Processing

Lignocellulosic By-product Streams

Using Organic Acids

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Voor mijn familie

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Chapter 1

Introduction

1

Introduction

This chapter will supply some background information on the topics mentioned in this thesis. It starts off broadly, explaining important terms and definitions in the biobased economy, after which the focus narrows to bioethanol, elaborating on fuel ethanol history, on production processes, and on state of the art lignocellulose pretreatment. After an explanation of the choices that were made for the research on organic acid pretreatment in this thesis, the Introduction states the research aims of this thesis, and ends by listing the general outline of the chapters to come.

Important terms and definitions

Biobased economy

In recent years, the term 'biobased economy' has become more and more common. It is meant to stress the difference with the 'oil-based economy' that we know today, in which oil and other fossil resources are used to meet our demands on energy, chemicals, and transportation fuels. In the 'biobased economy', renewable resources are applied, and although wind and solar energy can also be considered renewable, the term 'biobased' is mostly used to refer to the application of plant biomass for the production of electricity and bulk chemicals. Another way to describe the biobased economy is that it uses feedstocks that are continuously renewed by photosynthesis, as opposed to depleting fossil feedstocks that were accumulated long ago and over a long period of time.

Different countries and regions may have different reasons for joining the transition to a biobased economy. Usually, these are a combination of the following four, of which the first two could be grouped together under the term 'energy security'.

1. Diversification of resources for energy and bulk chemicals due to an expected oil shortage
2. Decrease dependency on oil delivering regions
3. Reduction of the emission of green house gasses
4. Rural economic development

1

Reason 1: Diversification of resources for energy and bulk chemicals due to an expected oil shortage

As the world population grows, as well as the energy usage per capita due to increased standard of living, more and more fossil resources are needed. As reserves are finite, their depletion may be predicted. Although some amount of oil will always be available, logic suggests that problems will arise as the supply starts to be outweighed by the demand, already some time before the point of near-depletion is reached. At some point, oil will simply become too expensive, and alternative resources will be needed.

An often used argument in this aspect is the Hubbert's peak oil theory [1], which explains how for any finite resource that is discovered, extraction will increase more or less exponentially, as more sources are discovered and more (effective) extraction facilities are built. After this first stage, the increase in extraction will slow down, mainly due to a decrease in new discoveries and limitations in extraction/production technology. At some point, a peak in production/extraction is reached, after which a decline would occur as more sources run out at a faster rate than that new discoveries are made (Figure 1.1) [1].

Predictions on the basis of the peak oil theory are subject to criticism. For example, it can be used to suggest a dramatic and catastrophic collapse scenario. This does not at all have to happen, but it does clarify that a transition to other resources is needed. Oil shales and tar sands are sometimes suggested and although their application is more complicated and therefore more expensive than today's oil, their reserves are very large [2]. Still, this would simply delay peak oil, and/or decrease the rate of decline after the peak. In short, it is generally accepted that cheap and easily available oil are, or will soon be, a thing of the past. Clearly, non-finite, or renewable resources would be preferable, and this is what the transition to the 'biobased economy' is about. The application (and therefore depletion) of resources that were produced and stored in the distant past will be replaced by the application of continuously renewed resources (therefore no depletion), implying a diversification of resources for the production of bulk chemicals and heat and power.

Reason 2: Decrease dependency on oil delivering regions

Most industrial countries are dependent on oil imports and would like to decrease this dependency. This may be true for the EU, but certainly also for the USA. This may be because of the wish to reduce price fluctuations, to be self-sufficient (one way of ensuring energy security), or to reduce the significant contribution oil imports make to the trade deficit [3, 4], but it is also driven by the wish to reduce the dependency on oil-exporting states, as a number of these are located in areas of increased political and military tension, such as the Middle-East [5].

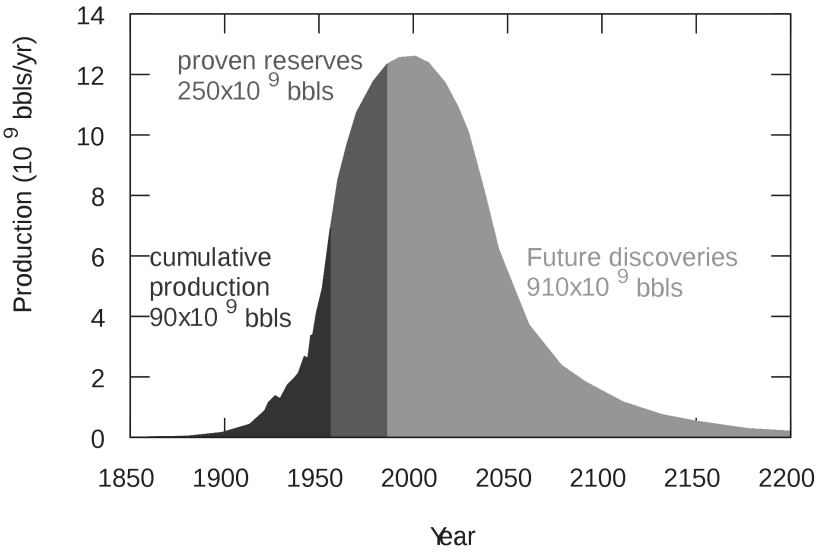

Figure 1.1. Peak oil graph (after Hubbert, 1956 [1]).

Reason 3: Reduction of the emission of greenhouse gasses

A well known argument for the change to a biobased economy is the associated reduction of the emission of greenhouse gasses. These gasses, mainly CO_2, but to a lesser extent also CH_4 and N_2O, contribute to the greenhouse effect, and therefore to global warming [6]. Since its creation in 1997, most governments have signed and ratified the Kyoto Protocol, which calls for the reduction of the emission of greenhouse gasses to below 1990 levels [7]. The protocol entered into force in 2005 and it has subsequently been confirmed in other conferences, such as in Bali, Indonesia and Copenhagen, Denmark.

Reason 4: Rural economic development

Although mentioned last, the need for rural development is a very valid reason for many countries to join the transition a biobased economy. Certainly, there are opportunities for developing nations to use the biobased economy to increase prosperity and employment in rural areas. But also first world countries can have their underdeveloped areas benefit from this transition. Some examples are: palm and Jatropha oil in Africa and Asia, sugar cane in Brazil, corn in the US, and sugar beet and rapeseed in France and Germany.

1 Biorefinery

Definitions of the term 'biorefinery' usually differ somewhat, but the general view is that it is a system in which renewable resources, usually plant based, are treated, separated, and modified (refined) in order to produce chemicals, transportation fuels, and heat and power. Sometimes, the production of food and animal feed is also included in the concept of biorefinery [8]. In essence, this is quite similar to the petroleum refinery. Obviously, there are differences, not the least being that oil and natural gas refinery does not involve food and feed production. Another difference is the processes and technology involved can greatly differ, as plant material greatly differs from oil, natural gas, or coal, both in composition and in structure.

Many by-product streams from food production are used for the production of animal feed. The simplest example might be wheat straw, a by-product that is used as forage, and for ground covering in stables. Staying with wheat as an example, when applying the biorefinery concept, many more applications are included than only food (grain to bread) and feed (straw as forage) (Figure 1.2). This shows how biorefinery opens possibilities, provided that the economics are optimised, for the creation of increasing the total valorisation in the plant based production chain, while saving on fossil resources.
In the related discussion of 'food versus fuel', the term biorefinery is also used regularly, as it entails the production of both food and renewable fuels from an area of agricultural land, hereby reducing competition for land between application for food/feed and for fuel (or other bulk chemicals) [9, 10].

Valorisation

In the area of biorefinery/biomass research of recent years, the term 'valorisation' is used to describe the creation of 'extra value' of a raw material, intermediate, or (by-)product stream. And 'extra value' is simply defined as the difference between the increased value of a product and the costs associated with this increase. In the case of biorefinery, costs usually refer to separation and purification costs, including matters such as capital expenditure and needed process energy. When reading literature on the subject of valorisation, it is advisable to keep in mind that there is usually a selection in the list of costs that have been taken into account.
In short, an essential part of any biorefinery system is valorisation. The total value of the created product streams should be larger than the combination of the original value, with the addition of the costs for processing, separation, and purification.

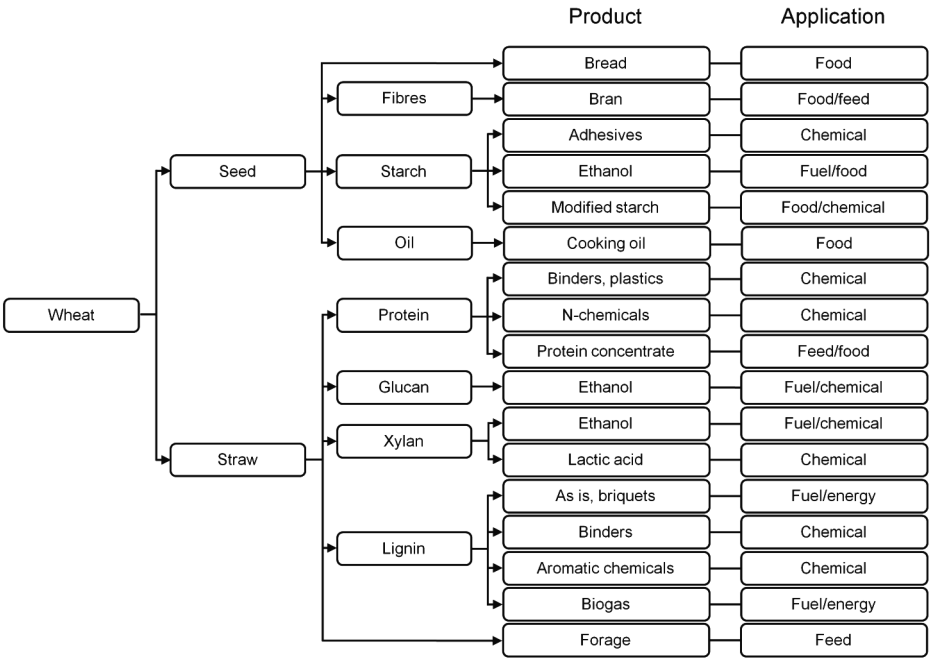

Figure 1.2. Simplified example of biorefinery of wheat; production of food, feed, chemicals, transportation fuel, and energy carriers.

Bioethanol

From past to present

Ethanol is not a new transportation fuel. The earliest cars, designed and built in the US and Germany, including the famous Model T built by Ford, could run on distilled grain ethanol. However, as ethanol from corn was heavily taxed in the US, gasoline was cheaper and therefore favoured. After US ethanol tax was lifted, corn prices were high, again favouring gasoline. Also in Germany, price differences between fuel ethanol and gasoline caused the latter to prevail. Finally, even though ethanol was technologically suitable to be used in combustion engines, and it could also have been used as an additive to solve the 'knocking' problem of gasoline engines in the 1920's, ethanol fuel was all but abandoned [11].

1

The oil crises of the 1970's renewed interest in ethanol as a transportation fuel, and tax incentives to promote blending gasoline with ethanol were introduced in the US [4, 12-14]. In this period, scientific research on lignocellulosic ethanol also emerged [15-17]. When the oil prices decreased again in the 1980's, interest for fuel ethanol also decreased. Because of the reasons mentioned in the first part of this introduction, fuel ethanol has gained enormous scientific and economic interest in recent years, and this continues to the present day.

Production numbers and policies

The production of bioethanol to be used for transportation fuel covers all motives for the transition to a biobased economy mentioned in the previous paragraph. This may be why today, bioethanol production is bigger than ever, and growing sharply. The two main fuel ethanol producing countries are Brazil and the United States, with 25 and 41 gigaliter (GL), respectively, in 2009 [18]. The US have passed Brazil as largest producer in 2006, but in 2008, they were still a net importer of fuel ethanol, while Brazil exported about a third of its production in that year [19, 20]. Of course, something else to consider is that US fuel ethanol consumption in 2008 was about 7 % of its gasoline consumption, while Brazilian consumption of fuel ethanol seems to have surpassed that of gasoline [21]. In the European Union, although also increasing, fuel ethanol production in 2008 and 2009 was only 3.0 and 3.9 GL, respectively, lagging far behind that of Brazil and the US [18-20].

The US plan to increase biofuel production to 136 GL per year in 2022. 79 GL of this may be corn ethanol and 57 GL is to be cellulosic ethanol; the latter being partly (for 19 GL) interchangeable with other biofuels, most likely biodiesel [20, 22, 23].
The European Union (EU) plans to increase the application of biofuels to at least 10 % of transportation gasoline and diesel by 2020 [24]. Although the EU directives do not mention production goals for specific biofuels, replacing 10 % of the gasoline consumption with bioethanol would entail an annual bioethanol production of around 22 GL, based on gasoline consumption of 145 GL per year from 2004-2008 [21]. Replacing 10 % of EU diesel fuel consumption would entail around 34 GL biodiesel production, based on annual EU diesel consumption from 2004-2008 [25].

First and second generation bioethanol

The process of making ethanol from lignocellulosic feedstock has become known as 'second generation' bioethanol production. The production of fuel bioethanol from cereal grains is now logically called 'first generation'. A lot of the growth in bioethanol production is expected to come from second generation processes, but the large majority of today's production uses first generation processes, and will continue to play a very large role in the coming years [20, 22-24].

A first generation bioethanol process uses fermentable sugars that are, or can be made, relatively easily available from the feedstock. The sugars can come straight from the plant, or from molasses, as is the case for sugar cane, or from the starch present in the cereal grain. In most cases, only mild technologies are needed to release the fermentable sugars, such as pressing, milling, or heating to mild temperatures in presence of amylases enzymes. After this, the sugars are fermented to ethanol, followed by a distillation process in order the yield the fuel ethanol.

Second generation bioethanol production uses lignocellulosic materials as feedstock. Common examples are agricultural by-products like corn stover, wheat straw, and rice straw, as these are relatively abundant worldwide and high in polymeric sugars. Another possibility is bagasse, the lignocellulosic by-product from first generation ethanol production from sugar cane, mostly available in Brazil. Materials such as wood and forestry by-products are also possible [26], or specially grown energy crops, usually perennial plants such as *Miscanthus giganteus* [27]. Streams such as fibre sludge from recycled paper production can also be applied [28].

Compared to first generation bioethanol production, the use of lignocellulosic by-product streams results in less competition between food and fuel application involving land use for high-quality edible carbohydrates [29]. Second generation processes also compare well to first generation with regard to reducing greenhouse gas emissions. First generation corn ethanol reduces greenhouse gas emissions by 20-29 %, compared to an energy equivalent amount of gasoline, while second generation bioethanol could result in a reduction of 70 to even 86 %, because much less fossil resources are used to cultivate the raw materials [30-32].

In the mentioned lignocellulosic materials, the sugars are present in polymeric form and part of the lignocellulose matrix, which makes them less easily available. In fact, lignocellulosic biomass requires pretreatment to facilitate the subsequent enzymatic hydrolysis of the cell wall polysaccharides to fermentable sugars [33, 34]. In Figure 1.3, a simplified comparison of first and second generation bioethanol production process is depicted.

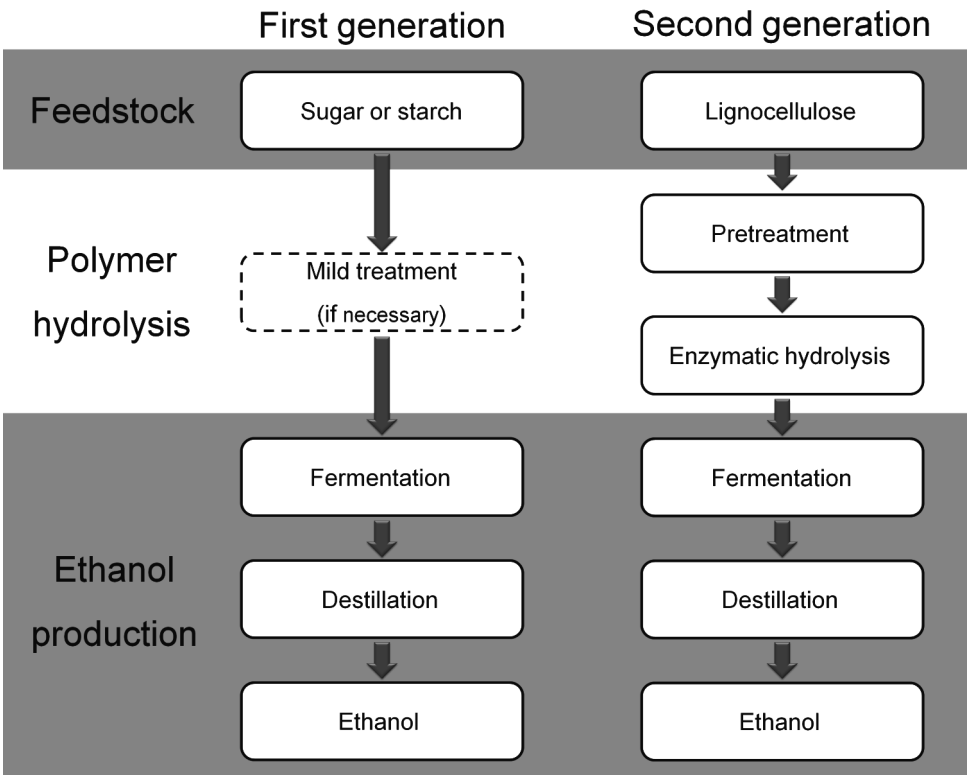

Figure 1.3. Simplified schematic, comparing first and second generation bioethanol production processes.

Lignocellulose pretreatment; state of the art

Many different pretreatment processes exist, usually treatments that include heat and a catalyst (acid or base), sometimes including a mechanical action. Several reviews exist which mention various pretreatments: biological, mechanical, thermal, chemical, and physical. These include examples like: acid hydrolysis, alkaline hydrolysis, steam explosion, ammonia fibre explosion, organic solvents, ozonolysis, ionic liquids, supercritical CO_2, liquid hot water, wet oxidation, ultrasound, and others [3, 33, 35, 36].
In general, this means that the lignin-carbohydrate matrix is disrupted, leading to a decreased lignin barrier, decreased cellulose cristallinity, increased surface area, and increased porosity of the pretreated material. A leading pretreatment technology uses dilute sulphuric acid (50-300 mM) at 100-200 °C. Acid pretreatment hydrolyses and solubilises the hemicellulose fraction, while disrupting the lignin, and rendering the

residual cellulose more accessible for cellulolytic enzymes (Figure 1.4). The cost associated with pretreatment of the raw material is significant; about 20 % of the total production costs of second generation bioethanol production are associated with the pretreatment, proving the important role pretreatment plays in the production of second generation bioethanol [3, 33, 37, 38].

Choices for this study

Maleic and fumaric acid

While the hot dilute sulphuric acid treatment of lignocellulose is seen as a promising and applicable pretreatment [33, 37, 38], it has some drawbacks. Firstly, during the treatment, free sugars resulting from the hydrolysis of polysaccharides (mostly hemicellulose) can degrade to furfural and 5-hydroxymethylfurfural [40-42]. These compounds inhibit yeast cells in the final sugar-to-ethanol fermentation [43-45], and their production means loss of fermentable sugars. This would lead to extra costs due to larger needed reactor volumes as well as due to less efficient use of feedstock, respectively. Both fermentation costs and

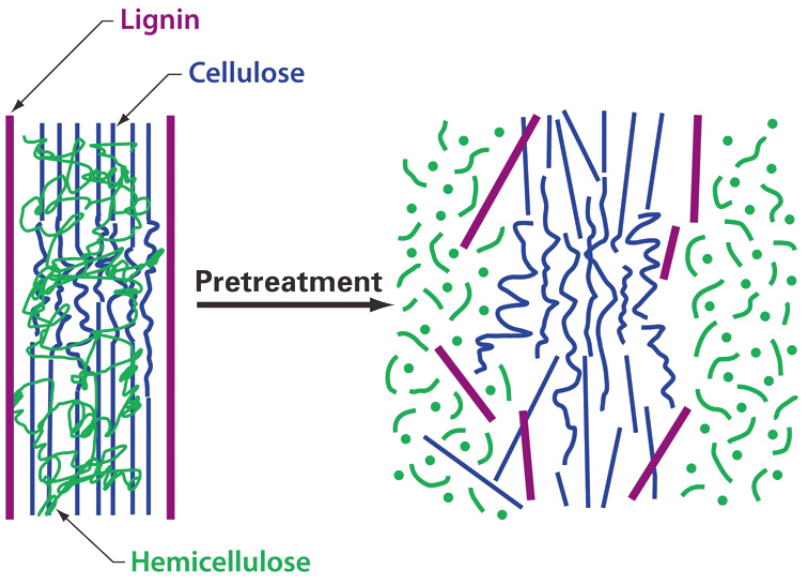

Figure 1.4. Schematic depiction of the effect of acid pretreatment on lignocellulosic biomass [33, 39].

raw material costs are relevant, when considering the total production costs of bioethanol [37]. Another downside of using sulphuric acid is the related production of gypsum. Gypsum may negatively affect downstream processing, but the value of the by-product stream will also be low, or even negative [3].

In research prior to this thesis, the organic maleic acid has been suggested as an alternative to sulphuric acid, since maleic acid catalysed the acid hydrolysis of cellobiose (dimer of glucose) and cellulosic material, while resulting in lower levels of glucose degradation than sulphuric acid [46, 47]. Fumaric acid is similar in structure to maleic acid, but it is somewhat weaker. As opposed to maleic acid, however, fumaric acid can be produced by fermentation. In principle, this can be done by using part of the sugar stream created by the hydrolysis of lignocellulosic polysaccharides; glucose as well as xylose [48-51]. If these organic acids were to be used in the pretreatment, the quality of the by-product stream would also improve significantly, as it might be more easily burned in co-firing installations, used as soil fertiliser, or applied in animal feed [3, 52, 53]. This would take biorefinery of lignocellulosic material a step further, and it would offer possibilities for valorisation of the raw material, although it should be taken into account that organic acids are generally more expensive than sulphuric acid [54, 55]. In short, application of maleic and fumaric acid in the acid pretreatment of lignocellulosic biomass might avoid all three mentioned downsides of using sulphuric acid.

Wheat straw

In the US, corn stover is the raw material of choice for the production of second generation bioethanol, since corn production (partly for first generation bioethanol) is over 300 million tonnes per year [56]. In the European Union, at 150 million tonnes per year, wheat production is more than twice as large as the production of corn (63 million tonnes per year) [57], making wheat straw a likely candidate to be used for second generation bioethanol production in the EU. This is also why wheat straw has been chosen for the research in this thesis.

EU wheat production uses about 26.5 million hectare (ha), or 28.9 % of the total harvested agricultural area, and wheat straw production is around 195 million tonnes per year. Assuming, firstly, that 1 to 2 tonne/ha of straw is left on the land in order to maintain soil quality and, secondly, that a 90 % yield of ethanol from carbohydrate is achieved, the total potential for EU bioethanol production from wheat straw lies between 52 and 61 GL per year [57-60]. This is about 32 % to 38 % of the 160 GL bioethanol needed to completely change from gasoline (145 GL/year average over 2004-2006) to E85 fuel (188 GL/year)

in the EU. This means that about 37.9 – 44.5 GL of gasoline can potentially be replaced with bioethanol from EU wheat straw, when using E85 [21, 61].

Jatropha curcas press cake

The main by-product from biodiesel and/or bio-oil production from *Jatropha curcas* seeds is de-oiled press cake, resulting from oil pressing and subsequent solvent extraction of remaining oil. As it is high in protein, there is a lot of extra valorisation to be obtained using the protein fraction of this type of feedstocks. *Jatropha* press cake (JPC) cannot be used directly for food or feed applications, because of the toxicity or anti-nutritional compounds that are present [62-66]. This is why a lot of the attention regarding valorisation is focused on the application of the protein fraction in the non-food sector: binders/glues, emulsifiers, protein films, plastics, and N-chemicals [67-75].
With worldwide *Jatropha curcas* oil production expected to increase enormously over the next two decades, the amounts of protein rich press cake are correspondingly large. As an example, for Indonesia alone (expected to be a major producer), estimations for production of biodiesel and bio-oil in 2025 vary from 4.7 to 6.4 and even 16 GL per year [76-79]. Assuming 6 GL and 50 % of which from *Jatropha curcas*, this would amount to an annual Jatropha seed production, resulting in around 6 million tonnes of de-oiled press cake per year. About a third of this, or 2 million tonnes per year, is protein. The polymeric sugar fraction of the press cake constitutes around 25 % (w/w) of the dry weight [75, 80], or 1.5 million tonnes per year, emphasising that this fraction can also add considerably to the valorisation of the production process of *Jatropha* oil.

Research aim

The main aim of this thesis is to study the performance of organic acids in the pretreatment of lignocellulosic material. More specifically, it would be interesting to see what the influence is of maleic and fumaric acids on the sugar degradation during the pretreatment, and how well these acids perform in the pretreatment itself, as alternatives to sulphuric acid. Points of interest are specific opportunities and financial benefits of the organic acid pretreatment, as are possible downsides which may lead to extra associated costs. Lastly, this study aims to look for opportunities for extending the application of the organic acid pretreatment to other, protein-containing, lignocellulosic raw materials.

1 Outline of this thesis

Chapter 2 is on the degradation of arabinose to furfural under dilute acid lignocellulose pretreatment conditions. The rate constants of arabinose degradation under hot acid conditions are determined at different temperatures, comparing fumaric and maleic acid with sulphuric acid and water as a control.

Chapter 3 deals with the dilute acid pretreatment of wheat straw, comparing fumaric and maleic acid with sulphuric acid, and water alone as a control. The influence of pretreatment temperature on the efficiency of the subsequent enzymatic hydrolysis is determined, as well as the influence of increasing the solids loading in the pretreatment reactor.

In **chapter 4**, the dilute maleic acid pretreatment of wheat straw is economically optimised. The variables used for the experimental design in this study are pretreatment time, pretreatment temperature and maleic acid concentration. Factors used for the optimisation are benefits from glucose and xylose, and costs resulting from furfural production, neutralisation needed for subsequent enzymatic hydrolysis, heating, and maleic acid replenishment.

Looking for higher-value applications of maleic acid, possibilities for increasing the valorisation of *Jatropha* press cake are studied in **chapter 5**. Raw material for the experiments in this study is the washed by-product of the alkaline protein extraction of de-oiled *Jatropha* press cake. The object is to solubilise the remaining protein fraction, comparing acidic conditions with a pH-neutral treatment, while also considering the pretreatment action on the lignocellulose fraction .

Finally, **chapter 6** is a general discussion chapter, in which results of the work in this thesis are discussed, and suggestions for future research are put forward. Lastly, this chapter presents the general conclusions of the thesis.

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Chapter 2

Differential effects of mineral and organic acids on the kinetics of arabinose degradation under lignocellulose pretreatment conditions

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2

Abstract

Sugar degradation occurs during acid catalysed pretreatment of lignocellulosic biomass at elevated temperatures, resulting in degradation products that inhibit microbial fermentation in the ethanol production process. Arabinose, the second most abundant pentose in grasses like corn stover and wheat straw, degrades into furfural. This paper focuses on the first order rate constants of arabinose (5 g/L) degradation to furfural at 150 and 170 °C in the presence of sulphuric, fumaric, and maleic acid and water alone. The calculated degradation rate constants (k_d) showed a correlation with the acid dissociation constant (pK_a), meaning that the stronger the acid, the higher the arabinose degradation rate. However, de-ionised water alone showed a catalytic power exceeding that of 50 mM fumaric acid and equalling that of 50 mM maleic acid. This cannot be explained by specific acid catalysis and the shift in pK_w of water at elevated temperatures. These results suggest application of maleic and fumaric acid in the pretreatment of lignocellulosic plant biomass may be preferred over sulphuric acid. Lastly, the degradation rate constants found in this study suggest that arabinose is somewhat more stable than its stereoisomer xylose under the tested conditions.

Introduction

Future oil shortages, increasing oil prices and international agreements are reasons for increased research on alternative routes to produce chemicals and transportation fuels. Fermentation technology can produce such liquid fuels, but the feedstock (fermentable sugars) and processing costs need to be sufficiently low to compete economically with oil derived fuels. In current first generation bioethanol production, relatively expensive sugar and starch derived from sugar cane and maize are used as feedstock. However, second generation processes will use relatively cheap and more abundant renewable lignocellulosic raw material, such as agricultural residues like corn stover, wheat straw, or forestry by-products. Using these by-product streams also results in less competition for high-quality edible carbohydrates.

