments HWE1-HWE8.
A correlation between lower final pH and higher extraction temperatures together with lower final xylose concentrations is found, see Fig. 4 and Tab. 4. This is probably due to a higher rate of xylose degradation at higher temperatures. Hot-water extraction experiments performed at 160-165°C (HWE1-5) gives after secondary hydrolysis extracted liquor containing around 65 g/L xylose. An extraction temperature between 170-180°C (HWE6-8) gives decreased final xylose concentration compared to extraction temperature between 160-165°C.

The final acetic acid concentration in the extracted liquid gives an indication how much xylan that is extracted and further how much xylose that is degraded since each xylose unit in birch wood, xylan has 0.7 acetate units attached to it (Theander and Nelson, 1988). If the acetic acid/xylose ratio is considered from extractions performed (see Tab. 4) acetic acid 60.05 g/mole and xylose 150.13 g/mole, it will give ratios for HWE1-HWE5 at an average of 0.86 and for HWE6-HWE8 at an average of 1.95, indicating degradation of xylose. Most of carbohydrates have increasing stability with decreasing pH, with the highest stability at pH 3-4 (Robyt, 1998). However, a pH below 3 is probably too severe in combination with a high temperature and longer hold time, see Tab.4 and Fig. 4 (Palmqvist and Hahn-Hägerdahl, 2000). If the extraction temperature is decreased, the final xylose concentration will decrease significantly as illustrated in Fig. 5. The hot-water extractions were performed with a L/DW ratio of 4 g/g.

4.3. Economic evaluation

Figure 6 shows production levels of electricity, heat and xylose as a function of plant size measured in electrical power output. The calculations are based on an annual plant operational time of 6000 hours.
Figure 6. Annual production levels of electricity, heat (left y-axis) and xylose (right y-axis)

Figure 7 shows the resulting electricity cost as a function of the power output at different annual operational hours.

![Figure 7. COE (€ per MWhel) in the power output range 200-800 kWel at different annual operational hours of the plant](image)

The electricity production costs vary in range of €85.6 to €196.2 per MWh el. No green electricity certificates have been accounted for. The resulting cost for heat production is in the range of €23.5 to €51 per MWh heat. As Fig. 7 illustrates, the annual operational time has a large influence on the electricity production cost, which underlines the importance to avoid fouling on heat exchanger surfaces.

Figure 8 shows the resulting costs for producing fermentable liquor mainly consisting of xylose and acetic acid.

![Figure 8. The cost to produce fermentable liquor with a xylose concentration of 65 g/L at different annual operational hours of the plant](image)

The results show that a fermentable liquor stream containing 65 g/L can be produced to within a cost range of €0.44 to €4.15 depending on plant size and number of annual operational hours. It is difficult to assess the competitiveness of this process as various degree of downstream processing is required to have a final sellable product. For comparison, it can be mentioned that in the year 2005, the international market price of xylose was US$ 5.0/kg (Murthy et al., 2005) which with the 2005 average exchange rate corresponds to approximately €6/kg.
5. DISCUSSION

The proposed integrated process seems to be a promising concept for small-scale production of heat, electricity and green chemicals. The hot-extraction process gives a high yield of xylose to a reasonable cost and the extracted wood chips are well suited as a fuel for CHP production. The low ash- and alkali contents might even make it possible to use it as a fuel in directly fired gas turbines in pulverised form. It would also be possible to use the fuel in ORC (Organic Rankine Cycles) for CHP production. These technologies should also be evaluated in order to investigate the influence on plant economics.

In a Swedish perspective, the estimated electricity production cost is too high at current conditions and requires higher electricity prices, subsidies and/or technological learning effect to reduce the specific investment. In this study, the latter varies in between €1700-€2500 per kWel, which agrees fairly well with the values presented by Kautz and Hansen (2007).

The free extraction liquor contains acetic acid at concentrations in the range of 25-30 g/L which can be inhibitory to organisms used in the fermentation processes. Therefore, it is likely that the acetic acid must be separated from the stream. This will on the one hand add a cost, but on the other, the acid can be used in the secondary hydrolysis process and/or sold as a product. The acetic acid may also be used for extraction of soft wood.

Furthermore, it may be possible to also extract parts of the cellulose of the birch wood to increase the lignin content and thereby the heating value of the extraction residue. The sugar stream would then also increase, which would be beneficial during fermentation.

There are some practical issues that need to be considered and solved. It would for example be beneficial if the extraction process could be operated continuously instead of in batch mode. The pressing process must also be designed in a way that recovers a large share of the fuel bound liquid. Large volumes of water are required in the extraction process, which may make it difficult to commercialize the concept in water-scarce regions unless the water can be recycled to a reasonable cost.

