Development of a lactic acid production process using lignocellulosic biomass as feedstock

Edwin C. van der Pol

Thesis committee

Promotor

Prof. Dr G. Eggink
Professor of Industrial Biotechnology
Wageningen University

Co-promotor

Dr R.A. Weusthuis
Associate professor, Bioprocess Engineering Group
Wageningen University

Other members

Prof. Dr G. Zeeman, Wageningen University
Prof. Dr G.J.W. Euverink, University of Groningen
Dr E. de Jong, Avantium, Amsterdam, The Netherlands
Dr D.Z. Machado de Sousa, Wageningen University

This research was conducted under the auspices of the Graduate School VLAG
(Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences)

Development of a lactic acid production process using lignocellulosic biomass as feedstock

Edwin C. van der Pol

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 18 March 2016
at 1.30 p.m. in the Aula.

E.C. van der Pol

Development of a lactic acid production process using lignocellulosic biomass as feedstock
168 pages.

PhD thesis, Wageningen University, Wageningen, NL (2016)
With references, with summaries in English and Dutch

ISBN 978-94-6257-673-5

TABLE OF CONTENTS

CHAPTER 1

General introduction and thesis outline

TRANSITION FROM A PETROCHEMICAL TO A BIOBASED ECONOMY

For millennia, people have relied on natural resources such as wood for energy production, e.g. for cooking and heating. This changed during the industrial revolution. Coal became a popular alternative for energy derived from natural resources. During the last century, crude fossil oil became a widely used feedstock for the production of fuels, energy and chemicals.

In recent years, with rising oil prices, decreasing oil reserves, and an increased consciousness of the impact of using of fossil resources on the environment such as global warming, the search for alternatives of oil derived products was intensified (Christensen et al. 2008, Lashof and Ahuja 1990). Important parameters for fossil oil alternatives are renewability and sustainability. The first steps in the transition from a petrochemical to a renewable (biobased) economy are already being implemented. An example is the production of ethanol from sugar cane and corn (Gray et al. 2006). Bioethanol is seen as a suitable biobased alternative for transportation fuels, and regulations in the European union are already in place enforcing the addition of 5% bioethanol to oil-derived gasoline (E5) (Bielaczyc et al. 2013). Renewable energy sources which are already used are biomass, wind, solar devices and hydroelectricity. Together, they produce 11% of the total energy consumed in the European union (EU report 2013).

Fossil oil is not only used for the production of transportation fuels and electricity, but also for the production of chemical intermediates for the petrochemical industry (Christensen et al. 2008). These intermediates are versatile, and can be used in numerous applications such as the production of plastics, fibres and rubbers. Examples of oil-derived chemicals are ethylene, acrylic acid and acrylamide (Haveren et al. 2007). To make a full transition towards a biobased economy, alternatives for crude oil derived petrochemicals such as plastics have to be found. Currently, around 5% of the worldwide oil production is used for the production of plastics (Bayer 2013). Polymerized lactic acid (PLA) may be a suitable alternative to oil-derived plastics

Table 1: *Different applications of lactic acid, either as final product or as feedstock for other processes. Some of the applications of lactic acid require a food grade production process (Bayer 2013, Dusselier et al 2013, Rohan 2015, Tiwari 2014).*

Lactic acid use	(potential) market size (kt/annum)	Purity required
Food market	300-400	>98%, food grade
Cosmetics	200-300	85%
PLA as replacement PET	60,000-70,000	>99%, optically pure
PLA as replacement PS	20,000-30,000	>99%. Optically pure
Conversion to Acrylic acid	4,000-7,000	>99%, optically pure
Conversion to 1,2-propanediol	1,200-1,600	>99%, food grade if used as food additive