Lignocellulosic biomass requires pretreatment to facilitate the hydrolysis of cell wall polysaccharides to fermentable sugars [1]. Pretreatment usually combines a catalyst (acid or base) in water with thermal treatment. For example, sulphuric acid pretreatment is used at 50-300 mM at 100-200 °C to hydrolyse hemicellulose, disrupt lignin, and render the residual cellulose more reactive when exposed to cellulolytic enzymes [1-4]. During the acid pretreatment at elevated temperature, degradation of the fermentable sugars occurs. Degradation products like furfural from pentoses and 5-hydroxymethylfurfural (HMF) from hexoses are inhibitory to yeasts in subsequent sugar-to-ethanol fermentation processes, which results in a lower efficiency of the ethanol production process [5-8].

At elevated temperatures, furfural degrades further into formic acid [9], while HMF degrades into both formic and levulinic acid [5, 6]. In warm season grasses like wheat and maize, the hemicellulose fraction of the structural polysaccharides largely consists of arabinoxylan or glucuronoarabinoxylan (GAX) [10-12]. Thus, arabinose is the second most abundant pentose present in biomass like corn stover and wheat straw. While lignocellulosic materials contain much less L-arabinose than D-xylose, the relative amounts of the sugars strongly depend on the raw material. For example, on a dry matter basis corn stover contains of 15 % xylan and 3 % arabinan, wheat straw contains 19 % xylan and 2 % arabinan, whereas wheat bran contains 19 % xylan and 15 % arabinan [13, 14]. Priority has been given to efforts to develop metabolically engineered microbes to ferment xylose to ethanol. However, recent efforts have been initiated to develop microbes able to convert arabinose to ethanol in order to increase yields proportionally [15-18]. In addition, arabinose is a pentose and, like xylose, can be degraded to furfural [9, 19-21]. If the degradation rate of arabinose is similar to or higher than that of xylose (or glucose), its presence and behaviour during the pretreatment may have an important

negative influence on the ethanol production process. Since feedstock constitutes a substantial fraction of the end product prices, improving yield is important to the economic success of commodity chemical and fuel production. While arabinose may not be the most important sugar defining ethanol yield, its significance cannot be overlooked in the development of lignocellulose conversion technologies.

Maleic acid has been described as a possible alternative to sulphuric acid in acid pretreatment [22], resulting in high glucose yields and in lower amounts of inhibitory by-products. The latter is explained by the fact that while sulphuric acid is strong, maleic acid is a weak acid and sugar degradation is acid-catalysed [19, 21, 23, 24]. In addition, xylose degradation has been shown to be much slower in the presence of maleic acid compared with sulphuric acid below 175 °C [25].

Application of sulphuric acid also leads to a large inorganic waste stream, mostly gypsum. Using organic acids in the pretreatment would increase the quality of the by-product stream. An organic by-product stream would logically be more easily applied in co-firing installations, in fertilising soil, and in animal feed [26, 27]. Thus there is interest in using organic acid to pretreat lignocellulosic biomass, including maleic, succinic, and acetic acid [22]. Fumaric acid is similar in structure to maleic acid (trans- and cis-butenedioic acid, respectively) and is stronger than succinic acid. Fumaric acid may be produced in situ by fermentation, and together with acid recycling [28-30] these are possible options to further improve the efficiency of the whole ethanol production process.

In acid pretreatment of lignocellulose, the dilemma is that intensifying the acid pretreatment conditions to reach a higher sugar yield, usually means a higher degree of sugar degradation. A compromise is needed between sugar yield and the level of sugar degradation. What is more important depends on the applications and value of the different (by-)product streams.

Generally speaking, less sugar degradation and furfural formation is better and therefore the advantage of organic acids versus sulphuric acid is two-fold: less sugar degradation and an organic by-product stream.

In this paper, the kinetics of the degradation of arabinose are studied in the presence of sulphuric, maleic, and fumaric acid, and of water alone. Experimental conditions such as temperature, reaction times, and arabinose concentration are similar to those found in the pretreatment of lignocellulose biomass like corn stover and wheat straw. To link to practical pretreatment, as well as to show relevance of arabinose and the chosen experiment conditions, conversion of arabinan to monomeric arabinose is determined using wheat straw as lignocellulosic feedstock in lab scale pretreatment.

Materials and Methods

All chemicals, except where noted below, were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Experimental set up of arabinose degradation

For assessing arabinose degradation in the presence of different acid catalysts, arabinose (Sigma A3131) was dissolved in de-ionised water or in 50 mM aqueous acid solutions to generate an arabinose concentration of 5 g/L (33 mM). The acids used were maleic (M-0375), fumaric (F-19353) and sulphuric acid (Mallinckrodt 2468), and all used chemicals were of research grade. Degradation at temperatures of 150 and 170 °C was examined with reaction times ranging from 10 to 60 minutes. For each reaction temperature, triplicate experiments were conducted for each of the de-ionised water/acid conditions.

Arabinose degradation kinetics measurement

Due to the increased pressure at elevated temperatures (a vapour saturation pressure of water of ~5 and 8 bars at 150 and 170 °C, respectively) [31] and the mechanical stress of rapid temperature changes on the reactors, all kinetics experiments were carried out in modified miniature glass reactor tubes. The reactor tubes were constructed using 12x32 mm crimp top HPLC vials (Alltech, Nicholasville, KY, USA) with the seal reinforced by the addition of a piece of 0.075 mm (0.003 inch) brass sheet fitted between the original seal and the crimp cap. Each reactor has a 2.0 mL total volume, with a 1.5 mL working volume (at room temperature) to allow head space for liquid thermal expansion. Temperature control was achieved utilising a Techne SBS-4 fluidised sand bath (Cole-Parmer, Vernon Hills, IL, USA). The heat-up time was considered to be insignificant due to the very small size of the reactor vials (1.5 mL content). After the selected reaction time, the reactor vials were cooled by quenching in 20 °C water. After the reactors were cooled down, the content was filtered through a 0.20 µm nylon filter (Fisherbrand), diluted to an appropriate concentration and further analysed by the HPLC system described below.

HPLC analysis in degradation experiments

Samples were analysed for arabinose, organic acids, and furfural concentrations by HPLC. Sample analysis utilised a Bio-Rad HPX-87H (300×7.8 mm) organic acid column (Bio-

Rad Laboratories Inc., Hercules, CA, USA) in a HPLC system consisting of a Rainin pressure module and Rainin solvent delivery system (Rainin Instrument, Oakland, CA, USA), Waters 717 plus autosampler, Waters 2414 refractive index detector, Waters 2487 dual λ absorbance detector set at 280 nm (Waters Corp., Milford, MA, USA), and a personal computer with Empower software (Waters Corp., Milford, MA, USA) for data processing and storage. The mobile phase was 5 mM sulphuric acid in distilled, de-ionised water filtered through 0.2 μm filters. The operating conditions for the HPLC column were 70 °C with a mobile phase flow rate of 0.6 mL/minute. Complete sample elution was be accomplished within 48 minutes per injection. Arabinose and organic acids were measured by refractive index and furfural by UV absorption. Standard curves were obtained by dissolving pure compounds (> 99 % purity) in the mobile phase. Fractional dilutions of the standard solution were prepared to give calibration curves against peak area for arabinose (0.125 – 4.000 g/L), organic acids (0.125 – 4.000 g/L), and furfural (Fluka 48070) (0.0116 – 0.148 g/L). When the linear regressions for the calibration curves were computed, R^2 values were > 0.9999 in all cases.

Preparation and analysis of wheat straw

Wheat straw (harvest September 2006, Delfzijl, The Netherlands) was milled twice; first in a Pallmann mill (4×30 mm sieve) and then in a Retsch mill (1 mm sieve). Milled straw was kept in a sealed plastic barrel at room temperature until used. Chemical composition was analysed as described by TAPPI methods [32-37], with minor modifications. Samples were extracted with ethanol:toluene 2:1, 96 % (v/v) ethanol and hot water (1 hour) at boiling temperature. The extracted samples were dried at 60 °C for 16 hours. Monomeric sugar and lignin content of the ethanol-extracted material was determined after a two-step hydrolysis with sulphuric acid (12 M for 1 hour at 30 °C; 1 M for 3 hours at 100 °C). The acid soluble lignin in the hydrolysate was determined by spectrophotometric determination at 205 nm. Monomeric sugars were measured by HPAEC-PAD (High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection). A Dionex system with Carbopak PA1 column with pre-column was used at 30 °C, with de-ionised water as mobile phase (1 mL/min) and fucose as internal standard. For comparison purposes, the Dionex HPLC method was also used for determination of monomeric arabinose in the liquid phase of both pretreated and enzymatically hydrolysed wheat straw. Dry matter content was 91.8 % (w/w) (24 h at 105 °C). On dry matter base, the straw composition was: 36.3 % cellulose, 23.2 % hemicellulose, 25.5 % lignin, 3.3 % protein, 7.8 % extractives, and 6.7 % ash (w/w). The arabinan content was 2.1 % (w/w).

Wheat straw pretreatment

Milled wheat straw (8.0 g) was mixed in poly-ethylene containers with 65.5 mL of acid solution (50 mM) or with de-ionised water, resulting in 10 % (w/w) straw solid loading. Acids used were maleic acid (M-153), fumaric acid (F-19353) and sulphuric acid (Fluka 84721). The straw/acid mixture was soaked for 20 to 24 hours at room temperature and then transferred to 316L stainless steel reactors (inner height × diam.: 90.0 × 40.0 mm; 5.0 mm wall), fitted with thermocouples. Four reactors at a time were heated in a Haake B bath with a Haake N3 temperature controller (Thermo Fisher Scientific, Waltham, MA, USA), filled with silicon oil (DC 200 fluid, 100 cSt, Dow Corning, Midland, MI, USA). Sample core temperature was recorded (Picotech data collector and software; Picotech, UK). Holding time was 30 minutes, starting from when desired core temperature was reached. Heating bath oil was preheated to 100 °C; the temperature difference between the oil and the inside of the reactor did not exceed 10 °C during heat up, and not more than 1 °C during the holding time. After the reaction time, the reactors were cooled by quenching in ice water. Duplicate experiments were conducted.

Enzymatic hydrolysis of pretreated wheat straw

After pretreatment, reactor contents were transferred to 250 mL baffled shake flasks. De-ionised water was added to dilute to 5 % (w/w), based on straw dry weight, taking into account water added during pH adjustment to 5.0 with 0.1 and 1 M NaOH solution, and water added with addition of 0.4 mL per g dry matter straw of GC220 cellulase enzyme mixture (Genencor, Rochester, NY, USA) at the start of the enzymatic hydrolysis. Flasks were left overnight for the pH to equilibrate. After pH fine tuning and enzyme addition, flasks were closed with airtight plugs and placed in an Innova 44 incubator shaker (50 °C, 150 rpm, 2 inch stroke; NBSC, Edison, NJ, USA). Samples of 1.5 mL were taken at t=0 and 72 h; after 5 minutes enzyme inactivation at 90 °C, samples were stored at -20 °C until arabinose analysis.

The arabinose yield was calculated as follows:

$$Arabinose\ yield\ (\%) \quad = \quad \frac{AH}{AS} \times 100\ (\%) \tag{1}$$

where AS is the arabinan content (%) of the dry straw (g arabinose/g dry matter straw), and AH is the arabinose content (%) of the hydrolysate supernatant (g arabinose/g dry matter straw).

Results and Discussion

Arabinose from wheat straw

Wheat straw was pretreated at 150 °C, in presence 50 mM of sulphuric, maleic, and fumaric acid, or water alone. The formation of arabinose monomers was measured after pretreatment and subsequent enzymatic hydrolysis, and expressed as percentage of arabinose yield (see Table 2.1). Maximal yield means that all of the 2.1 % (w/w) arabinosyl groups in the straw were hydrolysed.

The stronger the acid in the pretreatment, the more arabinosyl side chains were converted to arabinose. Up to 80 % arabinose yield was reached, after pretreatment with sulphuric acid; while using the organic acids results in a little less free arabinose. This shows that most of the arabinosyl side chains in the hemicellulose fraction of wheat straw are released as fermentable monomers. It also confirms the significance of arabinose contribution to improving the overall yield in lignocellulosic ethanol production. The 80 % arabinose yield under the tested conditions corresponds very well with literature values, for example on wheat bran [14].

The enzymatic hydrolysis did not increase the arabinose yield much, or not at all in the case of maleic and sulphuric acid pretreatment. Acid strength during pretreatment had more effect than subsequent enzymatic treatment. The fact that the 70 % yield during maleic acid pretreatment was not raised by the enzymatic treatment, while 80 % arabinose was released during the sulphuric acid pretreatment, suggests the possibility that some arabinosyl side chains were still remaining after the maleic acid pretreatment, and were not released during the enzymatic treatment. This would mean that it may not have been extensive arabinose degradation that was limiting the arabinose yield during the maleic

Table 2.1. Arabinose yield (%) from acid pretreated wheat straw.

	After pretreatment	*After enzymatic hydrolysis*
De-ionised water	15 (± 0.3)	33 (± 0.6)
50 mM fumaric acid	56 (± 0.8)	62 (± 0.5)
50 mM maleic acid	71 (± 0.6)	72 (± 1.1)
50 mM sulphuric acid	80 (± 0.5)	79 (± 1.3)

Acid pretreatment: 30 minutes at 150 °C; 50 mM acid; 10 % (w/w) dry straw solids loading.
Between brackets: deviation from average.

acid pretreatment, but that the subsequent enzymatic hydrolysis to arabinose itself was limited.

Arabinose degradation in solution

While in this study most of the arabinosyl groups are converted to arabinose during the acid pretreatment of wheat straw, degradation of the resulting sugar lowers the potential ethanol yield while also generating fermentation inhibiting furfural. Therefore, a closer examination of arabinose degradation to furfural in the presence of these acids was conducted. Arabinose degradation was examined in the presence of the same acids used for pretreatment: fumaric, maleic and sulphuric acid. As a control arabinose degradation was also measured in the presence of de-ionised water only. Samples were heated for 10, 20, 30 and 60 minutes at 150 and 170 °C. The degradation rate of arabinose is modelled as first-order with respect to arabinose and as zero-order with respect to the degradation product furfural, which leads to:

$$-\frac{dC_A}{dt} = k_d \cdot C_A \tag{2}$$

where C_A = arabinose concentration (g/L) and k_d = first-order degradation rate constant (min^{-1}).

All experiments were performed with an initial arabinose concentration of 5 g/L (33 mM). The measured residual arabinose concentration in the solution is expressed as a ratio over original concentration. The -ln(Ct/C0) versus time plot is shown in Figure 2.1 for a reaction temperature of 170 °C, with the slope of the graph representing the degradation rate constant k_d (min^{-1}). The calculated degradation rate constants for all experimental conditions can be found in Table 2.2. A student t-test was performed ($P<0.01$) to determine if differences between degradation rate constants were statistically significant [38].

At 150 °C, sulphuric acid has a larger degradation rate constant than the two organic acids (Figure 2.1 and Table 2.2). The difference between the rate constants of fumaric and maleic acid was not statistically significant ($P<0.01$). However, the presence of the organic acids resulted in a significantly smaller reaction rate constant than when water alone was present. At 170 °C, all degradation rate constants were larger than at 150 °C. Sulphuric acid (50 mM) showed a larger rate constant and therefore a higher degradation

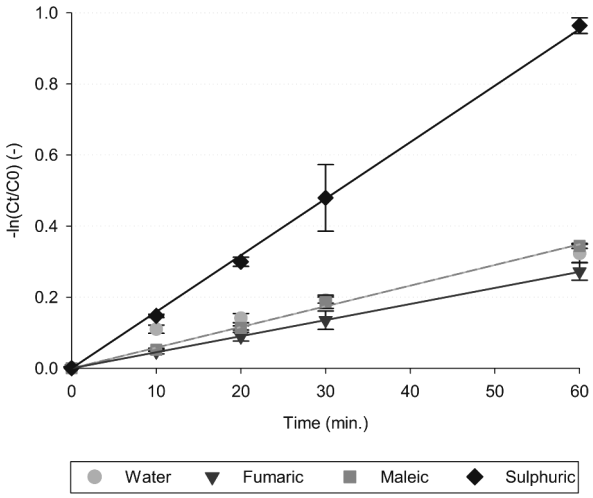

Figure 2.1. Arabinose degradation at 170 °C, all acids at 50 mM. Error bars: 95 % confidence interval.

rate than maleic acid. Maleic acid resulted in a larger rate constant than fumaric acid. When only water was present, the resulting rate constant was equal to that of maleic acid, significantly larger than that of fumaric acid and significantly smaller than that of sulphuric acid.

Comparing degradation rate constants from earlier studies on glucose and xylose (Table 2.2), at 170 °C in the presence of both 50 mM sulphuric and maleic acid, the degradation rate constants for arabinose were smaller than those found for xylose by Lu and Mosier [25]. The same is the case at 150 °C. The results of the present study indicate that arabinose is more stable than xylose under the tested conditions (acid catalysts and temperature). Concerning glucose, the stability of this sugar seems to lie in between those of arabinose and xylose, when maleic acid is present at 170 °C. At 170 °C in the presence of sulphuric acid, glucose appears to degrade more readily than xylose or arabinose. However, the glucose results from Mosier *et al*. [39] were obtained using stainless steel reactors while the xylose and arabinose results were obtained using glass reactors. Differences in degradation rate of xylose at similar pH in the presence of different inorganic catalysts have also been previously noted, with Fe^{3+} being a strong catalyst for the degradation of xylose [40]. Thus in comparing results from different studies,

Table 2.2. Calculated degradation rate constants k ($x10^{-3}$ min^{-1}) of arabinose degradation, compared with previously published data for glucose and xylose degradation rate constants (50 mM acid).

	Arabinose		Xylose		Glucose	
	150 °C	170 °C	150 °C	170 °C	150 °C	170 °C
De-ionised water	1.49 (± 0.12)	5.81 (± 0.53)	n.a.	n.a.	n.a.	n.a.
50 mM fumaric acid	0.61 (± 0.23)	4.52 (± 0.11)	n.a.	n.a.	n.a.	n.a.
50 mM maleic acid	0.92 (± 0.27)	5.81 (± 0.13)	1.83	11.04	1.86	8.48
50 mM sulphuric acid	2.56 (± 0.38)	15.9 (± 0.32)	5.02	19.08	8.48	38.7

Between brackets: limits of 95 % confidence interval. Xylose data from Lu and Mosier [25], Glucose data from Mosier *et al.* [39], n.a.: data not available

differences in the reactor construction and the possible influence of metal catalysts (especially steel) may bias the data.
In the present study, at 150 °C the degradation rate constants from maleic and fumaric acid were not significantly different, but they were at 170 °C (maleic acid being higher). One explanation for this is the difference in pH between fumaric and maleic acid solutions is greater at 170 °C than at 150 °C [41]. These results may also suggest that the activation energy for arabinose degradation in the presence of maleic acid is higher than the activation energy in the presence of fumaric acid [5, 25, 42, 43].

At an acid concentration of 50 mM, the arabinose degradation rate at 170 °C differs depending on the pK_a of the acid used (Table 2.3). The stronger the acid, the larger the resulting degradation rate constant of arabinose. However, at 170 °C, the degradation rate constant of arabinose in the presence of 50 mM maleic acid is equal to that when only water is present. This means that 50 mM maleic acid used shows no extra catalytic power to that of water and fumaric acid acts to stabilise arabinose. Similar behaviour has been noted for glucose degradation by Mosier *et al*. [39]. There it was found that even at 100 and 200 mM of maleic acid, the degradation rate was very close to that of water alone. The result that a presumed acid-catalysed degradation is catalysed by 50 mM of sulphuric (strong) acid and not by 50 mM of a weak acid suggests that the degradation is not a standard specific acid (H^+) catalysed reaction. Mosier *et al*. [39] found that a minimal amount of catalyst donated H^+ (not from water) was needed to increase degradation rates above the baseline (water alone). When sulphuric acid concentrations are below 25 mM the rate of degradation of glucose approached the rate caused by water alone. Possibly, a similar minimal amount is needed to catalyse arabinose degradation.

Table 2.3. pK_a values of acids and measured pH of reaction mixtures at room temperature (-) [31].

Acid	pK_a	pH at 50 mM acid, 5 g/L arabinose in water
Fumaric acid	3.02 / 4.38	2.20
Maleic acid	1.92 / 6.23	1.86
Sulphuric acid	-3 / 1.99	1.43

When 50 mM fumaric acid is present, the arabinose degradation rate constant at 170 °C is smaller than when only water is present. Here, the presence of the acid seems to diminish the catalytic behaviour of water. At 150 °C, both organic acids seem to diminish the catalytic behaviour of water. An explanation for this may have to do with an influence of the anion. The anion may influence the degradation by inhibiting the protonation of the hydroxyl group (Figure 2.2), which is the rate-limiting step in sugar degradation [24].

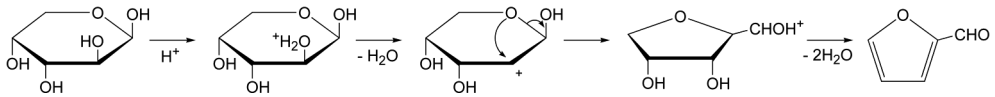

Figure 2.2. Degradation mechanism of arabinose to furfural (based on Nimlos *et al*. [21]).

It can be argued that it is the degradation in the presence of water alone that does not follow the general trend, and that all the tested acids are showing results that can be expected from a specific acid catalysis; larger rate constants as the pH decreases. The catalytic action of water alone can not be explained by acid catalysis and the increase in K_w at higher temperatures. When the temperature is raised from 25 °C to 150 °C, the pK_w decreases from 14 to 11.6 and the H^+ concentration increases by a factor 230 to around $1.5x10^{-3}$ mM [31]. However, this is still far from the H^+ concentration present in the reaction mixture with 50 mM fumaric acid at 25 °C, namely 6.3 mM. The K_a of carboxylic acids decreases as temperature rises from 25 to 150 °C or higher [41], but only by a factor of 3 to 4; not to the same extend as the increase of the K_w of water. Another mechanism for sugar degradation is a possibility. Nucleofilic attack of water and/or acid anion molecules on the sugar or the degradation intermediates (Figure 2.2) may account for these observations, but this remains undetermined. Further studies are needed to clarify the mechanism(s) of arabinose degradation in the presence of acids.

Conclusions

This study suggests using fumaric or maleic acid for biomass pretreatment instead of sulphuric acid has advantages. Mainly, because of the absence of catalytic action of the organic acids in arabinose degradation, less fermentation inhibiting furfural could improve the total efficiency of the ethanol production process. Indications are presented that the organic acids may even diminish the arabinose degradation, compared to water alone.
In addition, it is shown that during the maleic and fumaric acid pretreatment of wheat straw, most of the arabinosyl side chains in the hemicellulose fraction are released as fermentable arabinose to improve yields in ethanol production. The arabinose release is somewhat less than in the case of sulphuric acid, but the by-product stream is kept free of sulphur. Another interesting point is that arabinose degrades less readily than xylose and glucose. This difference may contribute to a better understanding of the mechanism of sugar degradation into fermentation inhibiting products during the pretreatment of cellulosic plant biomass.
All in all, it is clear from this study that careful selection of acid properties plays an important role in creating the most efficient acid pretreatment process.

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Chapter 3

Comparison of Dilute Mineral and Organic Acid Pretreatment for Enzymatic Hydrolysis of Wheat Straw

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3

Abstract

The efficiencies of fumaric, maleic, and sulphuric acid in wheat straw pretreatment were compared. As a measure for pretreatment efficiency, enzymatic digestibility of the lignocellulose was determined. Monomeric glucose and xylose concentrations were measured after subsequent enzymatic hydrolysis, as were levels of sugar degradation products furfural and hydroxymethylfurfural after pretreatment. The influence of pretreatment temperature and of wheat straw loading were studied. It is shown that, at 150 °C and 20 to 30 % (w/w) dry wheat straw, the pretreatment with dilute fumaric or maleic acid can be a serious alternative to dilute sulphuric acid pretreatment.

Introduction

Second generation bioethanol production uses relatively cheap, abundant, and renewable agricultural by-products, such as corn stover, wheat straw, or forestry residues. Furthermore, compared to first generation bioethanol, using lignocellulosic by-product streams results in less competition for high-quality edible carbohydrates between food and fuel application. In the European Union, with annual wheat production at more than 120 million tonnes, wheat straw is a likely candidate for use in second generation bioethanol production [1]. Lignocellulosic biomass requires pretreatment to improve cellulose accessibility to cellulolytic enzymes. Usually this entails a heat treatment in water in presence of a catalyst (acid or base). A common pretreatment uses dilute sulphuric acid (50-300 mM) at 100-200 °C to disrupt the lignin-carbohydrate matrix, and to facilitate enzymatic cellulose hydrolysis [2-7]. During hot acid pretreatment, some of the polysaccharides are hydrolysed, mostly hemicellulose. The resulting free sugars can degrade to furfural (from pentoses) and to 5-hydroxymethylfurfural (HMF; from hexoses) [8-10]. These compounds inhibit yeast cells and lead to decreased growth rate, ethanol production rate, and ethanol yield. In addition, their production means loss of fermentable sugars [11-13].

Maleic and fumaric acid have been suggested as alternatives for sulphuric acid in the pretreatment. Sugar degradation to furfural and HMF is described as acid catalysed, but neither maleic, nor fumaric acid promote such degradation reactions, resulting in lower amounts of degradation products [10, 14-19]. Another reason to replace sulphuric acid in the pretreatment is that it leads to large amounts of gypsum, which can negatively affect the downstream process, bu4t also results in a low-value by-product stream [6]. With organic acids, the quality of the by-product stream improves significantly, as it may be more easily burned in co-firing installations, used for fertilising soil, or applied in animal feed [20, 21].

In dilute acid pretreatments described in literature, solids loading usually varies from ca. 5 to 15 % (w/w) dry lignocellulosic biomass [16, 22, 23]. Substantially increased lignocellulose solids loading is preferred from an industrial point of view [24], as this reduces the amount of liquid phase per amount of feedstock, leading to lower energy demands and reduced reactor volume. In addition, a more concentrated product stream would reduce ethanol production costs, as well as water removal costs in the bioethanol separation/purification process.

In this study, we compared the efficiencies of water, fumaric, maleic, and sulphuric acid in the pretreatment of wheat straw at various temperatures. We investigated whether the dilute organic acids can pretreat wheat straw with an efficiency comparable to that of dilute sulphuric acid, while producing significantly less sugar degradation products. Furthermore, we investigated the effect of raising the solids loading, both on the efficiency of the pretreatment, as well as on the formation of sugar degradation products. As a measure of pretreatment efficiency, the enzymatic digestibility of the (hemi)cellulose was determined, calculating glucose and xylose yields from cellulose and hemicellulose conversion.

Materials and Methods

Preparation and analysis of wheat straw

Wheat straw (harvest September 2006, Delfzijl, The Netherlands) was milled twice; first in a Pallmann mill (4 × 30 mm sieve) and then in a Retsch mill (1 mm sieve). Milled straw was kept in a sealed plastic barrel at room temperature until used. Chemical composition was analysed as described by TAPPI methods [25-30], with minor modifications: (1) samples were extracted with ethanol:toluene 2:1, 96 % (v/v) ethanol and hot water (1 hour) at boiling temperature. (2) the extracted samples were dried at 60 °C for 16 hours. (3) monomeric sugar and lignin content of the ethanol-extracted material was determined after a two-step hydrolysis with sulphuric acid (12 M for 1 hour at 30 °C; 1 M for 3 hours at 100 °C). (4) acid soluble lignin in the hydrolysate was determined by spectrophotometric determination at 205 nm.
Monomeric sugars were measured by HPAEC-PAD (High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection). A Dionex system with Carbopak PA1 column with pre-column was used at 30 °C, with de-ionised water as mobile phase (1 mL/min) and fucose as internal standard. The Dionex HPLC method was also used for determination of monomeric sugars in the aqueous phase of both pretreated and enzymatically hydrolysed wheat straw. Dry matter content was 91.8 % (w/w) (24 h at 105 °C). The chemical composition of the used wheat straw is shown in Table 3.1.

Experimental set up of wheat straw pretreatment

All acids were of research grade and used as received (maleic acid: Aldrich M153; fumaric acid: Aldrich F19353; sulphuric acid: Fluka 84721). Milled wheat straw (8.00 g;

Table 3.1. Chemical composition (dry-weight basis) of the wheat straw used in this study.