6. CONCLUSIONS

The extracted wood-chips would serve very well as a fuel for combustion and gasification processes due to the relatively high heating value, low ash content and significantly lower concentrations of alkali metals than in the birch wood chips. This may lead to increased plant availability and better environmental performance, i.e. lower emissions of particulate matter.

The extraction experiments showed that it is possible to extract approximately 58.6% of the xylan (measured as xylose) hemicelluloses from birch wood chips with hot-water at a temperature of 160°C during 90 minutes. The final liquor contains mostly xylose after the secondary hydrolysis, but also other sugars at low concentrations that can be utilised by organisms for growth and product formation. The xylose concentration in the final liquor was approximately 65 g/L. If the acetic acid is separated, it can be used within the process and/or sold as a final product. It is also possible to extract the birch wood without any significant degradation of xylose resulting in decreased xylose concentration and accumulation of inhibitory compounds. An extraction temperature above approximately 170°C (90 minutes) degraded xylose resulting in decreased final xylose concentration together with formation of inhibitory compounds for organisms.

Extraction temperatures below 160°C together with shorter hold time results in a significant decrease of the final xylose concentration in extracted liquor. The next step should be to investigate a cost-effective process to separate liquor from inside the chips, recover more fermentable xylose, together with a cost-effective and environmental sustainable concentration in extracted liquor. The proposed integrated process seems to be a promising concept for small-scale production of heat, electricity and green chemicals. The hot-extraction process gives a high yield of xylose to a reasonable cost and the extracted wood chips are well suited as a fuel for CHP production. The low ash- and alkali contents might even make it possible to use it as a fuel in directly fired gas turbines in pulverised form. It would also be possible to use the fuel in ORC (Organic Rankine Cycles) for CHP production. These technologies should also be evaluated in order to investigate the influence on plant economics.

REFERENCES


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Paper III
Inhibition of Succinic Acid Production in Metabolically Engineered Escherichia Coli by Neutralizing Agent, Organic Acids, and Osmolarity

Christian Andersson, Jonas Helmerius, David Hodge, Kris A. Berglund, and Ulrika Rova
Div. of Biochemical and Chemical Process Engineering, Luleå University of Technology, SE-971 87, Luleå, Sweden

The economical viability of biochemical succinic acid production is a result of many processing parameters including final succinic acid concentration, recovery of succinate, and the volumetric productivity. Maintaining volumetric productivities \( >2.5 \text{ g L}^{-1} \text{ h}^{-1} \) is important if production of succinic acid from renewable resources should be competitive. In this work, the effects of organic acids, osmolarity, and neutralizing agent (\( \text{NH}_4\text{OH}, \text{KOH}, \text{NaOH}, \text{K}_2\text{CO}_3, \text{and Na}_2\text{CO}_3 \)) on the fermentative succinic acid production by Escherichia coli AFP184 were investigated. The highest concentration of succinic acid, 77 g \( \text{L}^{-1} \), was obtained with \( \text{Na}_2\text{CO}_3 \). In general, irrespective of the base used, succinic acid productivity per viable cell was significantly reduced as the concentration of the produced acid increased. Increased osmolarity resulting from base addition during succinate production only marginally affected the productivity per viable cell. Addition of the osmoprotectant glycine betaine to cultures resulted in an increased aerobic growth rate and anaerobic glucose consumption rate, but decreased succinic acid yield. When using \( \text{NH}_4\text{OH} \) productivity completely ceased at a succinic acid concentration of \( \approx 40 \text{ g L}^{-1} \). Volumetric productivities remained at 2.5 g \( \text{L}^{-1} \text{h}^{-1} \) for up to 10 h longer when K- or Na-bases where used instead of \( \text{NH}_4\text{OH} \). The decrease in cellular succinic acid productivity observed during the anaerobic phase was found to be due to increased organic acid concentrations rather than medium osmolarity. © 2009 American Institute of Chemical Engineers Biotechnol. Prog., 25: 116–123, 2009

Keywords: succinic acid, neutralizing agent, product inhibition, cell viability, osmotic stress

Introduction

Increased environmental concern and cost of petroleum has motivated the search for cost-effective alternatives for transforming relatively inexpensive biomass into fuels and chemicals through thermochemical and biochemical conversion. The key to success in the development of profitable industrial biochemical conversion technologies is the choice of target fermentations that can compete with the efficiency of the petrochemical industry. For this purpose, it is essential to develop fermentations that produce molecular building blocks, which can be used as precursors for the production of a number of high-value chemicals or materials. This building block concept follows much of the same strategy that is used by the petrochemical industry, i.e., production of high-value chemicals from a limited number of chemical intermediates. In 2004, U.S. Department of Energy (USDOE) identified 12 sugar-derived chemicals that could be produced from both lignocellulose and starch and serve as an economic driver for a biorefinery. Succinic acid is considered as one of the more promising of these building blocks. In addition to its direct use as a food ingredient and chemical, succinic acid has the potential to produce a wide range of products and derivatives, e.g., green solvents and biodegradable plastics. Succinate is traditionally manufactured from maleic anhydride through n-butane using petroleum as raw material.