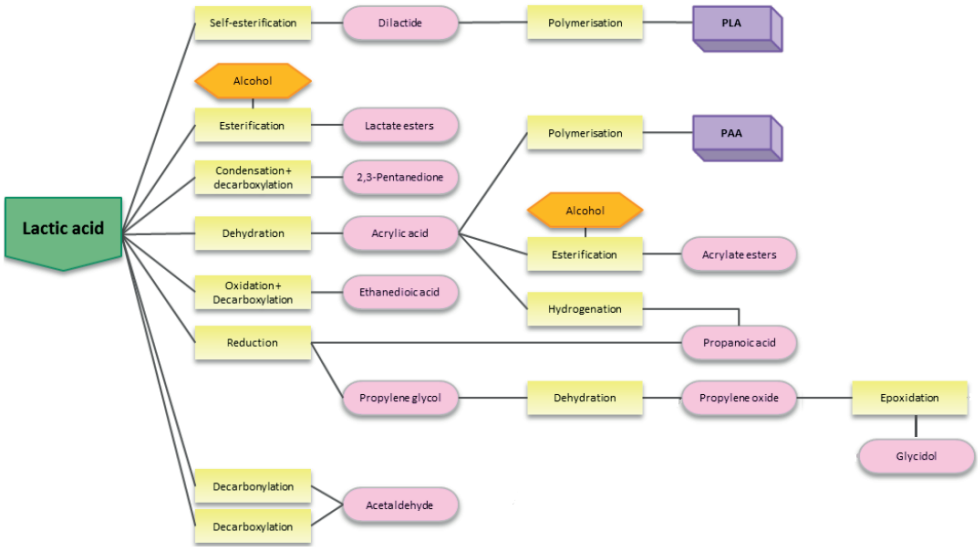


Figure 1: *Use of lactic acid as a chemical intermediate. Lactic acid can be a feedstock for many different chemical processes. Adapted from "From the Sugar Platform to biofuels and biochemicals" (E4tech et al. 2015)*

(Vennestrøm et al. 2011). Low-molecular weight PLA can be moulded into a bioplastic, which may replace polymers such as polyethylene (PE), while high-molecular weight PLA may replace polystyrene (PS) (Garlotta 2001). Furthermore, dehydration of lactic acid results in the formation of biobased acrylic acid, which can be polymerized and used for instance in the textile industry (Ghantani et al. 2013). Also, lactic acid can be converted to a wide range of other chemicals such as 1,2-propanediol for resin production or food/cosmetic additive, or to acrylic acid (Table 1 and figure 1) (Dusselier et al. 2013). Lactic acid is already produced on a large scale, with a worldwide production of 505 kilotons in 2013. However, most of this lactic acid is used for cosmetics and as preservative, and only a small fraction of the lactic acid currently produced is used for the synthesis of PLA (Tiwari 2014).

LIGNOCELLULOSE AS FEEDSTOCK FOR THE PRODUCTION OF BIOBASED CHEMICALS

First generation biobased chemicals are mainly produced from sugar or starch rich crops, such as sugarcane bagasse, corn, potatoes and sugar beets. Although these crops are renewable, their availability is relatively limited, since most of them are used for food applications (Srinivasan 2009). Second generation biobased chemicals are produced from alternative feedstocks which are not used as food, and which are available in large quantities, for instance lignocellulose (Fengel and Wegener 1983). Lignocellulose is the most abundant biomolecule

Table 2: *Composition of lignocellulose derived from different biomass resources, and the production yield which can be reached in tonnes of dry weight per hectare per year (van der Pol et al. 2014)*

	Yields	Composition (%)			
	T DW/ha/year	Cellulose	Hemicellulose	Lignin	Ash
Bagasse	7-13	36-45	25-28	17-20	1-3
Corn stover	2-6	36-41	26-30	16-21	2-6
Softwood	5-12	40-44	19-21	25-29	0-1
Hardwood	8-15	43-49	18-24	23-29	1-2
Straw (wheat)	3-8	38-48	23-29	13-19	5-9
Miscanthus	20-60	32-38	20-27	20-26	8-12

on earth, and can therefore supply the large quantities of feedstock required for the production of biochemicals (Claassen et al. 1999). It consists for 60-75% of polymerized sugars, which can be fermented to chemical intermediates after depolymerization (Fengel and Wegener 1983). Interesting sources of lignocellulose that do not compete for land use are corn stover, sugarcane bagasse and wood (waste). Other sources are wheat straw, barley straw or rice straw, although these are already used to a large extent for other purposes such as cattle feed. Energy crops, for example *Miscanthus*, may also be used as feedstock, since cultivation can achieve very high yields of up to 60 tonnes per hectare (table 2) (Heaton et al. 2008, Hoskinson et al. 2007).