Component	Content (% w/w)
Glucan	36.3
Xylan	19.0
Arabinan	2.1
Galactan, mannan, rhamnan	< 0.6 each
Uronic acids	2.1
Lignin	25.5
Extractives	7.8
Protein	3.3
Ash	6.7

7.34 g dry matter) was mixed in poly-ethylene containers with 65.5 mL of acid solution (50 mM) or with de-ionised water, resulting in 10 % (w/w) dry straw solids loading. The straw/acid mixture was soaked for 20 to 24 h at room temperature and then transferred to 316L stainless steel reactors (inner height × diameter: 90.0 × 40.0 mm; 5.0 mm wall), fitted with thermocouples. Four reactors at a time were heated in a Haake B bath with a Haake N3 temperature controller (Thermo Fisher Scientific, Waltham, MA, USA), filled with silicon oil (DC 200 fluid, 100 cSt, Dow Corning, Midland, MI, USA). Sample core temperature was recorded (Picotech data collector and software; Picotech, Cambridgeshire, UK). Pretreatments were performed at 130, 150, and 170 °C. Holding time was 30 minutes, starting from when desired core temperature was reached. Heating bath oil was preheated to 100 °C; the temperature difference between the oil and the inside of the reactor did not exceed 10 °C during heat up, and not more than 1 °C during the holding time. After the reaction time, the reactors were cooled by quenching in ice water.

In constant acid-to-straw experiments at 20 and 30 % (w/w) dry solids loading, pretreatment was carried out at 150 °C, with 8.00 g (7.34 g dm) straw with 28.7 mL of 114 mM, and 16.5 mL of 199 mM acid, respectively. In this way, acid-to-straw-ratio was kept constant at 5.17 % (w/w). In the case of fumaric acid, room temperature solubility was surpassed; an appropriate amount of solid fumaric acid was therefore added to the straw on top of the 50 mM solution before soaking. All experiments were conducted in duplicate.

Enzymatic hydrolysis of pretreated wheat straw

After pretreatment, reactor contents were transferred to 250 mL baffled shake flasks. De-ionised water was added to dilute to 5 % (w/w), based on straw dry weight, taking into account water added during pH adjustment to 5.0 with 0.1 and 1 M NaOH solution, and also taking into account water added in dosing 0.4 mL per g dry matter straw of GC220 cellulase enzyme mixture (batch 4900759148, 7608 IU/mL cellulase activity, Genencor, Rochester, NY, USA) at the start of the enzymatic hydrolysis, corresponding to 46 FPU per g dry matter straw [31]. This relatively high dosage is in the plateau region of the dose -effect curve of the enzyme mixture. This was to ensure the effect of the pretreatment on the sugar yield would be measured, not the effect of the enzyme concentration.
Flasks were left overnight for the pH to equilibrate. After pH fine tuning and enzyme addition, flasks were closed with airtight plugs and placed in an Innova 44 incubator shaker (50 °C, 150 rpm, 2 inch stroke; NBSC, Edison, NJ, USA). Samples of 1.5 mL were taken at t=0 and 72 h; after 5 minute enzyme inactivation at 90 °C, samples were stored at -20 °C until analysis.

The glucose yield from cellulose was calculated as follows:

$$Glucose\ yield\ (\%) \quad = \quad \frac{GH}{GS} \times 100\ (\%) \tag{1}$$

where GS is the amount of glucose present in the sample of dry straw (g glucose equivalents in cellulose), and GH is the amount of glucose (g) present in the aqueous phase of the sample, after pretreatment or enzymatic hydrolysis.

Xylose yield from hemicellulose conversion was calculated similarly, using xylan/xylose content. The fact that the form in which xylose is stored in hemicellulose is arabinoxylan rather than xylan is ignored.

HPLC analytical method for organic acids and sugar degradation products

Maleic acid, fumaric acid, furfural, and 5-HMF concentrations after pretreatment were measured by HPLC. Measurements were performed in the liquid phase prior to starting the enzymatic treatment. A Waters system with Shodex Ionpak KC-811 column at 30 °C with a Fast Fruit Juice Guard-Pak pre-column was used. Mobile phase (1 mL/min) was

3.65 mM phosphoric acid, internal standard was phenoxyacetic acid and peak detection was done with UV (210/280 nm).

Results and Discussion

Influence of pretreatment temperature

Organic acids can pretreat wheat straw with high efficiency (Figure 3.1). At 170 °C, maleic acid pretreatment results in nearly stoichiometric glucose yield after enzymatic hydrolysis, just as sulphuric acid (96 % and 98 % glucose yield, respectively). Also at 150 °C, maleic acid pretreatment is very effective with close to 80 % glucose yield after enzymatic digestion. Fumaric acid is less effective than maleic acid. A fumaric acid pretreatment at 150 °C results in a little more than half of all available glucose to become available for enzymatic hydrolysis, while at 170 °C, this pretreatment yielded an 85 % cellulose digestibility.

In general, increasing the temperature and lowering the pH are known to increase the pretreatment efficiency [22, 32, 33]. The trends as illustrated in Figure 3.1 are therefore as

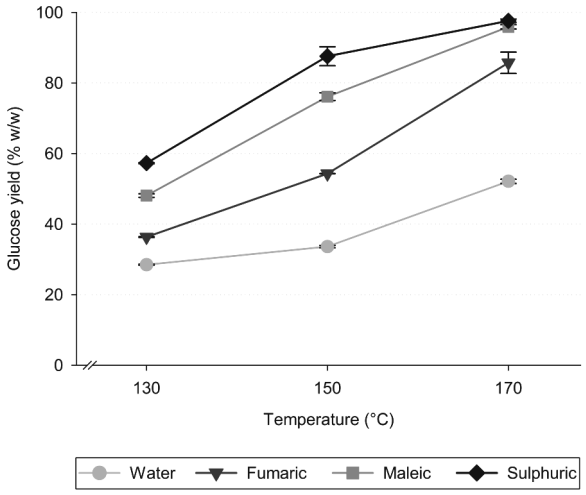

Figure 3.1. Glucose yield as function of pretreatment temperature, measured after enzymatic hydrolysis (72 h, 50 °C). 100 % = 0.40 g glucose/g dry matter straw. Error bars represent standard deviation.

expected: glucose yield from cellulose upon enzymatic hydrolysis increased with pretreatment temperature and with acid strength (i.e. water < fumaric < maleic < sulphuric).

A relatively small part of the glucan was already converted to monomeric glucose during the pretreatment (i.e. before enzymatic hydrolysis). Higher temperature and acid strength did increase this cellulose hydrolysis, but in these experiments glucose yield never exceeded 12 % (w/w), for sulphuric acid at 170 °C. Under these relatively severe conditions, less than 1.5 % of the total glucose was degraded to HMF. The resulting concentration of 3 mM HMF is not high enough to be inhibitory to yeasts in a glucose-to-ethanol fermentation process. HMF formation using the organic acids was negligible and comparable to pretreatment with only water (results not shown). Liu [34] reported yeasts capable of adaptation to 30 mM HMF without significant loss of productivity and yields in the fermentation process.

In Figure 3.2 and Figure 3.3, results for xylose yield from hemicellulose conversion are displayed. As opposed to cellulose, a large portion of the hemicellulose (up to ca. 80 % of total) was converted to monomeric sugars during the pretreatment, most notably when maleic or sulphuric acid were used at 150 or 170 °C (Figure 3.2). The conversion of hemicellulose during pretreatment is typical for dilute acid pretreatment and has been reported before by several authors when examining hemicellulose hydrolysis of corn stover and wheat straw [16, 22]. Generally, not all of the hemicellulose is hydrolysed completely to monomeric sugars. Some remains as insoluble hemicellulose, while another part is hydrolysed to oligomers. This phenomenon has been studied by several authors [23, 24, 35-37]. In the current study, the focus regarding hemicellulose conversion during pretreatment is on the production of monomeric xylose. Monomeric xylose has been shown to degrade to furfural in presence of dilute sulphuric acid, while xylose oligomer degradation under the same conditions was negligible. Some degradation of oligomeric xylose has been reported, but exclusively at neutral pH [38].

Pretreatment with sulphuric acid at 170 °C resulted in less free xylose than at 150 °C, due to more extensive degradation of xylose to furfural (Figure 3.4). When sulphuric acid was used at 170 °C, 23 % of the maximum xylose available was degraded to furfural, as opposed to half this amount when the two organic acids were used. At 150 °C, this difference is relatively even larger. Also at 150 °C, almost six times more monomeric xylose was released during the pretreatment with maleic acid than with the weaker fumaric acid, while only twice the amount of furfural was produced. In comparison, sulphuric acid pretreatment yielded only a little over 25 % more free xylose than maleic acid, while furfural formation using sulphuric acid increased almost by a factor three.

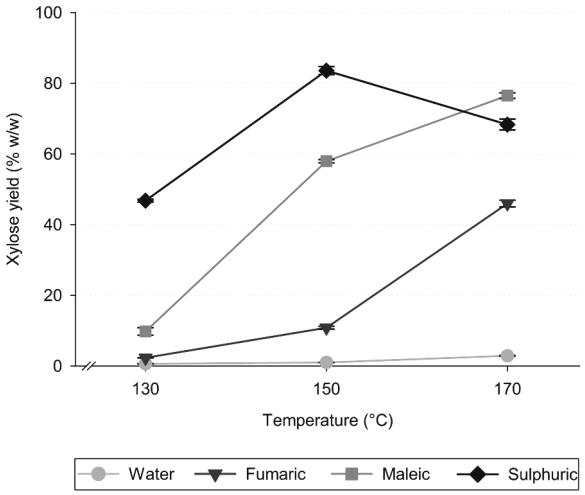

Figure 3.2. Xylose yield as function of pretreatment temperature, measured after pretreatment. 100 % = 0.21 g xylose/g dry matter straw. Error bars represent standard deviation.

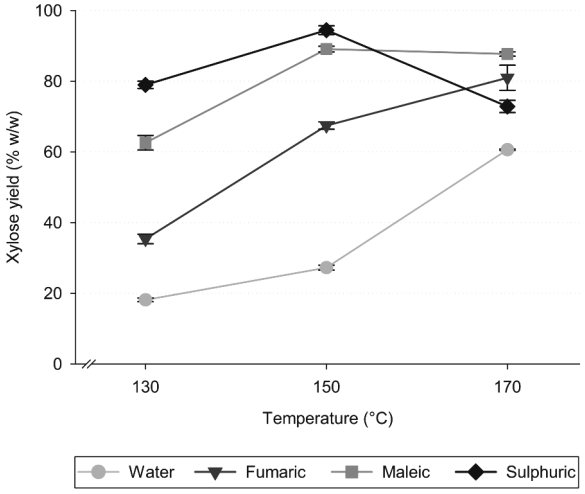

Figure 3.3. Xylose yield as function of pretreatment temperature, measured after enzymatic hydrolysis (72 h, 50 °C). 100 % = 0.21 g xylose/g dry matter straw. Error bars represent standard deviation.

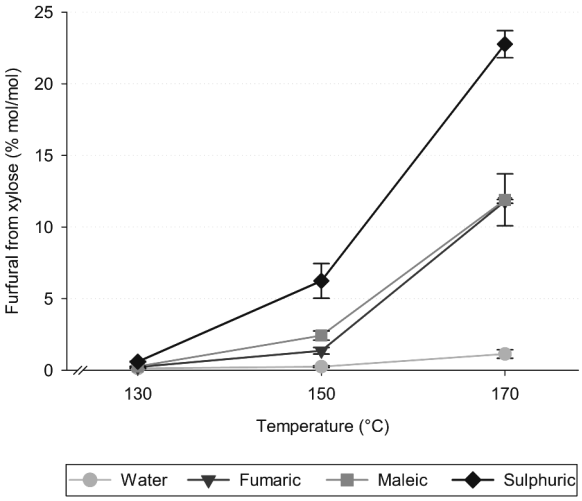

Figure 3.4. Furfural formation from xylose (% mol/mol) as function of pretreatment temperature. Error bars represent standard deviation.

When only water was used, hardly any free xylose was released and only trace amounts of furfural were produced during the pretreatment.
The above observations indicate that while the hydrolysis of hemicellulose during the pretreatment is acid catalysed (stronger acid causes more xylose release), furfural formation does not seem to be promoted by the presence of the organic acids. Sulphuric acid, however, seems to catalyse both hemicellulose hydrolysis as well as furfural formation. Although in this study the xylose degradation to furfural is not studied independently from hemicellulose hydrolysis during the pretreatment, these results do concur with earlier work on acid catalysed degradation of xylose and arabinose [16, 17]. A somewhat unexpected result is that similar amounts of furfural were produced during the 170 °C maleic and fumaric acid pretreatments. Assuming no catalysis of xylose degradation by either of the organic acids, furfural production was expected to be higher during the maleic acid pretreatment than when fumaric acid was used, due to the presence of more free xylose in the case of maleic acid (Figure 3.2 and Figure 3.4).

While the hot acid treatment causes a large fraction of the hemicellulose to be converted to monomeric sugar, the enzymatic treatment did further increase the xylose yield from what is shown in Figure 3.2 to what is shown in Figure 3.3. It would appear that the

enzyme mixture contains oligoxylanase and xylobiase activity. Using a pretreatment temperature of 150 °C, the final xylose yield after enzymatic hydrolysis from hemicellulose for maleic and sulphuric acid is similarly high: circa 90 to 95 %. When raising the pretreatment temperature to 170 °C, it is clear that both maleic and fumaric acid caused higher xylose yields (between 80 and 90 %) from hemicellulose than sulphuric acid did (circa 70 %), measured after the enzymatic treatment. Clearly, the mentioned xylose degradation to furfural during sulphuric acid pretreatment limited final xylose yield after enzymatic hydrolysis. The pretreatment with only water still resulted in final enzymatic xylose yield of around 60 %, when pretreatment was done at 170 °C.

Maleic acid pretreatment of lignocellulose is almost as effective as sulphuric acid in increasing enzymatic cellulose digestibility to glucose monomers, while yielding more xylose and less furfural. A similar conclusion is valid for fumaric acid, although it is less effective than maleic acid. Water is the least efficient, due to the fact it supplies the least H^+ ions to the acid catalysed hydrolysis, but it still results in a noticeable pretreatment of lignocellulose.

Raising the solids loading

Raising the solids loading in the pretreatment would decrease process cost, both by lowering reactor size and heating requirements during the pretreatment, and as well by creating a more concentrated product stream that is more efficient in downstream processing. This is assuming the effect of the acid pretreatment on increasing the enzymatic digestibility of the lignocellulose can be maintained at high solids loading. In the case of the organic acid pretreatment, it is also important to see if the effect of less sugar degradation product formation is maintained at high solids loading.

While the glucose yield was higher after 170 °C pretreatment (at 10 % solids loading), xylose yield for sulphuric acid pretreatment was lower, while furfural production was relatively high. Combined with the fact that the organic acids seemed to degrade or bind to the straw at 170 °C pretreatment (results not shown), it was decided to perform high solids loading experiments at 150 °C.

When the dry matter straw loading in the 150 °C pretreatment was raised from 10 to 20 and 30 % (w/w) while the acid concentration was maintained at 50 mM, this effectively meant the amount of acid available per amount of straw decreased. In these experiments, a strong decrease in enzymatic digestibility of cellulose and hemicellulose was observed for all acids studied, but not in the pretreatments with only water (results not shown). This suggests that it is not the higher solids loading that lowered the pretreatment efficiency, but more likely the lower acid-to-straw ratio as solids loading increases. During

pretreatment at high temperatures, uronic and acetic acids are released from the straw, which could catalyse the autohydrolysis of lignocellulosic material [23, 24, 35]. Therefore, it can be argued that, in the pretreatment with "only water", there are acids present and the amount available per amount of straw remains constant when the solids loading is raised. This can explain why no decrease in enzymatic digestion of cellulose and hemicellulose to monomeric sugars was observed.

With constant acid-to-straw ratio, raising the solids loading in 150 °C pretreatment from 10 to 20 and 30 % (w/w) did not lower glucose yield from cellulose, measured after the enzymatic hydrolysis (Figure 3.5). For the xylose yield from hemicellulose, the effect of raising the solids loading was somewhat different (Figure 3.6 and Figure 3.7). When water or fumaric acid were used, it was clear that higher solids loading pretreatment did not reduce xylose yield after enzymatic hydrolysis. But after sulphuric acid pretreatment, enzymatic xylose yield decreased by more than 20 %. This was largely caused by formation of circa 15 % more furfural (Figure 3.8).

Also illustrated in Figure 3.8 is that the effect of the organic acids causing much less furfural formation during the pretreatment than sulphuric acid does, persists at high solids

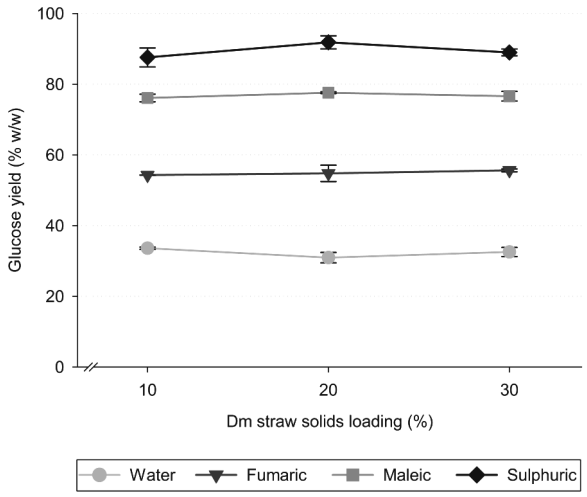

Figure 3.5. Glucose yield as function of dm straw loading, with equal acid:straw ratio, measured after enzymatic hydrolysis (72 h, 50 °C). Pretreatment temperature 150 °C. 100 % = 0.40 g glucose/ g dry matter straw. Error bars represent standard deviation.

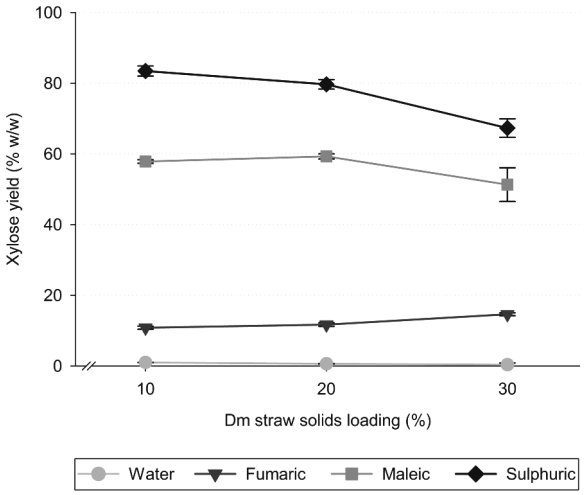

Figure 3.6. Xylose yield as function of dm straw loading, with equal acid:straw ratio, measured after pretreatment. Pretreatment temperature 150 °C. 100 % = 0.21 g xylose/g dry matter straw. Error bars represent standard deviation.

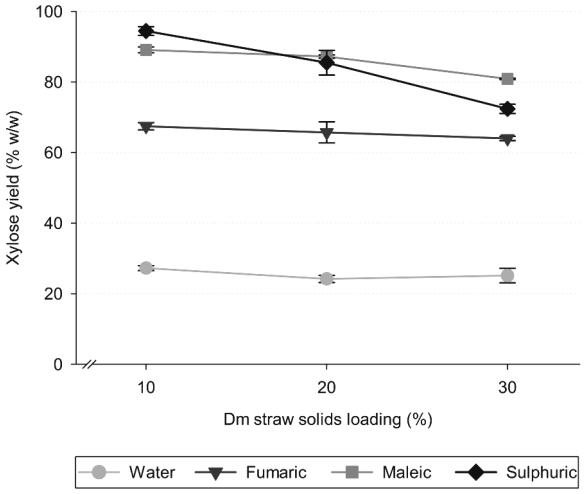

Figure 3.7. Xylose yield as function of dm straw loading, with equal acid:straw ratio, measured after enzymatic hydrolysis (72h, 50 °C). Pretreatment temperature 150 °C. 100 % = 0.21 g xylose/g dry matter straw. Error bars represent standard deviation.

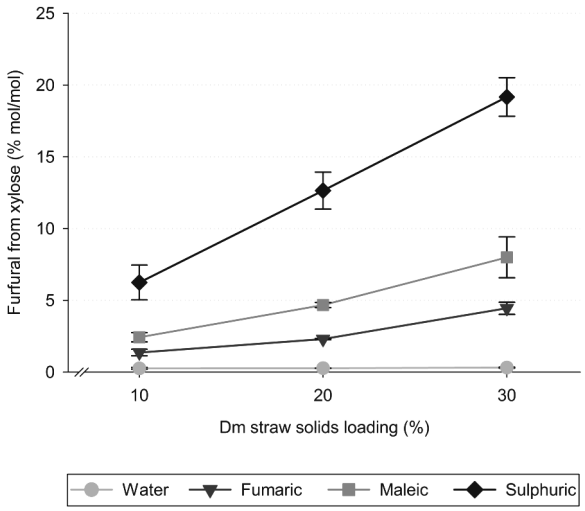

Figure 3.8. Furfural formation from xylose (% mol/mol) as function of dm straw loading, with equal acid:straw ratio. Pretreatment temperature 150 °C. Error bars represent standard deviation.

loading. However, furfural formation does increase when raising the solids loading, and for the maleic acid pretreatment this finally results in a slightly limited xylose yield after enzymatic digestion. Still, the final enzymatic xylose yield after a maleic acid pretreatment was higher than when sulphuric acid is used (Figure 3.7).

The reason for more sugar degradation at high solids loading can be twofold. Firstly, as the solids loading increases, the hemicellulose concentration increases. And although sugar degradation is usually assumed as first order, hemicellulose hydrolysis possesses kinetics of higher order [39]. The higher acid concentration combined with higher hemicellulose concentrations cause more hydrolysis into monomeric sugars that can be degraded. Secondly, higher sulphuric acid concentration in the aqueous phase will cause more degradation of monomeric xylose to furfural [15]. The relatively small amount of furfural formed in the maleic and fumaric acid pretreatment (3 and 5 times less than with sulphuric acid, respectively), again supports the idea that these acids do not catalyse sugar degradation, as was suggested earlier [15-17].

The sugar degradation during the sulphuric acid pretreatment at 30 % solids loading, resulted in 116 mM furfural in the aqueous phase. For maleic and fumaric acid, these furfural concentrations are 48 mM and 26 mM, respectively. Assuming enzymatic hydrolysis and subsequent (or simultaneous) sugar-to-ethanol fermentation at the similar

straw solids loading (30 % (w/w) as the pretreatment, these levels are likely to negatively influence the yeast fermentation. Since acetic acid is released during pretreatment and wheat straw is used at high concentrations, the combination of furfural and acetic acid (not measured) may be expected to negatively affect a subsequent ethanol production yield [40]. On the other hand, although inhibitory effects of furfural and HMF have been reported in the past, recent studies have focused on adaptation and increased tolerance to inhibitory compounds like HMF and furfural [34, 41-43].

All in all, it remains difficult to estimate the influence of the furfural concentrations produced in this study on the ethanol production by yeasts. One thing that can be stated is that even if the reported adaptation of the yeast is assumed, furfural concentration higher than 30 mM in the fermentor are best avoided. Minimal furfural production would obviously be best, since it would entail minimal sugar loss and therefore maximal feedstock utilisation. This all means that 20 % solids loading in the pretreatment, compared to 30 %, would probably result in less inhibition by furfural during ethanol production and a more efficient ethanol fermentation. Of course, there is probably room for optimisation.

To avoid high furfural concentrations in the sugar-to-ethanol fermentation, the final sugar-and-furfural containing hydrolysate can be mixed in with a sugar feedstock stream that contains no furfural at all. For example, a sugar stream from the first generation bioethanol production process, because no acid pretreatment at elevated temperatures is included in this process. Through mixing, low enough furfural concentrations can be reached. This is assuming that both processes are performed in each others vicinity.

Interestingly, after the organic acid pretreatment, part of the organic acid could no longer be measured in the aqueous phase. Most of this ‘lost’ acid was later detected after the enzymatic digestion of the pretreated material. This indicates that acid may be somehow selectively bound to the straw particles during the pretreatment, creating a lower acid concentration in the aqueous phase. From an economical perspective, the acid binding effect is important enough to be studied further, as it may negatively influence acid recycle. At a price of around 1750 US$ per tonne for maleic acid (maleic anhydride), maleic acid costs amount to a little over 90 US$ per tonne straw, assuming 5.17 % (w/w) acid-to-straw ratio and no recycle [44]. Sulphuric acid cost would be around 8.8 US$ per tonne straw, under the same assumptions [16, 45]. Clearly, organic acid recycle is of high importance to the economic feasibility of the process.

Conclusions

It has been made clear that efficient pretreatment of wheat straw is possible using maleic and fumaric acid. Furthermore, when raising the solids loading to 30 % (w/w), the acid pretreatment remains equally able to increase enzymatic digestibility of the feedstock. During the organic acid pretreatment, much less furfural is formed from xylose than when using sulphuric acid, and this effect persists when the solids loading is raised. However, at 30 % solids loading, the formation of furfural reaches undesirably high levels, not only in the case of sulphuric acid pretreatment, but to a lesser extent also in maleic acid pretreatment. To reach an acceptable furfural concentration, 20 % solids loading in the pretreatment would be advisable over 30 %, but there is room for optimisation. This study shows that the application of dilute organic acids in the pretreatment of lignocellulosic biomass like wheat straw can be effective and thus a serious alternative for the dilute sulphuric acid pretreatment.

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3

Chapter 4

Optimisation of the dilute maleic acid pretreatment of wheat straw

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Abstract

In this study, the dilute maleic acid pretreatment of wheat straw is optimised, using pretreatment time, temperature and maleic acid concentration as design variables. A central composite design was applied to the experimental set up. The response factors used in this study are: (1) glucose benefits from improved enzymatic digestibility of wheat straw solids; (2) xylose benefits from the solubilisation of xylan to the liquid phase during the pretreatment; (3) maleic acid replenishment costs; (4) neutralisation costs of pretreated material; (5) costs due to furfural production; and (6) heating costs of the input materials. For each response factor, experimental data were fitted mathematically. After data translation to €/tonne dry straw, determining the relative contribution of each response factor, an economic optimisation was calculated within the limits of the design variables.

When costs are disregarded, an almost complete glucan conversion to glucose can be reached (90 % from solids, 7-10 % in liquid), after enzymatic hydrolysis. During the pretreatment, up to 90 % of all xylan is converted to monomeric xylose. Taking cost factors into account, the optimal process conditions are: 50 min at 170 °C, with 46 mM maleic acid, resulting in a yield of 65 €/tonne dry straw, consisting of 68 €/tonne glucose benefits (from solids: 85 % of all glucan), 17 €/tonne xylose benefits (from liquid: 80 % of all xylan), 17 €/tonne maleic acid costs, 2.0 €/tonne heating costs and 0.9 €/tonne NaOH costs. In all but the most severe of the studied conditions, furfural formation was so limited that associated costs are considered negligible.

After the dilute maleic acid pretreatment and subsequent enzymatic hydrolysis, almost complete conversion of wheat straw glucan and xylan is possible. Taking maleic acid replenishment, heating, neutralisation and furfural formation into account, the optimum in the dilute maleic acid pretreatment of wheat straw in this study is 65 €/tonne dry feedstock. This is reached when process conditions are: 50 min at 170 °C, with a maleic acid concentration of 46 mM. Maleic acid replenishment is the most important of the studied cost factors.

Introduction

Second generation bioethanol production uses relatively cheap, abundant and renewable agricultural by-products, such as corn stover, wheat straw or forestry residues. Compared to first generation bioethanol production, the use of lignocellulosic by-product streams results in less competition for high-quality edible carbohydrates between food and fuel application.

With annual wheat production in the European Union (EU) at 120 million tonnes, it is the largest single cereal crop in the EU; with corn at 53 million tonnes per year. Wheat production uses about 25 million hectare (ha), or 28 % of the total harvested agricultural area, and wheat straw production is around 156 million tonnes per year. Assuming, first, that 1 to 2 tonne /ha of straw is left on the land in order to maintain soil quality and, secondly, that a 90 % yield of ethanol from carbohydrate is achieved, the total potential for EU bioethanol production from wheat straw lies between 39 and 48 gigalitre (GL) per year [1-4]. This is about 25 % to 30 % of the 160 GL bio-ethanol needed to completely change from gasoline (145 GL/year) to E85 fuel (188 GL/year) in the EU. This means that about 29 - 35 GL of gasoline can potentially be replaced with bioethanol from EU wheat straw, when using E85 [5, 6].