Succinic acid could also be produced by biochemical conversion of biomass using fungal or bacterial fermentations. Production of succinic acid has been demonstrated in a number of bacteria. A number of Escherichia coli (E. coli) mutants has recently been developed for succinate production. In this study, E. coli AFP184, a metabolically engineered strain with mutations in the glucose specific phosphotransferase system (ptsG), the pyruvate formate lyase system (pfl), and in the fermentative lactate dehydrogenase system (ldh), was used. AFP184 has been shown to produce succinic acid to final concentrations of 25–40 g \( \text{L}^{-1} \) with productivities in the range of 1.5–3 g \( \text{L}^{-1} \text{h}^{-1} \) from glucose, fructose, and xylose using a low-cost industrially relevant medium. The productivities, although initially very high, declined during the anaerobic phase of the fermentations. This loss of productivity and cell viability during the anaerobic production phase might be caused by organic acid inhibition, a well-known phenomenon in E. coli. The inhibitory effects of organic acids, osmolarity, and neutralizing agent (\( \text{NH}_4\text{OH}, \text{KOH}, \text{NaOH}, \text{K}_2\text{CO}_3, \text{and Na}_2\text{CO}_3 \)) on the fermentative succinic acid production by Escherichia coli AFP184 were investigated. The highest concentration of succinic acid, 77 g \( \text{L}^{-1} \), was obtained with \( \text{Na}_2\text{CO}_3 \). In general, irrespective of the base used, succinic acid productivity per viable cell was significantly reduced as the concentration of the produced acid increased. Increased osmolarity resulting from base addition during succinate production only marginally affected the productivity per viable cell. Addition of the osmoprotectant glycine betaine to cultures resulted in an increased aerobic growth rate and anaerobic glucose consumption rate, but decreased succinic acid yield. When using \( \text{NH}_4\text{OH} \) productivity completely ceased at a succinic acid concentration of \( \approx 40 \text{ g L}^{-1} \). Volumetric productivities remained at 2.5 g \( \text{L}^{-1} \text{h}^{-1} \) for up to 10 h longer when K- or Na-bases where used instead of \( \text{NH}_4\text{OH} \). The decrease in cellular succinic acid productivity observed during the anaerobic phase was found to be due to increased organic acid concentrations rather than medium osmolarity. © 2009 American Institute of Chemical Engineers Biotechnol. Prog., 25: 116–123, 2009

Keywords: succinic acid, neutralizing agent, product inhibition, cell viability, osmotic stress

Introduction

Increased environmental concern and cost of petroleum has motivated the search for cost-effective alternatives for transforming relatively inexpensive biomass into fuels and chemicals through thermochemical and biochemical conversion. The key to success in the development of profitable industrial biochemical conversion technologies is the choice of target fermentations that can compete with the efficiency of the petrochemical industry. For this purpose, it is essential to develop fermentations that produce molecular building blocks, which can be used as precursors for the production of a number of high-value chemicals or materials. This building block concept follows much of the same strategy that is used by the petrochemical industry, i.e., production of high-value chemicals from a limited number of chemical intermediates. In 2004, U.S. Department of Energy (USDOE) identified 12 sugar-derived chemicals that could be produced from both lignocellulose and starch and serve as an economic driver for a biorefinery. Succinic acid is considered as one of the more promising of these building blocks. In addition to its direct use as a food ingredient and chemical, succinic acid has the potential to produce a wide range of products and derivatives, e.g., green solvents and biodegradable plastics. Succinate is traditionally manufactured from maleic anhydride through n-butane using petroleum as raw material.