Lignocellulose is composed on a weight basis for 30-50% of cellulose, 18-30% of hemicellulose, 13-30% of lignin and up to 10% of ashes (Table 2). Cellulose is a rigid polymer of a few hundred to several thousand glucose molecules (Klemm et al. 2005). Hemicellulose polymers contain over 90% of sugars on a dry weight basis; mostly xylose, mannose, glucose, arabinose and galactose (Saha 2003). Furthermore, uronic acids, acetyl groups, ferulates and coumarins can be part of the hemicellulose structure. Lignin consists of polymerized phenolic compounds, mostly guaiacyl, syringyl or p-hydroxyphenyl derivatives, depending on the origin of the biomass (Toledano et al. 2010). Sugars in cellulose and hemicellulose are interesting as feedstock for fermentation processes, while phenolic monomers can be used as chemical intermediate in the chemical industry (Gosselink et al. 2012). Furthermore, lignin can be pyrolysed, creating an oil fuel which can be used to power combustion engines (Czernik and Bridgwater 2004).

THERMO-CHEMICAL PRETREATMENT OF LIGNOCELLULOSE

Lignocellulose is a recalcitrant composite material, of which the sugars present inside the structure cannot be directly consumed by most lactic acid producing micro-organisms.

Pretreatment of lignocellulose is therefore required to allow the conversion of lignocellulosic sugars to lactic acid by micro-organisms (Hendriks and Zeeman 2009). The goal of the pretreatment is to depolymerize sugar polymers present in the lignocellulose. Many different processes have been developed which result in monomerization of sugar polymers (Hendriks and Zeeman 2009, Mosier et al. 2005). Most pretreatment strategies combine a thermo-chemical process with enzymatic hydrolysis. Thermo-chemical pretreatment can increase the accessibility of cellulose and hemicellulose polymers, can decompose (part of) the hemicellulose, deacetylate the hemicellulose to increase accessibility, and/or can dissolve or decompose (part of) the lignin. Efficient decomposition of lignocellulose during chemical pretreatment is achieved by optimizing process parameters such as temperature, pressure, pH and addition of chemicals.

Several thermo-chemical pretreatment methods have been successfully tested on large scale: acid pretreatment, alkaline pretreatment, autohydrolysis and steam explosion (Eggeman and Elander 2005). Other methods which show promising results on laboratory and pilot plant scale are wet oxidation, ammonium fibre explosion (AFEX) and the organosolv procedure (Hendriks and Zeeman 2009). Every pretreatment method has its own specific effect on lignocellulosic structure, and can result in removal of lignin, degradation of hemicellulose, deacetylation of hemicellulose and/or substructure alteration (table 3). Due to the harsh conditions often applied during thermo-chemical pretreatment, by-products can be formed which can have a negative effect on other stages of the process such as enzymatic hydrolysis, fermentation and downstream processing (Table 3) (van der Pol et al 2014). The main inhibitory by-products observed in pretreated lignocellulose are phenolic compounds, small organic acids and furans (Palmqvist and Hahn-Hägerdal 2000).

Table 3: *effect of different pretreatment methods on the surface area, solibulisation, structure alteration of lignocellulose and by-product formation (adapted from Hendriks and Zeeman 2009 and Van der Pol et al. 2014). ++ = major effect, + = moderate effect, - = minor/no effect. *AFEX results in the formation of acetamide and phenolic amines, which are not taken into account in this table.*

Pretreatment method	Surface area increase	Solubilisation of		Altering Lignin structure	By-product formation		
		Hemicellulose	Lignin		Furans	Acids	Phenolics
Acid	++	++	+	++	++	+	+
Alkaline	++	-	++	++	-	++	++
Wet oxidation	++	-	++	++	-	++	++
AFEX	++	-	++	++	-	+*	+*
Steam explosion	++	++	-	+	+	+	-

BY-PRODUCT FORMATION AND EFFECT ON FERMENTATION

By-products formed during the pretreatment of lignocellulose can have a negative effect on the fermentability of lignocellulose derived substrates. Most of the lignocellulosic by-products formed can inhibit growth of micro-organisms, decrease productivities and/or can induce substantial lag phases (Palmqvist and Hahn-Hägerdal 2000). Three major types of lignocellulosic by-products can be distinguished: small organic acids, furans and phenolics (Palmqvist and Hahn-Hägerdal 2000).