Lignocellulosic biomass requires pretreatment in order to disrupt the lignin-carbohydrate matrix and to facilitate enzymatic cellulose hydrolysis by improving cellulose accessibility to cellulolytic enzymes. This usually means a treatment that combines heat and a catalyst (acid or base). A common pretreatment uses dilute sulphuric acid (50-300 mM) at 100-200 °C. The cost for pretreatment is significant; about 20 % of the total production costs of second generation bioethanol production [7, 8]. During hot acid pretreatment, some of the polysaccharides are hydrolysed, mostly hemicellulose [7, 9-13]. The resulting free sugars can degrade to furfural (from pentoses) or to 5-hydroxymethylfurfural (HMF; from hexoses) [14-16]. These compounds inhibit yeast cells and lead to decreased specific growth rates, specific ethanol production rates and ethanol yields. In addition, their production implies a loss of fermentable sugars [17-19].

Maleic acid and fumaric acid have been suggested as alternatives for sulphuric acid in the pretreatment. Both organic acids promote the hydrolysis of polysaccharides but, unlike sulphuric acid, neither promotes the degradation of free sugars to furfural and HMF. In recent work, both maleic and fumaric acid have been shown to be able to pretreat wheat straw; maleic acid somewhat outperforming fumaric acid. Using the two organic acids

resulted in much smaller amounts of degradation products compared with using sulphuric acid [16, 20-25].
Using organic acid in the pretreatment instead of sulphuric acid also significantly improves the quality of the by-product stream, as it may be more easily burned in co-firing installations, used as fertiliser or applied in animal feed [7, 26, 27].
Several authors have published on the optimisation of pretreatment of straw-type lignocellulose materials [28-31], focusing exclusively, however, on a maximum sugar yield and disregarding pretreatment economics. In the present study, we optimise the dilute maleic acid pretreatment of wheat straw on a monetary basis. We focus on the analysis of the optimisation of the maleic acid pretreatment alone – not as part of an integrated conversion process. This means that factors, such as capital costs, downstream processing costs and recycle costs, are not included in this study.
We study the influence of varying pretreatment time, temperature and maleic acid concentration on the following six factors of the resulting pretreatment:

1. glucose benefits from improved enzymatic digestibility of the raw material
2. xylose benefits from the solubilisation of xylan during the pretreatment
3. costs from replenishment of lost maleic acid
4. costs due to neutralisation of the pressed pretreated material
5. costs due to furfural formation from pentoses
6. heating costs of the input materials

The influence of these factors will be expressed in €/tonne straw. This means a weighing step is introduced that determines the relative contribution of each response factor to the resulting yield. In this manner, we can optimise the maleic acid pretreatment to yield the most value per tonne straw.

Materials and Methods

Experimental design and set up

Design-Expert 7.1.5 software (Stat-Ease Inc., Minneapolis, MN, USA) was used for the experimental design, model fitting and statistical data analysis. In order to reduce the number of experiments needed, a central composite factorial design was applied; the experimental conditions are mentioned in the 'Wheat straw pretreatment' section. Experimental data for each response factor were expressed in mathematical models. The starting point was a quadratic model which was then adjusted by backward elimination:

taking out terms that had no significant contribution ($P > 0.05$) one by one, and then recalculating the model with the remaining terms.

Preparation and analysis of wheat straw

Wheat straw (harvest September 2006, Delfzijl, The Netherlands) was milled twice; first in a Pallmann mill (4 × 30 mm sieve) and then in a Retsch mill (1 mm sieve). Milled straw was kept in a sealed plastic barrel at room temperature until used. Chemical composition was analysed in triplicate, as described by TAPPI methods [32-37], with minor modifications: (1) samples were extracted with ethanol:toluene 2:1, 96 % (v/v) ethanol and hot water (1 h) at boiling temperature; (2) the extracted samples were dried at 60 °C for 16 h; (3) monomeric sugar and lignin content of the ethanol-extracted material was determined after a two-step hydrolysis with sulphuric acid (12 M for 1 h at 30 °C; 1 M for 3 h at 100 °C); (4) acid soluble lignin in the hydrolysate was determined by spectrophotometric determination at 205 nm.

Monomeric sugars were measured by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD). A Dionex system with Carbopak PA1 column with pre-column was used at 30 °C, with de-ionised water as the mobile phase (1 mL/min) and fucose as the internal standard. The Dionex high-performance liquid chromatography (HPLC) method was also used for the determination of monomeric sugars in the aqueous phase of both pretreated and enzymatically hydrolysed wheat straw. Dry matter content was 91.8 % (w/w) (24 h at 105 °C). The chemical composition of the used wheat straw is shown in Table 4.1.

Wheat straw pretreatment

All chemicals were of research grade and used as received (maleic acid: Aldrich M153). Milled wheat straw (8.00 g; 7.34 g dry matter) was mixed in poly-ethylene containers with 65.5 mL of maleic acid solution (11, 50 or 89 mM), resulting in 10 % (w/w) dry straw solids loading. The straw/acid mixture was soaked for 20-24 h at room temperature and then transferred to 316L stainless steel reactors (inner height × diameter: 90.0 × 40.0 mm; 5.0 mm wall), fitted with thermocouples. Reactors were heated in a Haake B bath with a Haake N3 temperature controller (Thermo Fisher Scientific, Waltham, MA, USA), filled with silicon oil (DC 200 fluid, 100 cSt, Dow Corning, Midland, MI, USA). Sample core temperature was recorded (Picotech data collector and software; Picotech, Cambridgeshire, UK). Pretreatments were performed at 130 °C, 150 °C and 170 °C. Holding time was 10, 30 and 50 minutes, starting from when the desired core temperature

Table 4.1. Chemical composition (dry-weight basis) of the wheat straw used in this study.

Component	Content (% w/w)
Glucan	36.3
Xylan	19.0
Arabinan	2.1
Galactan, mannan, rhamnan	< 0.6 each
Uronic acids	2.1
Lignin	25.5
Extractives	7.8
Protein	3.3
Ash	6.7

was reached. Heating bath oil was preheated to 1 °C above the desired temperature. During the holding time, the temperature inside the reactors never differed more than 1 °C from the desired temperature. After the reaction time, the reactors were cooled by quenching in ice water.

Solid-liquid separation: press step

The pretreated material was pressed in a custom built hydraulic press. The inner diameter of the press was 40.0 mm and the free moving speed was 10 mm/s. Press time was 10 s, starting from when maximum pressure of 200 bar was reached. A filter was placed (0.5 mm thick, 39.0 mm diameter; +/- 400 holes of 0.8 mm diameter, evenly distributed) on the porous bottom of the press. Both press and filter were made of 316L stainless steel. Pressed pellets and pressed out aqueous phase were collected and stored at -20 °C until, respectively, enzymatic hydrolysis and analysis.

Enzymatic hydrolysis

After the pretreatment and press step, the resulting pellets were dried (48 h at 60 °C, under vacuum) and transferred to 250 mL baffled shake flasks. De-ionised water was added to dilute to 5 % (w/w), based on original straw dry weight, taking into account water added with the Na-azide solution (0.05 % [w/w] final concentration to prevent microbial growth), during pH adjustment to 5.0 with 0.1 and 1 M NaOH solution and with the enzyme addition. At the start of the enzymatic hydrolysis, 0.4 mL per g dry matter straw

of GC220 cellulase enzyme mixture was added (batch 4900759148, 7608 IU/mL cellulase activity, Genencor, Rochester, NY, USA), corresponding to 46 FPU/g original dry matter straw [38]. This relatively high dosage is in the plateau region of the dose-effect curve of the enzyme mixture. This was to ensure the effect of the pretreatment on the glucose yield would be measured, not the effect of the enzyme concentration.
Flasks were left overnight for the pH to equilibrate. After pH fine tuning and enzyme addition, flasks were closed with airtight plugs and placed in an Innova 44 incubator shaker (50 °C, 150 rpm, 2 inch stroke; NBSC, Edison, NJ, USA). Samples of 1.5 mL were taken at t=0 and 96 h; after 5 minute enzyme inactivation at 90 °C, samples were stored at -20 °C until analysis.

The glucose yield from cellulose was calculated as follows:

$$Glucose\ yield\ (\%) = \frac{GH}{GS} \times 100\ (\%) \quad (1)$$

where GS is the amount of glucose present in the sample of dry straw (g glucose equivalents in cellulose) and GH is the amount of glucose (g) present in the aqueous phase of the hydrolysate, after enzymatic hydrolysis of the pellet. Xylose yield was calculated similarly, using xylan/xylose content. The fact that the form in which xylose is stored in wheat straw hemicellulose is arabinoxylan rather than xylan was ignored.

Organic acid and sugar degradation product analysis

Maleic acid, fumaric acid, furfural, and 5-HMF concentrations after pretreatment were measured by HPLC. Measurements were performed in the liquid phase prior to starting the enzymatic treatment. A Waters system with Shodex Ionpak KC-811 column at 30 °C with a Fast Fruit Juice Guard-Pak pre-column was used. Mobile phase (1 mL/min) was 3.65 mM phosphoric acid, internal standard was phenoxyacetic acid and peak detection was done with ultraviolet (210/280 nm).

Analysis of oligomeric sugars

To assess the amount of oligomeric sugars present in the liquid phase after the pretreatment, a method closely resembling National Renewable Energy Laboratory (NREL) LAP-014 [39] was used. One millilitre samples were used for a secondary hydrolysis, in triplicate. This was performed using 4 % (w/w) H_2SO_4 at 121 °C during 10

min. Monomeric sugars as well as degradation products were measured, as described above, and, comparing these results with the values from just after the pretreatment, the original amounts of oligomeric sugars were calculated, taking into account the extra formation of degradation products during the secondary hydrolysis.

Titration of liquid phase

The titration of the liquid phase was performed using a Metrohm 718 stat titrino set (Metrohm, Herisau, Switzerland), with the titration vessel equipped with a thermostatic jacket (25 °C). Of each sample, 3 mL of the liquid phase was brought to pH 5.0 with 50 mM NaOH. Analyses were performed in duplicate.

Optimisation of benefits and cost factors

The costs and benefit factors used in this study are defined as follows:
(1) Glucose benefits: the benefits from glucose that is released from the pressed pretreated material, after enzymatic hydrolysis. This shows the effect of the pretreatment on (increasing) the enzymatic digestibility of the raw material.
(2) Xylose benefits: the benefits from the liquid phase, after the solid-liquid separation. Both oligomeric and monomeric xylose that are solubilised during the pretreatment are taken into account.
(3) Costs for the maleic acid that is not recovered in the liquid phase after the solid-liquid separation and, therefore, needs to be replenished.
(4) Costs for NaOH needed to neutralise the pressed pretreated material prior to the enzymatic hydrolysis. This in effect combines two sub-factors: (a) the amount of NaOH needed per volume of liquid phase to set the pH to 5 and (b) the total amount of this liquid phase still present in the pressed pellet, or in other words: the efficiency of the solid-liquid separation. These two sub-factors may depend differently on the process conditions.
(5) Costs that arise due to furfural formation from pentoses.
(6) Heating costs of the straw that enters the process and of the water that needs to be replenished.
All data is expressed in €/tonne straw, a weighing step that reveals the relative contribution of each factor. The total benefits (that is the economic yield of the pretreatment) was calculated by subtracting all costs from the glucose benefits. Within the limits of the design space, an optimisation was calculated.

Results and Discussion

Effect of pretreatment conditions on glucose benefits

The main goal of the pretreatment of lignocellulosic material is to increase the enzymatic digestibility. In order to study the influence of pretreatment time, temperature and maleic acid concentration on the pretreatment of wheat straw, the enzymatic digestibility of the solid pretreated material was determined, after solid-liquid separation. The resulting glucose yield, as determined after subsequent enzymatic hydrolysis, was translated to €/ tonne straw glucose benefits (with glucose at €200 per tonne [40]). The fact that a more concentrated glucose stream has a higher value per amount of glucose than a less concentrated stream is ignored.

The model for the experimental results is based on the significant effects of all three factors, extended with the squared factor of maleic acid concentration (see Equation 2). The quadratic model is significant ($P < 0.0001$) and fits the experimental data with $R^2_{adjusted} = 0.94$ (Appendix 4.1). In Figure 4.1, details of the response analysis of the results on glucose yield from the solid phase are shown as a three-dimensional surface.

$$Bene_{glu} = 53 + 4.0A + 12B + 10C - 9.8C^2 \quad (2)$$

with $Bene_{glu}$ = glucose benefits (€/tonne dry straw), $A = (t-t_C)/t_S$; $B = (T-T_C)/T_S$; $C = (M_A-M_{A,C})/M_{A,S}$; t = pretreatment time (min), T = pretreatment temperature (°C) and M_A = concentration maleic acid (mM); subscript C = centre value, subscript S= step value; t_C = 30 min; t_S= 20 min; T_C = 150 °C; T_S = 20 °C; $M_{A,C}$ = 50 mM; $M_{A,S}$ = 39 mM.

As shown in Equation 2 and Figure 4.1, within the studied design space, increasing the digestibility (expressed as glucose yield after subsequent enzymatic hydrolysis) is strongly dependent on increasing the pretreatment temperature. Increasing pretreatment time from 10 to 50 min also has a positive effect, but less so. The influence of the maleic acid concentration has a negative quadratic part, resulting in a maximum enzymatic glucose yield at 70.4 mM maleic acid (50 min at 170 °C). On the basis of the fit to the data, a maximum glucose yield of 71.87 €/tonne of straw is predicted, representing 90 % of all glucan present in the original straw.
During the pretreatment, high concentrations of maleic acid seem to favour the formation of monomeric glucose in the liquid phase (Table 4.2). Since most of the liquid phase is

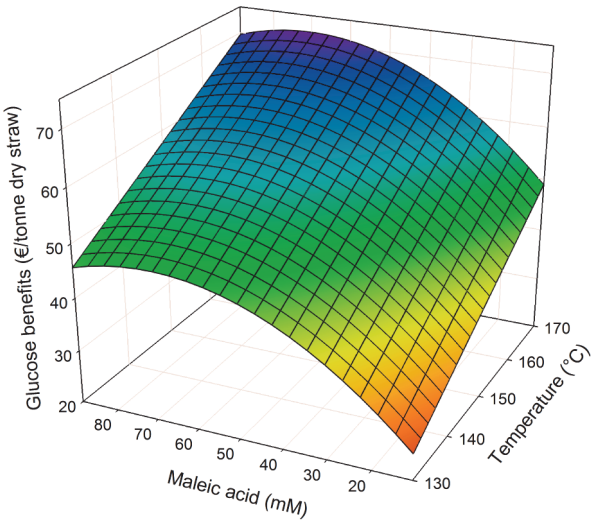

Figure 4.1. Three-dimensional response surface for glucose benefits (€/tonne dry straw) in relation to pretreatment conditions. Pretreatment time set at 50 min.

pressed out, raising the maleic acid concentration in the pretreatment will eventually result in a negative effect on the enzymatic glucose yield from the pressed material.
Within the experimental results, the maximum glucose yield from the pressed material was 82 % of the total of glucose present in the original straw. Under these conditions (50 min, 170 °C, and 89 mM acid), 10 % of the total glucose from the straw was detected as monomeric sugar in the liquid phase. Only 1 % of the total glucose had been degraded to HMF and only traces of oligo-glucans were present in the liquid phase, closing the glucose mass balance for 92 % (Table 4.2).

The glucose yields in this study are somewhat lower than in a previous work with maleic acid pretreatment of wheat straw, where 96 % of all glucan was retrieved as monomeric glucose after enzymatic hydrolysis [25]. However, the resulting maximum glucose yield of the present study (combining liquid and solid fraction to 92 % of total present glucose) is in line with other studies on pretreatment of straw-like lignocellulosic materials [28-31]. In fact, the model predicts a maximum glucose yield from the pretreated solids of 90 % of all glucan present in straw, while Table 4.2 implies that between 7 % to 10 % of all glucose would be present in the liquid phase. This suggests that 97 % to 100 % conversion

Table 4.2. Glucose mass balance of maleic acid pretreatment of wheat straw.

Run	Pre-treat-ment time (min)	Pre-treat-ment tem-perature (°C)	Maleic acid conc. (mM)	Glucose monomers in aqueous (%)	HMF in aqueous (%)	Glucose oligomers in aqueous (%)	Glucose in pressed material (%) Not cor-rected for a, b, and c	Glucose in pressed material (%) Corrected for a, b, and c	Total (%)
				a	b	c	d	e	a+b+c +e
1	10	130	11	1	0	2	24	24	27
2	50	130	89	2	0	5	54	54	61
5	50	170	11	1	0	6	63	63	70
6	10	170	89	8	1	1	79	78	88
7	10	170	11	1	0	5	46	46	52
8	50	170	89	10	1	0	82	81	92
9	10	130	89	1	0	6	45	45	52
11	50	130	11	1	0	4	36	36	41
14	30	130	50	1	0	6	44	44	50
15	50	150	50	4	0	4	71	71	79
16	10	150	50	2	0	6	61	61	69
17	30	150	89	6	0	2	72	72	81
18	30	150	11	1	0	4	36	36	41
19	30	170	50	7	1	1	82	81	91
cx6	30	150	50	3 (0.1)	0 (0.0)	5 (0.2)	66 (1.0)	66 (1.0)	74 (0.9)

cx6 = centre runs, six repeats. Average value given. Standard deviation between brackets. Percentages are expressed as glucose equivalents, as a fraction of total glucose equivalents present in untreated wheat straw.

of glucan to glucose can be reached, using maleic acid in the pretreatment. However, it also means that, in order to use more than 90 % of all glucose, the glucose that is solubilised in the liquid phase would need to be included.

Effect of pretreatment conditions on xylose benefits

During the pretreatment, a large part of the hemicellulose is solubilised. When the resulting xylose monomers and oligomers are taken into account for the optimisation, the

total benefits per tonne straw can be raised. For this study, we estimated the value of xylose at 100 €/tonne, 50 % of the value of glucose [40]. As with glucose, the fact that a more concentrated glucose stream has a higher value per amount of xylose than a less concentrated stream is ignored. In Table 4.3, the xylose mass balance is shown, clarifying that xylans are solubilised as oligomeric and monomeric xylose. For this study, both types are included as equally valuable in the xylose benefits calculation, ignoring the costs for an additional hydrolysis that might be needed to convert remaining oligomeric xylose to monomers.

The model for the experimental results is based on the significant effects of the pretreatment temperature and maleic acid concentration, extended with the squared factor of the latter (see Equation 3). The quadratic model is significant ($P < 0.0001$) and fits the experimental data with $R^2_{adjusted} = 0.90$ (Appendix 4.2). In Figure 4.2, details of the response analysis of the results on glucose yield from the solid phase are shown as a three-dimensional surface.

$$Bene_{xyl} = 14 + 3.0B + 5.1C - 5.2C^2 \quad (3)$$

with $Bene_{xyl}$ = xylose benefits (€/tonne dry straw), $B = (T-T_C)/T_S$; $C = (M_A-M_{A,C})/M_{A,S}$; T = pretreatment temperature (°C) and M_A = concentration maleic acid (mM); subscript C = centre value, subscript S = step value; T_C = 150 °C; T_S = 20 °C; $M_{A,C}$ = 50 mM; $M_{A,S}$ = 39 mM.

As shown in Equation 3 and Figure 4.2, within the studied design space, increasing the xylose yield is strongly dependent on increasing the maleic acid concentration, as well as the pretreatment temperature. Increasing pretreatment time from 10 to 50 min does not have a significant influence on the xylose yield. The influence of the maleic acid concentration has a negative quadratic part, resulting in a maximum xylose yield from the pretreated liquid phase at 68.9 mM maleic acid and 170 °C. Under these conditions, the model predicts a maximum xylose yield of 18.55 €/tonne of straw, representing 85 % of all xylan present in the original straw. The xylose that is solubilised under these conditions will largely be monomeric (Table 4.3) and about 5 % of all solubilised xylose remains in the liquid phase of the pressed pellet. It is clear that, in the maleic acid pretreatment, almost complete xylan conversion to xylose is quite possible but a part of this xylose will degrade to furfural. Obviously, the idea is to minimise loss of xylose to furfural formation.

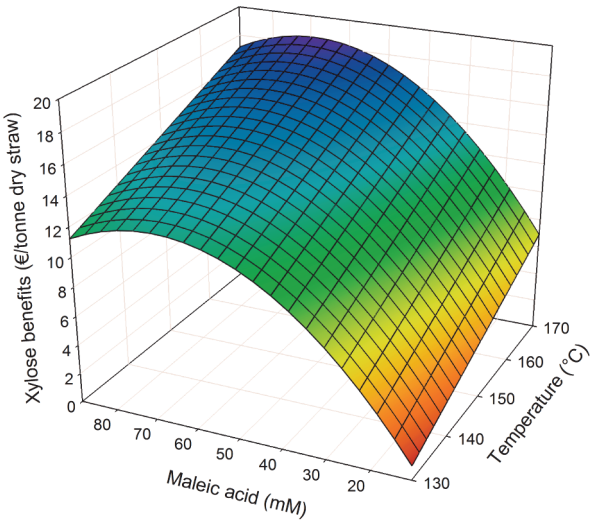

Figure 4.2. Three-dimensional response surface for xylose benefits (€/tonne dry straw) in relation to pretreatment conditions. Pretreatment time set at 50 min.

Within the experimental results, the maximum xylose yield from the liquid phase was 81 % of the total of xylose present in the original straw. That is, 86 % of all xylan was solubilised but, as not all liquid phase is pressed out, some solubilised xylose remains in the liquid phase of the pressed material. Under these conditions (10 min, 170 °C, and 89 mM maleic acid), 85 % of the total xylose from the straw was detected as monomeric sugar in the liquid phase. Only 1 % of the total xylose was present in oligomeric form, 7 % of all xylose had had been degraded to furfural and 6 % remained as polysaccharide in the pressed solid phase, closing the xylose mass balance for 100 % (Table 4.3). The xylose yields in this study are very comparable with previous work with maleic acid pretreatment of wheat straw and corn stover [21, 25].

Effect of pretreatment conditions on maleic acid costs

After the pretreatment, not all of the maleic acid is detected in the liquid phase that is pressed out during the solid-liquid separation. Some of the maleic acid is degraded, while another part continues downstream with the pressed solid phase. When recycling the maleic acid for use in further pretreatments of fresh straw, both these fractions of acid are

Table 4.3. Xylose mass balance of maleic acid pretreatment of wheat straw.

Run	Pre-treat-ment time (min)	Pre-treat-ment tem-perature (°C)	Maleic acid conc. (mM)	Xylose monomers in aqueous (%)	Furfural in aqueous (%)	Xylose oligomers in aqueous (%)	Xylose in pressed material (%) Not cor-rected for a, b, and c	Xylose in pressed material (%) Corrected for a, b, and c	Total (%)
				a	b	c	d	e	a+b+c +e
1	10	130	11	0	0	2	18	18	20
2	50	130	89	44	1	22	25	19	86
5	50	170	11	5	3	47	31	26	81
6	10	170	89	85	7	1	15	6	100
7	10	170	11	1	1	26	37	34	61
8	50	170	89	72	18	1	11	4	94
9	10	130	89	15	0	35	26	21	72
11	50	130	11	1	0	11	31	30	42
14	30	130	50	7	0	35	27	23	65
15	50	150	50	66	3	11	24	16	96
16	10	150	50	36	1	32	26	19	87
17	30	150	89	78	4	5	20	12	99
18	30	150	11	2	0	11	32	30	43
19	30	170	50	75	10	4	17	9	98
cx6	30	150	50	58 (2.2)	2 (0.1)	17 (1.0)	24 (0.3)	17 (0.2)	94 (1.4)

cx6 = centre runs, six repeats. Average value given. Standard deviation between brackets. Percentages are expressed as xylose equivalents, as a fraction of total xylose equivalents present in untreated wheat straw.

considered lost and need to be replenished. The experimental results of this study represent the cost of all maleic acid that is lost, expressed as €/tonne straw, with maleic acid at 1000 €/tonne [41]. For this study, it is assumed that all maleic acid that is detected can be recycled at no additional cost. Only the acid that needs to be replenished is considered.

The model used to describe the experimental results is based on the effects of all three factors, extended with parameters for interactions and squared factors (see Equation 4). A

square root transformation of the response factor was applied for improved model fit. The quadratic model is significant ($P < 0.0001$) and fits the data with $R^2_{adjusted} = 1.00$ (Appendix 4.3). In Figure 4.3, details of the response analysis of the results on acid loss from the liquid phase are shown as a three-dimensional surface.

$$\left(Costs_{MA}\right)^{0.5} = 2.9 + 0.23\,A + 0.56\,B + 1.6\,C + 0.27\,AB + 0.12\,AC + 0.38\,BC + 0.37\,B^2 - 0.45\,C^2 \quad (4)$$

with $Costs_{MA}$ = maleic acid costs (€/tonne dry straw), $A = (t\text{-}t_C)/t_S$; $B = (T\text{-}T_C)/T_S$; $C = (M_A\text{-}M_{A,C})/M_{A,S}$; t = pretreatment time (min), T = pretreatment temperature (°C) and M_A = concentration maleic acid (mM); subscript C = centre value, subscript S = step value; t_C = 30 min; t_S = 20 min; T_C = 150 °C; T_S = 20 °C; $M_{A,C}$ = 50 mM; $M_{A,S}$ = 39 mM.

As can be seen in Equation 4 and Figure 4.3, minimising costs due to acid loss within the studied design space is, for a large part, dependent on concentration of maleic acid that is present at the start of the pretreatment. As may be expected, the lower the maleic acid concentration, the lower the costs for replenishing any acid that is lost. However,

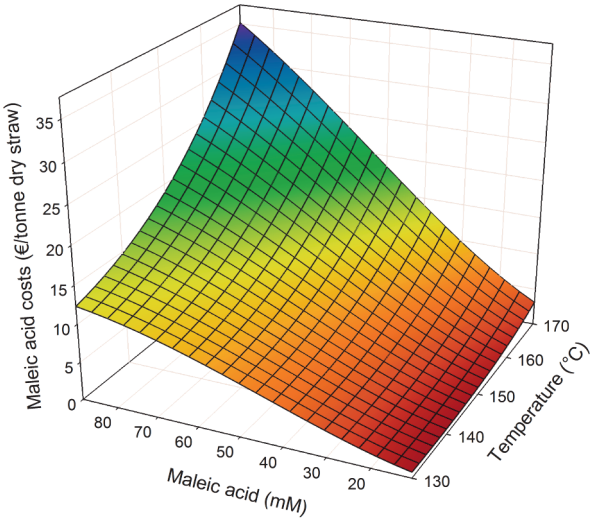

Figure 4.3. Three-dimensional response surface for maleic acid costs (€/tonne dry straw) in relation to pretreatment conditions. Pretreatment time set at 50 min.

increasing acid concentration results in higher costs for acid loss (results not shown). This may be not only because a higher concentration is left in the pellet after pressing but also because the isomerisation to fumaric acid itself is described as acid catalysed [42, 43].
Minimal acid loss within the studied design space is 0.54 €/tonne straw and occurs at a 10 min pretreatment with 11 mM maleic acid at 152 °C. In comparison, maximal acid costs occur when the pretreatment is most severe in this study: 50 min at 170 °C, with 89 mM maleic acid, results in a staggering 34.88 €/tonne straw.
It is striking that at shorter, less acidic pretreatments, there appears to be a small drop in acid loss when the temperature is raised from 130 °C to around 150 °C (max drop is about 0.95 €/tonne straw). This may be explained by an increase of the amount of liquid that is pressed out (results not shown), while the duration of the pretreatment was not long enough to allow for extensive maleic acid degradation (mostly isomerisation into fumaric acid) during the time of the pretreatment [44, 45]. When pretreatments are performed with more acid for longer time, more acid is degraded, no longer resulting in this reduction of acid costs around 150 °C.
Furthermore, for minimising acid costs, high pretreatment temperatures are best avoided (but the influence of increasing the temperature is less than increasing the acid concentration). Increasing pretreatment time only seems to have minimal influence.