Succinic acid could also be produced by biochemical conversion of biomass using fungal or bacterial fermentations. Production of succinic acid has been demonstrated in a number of bacteria. A number of Escherichia coli (E. coli) mutants has recently been developed for succinate production. In this study, E. coli AFP184, a metabolically engineered strain with mutations in the glucose specific phosphotransferase system (ptsG), the pyruvate formate lyase system (pfl), and in the fermentative lactate dehydrogenase system (ldh), was used. AFP184 has been shown to produce succinic acid to final concentrations of 25–40 g \( \text{L}^{-1} \) with productivities in the range of 1.5–3 g \( \text{L}^{-1} \text{h}^{-1} \) from glucose, fructose, and xylose using a low-cost industrially relevant medium. The productivities, although initially very high, declined during the anaerobic phase of the fermentations. This loss of productivity and cell viability during the anaerobic production phase might be caused by organic acid inhibition, a well-known phenomenon in E. coli. The inhibitory effects of organic acids, osmolarity, and neutralizing agent (\( \text{NH}_4\text{OH}, \text{KOH}, \text{NaOH}, \text{K}_2\text{CO}_3, \text{and Na}_2\text{CO}_3 \)) on the fermentative succinic acid production by Escherichia coli AFP184 were investigated. The highest concentration of succinic acid, 77 g \( \text{L}^{-1} \), was obtained with \( \text{Na}_2\text{CO}_3 \). In general, irrespective of the base used, succinic acid productivity per viable cell was significantly reduced as the concentration of the produced acid increased. Increased osmolarity resulting from base addition during succinate production only marginally affected the productivity per viable cell. Addition of the osmoprotectant glycine betaine to cultures resulted in an increased aerobic growth rate and anaerobic glucose consumption rate, but decreased succinic acid yield. When using \( \text{NH}_4\text{OH} \) productivity completely ceased at a succinic acid concentration of \( \approx 40 \text{ g L}^{-1} \). Volumetric productivities remained at 2.5 g \( \text{L}^{-1} \text{h}^{-1} \) for up to 10 h longer when K- or Na-bases where used instead of \( \text{NH}_4\text{OH} \). The decrease in cellular succinic acid productivity observed during the anaerobic phase was found to be due to increased organic acid concentrations rather than medium osmolarity. © 2009 American Institute of Chemical Engineers Biotechnol. Prog., 25: 116–123, 2009

Keywords: succinic acid, neutralizing agent, product inhibition, cell viability, osmotic stress
Effects can be due to both the difference between external and internal pH and specific effects on the metabolism caused by the organic acid anion.\textsuperscript{15–17} Acetate, for example, has been shown in \textit{E. coli} to inhibit enzymes in the methionine pathway leading to accumulation of homocysteine, which is inhibitory to growth.\textsuperscript{18} Another factor known to affect cell growth and product formation is high medium osmolarity due to unfavorable salts and sugar concentrations.\textsuperscript{19} Ethanologenic \textit{E. coli} have, for example, been shown to divert more carbon into production of osmolytes when osmotically stressed.\textsuperscript{20} Addition of a neutralizing agent to maintain a neutral fermentation pH might result in an osmotic environment unfavorable for succinic acid production. If biobased production of succinic acid should be economically feasible, the volumetric productivity should be kept higher than 2.5 g L\textsuperscript{−1} h\textsuperscript{−1} for as long as possible. A high final titre, although not as integral to the process as the volumetric productivity, is important for reducing the cost of downstream processing.\textsuperscript{1}

Comprehensive summaries of rates and yields for different strains have been published.\textsuperscript{21–22} In general, work done with \textit{C24} have, for example, been shown to divert more carbon into production of osmolytes when osmotically stressed.\textsuperscript{20} Additions of neutralizing agent to maintain a neutral fermentation pH might result in an osmotic environment unfavorable for succinic acid production. If biobased production of succinic acid should be economically feasible, the volumetric productivity should be kept higher than 2.5 g L\textsuperscript{−1} h\textsuperscript{−1} for as long as possible. A high final titre, although not as integral to the process as the volumetric productivity, is important for reducing the cost of downstream processing.\textsuperscript{1}

Fermentations

\textbf{Materials and Methods}

\textbf{Strain and seed culture preparation}

The \textit{E. coli} strain AFP184, which lacks functional genes coding for pyruvate formate lyase, fermentative lactate dehydrogenase, and the glucose phosphotransferase system,\textsuperscript{11} was used in this study. Cultures were diluted to 70% with glycerol and stored at −80°C. Seed cultures were prepared by inoculating 100 mL of sterilized medium (same medium as used for the batch fermentation, see below) in a 500-mL shake flask with 200 µL of the stock culture. The seed culture was incubated at room temperature (22°C) in an orbital shaker at 200 rpm for 16 h.