Effect of pretreatment conditions on sodium hydroxide costs

After the solid-liquid separation, the amount of NaOH needed to set the pH of the solid phase depended on two sub-factors: the amount of NaOH needed per volume of liquid to set the pH to 5 (comparable to 'acid number') and the volume of liquid remaining in the solid phase. In this study, both sub-factors reacted oppositely to changes in pretreatment time and temperature (results not shown), meaning that, as the solid-liquid separation gets somewhat more efficient when pretreatment conditions change, the same changes cause the remaining liquid phase to need more NaOH per volume to set the pH to 5. This may be due to acetic and uronic acids being released from the straw during the pretreatment (results not shown). In short, the changes in the two sub-factors due to changes in pretreatment time and temperature cancel each other out. This leads to the amount of NaOH needed to set the pressed pretreatment solids to pH 5 only being significantly dependent on the maleic acid concentration of the pretreatment. The model used to describe the experimental results as NaOH cost per tonne of straw (with NaOH costing 575 €/tonne [46]) is therefore linear, as can be seen in Equation 5. The model is significant ($P < 0.0001$) and fits the data with $R^2_{adjusted} = 0.98$ (Appendix 4.4). The costs

for NaOH within the studied design space vary between 0.15 and 1.81 €/tonne dry straw, for when 11 and 89 mM maleic acid was used, respectively.

$$Costs_{NaOH} = 0.98 + 0.83C \quad (5)$$

with $Costs_{NaOH}$ = NaOH costs (€/tonne dry straw), C= (M_A-$M_{A,C}$)/$M_{A,S}$; M_A = concentration maleic acid (mM); subscript C = centre value, subscript S = step value; $M_{A,C}$ = 50 mM; $M_{A,S}$ = 39 mM

Effect of pretreatment conditions of furfural production

The formation of furfural during the maleic acid pretreatment is very limited, confirming results of earlier studies [21, 25, 47]. Under most pretreatment conditions, only minor amounts of furfural are formed. In only one of the experiments (50 min, 170 °C, 89 mM acid), slightly more than 3 g/L of furfural was formed. This is close to 30 mM at which furfural and HMF have been reported to be inhibitory to yeast in the production of ethanol from glucose, although adaptation of yeast to similar concentrations has also been reported [48-50].

The model used to describe the experimental results is based on the effects of all three factors, extended with the interaction of temperature and acid concentration and the squared factor of the latter (see Equation 6). A logarithmic transformation of the response factor was applied for improved model fit. The quadratic model is significant ($P < 0.0001$) and fits the data with $R^2_{adjusted} = 0.94$ (Appendix 4.5).

$$\log(Fur) = -0.42 + 0.21A + 0.57B + 0.33C + 0.17BC - 0.22C^2 \quad (6)$$

with Fur = furfural concentration (g/L), A= (t-t_C)/t_S; B= (T-T_C)/T_S; C= (M_A-$M_{A,C}$)/$M_{A,S}$; t = pretreatment time (min), T = pretreatment temperature (°C) and M_A = concentration maleic acid (mM); subscript C = centre value, subscript S = step value; t_C = 30 min; t_S= 20 min; T_C = 150°C; T_S = 20°C; $M_{A,C}$ = 50 mM; $M_{A,S}$ = 39 mM.

In Figure 4.4, details of the response analysis of the results on furfural formation in the liquid phase are shown as a three-dimensional surface. The furfural concentration is depicted on a linear scale, emphasising, together with Equation 6, the very low furfural formation under most conditions, except the most extreme. The negative quadratic term in the equation somewhat lessens the influence of increasing the acid concentration.

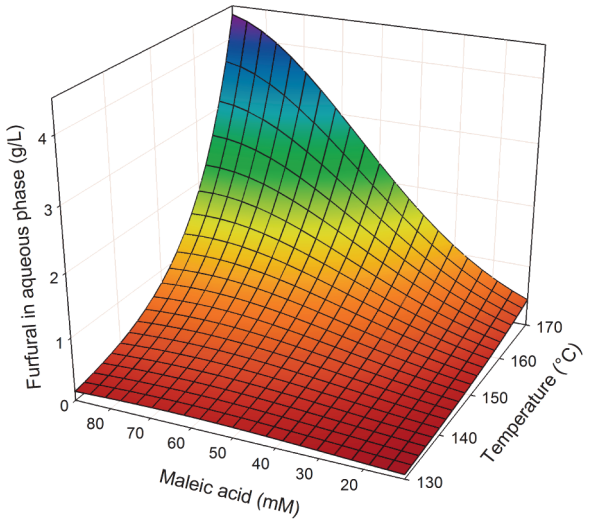

Figure 4.4. Three-dimensional response surface for furfural formation (g/L in liquid phase) in relation to pretreatment conditions. Pretreatment time set at 50 min.

Taking into account the experimental results, it can be assumed that the influence of furfural formation on the economics of the studied maleic acid pretreatment is zero, provided that the most extreme conditions are avoided. The furfural concentrations that are formed are usually low enough to avoid inhibition of yeast in the bio-ethanol production [48-52]. However, even though very limited in this study, furfural formation should not be completely disregarded. First, because production of furfural means loss of xylose, leading to a lower potential xylose yield and lower associated benefits. Secondly, if the liquid phase is reused for pretreatment of fresh material, build up of furfural could occur, possibly still resulting in non-negligible inhibitory furfural concentrations, and in higher associated process costs.

Effect of pretreatment conditions on heating costs

Heating lignocellulose means heating costs and heating wheat straw to 170 °C costs more than to 130 °C. Heating costs that are taken into account are those for the straw entering the pretreatment process, and also for the water that needs to be replenished, as some of it leaves the pretreatment process after the solid-liquid separation, as part of the solid phase

that continues to the enzymatic hydrolysis. As, in this study, the solid phase after solid-liquid separation usually consisted of around 40 % dry matter, with pretreatment time and temperature only having a negligible influence (results not shown), it is assumed that for every tonne of dry straw that enters the pretreatment 1.5 tonne of water needs to be included and it has to be heated as well. Considering the relatively small specific surface area of large scale process equipment, it is assumed that only negligible amounts of heating energy are needed during the holding time of the pretreatment, the solid-liquid separation and the recycle of water and acid. Using a calculated specific heat capacity of dry straw of 1.7 $Jg^{-1}K^{-1}$ and energy costs from coal values [53, 54], the heating costs for the process can be described with Equation 7.

$$Costs_{heating} = 1.78 + 0.27B \quad (7)$$

with $Costs_{heating}$ = heating costs (€/tonne dry straw), T = pretreatment temperature (°C); subscript C = centre value, subscript S = step value; T_C = 150 °C; T_S = 20 °C.

In short, the heating costs of the maleic acid pretreatment in the studied design space vary from 1.51 to 2.06 €/tonne dry straw, for 130 °C to 170 °C, respectively.

Optimal economic yield

Taking the benefits from glucose and xylose and subtracting the costs of maleic acid, heating and NaOH, results in an economic optimum of the maleic acid pretreatment at a reaction time of 50 min, at 170 °C, in presence of 46.21 mM maleic acid (Figure 4.5). This would mean a glucose yield of 68.03 €/tonne dry straw (85 % of all glucan), a xylose yield of 16.74 €/tonne dry straw (close to 80 % of all xylan), maleic acid costs of 16.55 €/ tonne dry straw, heating costs of 2.06 €/tonne straw and NaOH costs of 0.90 €/tonne straw. The total resulting economic yield at this optimum would then be 65.26 €/tonne straw. A pretreatment time of 10 or 30 min (results not shown) leads to a lower optimal economic yield (64.34 and 64.90 €/tonne straw, respectively), at 170 °C and at higher acid concentrations (53.07 and 49.72 mM, respectively). Pretreatment time does have an influence on the optimal economic yield, but less so than pretreatment temperature and maleic acid concentration.

Since the xylose benefits are somewhat speculative, and the contribution of glucose to the total benefits is about three to four times larger than that of xylose, an optimisation using only glucose benefits but still all cost factors is also performed (Figure 4.6).
The reasons for the xylose yield being somewhat speculative are: first, the estimated xylose value of half of that for glucose is quite uncertain; secondly, the fit of the xylose data is not as good as that for the glucose data; and, thirdly, in the case of glucose, the fermentable sugars are released during enzymatic hydrolysis and can relatively easily be transformed to ethanol by fermentation. For xylose, the solubilisation takes place at lower concentration in water that contains all acids and many other constituents. This implies a complication of the application of xylose and, therefore, adds uncertainty to its value.

Using only the benefits from glucose, the optimal conditions are again at a reaction time of 50 min, at 170 °C, but now in presence of 36.2 mM maleic acid (Figure 4.6). As the optimal conditions for both glucose and xylose yields are similar, it is as expected that not taking xylose benefits into account would result in an economic optimum at lower maleic acid concentration.
We therefore reach a glucose yield of 64.11 €/tonne dry straw (corresponding to 80 % of all glucan present in straw), maleic acid costs of 12.01 €/tonne dry straw, heating costs of 2.06 €/tonne straw and NaOH costs of 0.68 €/tonne straw. The total resulting economic yield at this optimum would be 49.35 €/tonne straw. Both the maleic acid concentrations of 36.2 and 46.2 mM would result in furfural concentration of 2 g/L or lower, which confirms the assumption of zero costs associated with furfural formation.

Logically, the glucose (and xylose) yields, when using the optimal economic pretreatment, are somewhat lower than maximally could be achieved. As a result of the rapidly increasing acid costs, it is more economical to settle for a less acidic pretreatment that yields a lower-than-maximum amount of glucose (and xylose). If 90 % of all glucans in the straw were yielded as glucose from the pretreatment solids, another €6.4 per tonne straw could be achieved. However, the acid costs would rise with an additional 15 to 27 €/ tonne straw (and the NaOH costs with 0.72 to 1.41 €/tonne straw).

A total yield of around 49 €/tonne of dry straw, or 65 €/tonne when taking xylose benefits into account, is fairly promising as theoretical sugar benefits are around 80 and 20 €/tonne dry straw for glucose and xylose, respectively. When taking into account feedstock costs of 25 to 33 €/tonne (corn stover [8, 55]), it is clear that process economics are somewhat challenging.

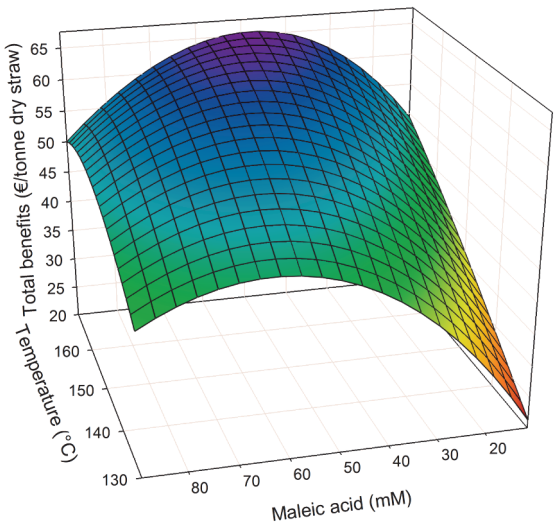

Figure 4.5. Optimisation of total benefits (€/tonne dry straw), including xylose, in relation to pretreatment conditions. Pretreatment time set at 50 min.

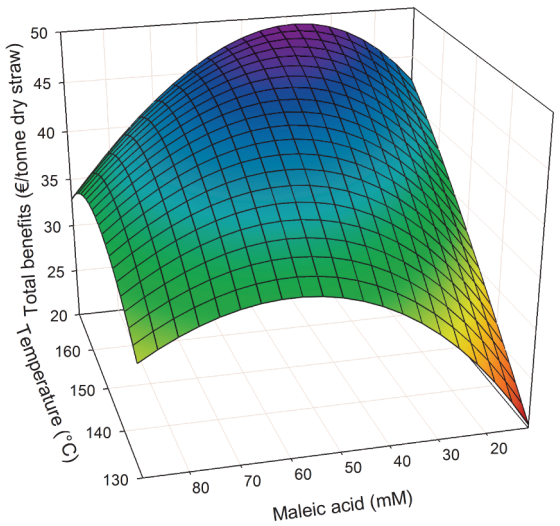

Figure 4.6. Optimisation of total benefits (€/tonne dry straw), not including xylose, in relation to pretreatment conditions. Pretreatment time set at 50 min.

In a comparable dilute sulphuric acid process, in which both glucose and xylose are assumed to be of similar value for ethanol production, the projected total ethanol production costs are 320 €/tonne ethanol. Enzyme costs are estimated at 24 €/tonne ethanol or 6 €/tonne feedstock. The pretreatment costs account for around 16 €/tonne feedstock, more than half of which (around 9 €/tonne feedstock) is due to the high capital cost of the pretreatment reactors that need to deal with the corrosive sulphuric acid [8]. Since the organic maleic acid is less corrosive, a lower capital cost for the pretreatment can be expected. Processing equipment, such as vessels and piping made of the more corrosion resistant 316L steel, can be 20 % to 50 % more expensive than when made of standard 304 steel [56]. Another matter that should be taken into account is the possibility that, when using sulphuric acid, extra features such as corrosion resistant storage vessels or more stringent safety and handling measures, compared to the application of the weaker (and solid) maleic acid may be needed. We estimate the extent of this difference to be significant but this, of course, greatly depends on the process conditions of the maleic acid pretreatment. On the other hand, maleic acid is more expensive than sulphuric acid, but the much reduced sugar degradation and the organic by-product stream from maleic acid offer advantages over the sulphuric acid treatment. Clearly, more study is needed in order to compare the economics of these two pretreatments.

Several options exist in order to raise the economic yield of the maleic acid pretreatment. First, the sugar yield could be somewhat raised by increasing the severity of the pretreatment [57]. However, as shown earlier, this should be done without raising the maleic acid concentration or the pretreatment temperature (to a higher value than in the studied design space), since both would quickly increase acid costs (also a higher temperature would increase heating costs and a higher maleic acid concentration would increase NaOH costs). This leaves only the pretreatment time to be increased to outside the studied design space, as this is the process variable which least affects maleic acid costs (in the studied design space). Heating costs would not increase, assuming that maintaining a certain temperature for longer requires much less energy than heating to a higher temperature. However, it should not be overlooked that increasing the process time will lead to higher process costs, since process throughput will be reduced, decreasing plant output per unit of capital investment.

A second option is to lower acid costs. For example, it may not be necessary to replenish all of the maleic acid that is lost in the pretreatment. The fumaric acid that results from the isomerisation of maleic acid during the pretreatment also possesses pretreatment potential, albeit less than maleic acid [25, 47]. In this study, up to 12 % of maleic acid is

transformed to fumaric acid (results not shown). It may, therefore, be possible to save a significant part of the maleic acid costs in this manner. However, applying this strategy in the recycle and re-use of the acids in a (semi-) continuous process implies a mixed fumaric-maleic acid pretreatment. It is not known to what extent the pretreatment efficiency of fumaric acid that is formed can simply be added to that of the maleic acid which is already present. This remains to be studied further, as it falls outside of the scope of the current study. Another way to lower acid costs would be to use an acid that is cheaper per tonne pretreated straw and/or to make acid *in situ*. For example, the xylose that is released in the pretreatment may be converted to lactic acid [58], which may be used in the pretreatment (although it is weaker than maleic acid).

The third option is to increase the process economics of the dilute organic acid pretreatment by moving to another raw material altogether, in order to increase the total valorisation. When a feedstock contains protein as well as lignocellulose, this protein may be hydrolysed with a mild acid treatment and separated in order to create more value. This may be done by the application of the protein rich stream to animal feed or, potentially even more economically attractive, it can be used in the production of chemicals. The so called 'green chemistry' can use these streams as raw material for the production of nitrogen-containing chemicals from amino acids [59-61]. After protein extraction, the remaining lignocellulose fraction can be treated to yield fermentable sugars. Although the raw material might be more expensive, and the total process would be more complex, the total potential for valorisation is also greater. In short, this implies the application of a more detailed biorefinery concept.

Something to keep in mind is that the production of second generation bioethanol can have several goals/reasons. For example, if first generation bioethanol production grows, as it is expected to do in the USA [62, 63], large quantities of lignocellulose will become available. The current process can make use of that by-product stream, especially in areas where it is not economical to transport the straw over long distances. In short, in an area with a lot of first generation ethanol plants, lignocellulosic material such as wheat straw or corn stover may become very cheap and easily available. Moreover, in order to make first generation bioethanol more sustainable, it can be considered essential to use the lignocellulosic by-product streams for second generation bioethanol production. Speaking in terms of carbon dioxide emissions, the additional (second generation) bioethanol would certainly speed up paying off carbon debts resulting from land use change in first generation bioethanol production [63, 64].

A final point of interest is the application of a high solids loading in the pretreatment. In the current study, 10 % (w/w) solids loading is applied. In previous work, it has been shown that raising the solids loading from 10 to 30 % (w/w) does not negatively affect the pretreatment efficiency, as long as the acid concentration is raised accordingly [25]. However, combining these findings with the current study, a higher maleic acid concentration would result in higher acid costs. This is partly due to more maleic acid being isomerised or degraded, as well as to the fact that a relatively smaller fraction of the total liquid phase can be pressed out. For example, 30 % (w/w) solids loading implies that only about a third of the total liquid phase can be pressed out in the solid-liquid separation (to 40 % w/w dry matter). Increasing the solids loading, on the other hand, would lead to a lower processing cost per tonne of raw material. Obviously, also considering the effects on xylose concentration and furfural formation, there is a trade off concerning the pros and cons when raising the solids loading.

This study uses an extensive, but not exhaustive number of factors that influence the optimisation of the maleic acid pretreatment of wheat straw. Since the focus is on the maleic acid pretreatment alone, and not on the whole integrated conversion process of wheat straw to bioethanol, some factors are not taken into account. These include factors like capital costs, enzyme costs, recycle costs and the possible benefits from the organic by-product stream that results from a conversion process with a maleic acid pretreatment.

Conclusions

It is shown that the optimal process conditions in this study for the dilute maleic acid pretreatment of wheat straw are 50 min at 170 °C with a maleic acid concentration of 46 mM. These conditions resulted in a theoretical optimal yield of 65.26 €/tonne dry straw, consisting of €68.03 glucose benefits, €16.74 xylose benefits, €16.55 maleic acid costs, €2.06 heating costs and €0.90 NaOH costs. The most important cost factor in this study is the cost of replenishing maleic acid. In all but the most severe of the studied conditions, furfural formation was so limited that the associated costs are considered negligible. At the economic optimum, the total glucose yield from the pretreatment solids is 85 % of the glucan originally present in the wheat straw. Xylose yield from the liquid phase after pretreatment is close to 80 % of all xylan present. After this pretreatment, 7 % to 10 % of the glucose is expected to be present in monomeric and oligomeric form in the liquid phase. Nearly all xylose present in the liquid phase is in a monomeric form. Using the dilute maleic acid pretreatment and subsequent enzymatic hydrolysis, almost complete

conversion of wheat straw glucan and xylan is possible and very high yields (approximately 90-95 %) can be achieved.

Abbreviations

GL = gigalitre; ha = hectare; HMF = 5-hydroxymethylfurfural; HPLC = high-performance liquid chromatography

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Appendices

Appendix 4.1. 'Glucose benefits', information on model, ANOVA, and R^2 of response factor.

Glucose benefits

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High
Intercept	53.1117	1	1.115815	50.70113	55.52227
Block 1	0.745003	2			
Block 2	-0.429903				
Block 3	-0.315099				
A-Pre time	4.028366	1	1.028159	1.807163	6.249569
B-Pre Temperature	12.00357	1	1.028159	9.782372	14.22478
C-Maleic acid conc	10.35827	1	1.028159	8.137071	12.57948
C^2	-9.830231	1	1.592817	-13.2713	-6.389159

ANOVA for Response Surface Reduced Quadratic Model
Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F		
Block	67.156386	2	33.57819				0.957 R2
Model	3078.7129	4	769.6782	72.80959	< 0.0001	significant	0.944 R2 adj
A-Pre time	162.27732	1	162.2773	15.35102	0.0018		
B-Pre Temperature	1440.858	1	1440.858	136.3015	< 0.0001		
C-Maleic acid conc	1072.9383	1	1072.938	101.4972	< 0.0001		
C^2	402.63934	1	402.6393	38.08865	< 0.0001		
Residual	137.42445	13	10.57111				
Lack of Fit	136.02462	10	13.60246	29.15179	0.0091	significant	
Pure Error	1.3998245	3	0.466608				
Cor Total	3283.2938	19					

Appendix 4.2. 'Xylose benefits', information on model, ANOVA, and R^2 of response factor.

Xylose benefits

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High
Intercept	14.31705	1	0.601537	13.02688	15.60722
Block 1	0.964818	2			
Block 2	-0.522899				
Block 3	-0.441919				
B-Pre Temperature	2.974675	1	0.554282	1.785857	4.163492
C-Maleic acid conc	5.132318	1	0.554282	3.943501	6.321135
C^2	-5.245643	1	0.85869	-7.08735	-3.403935

ANOVA for Response Surface Reduced Quadratic Model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F		
Block	17.770819	2	8.885409				0.916 R^2
Model	466.54698	3	155.5157	50.61883	< 0.0001	significant	0.898 R^2 adj
B-Pre Temperature	88.486896	1	88.4869	28.80162	< 0.0001		
C-Maleic acid conc	263.40689	1	263.4069	85.73637	< 0.0001		
C^2	114.6532	1	114.6532	37.3185	< 0.0001		
Residual	43.012042	14	3.072289				
Lack of Fit	42.956074	11	3.905098	209.3223	0.0005	significant	
Pure Error	0.0559677	3	0.018656				
Cor Total	527.32984	19					

Appendix 4.3. 'Maleic acid costs', information on model, ANOVA, and R^2 of response factor.

(Acid costs)^0.5

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High
Intercept	2.872143	1	0.034905	2.793184	2.951103
Block 1	-0.163568	2			
Block 2	0.247284				
Block 3	-0.083716				
A-Pre time	0.227573	1	0.029618	0.160574	0.294573
B-Pre Temperature	0.560977	1	0.029618	0.493977	0.627977
C-Maleic acid conc	1.575389	1	0.029618	1.508389	1.642389
AB	0.245813	1	0.033113	0.170905	0.32072
AC	0.119687	1	0.033113	0.044779	0.194595
BC	0.382614	1	0.033113	0.307706	0.457522
B^2	0.370056	1	0.053717	0.248539	0.491573
C^2	-0.448037	1	0.053717	-0.569554	-0.32652

ANOVA for Response Surface Reduced Quadratic Model
Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F		
							0.997 R^2
Block	0.548	2	0.273993				0.995 R^2 adj
Model	30.941	8	3.867628	440.905	< 0.0001	significant	
A-Pre time	0.518	1	0.517896	59.03949	< 0.0001		
B-Pre Temperature	3.147	1	3.146953	358.7489	< 0.0001		
C-Maleic acid conc	24.819	1	24.8185	2829.28	< 0.0001		
AB	0.483	1	0.483391	55.10597	< 0.0001		
AC	0.115	1	0.1146	13.06429	0.0056		
BC	1.171	1	1.171148	133.5095	< 0.0001		
B^2	0.416	1	0.416302	47.4579	< 0.0001		
C^2	0.610	1	0.610241	69.56676	< 0.0001		
Residual	0.079	9	0.008772				
Lack of Fit	0.0636075	6	0.010601	2.07317	0.2934	not significant	
Pure Error	0.0153406	3	0.005114				
Cor Total	31.567956	19					

Appendix 4.4. 'NaOH costs', information on model, ANOVA, and R^2 of response factor.

NaOH costs

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High
Intercept	0.982634	1	0.020664	0.938828	1.02644
Block 1	0.009952	2			
Block 2	0.002787				
Block 3	-0.012739				
C-Maleic acid conc	0.825155	1	0.028957	0.763769	0.886541

ANOVA for Response Surface Reduced Linear Model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F		
Block	0.0019066	2	0.000953				0.981 R^2
Model	6.8088067	1	6.808807	812.025	< 0.0001	significant	0.979 R^2 adj
C-Maleic acid conc	6.8088067	1	6.808807	812.025	< 0.0001		
Residual	0.1341596	16	0.008385				
Lack of Fit	0.1207683	13	0.00929	2.081176	0.2986	not significant	
Pure Error	0.0133913	3	0.004464				
Cor Total	6.9448729	19					

Appendix 4.5. 'Furfural formation', information on model, ANOVA, and R^2 of response factor.

Log10(Furfural concentration)

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High
Intercept	-0.421494	1	0.046107	-0.521953	-0.321035
Block 1	0.042894	2			
Block 2	-0.009522				
Block 3	-0.033372				
A-Pre time	0.213288	1	0.042485	0.12072	0.305855
B-Pre Temperature	0.566968	1	0.042485	0.474401	0.659536
C-Maleic acid conc	0.328423	1	0.042485	0.235856	0.42099
BC	0.166457	1	0.0475	0.062964	0.26995
C^2	-0.22436	1	0.065818	-0.367764	-0.080955

ANOVA for Response Surface Reduced Quadratic Model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F		
Block	0.0172938	2	0.008647				0.960 R^2
Model	5.1794656	5	1.035893	57.39058	< 0.0001	significant	0.943 R^2 adj
A-Pre time	0.4549159	1	0.454916	25.20326	0.0003		
B-Pre Temperature	3.214532	1	3.214532	178.0916	< 0.0001		
C-Maleic acid conc	1.0786159	1	1.078616	59.75751	< 0.0001		
BC	0.2216629	1	0.221663	12.28057	0.0043		
C^2	0.2097389	1	0.209739	11.61996	0.0052		
Residual	0.2165986	12	0.01805				
Lack of Fit	0.2157627	9	0.023974	86.04659	0.0018	significant	
Pure Error	0.0008358	3	0.000279				
Cor Total	5.413358	19					

Appendix 4.6. 'Heating costs', information on model, ANOVA, and R^2 of response factor.

Heating costs

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High
Intercept	1.784617	1	3.48E-07	1.784616	1.784617
Block 1	-2.74E-07	2			
Block 2	7.83E-07				
Block 3	-5.09E-07				
B-Pre Temperature	0.274556	1	4.88E-07	0.274555	0.274557

ANOVA for Response Surface Reduced Linear Model
Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F		
Block	0	2	0				1.000 R^2
Model	0.7538101	1	0.75381	63660000	< 0.0001	significant	1.000 R^2 adj
B-Pre Temperature	0.7538101	1	0.75381	63660000	< 0.0001		
Residual	0	16	0				
Lack of Fit	0	13	0				
Pure Error	0	3	0				
Cor Total	0.7538101	19					

4

Chapter 5

Valorisation of *Jatropha curcas*; secondary protein solubilisation under acidic and neutral conditions

This chapter has been submitted as: A.M.J. Kootstra, H.H. Beeftink, J.P.M. Sanders; Valorisation of *Jatropha curcas*; secondary protein solubilisation under acidic and neutral conditions.

Abstract

In this study, we investigated the possibilities for increasing the valorisation of De-Oiled *Jatropha* Press Cake (DO-JPC). The studied raw material is the by-product of the alkaline protein extraction of the DO-JPC: NaOH Extracted DO-JPC (NEDO-JPC). Protein solubilisation of NEDO-JPC was performed under neutral and acidic conditions (pH 2, 100 mM maleic acid), at elevated temperature (100, 120, and 140 °C), and at 5 % (w/w) dry solids loading. After the treatment, the amount of solubilised protein was determined, as well as the solubilisation of polymeric sugars and formation of sugar degradation products furfural and 5-hydroxymethylfurfural. Although a clear influence is shown for temperature, no difference in protein solubilisation was found between treatments at pH 7 and pH 2. A maximum of 25 % (w/w) of the available protein was solubilised, at 140 °C. The lignocellulose fraction of NEDO-JPC proved relatively recalcitrant to acid hydrolysis, suggesting a more intense treatment to be necessary to sufficiently increase accessibility for cellulolytic enzymes in a lignocellulosic bioethanol process. At €8.00 per tonne DO-JPC, it is concluded that the possibilities for valorisation of the protein fraction of NEDO-JPC at neutral and acid pH are limited, leaving the lignocellulose fraction as a source of valorisation to be investigated.

Introduction

Jatropha curcas

Jatropha curcas L. is grown in Latin America (Brazil, Mexico), Africa (Madagascar, Zambia, Tanzania), and Asia (India, China, Indonesia) [1, 2]. Traditional applications of the plant include erosion control, medicine, fertiliser, and living fence, with the oil from its seeds mainly used for soap production. With the increasing interest for biofuels in recent years, biodiesel production has been suggested as a main application of *Jatropha curcas* oil, because the properties and performance of *Jatropha* biodiesel have been found to compare well to mineral diesel [3, 4]. Attention for biofuels, opportunities for rural development in developing nations, combined with claims of ability to grow on marginal land and of very high oil yields, have lead to an enormous interest and increase in planned production area of this crop [1-5].

Possibly, combining the last two mentioned properties erroneously (able to grow on marginal land and high oil yield) contributed to the enthusiasm concerning this crop and recently, interest in *Jatropha* has returned to a more moderate level. Presently, *Jatropha* oil production is still considered promising, but basic plant science research remains to be performed [1, 2, 6-9]. In 2008, production from plantations was still limited, with about 900,000 hectares worldwide planted with *Jatropha*, most of which in Asia, but large areas were being established [2, 8].