\textbf{Fermentations}

\textbf{Media and Growth Conditions.} All batch fermentations, consisting of an aerobic growth phase (8–9 h) and an anaerobic production phase (30–100 h), were conducted in 1 L bioreactors (Biobundle 1L, Applikon Biotechnology, the Netherlands) with a total starting volume of 700 mL (including 35 mL seed culture and 200 mL glucose solution). The growth medium contained the following components in g L\textsuperscript{−1}: K\textsubscript{2}HPO\textsubscript{4} 1.4; KH\textsubscript{2}PO\textsubscript{4} 0.6; (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} 3.3; MgSO\textsubscript{4} \textsubscript{7H}2O 0.4; corn steep liquor (CSL; 50% solids, Sigma-Aldrich); 15; and antifoam agent (Antifoam 204, Sigma-Aldrich). The bioreactor was sterilized with the medium at 121°C for 15 min; thereafter, 200 mL of a separately sterilized glucose solution (350 g L\textsuperscript{−1}) and 35 mL seed culture were aseptically added, resulting in a final volume of 700 mL and a total glucose concentration of 100 g L\textsuperscript{−1}. The fermentation temperature and pH were controlled at 37°C and 6.5–6.7, respectively. During the aerobic phase pH control was achieved by automatic addition of NH\textsubscript{4}OH (15%, v/v; NH\textsubscript{4}H\textsubscript{2}O solution). The dissolved oxygen concentration (%DO) was measured by a pO2 electrode. The agitation speed was varied between 500 and 1000 rpm. During the aerobic growth phase, the culture medium was aerated with an air flow of 5 L min\textsuperscript{−1}. When the optical density at 550 nm (OD\textsubscript{550}) reached a value of 30–35 (after 8–9 h), the anaerobic production phase was initiated by withdrawing the air supply and sparging the culture medium with CO\textsubscript{2} at a flow rate of 0.8 L min\textsuperscript{−1}. The agitation speed was set to 500 rpm for the anaerobic phase, during which succinic acid was produced. To sustain a production rate of 40 mL sterilized glucose solution (600 g L\textsuperscript{−1}) was added after 23 and 40 h of total fermentation time. The anaerobic phase proceeded for ~100 h, or until the succinic acid production ceased. During the fermentations, samples were aseptically withdrawn for analysis of optical density, viable cells, sugars, and organic acid concentrations.

\textbf{Fermentations with Different Neutralizing Agents.} To compare the effects of different neutralizing agents on succinic acid productivity, final titre, and cell viability, different bases NH\textsubscript{4}OH (15%, v/v; NH\textsubscript{4}H\textsubscript{2}O solution), KOH (10 M), NaOH (10 M), K\textsubscript{2}CO\textsubscript{3} (4 M), or Na\textsubscript{2}CO\textsubscript{3} (2 M), were used for pH control during the anaerobic phase. All fermentations were carried out in triplicate except for the fermentations using NH\textsubscript{4}OH or K\textsubscript{2}CO\textsubscript{3}.

\textbf{Effects of Increased Osmolarity, Succinic Acid Concentration, and Glycine Betaine.} During the anaerobic phase, succinic acid or Na\textsuperscript{+} in the form of a sodium phosphate buffer, pH 6.6, were added to fermentations, which used 2 M Na\textsubscript{2}CO\textsubscript{3} as the anaerobic neutralizing agent.
A stock solution of succinic acid, 140 g L$^{-1}$, was prepared and neutralized with KOH. Addition of succinic acid or Na$^+$, 30 mL, respectively, were made 4 h into the anaerobic phase and then every 2 h until a total of 150 mL had been added, corresponding to 30 g L$^{-1}$ of succinic acid or a sodium concentration of 12.5 g L$^{-1}$. In the later case, this represents the amount of sodium required to neutralize 30 g L$^{-1}$ of succinic acid.

Fermentations with an initial glucose concentration of 100 or 150 g L$^{-1}$, respectively, and neutralized with 2 M Na$_2$CO$_3$ were supplemented with glycine betaine to a final concentration of 50 mM, which based on cell concentration is on the same order of magnitude as previous work. Three glucose additions (3 x 24 g) were done during the anaerobic phase of the fermentation with a starting glucose concentration of 100 g L$^{-1}$. A standard fermentation without glycine betaine addition and with an initial glucose concentration of 100 g L$^{-1}$ and two glucose feedings (24 g) was included as a reference.

Analysis

Cell concentration was monitored by spectrophotometry using OD$_{550}$ correlated to dry cell weight. To establish the number of viable cells, 10-fold serial dilutions of the fermentation samples in 0.9% w/v NaCl were plated on tryptone soy agar plates and incubated overnight at 37°C. The number of colonies was calculated and the number of viable cells was expressed as cells per liter fermentation broth. All dilutions were made in duplicate. Organic acids and sugars were detected and quantified by HPLC as previously described.