Jatropha valorisation

With the main product of *Jatropha* being biodiesel and/or bio-oil, the main by-product is de-oiled press cake, resulting from the oil pressing and subsequent solvent extraction of remaining oil. If the seeds are dehulled before pressing, the lignin-rich shells are mainly burned as fuel [5]. *Jatropha* press cake (JPC) is rich in protein, but it cannot be used directly for food or feed applications, because of the toxicity or anti-nutritional value of certain components (phorbol esters, trypsin inhibitor, lectin, and phytate). However, because of the favourable amino acid composition of the protein fraction, scientific attention is paid to research towards low-toxicity types of *Jatropha*, as well as detoxification technologies that can produce digestible *Jatropha* protein concentrates for animal feeds [10-14]. Another opportunity for the creation of more value from the press cake (valorisation of the *Jatropha* oil production process), lies in the application of the protein fraction in the non-food sector: binders/glues, emulsifiers, protein films & plastics,

and N-chemicals [15-23]. Also, the polymeric sugar fraction might be used for the production of bioethanol, in a second generation process similar to that for ethanol production from corn stover or wheat straw [24, 25]. This would be another way of valorising the entire process.
The application of the biorefinery concept to a complex raw material like *Jatropha* press cake obviously implies a more complex and therefore likely more expensive fractionation process. However, the potential gains in valorisation (creating more value) and fossil fuels savings are also much larger. In Figure 5.1, a simplified example of a biorefinery of *Jatropha curcas* seeds is shown.

Jatropha in Indonesia

Indonesia, with over 38 million hectares of agricultural surface theoretically suitable, has a large potential for *Jatropha* production [2, 26]. In 2006, plans were announced to increase production of biofuels, bioethanol, and biodiesel/bio-oil from *Jatropha curcas* oil and palm oil, possibly to 5 % of the total energy supply by 2025 [27]. In 2008, 75.500 ha of *Jatropha* had been planted. The estimates for envisaged biodiesel/bio-oil production in Indonesia by 2025 vary from 4.7 to 6.4 and even 16 GL per year. Assuming 6 GL and 50 % of which from *Jatropha*, this means about 3 GL of oil, from about 9 million tonnes of *Jatropha* seeds [2, 28-30]. An annual *Jatropha* seed production of 9 million tonnes would result in around 6 million tonnes of de-oiled *Jatropha* press cake (DO-JPC) per year. The protein (+/- 30 % w/w, or 2 million tonnes per year) and polymeric sugar fractions (+/- 25 % w/w, or 1.5 million tonnes per year) [20, 31] of this large by-product stream provide opportunities for additional valorisation of the total *Jatropha* oil process.

Protein extraction and lignocellulose pretreatment

Alkaline extraction is a commonly used technology for protein isolation from plant material, including *Jatropha curcas* [11, 32]. During this treatment, 70-75 % of the total protein from the raw material – such as *Jatropha* press cake (containing both shells and kernels) – can be solubilised. However, some protein remains (25-30 % of total protein), even after repeated extractions [20]. We hypothesise that by applying a hot acid treatment to the residue of the alkaline extraction (with a wash step in between), more protein is solubilised, due to acid hydrolysis. The protein solubilisation may be in the form of smaller proteins, peptides or even free amino acids.
Using the biorefinery concept of using as many of the components as possible of the raw material for products to create extra value, the idea is to have a subsequent heating step, to

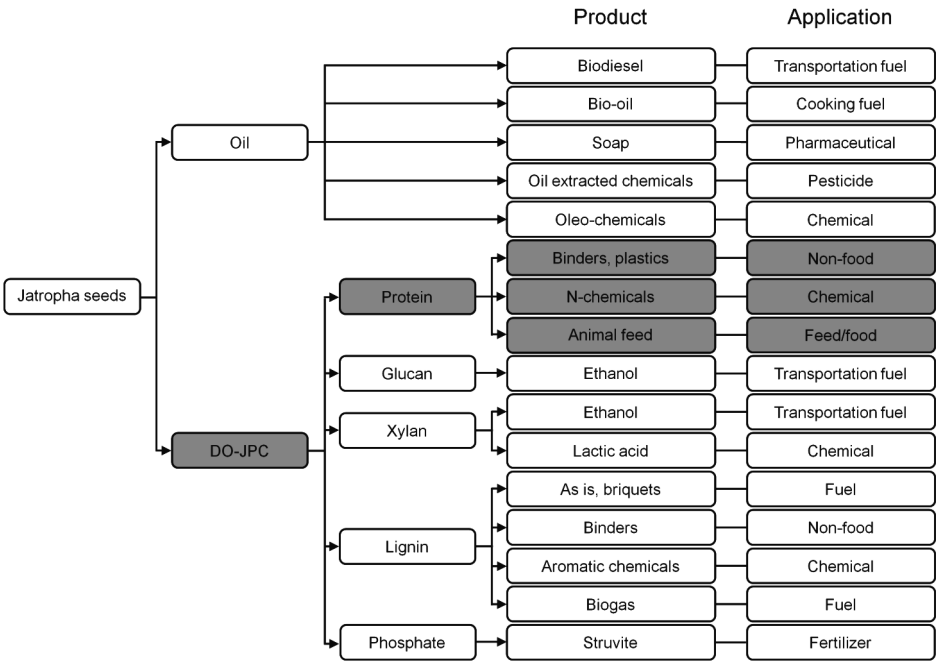

Figure 5.1. Simplified example of biorefinery for *Jatropha curcas* seeds. Protein valorisation in grey.

increase lignocellulose accessibility to cellulolytic enzymes, following the acid protein hydrolysis. In earlier work, it has been shown that maleic acid performs well in this treatment of lignocellulosic material for ethanol production. A major advantage of maleic acid over mineral acid being that very limited amounts of furfural and HMF were formed from monomeric sugars [33-35]. Considering this, it makes sense to use maleic acid for the protein solubilisation of NEDO-JPC as well. Another possibility may be to use lactic acid, as this is one of the possible product of the xylose fermentation in the biorefinery of *Jatropha curcas* we suggest in Figure 5.1 [36].

Research aim

In this study we apply a hot maleic acid treatment (100, 120 and 140 °C) to increase the solubilisation of protein from NEDO-JPC, in order to increase the total valorisation of the de-oiled *Jatropha* press cake. Compared to conditions generally applied in dilute acid lignocellulose pretreatment [33, 34, 37], relatively mild temperatures were chosen, in

order to minimise heating damage to the protein fraction. As controls, and for comparison, we included similar treatments at pH 7, and we determined the amount of soluble protein in untreated material. In short, the research questions of this study are as follows. Firstly, to what extent is extra protein solubilised in the maleic acid treatment at pH 2 compared to the treatment at pH 7 of NaOH-extracted de-oiled *Jatropha* press cake? And secondly: what extra valorisation can be reached with this process?

Materials and Methods

Preparation of NaOH extracted de-oiled Jatropha press cake

The *Jatropha* press cake was obtained in September 2008 from the Energy Technology Center (B2TE) of the Agency for the Assessment and Application of Technology (BPPT), Serpong, West-Java, Indonesia. The de-oiling was performed in December 2008 by continuous hexane extraction, at Pilot Pflanzenöltechnologie Magdeburg e.V. (PPM), Magdeburg, Germany. The de-oiled *Jatropha* press cake (DO-JPC) was stored at 4 °C, until used. 1 kg DO-JPC was mixed (10 % (w/w) dry solids loading with 55 mM NaOH, and left for a 2 hour extraction at 25 °C. After centrifugation (10816 × g, 15 minutes., SLA 3000 rotor, Sorvall RC 6+ centrifuge), 8 L of aqueous phase could be decanted. The pellet was washed with 8 L of water and centrifuged again, in order to rinse out remaining soluble protein and NaOH. This washing treatment was performed three times in total, leading to an estimated remaining NaOH concentration in the aqueous phase of the pellet of < 1 mM. The resulting material is NaOH extracted de-oiled *Jatropha* press cake (NEDO-JPC), and was stored in plastic containers at -20 °C, until used. See Figure 5.2 for the different processing stages from *Jatropha curcas* to NEDO-JPC.

Analysis of chemical composition of NEDO-JPC

Chemical composition of NEDO-JPC was analysed in triplicate, as described by TAPPI methods, [38-43] with minor modifications: (1) samples were extracted with ethanol:toluene 2:1, 96 % (v/v) ethanol and hot water (1 hour) at boiling temperature. (2) the extracted samples were dried at 60 °C for 16 hours. (3) monomeric sugar and lignin content of the ethanol-extracted material was determined after a two-step hydrolysis with sulphuric acid (12 M for 1 hour at 30 °C; 1 M for 3 hours at 100 °C). (4) acid soluble lignin in the hydrolysate was determined by spectrophotometric determination at 205 nm. (5) Ash content was determined at 575 °C. (6) Kjeldahl factor 6.25 was used.

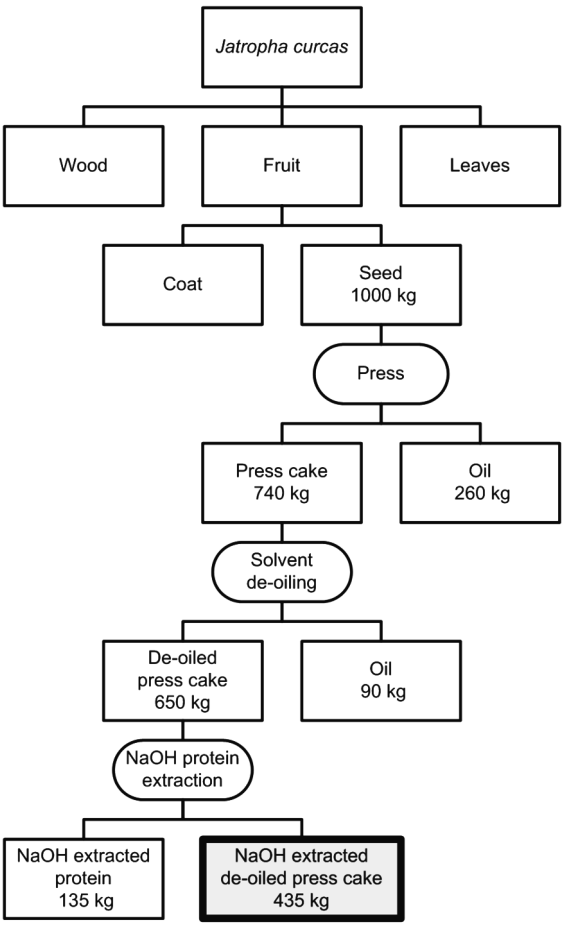

Figure 5.2. Different processing stages from *Jatropha curcas* to NEDO-JPC, the raw material of this study. Estimates for weights of fractions start from 1 tonne dry *Jatropha* seeds (35 % (w/w) oil [1, 2]). Note 1: Press cake before de-oiling: 12 % (w/w) oil; Note 2: Loss of +/- 12 % (w/w) material (other than protein) in NaOH extraction and subsequent wash steps.

Monomeric sugars were measured by HPAEC-PAD (High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection). A Dionex system with Carbopak PA1 column with pre-column was used at 30 °C, with de-ionised water as mobile phase (1 mL/min) and fucose as internal standard. The Dionex HPLC method was also used for determination of monomeric sugars in the aqueous phase of the acid treated NEDO-JPC.

Dry matter content was 19.9 % (w/w) (24 h at 105 °C). The chemical composition of the used NEDO-JPC is shown in Table 5.1.

Treatments at neutral and acidic pH

All chemicals were of research grade and used as received (maleic acid: Aldrich M153). For the experiments at pH 2, NEDO-JPC (15.0 g wet; 3.0 g dry matter) was mixed in poly -ethylene containers with 28.5 mL of maleic acid solution (200 mM) and 16.5 mL water, resulting in 5 % (w/w) dry NEDO-JPC solids loading and 100 mM maleic acid. For the experiments at pH 7, water was added to the NEDO-JPC. Since the washed NEDO-JPC was still somewhat alkaline (pH 7.5-8), the pH was set using a small amount of maleic acid (3 to 4 mM). The NEDO-JPC/acid mixture was soaked for 20 to 24 h at room temperature and then transferred to a Parr 316L stainless steel reactor (inner diameter × height: 33 × 120 mm; 9.0 mm wall; 0.1 L volume), fitted with a top stirrer and a thermocouple. The reactor was heated with a Parr A2237HCEE heater. The system was controlled using Parr 4842 and 4843 controllers (Parr Instruments Co., Moline, Il., USA). Treatments were performed at 100, 120, and 140 °C. Holding time was 30 min, starting

Table 5.1. Chemical composition (dry-weight basis) of the NEDO-JPC used in this study.

Component		Content (% w/w)
Protein		8.2
Total polymeric sugars		25.1
Of which	Glucan	12.0
	Xylan	10.4
	Arabinan	0.7
	Galactan	0.7
	Mannan	0.9
	Rhamnan	0.4
Acid insoluble lignin		48.2
Acid soluble lignin		1.3
Uronic acids		4.2
Extractives		1.8
Ash		7.5
Total		96.3

from when desired core temperature was reached. After the reaction time, the heater was removed, and the reactor was cooled by quenching in water.

Analysis of oligomeric sugars

To assess the amount of oligomeric sugars present in the liquid phase after the treatment, a method closely resembling National Renewable Energy Laboratory (NREL) LAP-014 [44] was used. One millilitre samples were used for an acid hydrolysis, in triplicate. This was performed using 4 % (w/w) H_2SO_4 at 121 °C during 10 min. Monomeric sugars as well as degradation products were measured, as described above and below, and, comparing these results with the values from just after the pretreatment, the original amounts of oligomeric sugars were calculated, taking into account the extra formation of degradation products during the secondary hydrolysis.

Analysis of sugar degradation products

Furfural and 5-HMF concentrations in the liquid phase were measured by HPLC. A Waters system with Shodex Ionpak KC-811 column at 30 °C with a Fast Fruit Juice Guard-Pak pre-column was used. Mobile phase (1 mL/min) was 3.65 mM phosphoric acid, internal standard was phenoxyacetic acid and peak detection was done with UV (210/280 nm).

Results and Discussion

Protein solubilisation of NEDO-JPC

Upon 140 °C treatment, a maximum of 25 % of the available protein in NEDO-JPC was solubilised (Figure 5.3). Protein solubilisation by hydrolysis was clearly enhanced at higher temperatures, as was expected. Quite surprisingly however, there was no clear difference in protein solubilisation between neutral and acidic treatments, although acid conditions were expected to promote hydrolysis. Hot acid is known to cleave peptide bonds, resulting in breakdown of protein into smaller, more soluble peptides and free amino acids, depending on the intensity of the treatment [45]. The present results suggest there was no noticeable level of acid hydrolysis of the protein taking place.
Solubilisation of 25 % of the available protein is not particularly high. Apparently, the easily extractable protein has already been solubilised in the preceding alkaline treatment

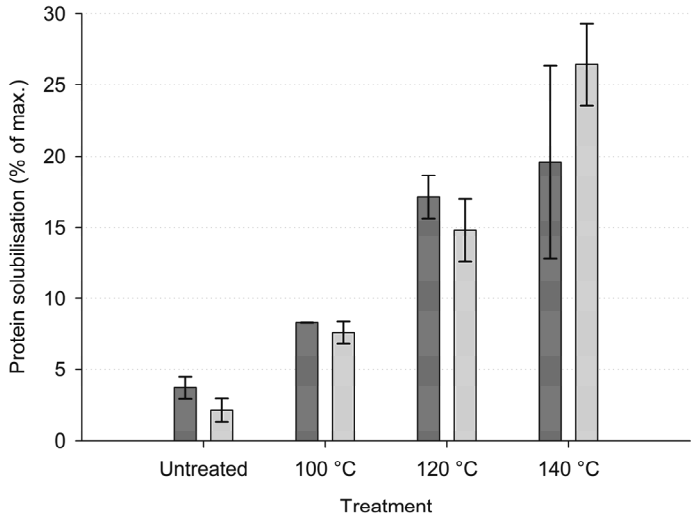

Figure 5.3. Protein solubilisation of NEDO-JPC at pH 7 (dark grey) and pH 2 (light grey). Error bar = standard deviation.

of DO-JPC. Possibly, the more recalcitrant fraction consists of the protein present in the shells. On visual inspection, the solids of NEDO-JPC may be assumed to consist largely of shell material. Both these ideas are supported by the fact that the chemical compositions of NEDO-JPC (Table 5.1) and *Jatropha* seed shells [14] are very similar.

Hot maleic acid treatment of NEDO-JPC (100 mM acid, pH 2, 30 min. at 100, 120, and 140 °C) thus did not increase the solubilisation of protein, compared to heating at neutral pH, although the reason is unclear. In terms of valorisation of *Jatropha* press cake, treatment at neutral pH would be economically superior, as it would yield similar results, while saving on acid costs.

Sugars released during treatment of NEDO-JPC

Conversion of glucan to mono- and oligomeric glucose

While only small amounts of monomeric and oligomeric glucose were released, there clearly are trends with respect to pH and temperature (Figure 5.4 and Figure 5.5).
Increasing the temperature resulted in more hydrolysis of glucan into oligomeric glucose. But only under acidic conditions did the oligomers break down to monomeric glucose,

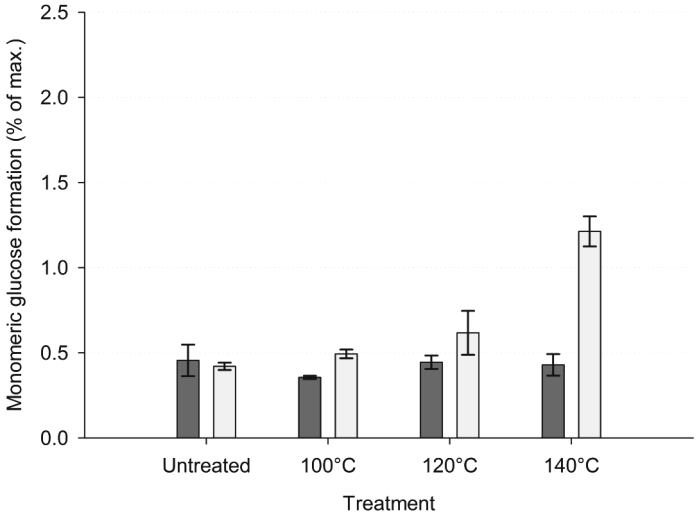

Figure 5.4. Monomeric glucose (% of max.) in aqueous phase, at pH 7 (dark grey) and pH 2 (light grey). Error bar = standard deviation.

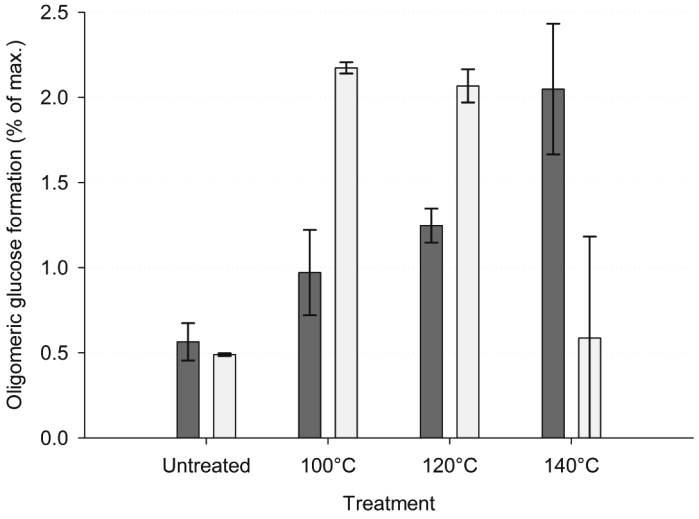

Figure 5.5. Oligomeric glucose (% of max.) in aqueous phase, at pH 7 (blue) and pH 2 (red). Error bar = standard deviation.

and more clearly so at higher temperatures. This agrees with earlier results on maleic acid pretreatment of wheat straw [33, 34]. Comparing 120 and 140 °C at pH 2, there was a 1.5 % decrease in oligo-glucose (Figure 5.5), while only a 0.5 % increase in mono-glucose (Figure 5.4). No clear explanation is available for this. Possibly, other (degradation) products were formed but have not been measured.

Conversion of xylan to mono- and oligomeric xylose

A little more xylan than glucan was solubilised, but the general trend is the same for both polymers. With increasing temperature, xylan hydrolysis to oligomeric xylose increased. But only under acidic conditions did the oligomers break down to monomeric xylose, and more clearly so as the temperature increased (Figure 5.6 and Figure 5.7). It remains unexplained why the decrease in oligomeric xylose did not completely correspond with the smaller increase of monomeric xylose, after 120 °C (and 140 °C) treatment at pH 2. After a 140 °C treatment at pH 2, around 16 % of all xylose was detected as monomeric xylose. Compared to earlier results obtained with wheat straw (>75 % of xylan solubilised as monomeric xylose) [33, 34], this is considerably less and it suggests that the lignocellulose fraction in NEDO-JPC is more recalcitrant to acid hydrolysis than raw materials like wheat straw and corn stover. In order to sufficiently increase accessibility for cellulolytic enzymes in a lignocellulosic ethanol process, a more intense hot-acid pretreatment may be needed.

Formation of sugar degradation products

Only traces of degradation products HMF and furfural were formed during most treatments (Figure 5.8 and Figure 5.9). Only at 140 °C and pH 2, a noticeable amount was detected. However, this was still very little (furfural: 0.90 % of all pentose; HMF: 0.13 % of all hexose). Compared to wheat straw pretreatment, considerably less sugar degradation products were formed [34]. This is not surprising, since there were also a lot less free monomeric sugars solubilised. Clearly, the low level of monomeric sugars was not caused by extreme sugar degradation.

Value of the solubilised protein

To quickly assess valorisation due to protein solubilisation, we introduce the following simple calculation: soy bean meal costs 300 €/tonne and contains 50 % protein, which means soy protein is worth about 600 €/tonne protein. For simplicity reasons, we assume

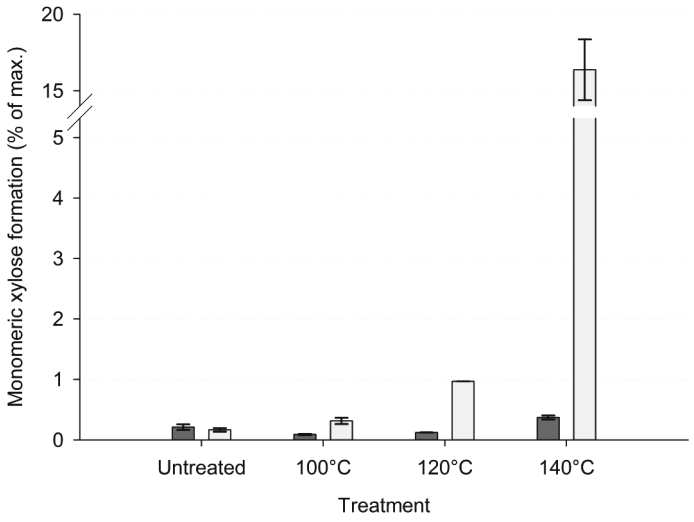

Figure 5.6. Monomeric xylose (% of max.) in aqueous phase, at pH 7 (dark grey) and pH 2 (light grey). Error bar = standard deviation.

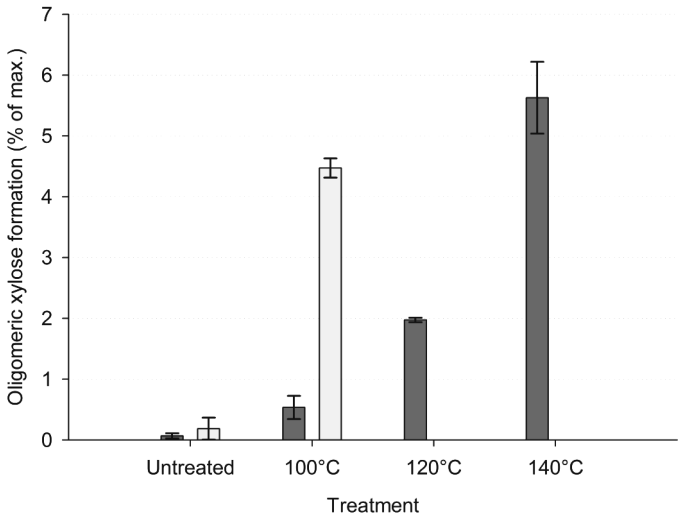

Figure 5.7. Oligomeric xylose (% of max.) in aqueous phase, at pH 7 (dark grey) and pH 2 (light grey). Error bar = standard deviation.

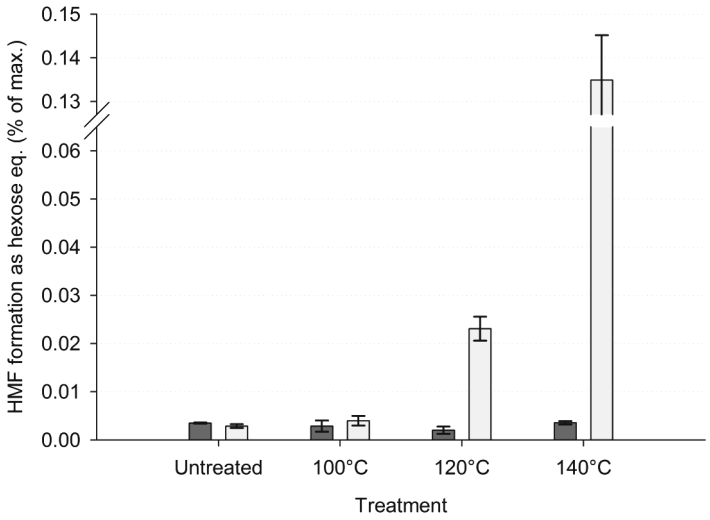

Figure 5.8. HMF formation, as hexose eq. (% of max.), during treatment at pH 7 (dark grey) and pH 2 (light grey). Error bar = standard deviation.

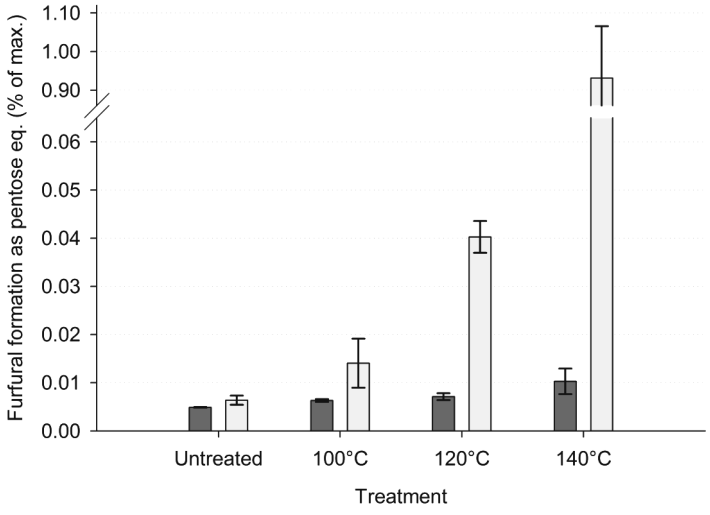

Figure 5.9. Furfural formation, as pentose eq. (% of max.), during treatment at pH 7 (dark grey) and pH 2 (light grey). Error bar = standard deviation.

the costs for concentration are balanced out by the increased value, and that protein from *Jatropha* has the same value as that of soy. In Table 5.2, it is shown that with the solubilisation of 25 % of the available protein in NEDO-JPC, a maximum protein valorisation of €8 per tonne of DO-JPC was reached. Valorisation of all available protein in NEDO-JPC would entail €32 per tonne of DO-JPC (or €49 per tonne of NEDO-JPC). Compared to the 126 to 168 €/tonne DO-JPC that could be achieved with the alkaline extraction of 70 % of the protein present in DO-JPC, 8 €/tonne is a quite limited contribution to valorisation. The prospect of valorisation would deteriorate further if the treatment costs would be taken into account.

In the present hot-acid treatment, heating costs are likely to be significant part of the protein solubilisation costs. Using a estimated heat capacity for dry NEDO-JPC of 1.4 $Jg^{-1}K^{-1}$ [46], and the inclusion of 1.5 tonne process water per tonne of dry matter, heating from 20 to 140 °C costs 924 MJ/tonne dry NEDO-JPC. Conversion to 'coal value' (coal calorific value: 30 GJ/tonne; price 47 €/tonne [47]) leads to heating costs of €1.5 per tonne dry NEDO-JPC, or 1.0 €/tonne dry DO-JPC. Seeing that solubilisation of 25 % of the protein present in NEDO-JPC can lead to a maximum valorisation of 8.0 €/tonne DO-JPC, heating costs of 1.0 €/tonne are quite substantial, but not extreme. However, matters like capital expenditure, operational costs, and process costs such as for solid/liquid separation and protein precipitation are not taken into account.
Considering the valorisation of NEDO-JPC, the maximum contribution of the available glucose and xylose (e.g. for production of bioethanol or lactic acid, see Figure 5.1) can be

Table 5.2. Valorisation of DO-JPC and NEDO-JPC based on protein content.

Material	Protein (% w/w)	Value protein (€/tonne material)	Value protein (€/tonne DO-JPC)
Protein concentrate	100	600	-
DO-JPC	30-40	180-240	180-240
NEDO-JPC	8.2	49.2	32

Material	Protein solubilisation (% of max)	Value solubilised protein (€/tonne material)	Value solubilised protein (€/tonne DO-JPC)
DO-JPC	70	126-168	126-168
NEDO-JPC	25	12.30	8.00

Note: The influence of protein concentration on the value per amount of protein is ignored.

estimated. When using a glucose value of 200 €/tonne [48], and a xylose value of 100 €/tonne, and assuming that both sugars can be fully solubilised and extracted, the maximum contribution of these sugars would be 34 €/tonne NEDO (24 €/tonne from glucose, and 10 €/tonne from xylose), or 22.7 €/tonne DO-JPC. This estimate ignores processing costs and the fact that a more concentrated sugar stream has a higher value per amount of sugar than a less concentrated stream. Combining complete extraction of both the protein as well as fermentable sugars results in a maximum theoretical valorisation of 83 €/tonne NEDO-JPC. This would then leave lignin and ash as residual fractions. For the estimation of the burning value of NEDO-JPC, it is assumed to consist entirely of *Jatropha* shells (calorific value 15.7 GJ/tonne [31]). The shell value in replacing coal then is 24.6 €/tonne shells (or NEDO-JPC), or 16.4 €/tonne DO-JPC.

Ideally, it would be preferable to extract all protein as well as all sugars separately from the NEDO-JPC. Because of the limited solubilisation of protein displayed in this study, it may preferable to put more emphasis on valorisation of the polymeric sugar fraction of NEDO-JPC, while trying to include solubilised protein value if and where possible. This could entail a process combining a mild-temperature-neutral-pH treatment aiming to extract protein, followed by a more intense treatment in which acid is added and temperature is raised, in order to sufficiently pretreat the lignocellulose fraction for application in second generation bioethanol production, after subsequent enzymatic hydrolysis. The remaining lignin-rich material could, as long as an economically superior application is not available, be burned, applied in biogas installations, or used in the production of syngas or synthetic natural gas. There is some doubt whether *Jatropha* shells can be applied in sustained combustion [31]. This has to do with a high ash content (14.8 % w/w,), although lower values (4.7 % w/w) have also been reported [14]. Still, it may be that application of *Jatropha* shells as fuel lies more with household cooking fuel, such as briquettes [31].

Suggestions for future work

Concerning fermentable sugars, and the recalcitrance of *Jatropha* press cake lignocellulose that is suggested in this study, it is certainly interesting to further study valorisation opportunities in this area. It would also be interesting to see how much protein valorisation could be achieved, while the focus is shifted towards fermentable sugars. The final by-product stream that remains, containing mostly lignin and ash, also requires further study. In addition to being burned or otherwise applied to create energy, using lignin as raw material for producing glues/binders or phenolic chemicals offers opportunities for further valorisation [49, 50]. The same goes for the production of

struvite or other fertiliser from the ash, recycling the nutrients such as phosphorus present in *Jatropha* seed cake and shells to agriculture [1, 31].
If the *Jatropha* seeds would be dehulled before pressing, the shells would not enter the oil pressing stage and the DO-JPC would remain shell-free. The shells would also remain dry, as they would not enter the protein solubilisation steps, and could therefore directly be used for fuel applications, in case further research shows that it is difficult to valorise the sugar fraction as fermentable sugars [31]. Of course, it is important that dehulling does not negatively affect the oil pressing process [31].
Another interesting feature to look at considering the protein valorisation, is the amino acid composition. It may be that, with the different protein solubilisation technologies, there are possibilities for creating different streams with different amino acid composition, which would certainly be interesting for the application of amino acids for the production of N-chemicals.
Lastly, considering application towards feed and food it would be interesting to see if and how the protein solubilisation technologies can be used to decrease the toxicity and anti-nutritional value of some components of the *Jatropha* press cake.

Conclusions

Up to 25 % of the protein in NEDO-JPC can be solubilised, meaning that a maximum additional valorisation of €8 per tonne DO-JPC can be achieved by protein solubilisation (100 % solubilisation of all NEDO-JPC protein would yield €32 per tonne DO-JPC). Regarding protein solubilisation, hot-acid treatment with maleic acid is not superior to hot -neutral treatment. The lignocellulose fraction of NEDO-JPC seems relatively recalcitrant to acid hydrolysis; a more intense hot-acid pretreatment may be needed to sufficiently increase accessibility for cellulolytic enzymes in a lignocellulosic ethanol process. Combining protein and sugar, the theoretical maximal valorisation is €83 per tonne NEDO-JPC, with lignin and ash as the two main residual fractions.

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Abbreviations

JPC: *Jatropha* press cake. The by-product from oil pressing *Jatropha* seeds. It contains both kernel and shell.
DO-JPC: De-oiled *Jatropha* press cake. The raw material that is the source of the NEDO-JPC used in this study.
NEDO-JPC: NaOH extracted DO-JPC. NEDO-JPC is the material used in this study.

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Chapter 6

General Discussion

Introduction

The main aim of this thesis is to study the performance of organic acids in the pretreatment of lignocellulosic material for bioethanol production. More specifically, this thesis focuses on how maleic or fumaric acid perform in the hot acid pretreatment of wheat straw, concerning fermentable sugar yields and sugar degradation, and compared to sulphuric acid. Another question that is addressed is what other opportunities there are for extending the application of the organic acid pretreatment to other, protein-containing, lignocellulosic raw materials. This general discussion starts off evaluating the different research chapters, after which the results are discussed in a more general manner, in relation to literature. The discussion is finalised by some suggestions for future research, and the general conclusions.

Present work

Arabinose degradation

In chapter 2, it is suggested that using fumaric or maleic acid for lignocellulose pretreatment has advantages over sulphuric acid. This is mainly because, contrarily to sulphuric acid, both organic acids were found not to display any catalytic activity towards the degradation of arabinose. Less fermentation inhibiting furfural would increase the total efficiency of the ethanol production process, regarding both yield per amount of input feedstock as well as volumetric productivity. However, the organic acids were found to catalyse the hydrolysis of the arabinosyl side chains of the hemicellulose in wheat straw. This combination of results is very positive and makes fumaric and maleic acid potentially interesting candidates for application in biomass pretreatment.

The significance of arabinose for improving the overall yield per amount of feedstock in lignocellulosic ethanol production was confirmed. Most of the arabinosyl side chains were hydrolysed to free monomers. Furthermore, arabinose was shown to be more stable than xylose or glucose in the degradation reaction [1, 2]. This means that arabinose could contribute to the final ethanol yield, while its contribution to the final level of sugar degradation products could be less than proportional compared to monomeric xylose and glucose.

In this chapter, it is shown that fumaric and maleic acids show potential for lignocellulose pretreatment. Their application results in a decrease in formation of sugar degradation

products furfural and 5-HMF. The extent of this decrease remains somewhat unclear, since stronger acids do show more catalytic power in the hydrolysis of polysaccharides, and higher concentrations of free xylose and arabinose could still lead to production of more furfural and HMF, even without catalysed sugar degradation. This is why a more extensive study of the dilute organic acid pretreatment of wheat straw was performed, as described in chapter 3.

Comparing dilute mineral and organic acid pretreatment

In chapter 3, the dilute organic acid pretreatment of wheat straw is studied, comparing fumaric and maleic acids with sulphuric acid, with water alone as control. The main goal was to see if an efficient organic acid pretreatment of wheat straw is possible, which is why not only the influence of the pretreatment temperature was taken into account, but also the influence of a high lignocellulose solids loading was studied. In this study, there were two main focus points. Firstly, research focused on the formation of monomeric sugars during the enzymatic hydrolysis that follows the pretreatment. Secondly, the focus was on the formation of sugar degradation products during the pretreatment, in order to see to what extent the absence of catalytic activity of fumaric and maleic acid on sugar degradation as seen in chapter 2 would result in lower levels of sugar degradation products in a practical biomass pretreatment with these organic acids.

An efficient pretreatment of wheat straw was found to be possible with maleic and fumaric acid. Fumaric acid was somewhat less effective, but both maleic and sulphuric acid pretreatments resulted in almost complete enzymatic digestibility of wheat straw, approaching maximal theoretical values for monomeric glucose (96 and 98 %, respectively). Raising the wheat straw solids loading to 30 % (w/w) did not negatively affect the pretreatment, as far as enzymatic digestibility of the feedstock is concerned. Compared to sulphuric acid, much less sugar degradation products were formed during the organic acid pretreatments, both at low and high solids loading. At high solids loading, however, furfural levels increased to an undesirable level, albeit still much lower than with sulphuric acid. Although strongly reduced compared to dilute sulphuric acid pretreatment, furfural formation does remain a point of attention (or optimisation) in the organic acid pretreatment. The strongly reduced xylose degradation with organic acids was reflected in higher xylose yields, compared to sulphuric acid. A possible downside of the use of fumaric and maleic acid was also found. Neither acid is completely heat stable, which could lead to extra costs because of acid replenishment.

This chapter showed that the application of dilute organic acids can be effective in the pretreatment of lignocellulosic biomass like wheat straw. Compared to sulphuric acid,

maleic acid pretreatment resulted in similar amounts of glucose, more xylose, and much less formation of sugar degradation products. These results make maleic acid a serious alternative for the dilute sulphuric acid pretreatment, and have lead to the optimisation of the maleic acid pretreatment in chapter 4.

Optimisation of maleic acid pretreatment of wheat straw

In chapter 4, the dilute maleic acid pretreatment of wheat straw was economically optimised, using pretreatment time, temperature and maleic acid concentration as design variables. The optimisation factors taken into account were benefits from glucose and xylose, and costs resulting from maleic acid replenishment, heating, neutralisation, and furfural production. A solid-liquid separation step was introduced after the pretreatment. This resulted in a liquid phase containing solubilised hemicellulose (oligo- and monomeric xylose) and any sugar degradation products, and a solid pellet containing most of the cellulose (polymeric glucose) and lignin. The pellet was used for the enzymatic digestion.

Disregarding costs, almost complete conversion of cellulose glucan could be reached, after enzymatic hydrolysis. During the pretreatment, up to 90 % of all xylan in the hemicellulose was converted to monomeric xylose.

Taking costs in to account, optimal process conditions were found to be: 50 min at 170 ° C, with 46 mM maleic acid. These pretreatment conditions led to the maximum net yield of €65 per tonne dry wheat straw. This consisted of €68 glucose benefits (from pellet: 85 % of all glucan), €17 xylose benefits (from liquid: 80 % of all xylan), €17 maleic acid costs, €2.0 heating costs and 0.90 €/tonne neutralisation costs. Furfural formation during the maleic acid pretreatment experiments was so limited that associated costs could be considered negligible, except under the most severe conditions. Clearly, replenishment of maleic acid is the most important of the studied cost factors.

The €65 per dry tonne wheat straw that is the maximum yield shown in this chapter, makes the maleic acid pretreatment process likely to be viable, but the opportunities for valorisation for this feedstock are limited. One option to increase the process economics of the dilute maleic acid treatment is to move to another raw material altogether. When a feedstock contains protein as well as lignocellulose, dilute acid treatment may be used to (partly) hydrolyse and/or solubilise these fractions, as a way to increase the total final valorisation. This part of the study is described in chapter 5.

Application of hot maleic acid treatment in valorisation of Jatropha press cake

In chapter 5, in search of higher-value applications of maleic acid, possibilities for increasing the valorisation of *Jatropha* press cake were studied. The raw material in this study was the washed by-product of the alkaline protein extraction of de-oiled *Jatropha* press cake. In order the solubilise the remaining protein fraction, this NEDO-JPC was heated in presence of 100 mM maleic acid (pH 2), as well as under neutral conditions. In this manner, the acid treatment might also be considered a pretreatment for the lignocellulose fraction.

The results showed that up to 25 % of the remaining protein in NEDO-JPC could be solubilised. Disregarding costs, this would result in an additional valorisation of €8 per tonne de-oiled *Jatropha* press cake. The hot maleic acid treatment of NEDO-JPC did not result in more protein solubilisation than hot-neutral treatment. This would mean the treatment at neutral pH would be preferable, since it would yield the same amount of protein, while saving on acid costs.

Considering the lignocellulose fraction, the acid treatment clearly had more effect than the neutral treatment. But there were strong indications that the lignocellulose fraction is relatively recalcitrant to acid hydrolysis. Therefore, a more intense hot-acid pretreatment would probably be needed to sufficiently increase accessibility for cellulolytic enzymes in a lignocellulosic ethanol process.

In this chapter, it is shown that the performance of the hot maleic acid treatment on in the valorisation of *Jatropha* press cake was quite limited, concerning protein solubilisation and lignocellulose pretreatment.

Relation to literature and societal impact

Acid pretreatment of lignocellulosic material

In the field of lignocellulose pretreatment in recent years, a lot of attention has been addressed to acid pretreatment, focussing on maximising sugar yield. These studies often use dilute sulphuric acid pretreatment for raw materials like cereal straw, such as from wheat, corn, rice, rye, and barley. Yields of fermentable sugars after subsequent enzymatic hydrolysis is usually over 90 % of theoretical maximum [3-7]. Some organic acids have also been applied, such as acetic, formic, lactic, and oxalic acid. Also combinations of acids are applied. Fermentable sugar yields following pretreatment with

these acids vary somewhat, with dilute formic acid being little effective, but the most successful ones generally reach 80 or 90 % glucose yields after subsequent enzymatic hydrolysis [8-12]. A direct and full comparison between these pretreatment results is not possible, because of differing experimental conditions, but in general, the process optima for reducing the formation of sugar degradation products to increase hydrolysate fermentability on one hand and for optimising glucan conversion in the enzymatic hydrolysis on the other hand are contradictive, as is usually the case with acid pretreatment.

Still, the sugar yields following maleic or fumaric acid pretreatment of wheat straw as described in this thesis compare very well to the work of these other authors. In fact, in several recent literature reviews, the organic acid pretreatment with maleic acid advocated in this thesis is recognised as an alternative for using dilute sulphuric acid, mainly because of the high sugar yields and low sugar degradation [3, 13, 14]. Furthermore, the work described in this thesis on organic acid pretreatment of lignocellulosic material has lead to two patent applications [15, 16].

Foust *et al.* [4] have done a very nice study on the economics of the conversion of lignocellulose to ethanol. They compared the thermochemical conversion of wood chips with the biochemical conversion of corn stover, with the latter including a sulphuric acid pretreatment. In their calculations, the authors used a glucan-to-glucose conversion of 80 to 95 %, and a xylan-to-xylose conversion of 63 to 80 %. These numbers are quite easily met in the pretreatment using maleic and fumaric acid, as shown in this thesis.

Sugar degradation and formation of degradation products

Apart from the study on arabinose degradation described in chapter 2, a small number of studies have focused on sugar degradation in presence of organic acids. Xylose and glucose degradation in presence of maleic acid have been studied, as mentioned in chapter 2 [2 , 17]. Also, a more extensive study has been performed on xylose degradation by maleic acid, in which the authors suggest that, the dicarboxylic maleic acid has a double action. Because maleic acid is a weak acid, only part of the protons are dissociated from the two carboxylic groups under normal pretreatment conditions. While dissociated protons act as specific acid catalysts (quick reaction), the undissociated acid itself may act as a general acid catalyst (slow reaction), which means that the catalysis (proton transfer) also becomes dependent on the undissociated acid, and not only on the proton concentration [18]. Very interestingly, the same authors, using a buffered system to study xylose degradation at pH 0.8-5.8, suggested that in addition to protons [H^+], also hydroxyls [OH^-], and the water as solvent, also have an important influence on the

degradation rate constant of xylose, even under acidic conditions. Furthermore, the pH at which minimal xylose degradation occurred in the applied buffered system, was 2.2 [19, 20]. This might provide some insight in why 50 mM of maleic acid (pH 1.86) had no extra catalytic effect on arabinose degradation as described in chapter 2, and why 50 mM fumaric acid (pH 2.20) was causing even less arabinose degradation than water alone. Of course, the experimental conditions of the work in chapter 2 were different (type and concentration of sugar, non-buffered system) from the mentioned study. One thing that does not comply with the finding that solvent and OH^- ions are so important in sugar degradation is that in chapter 2, the arabinose degradation rate constant in presence of water alone was very close to that when in presence of maleic and fumaric acid. In presence of water alone, it would have been expected to be larger. If this has to do with buffered/non-buffered systems can only be suggested.

In short, acid catalysis is not the only important contributor to sugar degradation. In fact, catalysis by solvent and hydroxyls may contribute much more to pentose degradation, even under fairly acidic conditions (pH 5.8-2.2). However, the observation that xylose degradation was minimal at pH 2.2 opens possibilities for the organic acid pretreatment to minimise sugar degradation. However, this is keeping in mind that at a somewhat higher pH, the degradation may increase again. Another interesting thing to mention is that the degradation products of the solvent and base catalysed xylose degradation are not the yeast inhibiting furfural. Instead, compounds are mentioned such as xylose isomerisation products (lyxose, arabinose), organic acids (pyruvic, formic, acetic, citric, lactic), and resin type compounds, amongst others [20-22]. But even disregarding degradation products, loss of fermentable sugar means less efficient feedstock use, resulting in higher bioethanol production costs [4].

Theoretical bioethanol production from straw in the European Union

From 2004 to 2008, the EU used around 145 GL of gasoline annually, a number that seems to be slowly decreasing [23]. In order to determine whether the EU has the potential of producing enough fuel ethanol from domestic straw feedstocks to be completely self-sufficient, a simple calculation can be made, using 145 GL as annual EU gasoline consumption. Total EU cereal production in 2008 was 318 million tonnes, with 88 % or 280 million tonnes of which accounted for by wheat, barley and corn [24]. In Table 6.1, a summary on production numbers on these cereals, their straw and the potential bioethanol production is shown.

Table 6.1. Potential bioethanol production from EU straw [7, 25-29].

	Wheat	Barley	Corn	Total
Cereal produced in 2008 (million tonnes)	150	65.7	62.9	279
Straw to cereal ratio (-)	1.3	1.2	1.0	-
Total straw (million tonnes)	195	78.8	62.9	337
Straw to be left on land (tonne/ha)	1.7	1.7	2.7	-
Area harvested (million ha)	26.5	14.5	8.88	49.9
Total straw theoretically available (million tonnes)	150	54.2	38.8	243
Theoretical ethanol yield (L/tonne straw)	376	407	385	-
Total theoretical ethanol yield (GL)	56.5	22.1	15.0	93.5

As pure fuel ethanol (E100) contains 65.8 % of the energy (lower heating value; LHV) that gasoline does, it would take 220 GL ethanol to completely replace the annual 145 GL gasoline with E100 [30]. However, in order to facilitate cold operation in flexible fuel cars, 15 % (V/V) gasoline is mixed with ethanol, creating E85. The theoretical ethanol yield from straw of 93.5 GL could be used to produce a maximum of 110 GL E85 fuel, effectively replacing 61.5 GL gasoline; 42.4 % of the annual EU gasoline consumption of 145 GL. (It is worth mentioning that, while the theoretical energy content of E85 fuel is 71 % of that of gasoline, the US Department of Energy (US-DOE) uses 77 %. This is because, in practice, some other additives are also present in E85, even some gasoline used as denaturant in ethanol. Furthermore, the ethanol content of E85 is sometimes lowered to 70 % during winter in cold climates [30].)

Clearly, using all of the straw in the EU for bioethanol production, would not lead to bioethanol self-sufficiency in the EU. For comparison, it should be considered that EU net crude oil imports are about 10 times larger than the amount it produces [31]. Other lignocellulosic feedstocks, such as domestic waste, forestry residues, and energy crops would be needed. Of course, imports of biomass as well as bioethanol will also be a large part of the picture. Another possibility lies in the production of other biofuels that are that can replace gasoline, while using different feedstocks. An example is biomethanol, for which a very large production plant was opened in the Netherlands in 2010, capable of producing 250 ML of biomethanol annually, using crude glycerin as feedstock, a by-product from biodiesel production [32, 33].

Recycle of organic acid

In this thesis, the importance of reducing acid costs is stressed. This is especially valid for organic acids, as these are generally more expensive than sulphuric acid, as mentioned in chapters 4 and 5. Recycling acids is therefore important. Preferably, this should be done without recycling the entire liquid phase, as the presence of the dissolved oligomeric and monomeric sugars would eventually lead to increased formation of sugar degradation products. One method is to create insoluble acid salts and harvest these, after which re-acidification with a strong acid takes place, usually sulphuric acid. This may be applied, since it still keeps the pretreatment free from sulphuric acid, hereby reducing formation of sugar degradation products. However, it does create another sulphuric acid containing stream. Alternatively, several separation methods can be applied to selectively recycle organic acids. Examples are: adsorption [34], crystallisation using cooling [35, 36] or template induction [37], and nanofiltration [38]. These separation methods have their origin in fermentation technology, as 'in situ product recovery or removal'. However, all mentioned examples are performed at temperatures that are lower than those used in the pretreatment, which seems to be a major disadvantage of these technologies when applied to hot solutions. Continuously cooling and reheating the liquid phase would require a lot of energy, resulting in high recycle costs.

Animal feed production and biorefinery

There are opportunities for animal feed producers around the subject of this thesis. For example: using pretreated straw-like material as feed. Treatments aiming to increase the enzymatic digestibility of strawlike materials in second generation bioethanol processes can also increase the digestibility of these materials for ruminants [39, 40]. This would entail a higher feed efficiency of cereal straw and similar lignocellulosic materials.
Furthermore, any organic acid still present after the pretreatment process would not be problematic. Many organic acids that have been suggested for organic acid pretreatment can be digested by animals, with the exception of maleic acid. In fact, many methods for the storage of forage or fodder include lowering the pH with lactic, acetic, formic, or propionic acid, by addition, or by partial fermentation. In addition to their energetic value, animal growth promoting properties are awarded to some organic acids, such as citric, formic, lactic and fumaric acid. Specifically for fumaric acid, there are indications that its presence in feed can inhibit growth of methanogenic microorganisms, hereby possibly reducing methane formation in ruminants, although this has not yet been confirmed *in vivo* [41-45].

Another thing to consider is the key position animal feed producers can take in biorefinery systems. They can act not only as a user of the by-product streams that are generated in the different processes, but also as a driving force, more in the beginning of the biorefinery. The main important component of feedstocks in this aspect is the protein fraction, as distribution and application of proteins is becoming more and more important in the world feed & food supply, as the worlds' population continues to increase while the average living standard also increases. This leads to more production of animal feed and higher demand for feed protein sources [46, 47]. In the case of protein rich compound feeds, the focus is mostly on the amino acids lysine, methionine, and cysteine, as these are usually the first amino acids in animal feed to be growth limiting [48].
Already experienced and economically involved in this type of by-product streams, while also having the logistics in place needed to deal with these large product streams, it might be very advantageous for animal feed producers to move to the beginning of the biorefinery chain. With the right technology, they could create streams which are more concentrated in certain desired amino acids; protein/amino acid concentrates. On the one hand, these could be applied in animal feed, while on the other hand other concentrates, or the remaining materials could be sold towards other applications, such as using as feedstock for the production of protein-based products & N-chemicals (proteins), second generation bioethanol (lignocellulose), and phenolic chemicals (lignin) [49-56]) This would lead to a much more efficient use of protein containing feedstocks (both for animal feed and chemicals) and also to decreased manure production because of more efficient animal feed.

Large scale *Jatropha curcas* production

The enormous production of *Jatropha curcas* biodiesel that is planned, as mentioned in chapter 5, does not only entail positive consequences. It may be true that on the one hand, large scale *Jatropha* production of biodiesel in combination with a functional biorefinery of the whole plant, can mean social and economic benefits for a large amount of people in rural parts of the developing world [57]. On the other hand however, if millions of hectares are to be planted, these plantations will need very large amounts of manual labour. For example, *Jatropha* fruits do not ripen all at once on one plant, so the same field needs to be 'partially harvested' several times per year by hand, hereby increasing the amount of labour needed. In total, including weeding, irrigation, fertilisation, pruning, and harvest, it is estimated that 70 person days per hectare per year are needed for a *Jatropha curcas* plantation. [58]. This means that for a large plantation of 10,000 hectares,

about 2,000 full time workers would be needed. Local farmers cannot supply all the labour, so workers need to attracted from elsewhere. It is therefore quite imaginable that in a region where a million hectares of *Jatropha* plantation is planned, entire villages and cities will need to be built, just to house the workers and their families. Another matter to consider is how to prevent extremely bad working conditions in these plantations. *Jatropha curcas* seeds are quite toxic, and getting in contact with them, or with the oil they contain, can negatively affect health [59]. Plant science may be able to solve some of these issues, by increasing productivity, developing of non-toxic varieties, and synchronising the ripening stage, so that all fruits can be harvested at once [59-61]. In short, large scale *Jatropha curcas* production may bring economic and social benefits, but great care should be taken not to let it cause problems, social nor health related.

Food versus fuel

The Food versus Fuel discussion deals with the competition for feedstocks between fuel and food/feed application. First generation bioethanol production can be criticised for using cereal grains as feedstock, which could in principle also be applied to produce food and feed, although it does produce a protein-rich by-product stream (DDGS) that can be used to feed animals [39, 62]. Second generation bioethanol production uses the lignocellulose fraction of the plant, and because these parts are not edible, this is regarded as much more positive in the mentioned discussion. Still, a large part of the food versus fuel discussion has to do with land use. The more efficiently the land allocated for fuel production can be used, the better [63].

Application of organic acids in the pretreatment of straw would lead to (by-)product streams that may be applicable in animal feed, as mentioned before. In this way, biorefinery plays a positive role in the Food versus Fuel discussion. In other words, when assuming a biorefinery system in which organic acids are applied for converting straw to bioethanol and animal feed, this could be considered more a case of Food ánd Fuel, in stead of Food versus Fuel, as it reduces competition for land use between the mentioned applications.

Suggestions for future research

From the work an arabinose degradation presented in chapter 2, in combination with both the discussion on specific & general catalysis as well as that on solvent and base catalysed sugar degradation which may occur, even under acidic conditions, it is clear that these

phenomena should be studied further. In the organic acid pretreatment, in which conditions are usually less acidic than when sulphuric acid is applied, opportunities exist for minimising degradation of xylose and arabinose, even separately from furfural formation. Both would increase the overall efficiency of the ethanol production. Of course, it should not be forgotten that polysaccharide hydrolysis is also important. It is acid catalysed, and supplies the degradation reactions with substrate, so to speak. A lot of free pentoses may still lead to more degradation/furfural formation than when less free pentoses are available under conditions that favour degradation.

In chapter 3, it is shown that furfural production when using maleic acid is quite high at 30 % (w/w) solids loading, albeit still lower than when using sulphuric acid. It would be interesting to optimise the maleic acid pretreatment with regard to the solids loading, as it is suggested to be between 20 and 30 % (w/w). Also, it might be possible to lower furfural production by using a counter current pretreatment reactor, removing solubilised (oligo-) pentoses from the process before they are degraded.

In chapter 4, costs for acid replenishment were the largest of the studied cost factors. Therefore, further study on how to reduce these costs is certainly justified. One way of reducing acid replenishment costs would be to take into account the isomerisation of maleic acid to fumaric acid.. In a continuous system, the result would be a mixed maleic/ fumaric acid pretreatment. Any formed fumaric acid may still have an effect on the pretreatment, resulting in less maleic acid that needs to be replenished. The influence of this mixed acid pretreatment is not yet known and deserves study. Possibly, the maleic acid replenishment can be done by adding fumaric acid. Fumaric acid can be produced in situ (refs) from a part of the released sugar stream.
Regarding the optimisation of the maleic acid pretreatment, further study should focus more on the optimisation of the integrated conversion process of wheat straw to bioethanol, using maleic acid in the pretreatment. Matters to be taken into account might include costs for acid recycling and re-use of process energy, as well as costs for capital expenditure and enzymes. For the benefit side, there is the organic by-product stream to take into account, as well as how to apply the lignin fraction. Other matters of influence would be increasing the solids loading in the pretreatment as well as in the enzymatic hydrolysis, and the influence of increased concentration on value of the sugar stream (mainly meaning decreased downstream processing costs).