Results

Fermentations with different neutralizing agents

Standard dual-phase fermentations were carried out with NH$_4$OH, KOH, Na$_2$CO$_3$, NaOH, and Na$_2$CO$_3$ as neutralizing agents. The total cell mass (gram dry cells in the culture) generated was similar for all neutralizing agents (data not shown) and no growth was detected in the anaerobic phase in any of the fermentations carried out. Instead, the optical density continuously decreased due to dilution and cell lysis. All four alkali-bases resulted in similar fermentation profiles (Figures 1a,b), where the highest final succinic acid concentration, 77 g L$^{-1}$, was achieved when Na$_2$CO$_3$ was used as base. Using NaOH resulted in 69 g L$^{-1}$, K$_2$CO$_3$ in 64 g L$^{-1}$, and KOH in 61 g L$^{-1}$. The only by-product formed was acetic acid and the lowest concentration, 4.6 g L$^{-1}$, was obtained when Na$_2$CO$_3$ was used. Fermentations with NH$_4$OH, NaOH, and K$_2$CO$_3$ resulted in acetic acid concentrations of 5.5, 5.7, 6.0, and 5.1 g L$^{-1}$, respectively. All acetic acid was produced during the anaerobic phase. When NH$_4$OH was used for neutralization a maximum succinic acid concentration of 43 g L$^{-1}$ was obtained and succinic acid production completely stopped after a total fermentation time of 32 h (Figure 1a). Yields were in the range of 0.8 g succinic acid per gram glucose consumed in the anaerobic phase (1.22 mole mole$^{-1}$) for all fermentations (Table 1).

Viable cell concentrations for fermentations using NaOH or KOH as the pH regulator showed similar trends. During the first 20 h of anaerobic conditions, the viability of the cultures decreased significantly, but for the remainder of the fermentations only small losses in viability occurred.
The productivity in NH4OH fermentations was well below 1 g/L after 20 h of total fermentation time (Figure 2a). At the onset of the anaerobic phase, volumetric productivities reached initial values of 3–3.5 g L−1 h−1, but decreased significantly after ~20–25 h of total fermentation time for fermentations neutralized with KOH, NaOH, K2CO3, and Na2CO3 (Figure 2b). At this time, the productivity in NH4OH fermentations was well below 1 g L−1 h−1.

**Effects of increased osmolarity, succinic acid concentration, and glycine betaine**

Fermentations in which 150 mL of either a 140 g L−1 succinic acid solution or a sodium phosphate buffer were added gradually during the anaerobic phase were carried out. The amount of succinic acid produced when the buffer was added was significantly higher than when the succinic acid solution was added (Figures 3a,b). In neither of the fermentations were the viable cell concentration negatively affected (Figure 3c). Externally added succinic acid resulted in a decreased anaerobic productivity per viable cell as the total succinic acid concentration increased (Figure 3d).

Fermentations with varying initial glucose concentration (100 or 150 g L−1) with or without addition of the osmoprotectant glycine betaine to a concentration of 50 mM were carried out using Na2CO3 for neutralization. A standard fermentation without glycine betaine addition and with an initial glucose concentration of 100 g L−1 resulted in final succinic acid concentrations of 59 and 65 g L−1, respectively, (Table 2). When glycine betaine was added, the aerobic growth time needed to obtain an optical density of 1014 (a) and volumetric productivity (g L−1 h−1) (b) as functions of time.

**Discussion**

**Effects of neutralizing agent on succinic acid production**

When constructing processes for biobased production of fuels and chemicals it is important to consider how changes
in the fermentation phase might affect downstream processing operations. Separation of the products as well as recovery and recycling of chemicals used in the process is essential in order to obtain good plant economics. An efficient downstream process configuration for the recovery of succinic acid that permits internal recycle of chemicals has previously been demonstrated. The method involves formation of diammonium succinate, which is accomplished by using \( \text{NH}_4\text{OH} \) as neutralizing agent during the fermentation. High concentration of ammonia has been shown to negatively effect \( E. \text{coli} \) growth, and it is possible that ammonia can account for the observed decrease in succinate productivity by \( E. \text{coli} \) AFP184 during the anaerobic phase. The effects on succinate production when replacing the neutralizing agent were studied. The main requirements of the selected bases were that they should be low-cost and be compatible with the proposed recovery process; hence, different monovalent alkali-bases were selected. As a macronutrient for \( E. \text{coli} \) growth, potassium is involved in a number of fundamental biological processes including maintaining the osmotic balance of cells. Sodium hydroxide (NaOH) is a widely available low-price commodity chemical, and \( E. \text{coli} \) has been reported to grow in media containing high concentrations of sodium. Divalent bases of alkaline earth metals such as calcium hydroxide or carbonate could be considered. The use of calcium bases, however, would interfere with the recovery and recycling operations. Therefore, divalent alkaline earth metal bases were excluded from the scope of this investigation.

![Figure 3. Fermentations with succinic acid or salt-buffer addition.](image-url)

(a) Total (●), produced (▲), and added (◆) succinic acid concentration when additional succinic acid was added. (b) Succinic acid concentration equivalent to the amount of added sodium ions (●) and produced succinic acid (▲). (c) Viability cells per liter \( (g \text{ cell}^{-1} \text{ h}^{-1}) \times 10^{12} \) for fermentation with succinic acid (●) and Na\(^+\)-buffer (◆) addition. Viable cell counts were done in duplicate and the values are averages of the data range (error bars). The broken lines indicate the transition to the anaerobic phase. In all figures, (◆) represents a control using 2 M Na\(_2\text{CO}_3\).