During the study in chapter 5 on solubilisation of *Jatropha* press cake protein, some questions remained unanswered. For example, why did the pH 2 treatment not result in more protein solubilisation than the neutral treatment? And what would happen if the temperature of the treatment was increased over 140 °C? Would a lot more protein be solubilised? And in what from? In general, the valorisation of *Jatropha* press cake definitely deserves more research. The amounts of this feedstock that can be expected to become available in the near future are enormous, and therefore so are opportunities for valorisation.

Regarding protein valorisation, an interesting feature to look at is the amino acid composition. It may be that, with the different protein solubilisation technologies, there are possibilities for creating different streams with different amino acid composition, which would certainly be interesting for the application of amino acids for the production of N-chemicals. Considering protein application in feed and food it would be interesting to see if and how the protein solubilisation technologies can be used to decrease the toxicity and anti-nutritional value of some components of the *Jatropha* press cake.

Regarding lignocellulose, it is suggested in chapter 5 that this fraction of *Jatropha* press cake is quite recalcitrant to acid pretreatment. Valorisation possibilities of the lignocellulose fraction of *Jatropha* press cake should be studied further, starting with what would be a sufficiently intense acid pretreatment in order to increase the enzymatic digestibility of *Jatropha* press cake lignocellulose to an acceptable level in a lignocellulosic ethanol process (or for another application for the released sugars). It would also be interesting to see how much protein valorisation could be achieved; at the same time, if possible.

If the *Jatropha* seeds were dehulled before the oil pressing stage, the press cake would remain shell-free. The shells are low in protein and high in lignocellulose, while the opposite is true for the kernels, which makes the dehulling step an easily applicable separation method for valorisation purposes. If efficient acid pretreatment of the lignocellulose fraction of the shells is possible, having ‘pure’ shells as feedstock would simplify the process and facilitate higher solids loading than if the shells are mixed in with the kernel material. The shells would also remain dry, as they would not enter the protein solubilisation steps, and could therefore directly be used for fuel applications, in case further research shows that it is difficult to valorise the lignocellulose fraction as fermentable sugars. Of course, it is important that dehulling does not negatively affect the oil pressing process [64].

In a biorefinery using mild organic acid processing of lignocellulosic materials, many possibilities open up for animal feed applications and producers, as previously suggested. Regarding lignocellulosic raw materials, both the application of the by-product stream of the organic acid pretreatment as animal feed, as (pre)treatments focussed on increasing the feed value of the raw material itself should be researched. Possible research topics would be the digestibility for ruminants (and possibly for other animals), but also sugar degradation, or the influence of the organic acids, and other compounds that may result from the treatment. Similar suggestions can be made for protein containing lignocellulosic materials, with regard to producing protein or amino acid concentrates. Something to keep in mind would be the influence of the treatment of some toxic and anti-nutritional components, such as in *Jatropha* press cake. In short, a higher feeding value of the raw material, could mean an increased valorisation of the agricultural processes involved, and a more efficient use of agricultural land.

Finally, as a general suggestion, biorefinery research could apply a more influential approach to its raw materials, by focussing less on a by-product stream as it is available now, and more on how it would (be arranged to) result from an integrated biorefinery system. In other words, the 'main' process might be altered somewhat further upstream, in order to increase the valorisation potential of the by-product(s). The dehulling of *Jatropha* seeds mentioned before would be a simple example. Another would be protein valorisation from Dried Distiller's Grains and Solubles (DDGS), a protein-rich dried by-product from first generation bioethanol production, now mainly used 'as is' as animal feed. As processes to be used to extract the protein fraction would be likely to involve adding water, it would be beneficial to use the wet by-product stream: Wet Distiller's Grains (WDG). This would save costs related to both drying as well as to subsequent water addition. Furthermore, it might also result in a higher quality product, as the protein fraction would suffer less heat damage. Of course, this would imply that the valorisation process takes place close to the location where the WDG is produced, as costly transportation and storage of an aqueous stream should be prevented.

General conclusions

In this thesis, it was shown that organic acids can be successfully applied in processing lignocellulosic material. Using wheat straw, pretreatments with dilute maleic and fumaric acid were shown to be effective alternatives to that with dilute sulphuric acid, which is a leading pretreatment method in the production of second generation bioethanol. Neither of

the organic acids catalysed the degradation of arabinose to the yeast inhibiting furfural, while sulphuric acid did. In the maleic and fumaric acid pretreatment of wheat straw, this resulted in much less furfural formation from xylose. Regarding sugar yields, maleic acid even outperformed sulphuric acid under certain conditions, with similarly close-to-complete glucan conversion, while resulting in higher xylose yield, due to the much reduced xylose degradation.

Optimisation of the maleic pretreatment of wheat straw resulted in a maximum financial yield of 65 €/tonne dry straw, with sugar yields of 85 €/tonne straw, and with acid replenishment as the most important cost factor, at 17 €/tonne dry straw.

Application of a hot maleic acid treatment in order to extract extra protein from the de-oiled press cake of *Jatropha curcas*, which had already undergone an alkaline protein extraction, had only limited success. No effect of the acid was found on the amount of extra extracted protein. Maleic acid did have a small effect on the lignocellulosic fraction of this feedstock, indicating the need for a more severe pretreatment in order to facilitate an efficient enzymatic hydrolysis of the polysaccharides to fermentable sugars.

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Summary

In the transition from an economy based on fossil resources to a biobased economy, the goal is to replace these fossil resources with renewable ones. An often used term in this area of research is biorefinery, which describes the concept of processing organic raw materials in such a way as to produce an economically optimised combination of chemicals, materials, and heat & power.

A lot of the planned growth in the production of bioethanol, to be used as transportation fuel to replace gasoline, is to come from second generation processes, which use lignocellulosic raw materials like cereal straw, bagasse, or forestry by-products. This type of raw material requires a fair amount of processing in order to make the fermentable sugars available for fermentation. Usually, a pretreatment is required to facilitate the enzymatic hydrolysis of the cell wall polysaccharides to fermentable sugars. A common pretreatment method is to heat the material in presence of dilute sulphuric acid, hereby disrupting the lignin-carbohydrate complex, hydrolysing part of the polysaccharides (mostly hemicelluloses) and making the remaining cellulose more accessible to the cellulolytic enzymes. The sulphuric acid pretreatment has some unwanted effects. Firstly, the hydrolysis of polysaccharides during the pretreatment leads to free sugars, which then degrade to furfural and 5-hydroxymethylfurfural. These compounds are inhibitory to yeasts later on in the ethanol fermentation, leading to increased costs, and to a lower ethanol yield per amount of feedstock. Secondly, the use of sulphuric acid results in large amounts of gypsum, which may negatively affect downstream processing, and results in a low-value by-product stream. If organic acids were to be applied in the pretreatment, the quality of the by-product stream would improve significantly, as it may be more easily burned in co-firing installations, used for fertilising soil, or applied in animal feed.

In research prior to this thesis, the organic maleic acid has been suggested as an alternative to sulphuric acid, since maleic acid catalysed the hydrolysis of cellobiose (dimer of glucose) and pure cellulose, while resulting in lower levels of glucose degradation than sulphuric acid. Fumaric acid is similar in structure to maleic acid, but it is somewhat weaker. As opposed to maleic acid, however, fumaric acid can be produced by fermentation.

The aim of this thesis is to study the performance of organic acids in the pretreatment of lignocellulosic material. More specifically, this study focuses on what the influence is of maleic and fumaric acids on the sugar degradation during the pretreatment, and how well these acids perform in the pretreatment itself, as alternatives to sulphuric acid. The optimisation of the maleic acid pretreatment takes into account not only specific

opportunities of the organic acid pretreatment leading to financial benefits, but also possible downsides which may lead to extra associated costs. Lastly, this study aims to look for opportunities for extending the application of the organic acid pretreatment to other, protein-containing lignocellulosic raw materials.
As wheat is the largest cereal crop in the European Union, wheat straw was chosen as lignocellulosic raw material for the work in this thesis. For the work on protein containing lignocellulosic raw material, the by-product from biodiesel production from the oil-seeds of *Jatropha curcas* was chosen, the de-oiled press cake.

In the research described in this thesis, both maleic and fumaric acid are found not to catalyse the degradation of arabinose, the second pentose present in wheat straw, to the yeast inhibiting furfural, while sulphuric acid clearly does (Chapter 2). Furthermore, the organic acids are found to catalyse the hydrolysis of the arabinosyl side chains of the hemicellulose in wheat straw. This combination of results make fumaric and maleic acid potentially interesting candidates for application in lignocellulose pretreatment. Furthermore, arabinose is shown to be more stable than xylose or glucose in the degradation reaction. Therefore, arabinose can contribute to the final ethanol yield from wheat straw, while its contribution to the formation of sugar degradation products may be less than proportional, compared to monomeric xylose and glucose.

A more extensive study of the dilute organic acid pretreatment of wheat straw is described in Chapter 3, comparing fumaric and maleic acids with sulphuric acid, with water alone as control. In order to see if an efficient organic acid pretreatment of wheat straw is possible, not only the influence of the pretreatment temperature is studied, but also the influence of a high straw solids loading is taken into account. Efficient pretreatment of wheat straw is found to be possible with maleic or fumaric acid. Fumaric acid is somewhat less effective, but both maleic and sulphuric acid pretreatments result in almost complete enzymatic digestibility of wheat straw, approaching maximal theoretical values for monomeric glucose (96 and 98 %, respectively). High solids loading (30 % (w/w)) does not negatively affect the pretreatment, as far as enzymatic digestibility of the feedstock is concerned. Compared to sulphuric acid, much less sugar degradation products are formed during the organic acid pretreatments. The strongly reduced xylose degradation in presence of the two organic acids is reflected in higher xylose yields. However, a possible downside of the use of fumaric and maleic acid is also found. Neither acid is completely heat stable, which could lead to extra costs because of acid replenishment.

In Chapter 4, the dilute maleic acid pretreatment of wheat straw is optimised, using pretreatment time, temperature, and maleic acid concentration as design variables. The dependent variables taken into account for the optimisation are benefits from glucose and xylose, and costs resulting from maleic acid replenishment, heating, neutralisation, and furfural production. Following the pretreatment, a solid-liquid separation step is introduced, after which only the pellet is used for the enzymatic hydrolysis. It is shown that, disregarding costs, almost complete conversion of glucan of the pellet can be reached in the subsequent enzymatic hydrolysis. Furthermore, up to 90 % of all xylan in the hemicellulose can be converted to monomeric xylose, during the pretreatment.
When costs are taken into account, the maximum net yield is found to be €65 per tonne dry wheat straw, resulting from €68 glucose benefits, €17 xylose benefits, €17 maleic acid costs, €2.0 heating costs, and €0.90 neutralisation costs. Furfural formation is found to be so limited that associated costs can be considered negligible, except under the most severe conditions. Clearly, replenishment of maleic acid is the most important of the studied cost factors.
The process conditions leading to the maximum net yield are: 50 min at 170 °C, with 46 mM maleic acid. These conditions lead to 80 % of all the xylan from the straw being solubilised in the liquid phase, while enzymatic hydrolysis of the pellet leads to 85 % of all the straw glucan to be retrieved as glucose.

In Chapter 5, a possibility for increasing the valorisation of *Jatropha* press cake is studied, in search of higher-value applications of the maleic acid treatment. The washed by-product of an alkaline protein extraction of de-oiled *Jatropha* press cake is the raw material in this study. A hot maleic acid treatment (100 mM, pH 2) is compared to a hot neutral treatment, focussing on the solubilisation of extra protein from the raw material. Up to 25 % of the remaining protein is solubilised, at 140 °C, leading to an estimated €8 of additional protein value per tonne press cake, when disregarding costs. Moreover, the hot maleic acid treatment does not result in more protein solubilisation than the hot-neutral treatment, making the latter preferable, since it results in the same amount of solubilised protein, while saving on acid costs.
As a pretreatment for the lignocellulose fraction, the hot maleic acid treatment clearly has more effect than the hot neutral treatment. However, the maximum effect is still limited, indicating that the lignocellulose fraction of *Jatropha* press cake is relatively recalcitrant to acid hydrolysis and that a more intense hot-acid pretreatment is probably needed to sufficiently facilitate enzymatic hydrolysis in a lignocellulosic ethanol process.

In Chapter 6, the key results of this thesis are discussed, as well as more general matters. These include a comparison of the acid pretreatment results described in this thesis to those of others, as well as a possible explanation for the observed absence of acid catalysis of the sugar degradation by the organic acids. Another point brought forward is that the maximum bioethanol production from all EU straw would replace 42 % of EU gasoline consumption, followed by remarks on acid recycling, opportunities for the animal feed industry, large scale *Jatropha* production, and the application of organic acids having a positive effect regarding the 'food versus fuel' discussion.
The suggestions for future research include further study of the acid catalysis of sugar degradation, optimisation of maleic acid pretreatment using high solid loadings and process integration, reducing acid costs by including isomerisation reactions from maleic to fumaric acid and vice versa, dehulling of *Jatropha* seeds before oil pressing, exploring possibilities for organic acid treatment for animal feed production, and lastly, the suggestion for biorefinery research in general to focus more on improving its feedstocks, by slightly changing processes further upstream.

The general conclusion of this thesis is that organic acids can be successfully applied in processing lignocellulosic material. Maleic acid, and to a lesser extent fumaric acid, perform well in the pretreatment of wheat straw, resulting in high sugar yields and a much reduced sugar degradation, compared to sulphuric acid. Optimisation of the maleic acid pretreatment of wheat straw results in a maximum financial yield of €65 per tonne of dry straw, with acid replenishment as the most important cost factor, at €17 per tonne of dry straw.
Application of maleic acid in order to extract extra protein from the de-oiled press cake of *Jatropha curcas*, which had already undergone an alkaline protein extraction, does not lead to extra solubilised protein.

Samenvatting

In de overgang van een economie die gebaseerd is op fossiele grondstoffen naar een 'biobased' economie, moeten deze fossiele grondstoffen worden vervangen door hernieuwbare. Een veel gebezigde term in deze context is bioraffinage; hiermee wordt gedoeld op de omzetting van een mengsel van organische grondstoffen in een aantal economisch geoptimaliseerde combinaties van chemicaliën, materialen, warmte en elektriciteit.

Veel van de geplande toename in bioethanolproductie, bedoeld als vervanger van benzine als transportbrandstof, zal gaan komen van tweede-generatie-processen, waarbij lignocellulose-achtige materialen zoals stro, bagasse, en bijproducten uit de bosbouw worden gebruikt. Dit type grondstof moet worden behandeld om de aanwezige fermenteerbare suikers beschikbaar te krijgen voor fermentatie. Zo is doorgaans een voorbehandeling nodig om de enzymatische hydrolyse van de polysachariden uit de celwand te vergemakkelijken. Een veel toegepaste voorbehandeling is het verwarmen van het materiaal in aanwezigheid van verdund zwavelzuur, waarbij het lignine-koolwaterstof-complex gedeeltelijk wordt verbroken, een deel van de polysachariden oplosbaar wordt (vooral de hemicellulose), en de resterende cellulose beter toegankelijk wordt voor de cellulolytische enzymen. Het gebruik van heet zwavelzuur heeft wel een aantal onbedoelde en ongewenste gevolgen. Ten eerste leidt de hydrolyse van polysachariden tot monomere suikers, die dan weer kunnen afbreken tot furfural en 5-hydroxymethylfurfural. Deze stoffen remmen de gisten in de ethanolfermentatie, wat leidt tot hogere kosten en een lagere ethanolopbrengst per hoeveelheid grondstof. Ten tweede leidt het gebruik van zwavelzuur tot grote hoeveelheden gips, wat negatieve gevolgen heeft voor het verdere proces, en leidt tot een laagwaardige bijproductstroom. Als organische zuren zouden worden toegepast in de voorbehandeling, zou dit de waarde van de bijproductstroom positief beïnvloeden. Zo zou deze bijvoorbeeld gemakkelijker kunnen worden verbrand om warmte en elektriciteit op te wekken, of toegepast als kunstmest, of toegepast in diervoeder.

In eerder onderzoek is maleïnezuur voorgesteld als alternatief voor zwavelzuur, omdat maleïnezuur de hydrolyse katalyseerde van cellobiose (dimeer van glucose) en van zuivere cellulose, terwijl het resulteerde in minder afbraak van glucose in vergelijking met zwavelzuur. Fumaarzuur lijkt qua structuur op maleïnezuur, maar is wat zwakker. In tegenstelling tot maleïnezuur kan fumaarzuur door middel van fermentatie geproduceerd worden.

Het doel van dit proefschrift is om de voorbehandeling van lignocellulose-achtige grondstoffen met organische zuren te bestuderen. Meer specifiek gesteld, richt dit onderzoek zich op de invloed van maleïnezuur en fumaarzuur op de suikerafbraak tijdens de voorbehandeling, en op de prestatie van deze zuren in de voorbehandeling zelf, vergeleken met zwavelzuur. De optimalisatie van de voorbehandeling met maleïnezuur houdt niet alleen in het maximaliseren van de suikeropbrengst en de gerelateerde baten, maar houdt ook rekening met nadelige kanten van de voorbehandeling die hogere kosten met zich meebrengen. Als laatste richt dit onderzoek zich op mogelijkheden om de toepassing van maleïnezuur uit te breiden naar andere, eiwithoudende lignocellulose-achtige grondstoffen. Omdat tarwe de meest verbouwde graansoort is in de Europese Unie, is tarwestro gekozen als grondstof voor het werk over voorbehandeling van lignocellulose in dit proefschrift. Voor het werk over eiwithoudende lignocellulose-achtige grondstoffen is gekozen voor het bijproduct van biodieselproductie uit oliezaden van *Jatropha curcas*; de ont-oliede perskoek.

In het onderzoek in dit proefschrift blijken zowel maleïnezuur als fumaarzuur niet de afbraak van arabinose, de tweede pentose aanwezig in tarwestro, naar het gistremmende furfural te katalyseren, terwijl zwavelzuur dat duidelijk wel doet (Hoofdstuk 2). Beide organische zuren blijken wel de hydrolyse te katalyseren van arabinose-zijketens van het hemicellulose in tarwestro. Deze combinatie van resultaten maken fumaarzuur en maleïnezuur interessante kandidaten voor toepassing in de voorbehandeling van lignocellulose. Verder wordt aangetoond dat arabinose stabieler is dan xylose en glucose in de afbraakreactie. Arabinose kan dus bijdragen aan de ethanolopbrengst uit tarwestro, terwijl de bijdrage aan de vorming van suikerafbraakproducten minder dan proportioneel kan zijn, vergeleken met die van monomere xylose en glucose.

Een uitgebreidere studie naar voorbehandeling met verdund organisch zuur wordt beschreven in Hoofdstuk 3, waarbij fumaar- en maleïnezuur worden vergeleken met zwavelzuur, met water als controle. Om te bekijken of een efficiënte voorbehandeling van tarwestro met organisch zuur mogelijk is, wordt niet alleen de invloed van de voorbehandelingtemperatuur bestudeerd, maar ook de invloed van hoger gehalte aan stro. Er wordt gevonden dat een efficiënte voorbehandeling van tarwestro mogelijk is met maleïnezuur en fumaarzuur. Fumaarzuur is iets minder effectief, maar zowel maleïnezuur als zwavelzuur resulteren in een vrijwel volledige enzymatische verteerbaarheid van tarwestro. De omzetting naar monomere glucose is respectievelijk 96 en 98 % van wat theoretisch maximaal haalbaar is.

Het verhogen van het gehalte stro naar 30 % (w/w) heeft geen negatieve gevolgen voor de voorbehandeling, gelet op de enzymatische omzetting van de grondstof. Vergeleken met zwavelzuur worden veel minder suikerafbraakproducten gevormd tijdens de organisch-zure voorbehandelingen. De sterk gereduceerde afbraak van xylose bij gebruik van de twee organische zuren vertaalt zich in hogere opbrengsten aan xylose. Er wordt echter ook een mogelijk nadeel gevonden van het gebruik van fumaarzuur en maleïnezuur. Geen van beide zuren is helemaal hittestabiel, wat kan leiden tot hogere kosten doordat het zuur zou moeten worden aangevuld.

In Hoofdstuk 4 wordt de voorbehandeling met verdund maleïnezuur geoptimaliseerd, met voorbehandelingtijd, -temperatuur, en maleïnezuurconcentratie als variabele grootheden. De afhankelijke variabelen die worden meegenomen in de optimalisatie zijn de baten uit glucose en xylose, en kosten van het aanvullen van maleïnezuur, verhitting, neutralisatie, en furfuralproductie. Na de voorbehandeling wordt een vast-vloeibaarscheiding toegepast, waarna alleen vaste fase wordt onderworpen aan enzymatische hydrolyse. Er wordt aangetoond dat, niet lettend op de kosten, een bijna volledige omzetting van het glucaan uit de vaste fase kan worden bereikt in de enzymatische hydrolyse. Verder kan tijdens de voorbehandeling tot 90% van alle xylaan in het hemicellulose worden omgezet naar monomere xylose.
Wanneer wel rekening word gehouden met kosten, komt de maximale netto opbrengst uit op €65 per ton tarwestro, bestaande uit €68 glucosebaten, €17 aan xylosebaten, €17 maleïnezuurkosten, €2.0 verhittingskosten, en €0.90 kosten voor neutralisatie. Er wordt zó weinig furfural gevormd dat hieraan gerelateerde kosten verwaarloosbaar worden geacht, behalve onder de meest extreme procesomstandigheden. Het is duidelijk dat, van de bestudeerde kostenfactoren, de kosten van het aanvullen van het maleïnezuur het meest van belang zijn.
De procesomstandigheden die leiden tot de maximale netto opbrengst zijn: 50 minuten bij 170 °C, met 46 mM maleïnezuur. Deze omstandigheden leiden ertoe dat 80 % van alle xylaan uit het stro oplost in de vloeibare fase van het voorbehandelde mengsel, terwijl de enzymatische hydrolyse van de vaste fase ertoe leidt dat tot 85 % van alle glucaan uit het stro wordt omgezet in glucose.

Op zoek naar een hogere meerwaarde van de behandeling met maleïnezuur op het gebied van eiwithoudende lignocellulose-achtige grondstoffen, wordt in Hoofdstuk 5 een mogelijke toename van de valorisatie van *Jatropha*-perskoek bestudeerd. De grondstof voor dit onderdeel is het gewassen bijproduct uit de alkalische eiwitextractie van ont-

oliede *Jatropha*-perskoek. Een behandeling met verhit maleïnezuur (100 mM, pH 2) wordt vergeleken met een neutrale verhitting, waarbij wordt gekeken naar het oplosbaar worden van extra eiwit uit de grondstof. Tot 25 % van het resterende eiwit wordt oplosbaar bij een behandeling bij 140 °C, wat resulteert in een geschatte €8 additionele eiwitwaarde per ton perskoek, geen rekening houdend met kosten. De verhitting in aanwezigheid van maleïnezuur leidt niet tot meer oplosbaar eiwit dan de neutrale behandeling. Dit betekent dat de neutrale behandeling de voorkeur verdient, aangezien er evenveel eiwit oplosbaar wordt, terwijl er wordt bespaard op zuurkosten.
Als voorbehandeling van de lignocellulosefractie heeft de verhitting met maleïnezuur duidelijk meer effect dan de neutrale verhitting. Het effect is echter vrij klein, wat erop wijst dat de lignocellulose in *Jatropha*-perskoek relatief goed bestand is tegen zure hydrolyse en dat waarschijnlijk een intensievere zure verhitting nodig is om de enzymatische hydrolyse voldoende te vergemakkelijken, wanneer lignocellulose uit *Jatropha*-perskoek voor ethanolproductie zou worden gebruikt.

In Hoofdstuk 6 worden de belangrijkste resultaten van dit proefschrift bediscussieerd. Ook algemenere zaken komen aan bod, zoals een vergelijking van de resultaten van dit proefschrift met die van anderen, en een mogelijke verklaring voor het achterwege blijven van zure katalyse van de suikerafbraak in aanwezigheid van de gebruikte organische zuren. Een ander discussiepunt is dat, indien gebruik wordt gemaakt van al het stro binnen de EU, in theorie genoeg ethanol kan worden geproduceerd om 42 % van de benzineconsumptie in de EU te vervangen. Dit wordt gevolgd door discussie over hergebruik van zuur, kansen en mogelijkheden voor de diervoederindustrie, grootschalige productie van *Jatropha*, en het positieve effect dat het gebruik van organische zuren kan hebben op de ‘food versus fuel’ discussie.
Suggesties voor verder onderzoek richten zich op de katalyse van suikerafbraak, de optimalisatie van de voorbehandeling met maleïnezuur met hoger strogehalte en procesintegratie, het terugbrengen van zuurkosten door rekening te houden met isomerisatie van maleïnezuur naar fumaarzuur en vice versa tijdens de voorbehandeling, het pellen van de *Jatropha*-zaden vóór de oliepersstap, en de mogelijkheden om behandelingen met organische zuren te gebruiken voor de productie van diervoeding. Als laatste wordt de suggestie gedaan aan het bioraffinageonderzoek in het algemeen, om zich meer te richten op het verbeteren van de te gebruiken grondstoffen, door kleine veranderingen eerder in het totale proces.

De algemene conclusies van dit proefschrift houden in dat organische zuren met succes kunnen worden toegepast bij het verwerken van lignocellulose-achtige grondstoffen. Maleïnezuur en, in mindere mate, fumaarzuur presteren goed in de voorbehandeling van tarwestro, en resulteren in hoge suikeropbrengsten en een sterke afname van de suikerafbraak, in vergelijking tot zwavelzuur. De optimalisatie van de voorbehandeling van tarwestro met maleïnezuur resulteert in een maximale opbrengst van €65 per ton droge stro, met de kosten voor de aanvulling van maleïnezuur als belangrijkste kostenfactor: €17 per ton droge stro.
De toepassing van maleïnezuur om extra eiwit vrij te maken uit ont-oliede *Jatropha*-perskoek, die al een alkalische eiwitextractie had ondergaan, leidt niet tot meer opgelost eiwit.

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Curriculum vitae

Anne Maarten Joost (Maarten) Kootstra was born on October 26, 1972, in ‘s Hertogenbosch, the Netherlands. In 1991, he graduated from secondary grammar school Gymnasium Beekvliet, (Sint Michielsgestel, NL) after which he studied Food Technology at Wageningen University, focussing on Food Science, Dairy Science and Food Microbiology. After obtaining his Masters degree in 1998, he worked at Nutreco (Boxmeer, NL) as a Scientist Animal Feed Technology and at Unilever Research (Vlaardingen, NL) as a Scientist Food Processing. From 2001 to 2005, he worked as a Scientist Food Technology at Food & Biobased Research (formerly known as Agrotechnology & Food Innovations, Wageningen, NL). In 2005, he started his PhD research at Bioprocess Engineering, and Valorisation of Plant Production Chains, at Wageningen University, the results of which are described in this thesis.

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Patents

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Overview of completed training activities

Discipline specific activities

'Visiting scholar' at Laboratory of Renewable Resources Engineering (LORRE) Purdue University, West-Lafayette, Indiana, USA, 2006
Advanced course 'Bioreactor design', VLAG, 2006
Advanced course 'Reaction kinetics in food science', VLAG, 2007

General courses

Interview training, Meijer & Meijaard, 2005
Scientific writing, Language Services, 2008
Presentation skills, Language Services, 2009
Networking training, Meijer & Meijaard, 2010

Conferences and Symposia

Netherlands Biotechnology Congress, Ede, the Netherlands, 2006
Renewable Resource Congress, Gent, Belgium, 2007; Rotterdam, the Netherlands, 2008
Netherlands Process Technology Symposium, Veldhoven, the Netherlands, 2007/2008/2009
Biotechnology Seminar, Kobe University, Japan, 2008
Symposium Tokyo University of Agriculture & Technology, Japan, 2008
Biotechnology Seminar, Osaka University, Japan, 2008

Optionals

Preparation of research proposal, 2005
Brainstorm week Process Engineering, 2005
Course 'Renewable resources for the bulk chemical industry', ORC 90306, 2006
Open day Biotechnology, Wageningen University, 2007+2008
Process Engineering Brainstorm day, 2008+2009
Process Engineering PhD study tour to Japan, 2008
Bilateral Research Activities Programme; Work visit to New Zealand, 2009

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