### Table 2. Summary of Fermentations Parameters for Fermentations with Glycine Betaine and/or Higher Initial Glucose Concentration

<table>
<thead>
<tr>
<th>Fermentation</th>
<th>( S_A ) (g L(^{-1}))</th>
<th>( Y_{p/s} ) (g g(^{-1}))</th>
<th>Time (hours)</th>
<th>( Q_p ) (g L(^{-1}) h(^{-1}))</th>
<th>( Q_C ) (g L(^{-1}) h(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference(^{c})</td>
<td>72</td>
<td>0.73</td>
<td>80</td>
<td>1.22</td>
<td>1.62</td>
</tr>
<tr>
<td>Betaine</td>
<td>65</td>
<td>0.57</td>
<td>72</td>
<td>2.98</td>
<td>1.20</td>
</tr>
<tr>
<td>Glucose 150 g L(^{-1})</td>
<td>65</td>
<td>0.79</td>
<td>80</td>
<td>2.41</td>
<td>1.07</td>
</tr>
<tr>
<td>Glucose 150 g L(^{-1}) and betaine</td>
<td>59</td>
<td>0.66</td>
<td>48</td>
<td>2.48</td>
<td>1.72</td>
</tr>
</tbody>
</table>

*\( S_A \) is the mass yield of succinic acid based on the glucose consumed in the anaerobic phase, and \( Q_p \) is the volumetric productivity of succinic acid during the anaerobic phase after termination of fermentation. \( T_{20} \) is the total fermentation time.

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From the fermentation profiles (Figure 1), it is clear that using potassium or sodium bases for neutralization would be beneficial for the final succinic acid concentration. Compared with fermentations neutralized with NH₄OH, fermentations neutralized with potassium or sodium bases obtained increased final titers by at least 50% and in the case of Na₂CO₃ almost 100%. It should be noted that unlike NH₄OH fermentations, succinate production in alkali neutralized fermentations never ceased. Other studies have demonstrated that ammonia challenges the integrity of the outer membrane of E. coli, reducing growth²⁷ and at concentrations >3 g L⁻¹ ammonia is known to inhibit growth.²⁸ Using NH₄OH as a neutralizing agent generated ammonia concentrations of more than 10 g L⁻¹ after 16 h anaerobic succinate production. Both the concentration of viable cells and succinate productivity (Figures 1c and 2) decrease rapidly, indicating that concentrations of this magnitude are toxic to the organism.

With regards to productivity and final acid concentrations the difference between using NaOH or KOH was marginal (Figures 1 and 2). However, using Na₂CO₃ and K₂CO₃ resulted in an increased volumetric productivity compared with NaOH and KOH. The increased productivity is likely caused by an increased availability of hydrogen carbonate (HCO₃⁻) from the addition of the base chemical. The enzyme phosphoenolpyruvate (PEP) carboxylase catalyzing the carboxylation of PEP to oxaloacetate uses HCO₃⁻ as a substrate for the reaction.²⁹ The higher productivity during neutralization with CO₃⁻ bases indicates that the medium is not saturated by the sparged CO₂. The additional availability of HCO₃⁻ seem to result in an increased metabolic flux toward succinate. It is clear from the results that the preferred neutralizing agent would be Na₂CO₃, because it generated the highest productivities, final titers and had the lowest by-product formation. However, if considering the yield, the choice of base is not as obvious as all fermentations resulted in an average yield of 0.8 g succinate per gram glucose consumed during the anaerobic phase, which constitutes 71% of the theoretical maximum (1.12 g g⁻¹).³⁴,³⁵

Effects of organic acids and osmotic stress on succinic acid production

From the experiments carried out with addition of either succinic acid or sodium buffer, it is clear that the main reason for the decrease in productivity per viable cell is the increase in succinic acid concentration during the anaerobic production phase (Figure 3). The osmolarity of the medium appears to have only marginal effects on succinate productivity. This conclusion is further substantiated by the results from the fermentations supplemented with glycine betaine (Table 2). There are numerous studies and reviews on osmotic stress and osmotolerance in bacteria.²⁶⁻³¹ E. coli subjected to osmotic stress respond by accumulating compatible solutes, such as glycine betaine, proline, and trehalose.³²⁻³³ Of these, the solute offering the highest osmoprotection in E. coli is glycine betaine.³⁴ E. coli can only accumulate glycine betaine or proline if supplemented in the medium or by a two-step oxidation of externally provided choline. CSL contain some proline, but no glycine betaine. In the fermentations supplemented with glycine betaine, the cellular succinic acid productivity was not affected (data not shown), but the anaerobic glucose consumption rate was increased resulting in a decreased succinate yield suggesting synthesis of other fermentation products (Table 2). However, the acetic acid concentration was not increased and no other fermentation products were detected. Using a higher initial glucose concentration (150 g L⁻¹) and hence a higher medium osmolarity, increased the duration of the aerobic growth phase necessary to reach an OD₅₇₀ of 35. With glycine betaine added to the growth medium this effect was cancelled. Although the medium osmolarity does not seem to affect succinate productivity, it could still be proposed that the viable cell loss could be due to the high osmolarity of the medium that is generated during the anaerobic phase (>1.5 osmoles per liter from neutralized succinate alone). The repressed growth observed when high glucose concentrations were used also point to osmolyte having a negative impact on the cellular metabolism. Under anaerobic conditions, or in media deprived of other osmoprotectants, E. coli synthesizes trehalose intracellularly.³⁵,³⁶ It has been shown that increased intracellular concentrations of trehalose in ethanologenic E. coli did not improve growth in the presence of formate, lactate, or acetate, suggesting that another mechanism than osmotic stress is responsible for growth inhibition in cultivations with weak organic acids.³⁷ The same result was observed in this study, i.e., glycine betaine did not improve the anaerobic cell viability, suggesting that in this study the reduced viability in the anaerobic phase is related to the succinic acid concentration and not the medium osmolarity. Studies with ethanologenic E. coli in high osmolarity CSL media with xylene as the sugar source have shown that addition of betaine improved growth, but did not significantly affect ethanol production.³⁸ In contrast, studies with E. coli engineered for lactic acid production has shown that betaine greatly increased the volumetric lactic acid productivity.³⁹ In this investigation glycine betaine improved the aerobic growth at high osmolarities, but affected the anaerobic succinic acid yield negatively. Response and effectiveness of betaine as an osmolyte has been reported to be dependent on the nature of the fermentation process, i.e., media composition and growth conditions,³⁵ which might explain the different results obtained.

It can be concluded that addition of glycine betaine and most likely any other agent increasing the organism’s osmo-tolerance does not benefit succinic acid production by AFP184. These results suggest that the osmolytic and ionic strength (salt concentration) of the medium are of little importance for cellular succinic acid productivity. Not even at osmolarities of the same magnitude as when succinic acid concentrations are 60 g L⁻¹ (>1.5 Osmoles) does the productivity per viable cell decrease, instead the produced organic acids are responsible for the reduced productivity. Organic acid toxicity is well known to affect cell growth and limit product formation in E. coli and if the main product is the organic acid itself, it also limits any industrial productivity. During anaerobic succinate production organic acids can affect cells both by lowering the cytoplasmic pH (pHₐ), which can have detrimental effects on the function of cellular proteins and enzymes and by the increased intracellular concentration of the acid anion. The extracellular pH in this study was controlled between 6.5 and 6.7. In this pH interval, succinic acid with pKa of 4.19 and 5.57 will be present in its dissociated form (>99.6%) and will thus not channel protons into the cytoplasm lowering the pHt. In contrast, it has been suggested that dissociated lactate and acetate can traverse the E. coli cell membranes catalytically dissipating the proton motive force. If this also applies for
succinate it purports that accumulation of the succinate anion in the cytoplasm would be responsible for the observed decrease in productivity. Although acetate is a potent inhibitor of E. coli growth, the concentrations obtained in this study are not high enough to solely be the cause of the observed decrease in glucose consumption and succinate productivity. Rather the total load of organic acids must be considered. The inhibitory potential of different organic acids varies and is related to the hydrophobicity of the acid. Acetic acid would thus be a stronger inhibitor than succinic, but the data obtained in this work does not indicate that the acetate concentrations achieved would be detrimental to succinate production. Nevertheless, in an effort to maximize succinate productivity and yield as well as from a downstream perspective, it is desirable to minimize the amount of produced acetate.

Conclusions

In this study we have demonstrated that replacing the neutralizing agent can provide substantial process improvements in the form of increased duration of high volumetric productivity and increased final titer. Compared with fermentations neutralized with NaOH, it was possible to achieve an almost 100% increase in final succinic acid concentration using Na2CO3. The decrease in cellular succinic acid productivity observed during the anaerobic phase was found to be due to increased acid concentrations rather than medium osmolarity. It was also observed that the cell viability decreased during the anaerobic phase irrespective of the base used. The viable cell loss is attributed to increased acid concentrations coupled with a possible cytoplasmic accumulation of succinate. Further studies will be directed toward increasing the duration of high anaerobic succinic acid production by investigating different methods to circumvent the impairing effects of the generated organic acid load.

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