Small organic acids can passively diffuse through cell walls and membranes (Axe and Bailey 1995). Since the intracellular pH has to be closely regulated for optimal intracellular enzyme activities, these acids and/or accompanying protons that cause a drop in pH are actively removed from the cells at the expense of ATP (Verduyn et al. 1992, Orij et al. 2011), resulting in increased maintenance energy requirements. Due to the reduction in available energy, the presence of organic acids limit growth rate and/or productivity of micro-organisms. However, the production of lactic acid or other fermentation products during anaerobic fermentations is often not reduced by moderate acid concentrations. Since the formation of fermentation products such as lactic acid is the only process generating metabolic energy, the increased maintenance requirements may even result in a slight increase of product yields (Taherzadeh et al. 1997).

Furans may damage different parts of the cell. Most of these damages are related to the aldehyde group of the furans, which is able to transform water and oxygen to potentially toxic reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (Allen et al. 2010). ROS can cause damage to different parts of the cell, such as DNA, mitochondria, vacuole membranes, the cytoskeleton and nuclear chromatin. The aldehyde group itself can also damage DNA and inhibit different enzymes such as dehydrogenases (Modig et al. 2002). Cells exposed to furans can show an extensive lag phase before growing, may have a lower growth rate and lower productivity, and in some cases show a decrease in conversion yield of sugars to the desired product (Palmqvist and Hahn-Hägerdal 2000).

Phenolics can change the fluidity of membranes, causing leakage of vital compounds through the cell membrane (Fitzgerald et al. 2004). Most phenolics also contain reactive side groups such as aldehydes, which can form ROS or interact with DNA (Feron et al. 1991). Phenolics can induce lag phases, reduce growth rates and productivities, and can lower production yields (Palmqvist and Hahn-Hägerdal 2000).

Three options have been proposed as a solution to overcome the problems related to by-products, which occur during the fermentation process of pretreated lignocellulose to lactic acid:

- Thermo-chemical pretreatment methods can be improved to reduce the formation of by-products. This may be difficult to accomplish, since a reduction of by-product formation should not lead to a reduction in accessibility of (hemi)cellulose by enzymes. Furthermore,

the formation of some of the by-products cannot be prevented, since they are part of the hemicellulosic structure, and are released when hemicellulosic sugars are monomerized. Examples are acetic acid, ferulic acid and coumaric acid. Formation of other compounds which are not part of the lignocellulose structure, for instance furfural, may be significantly reduced without reducing sugar monomerization/accessibility by choosing the right pretreatment conditions (Bustos et al. 2004)

- By-product removal can be applied directly after the thermo-chemical pretreatment of lignocellulose. Examples are washing pretreated lignocellulose with superheated water, extraction of by-products using active charcoal or lime, or using microbial detoxification. By-product removal can be an effective method to reduce the amount of by-products present in the lignocellulose derived substrates, however it adds to the complexity of the process, thus increasing costs, and it may result in a (slight) loss of material (van der Pol et al. 2015, Zhao et al. 2013).
- Micro-organisms can be improved to withstand higher concentrations of by-products. This improvement can be achieved via methods such as genetic engineering, evolutionary engineering or mutagenesis. Although it may be difficult and time consuming to implement genetic engineering and evolutionary engineering in some cases, and not many targets for genetic engineering are identified, it may provide a powerful tool to reduce the effect of by-products on the micro-organism.

Each combination of pretreatment method, lignocellulose source and microorganism used may require a different solution to acquire an economically feasible process with sufficient yields and productivity. Ideally, one would like to adapt the micro-organism when this does not results in an impaired production or growth, since this method will not result in additional process costs. A more detailed description of by-products formed during different pretreatment, the effect of by-products on fermentation towards bioethanol, and strategies to overcome by-product toxicity for ethanol producing micro-organisms is given in chapter 2.

ENZYMATIC HYDROLYSIS AND FERMENTATION OF PRETREATED LIGNOCELLULOSE TO LACTIC ACID

After thermo-chemical pretreatment of lignocellulose, a solid substrate is obtained, in which most sugars are still present in a polymerized form. Enzymatic hydrolysis is an efficient method to depolymerize those sugars. Complex mixtures of enzymes containing for instance cellulases, cellubiohydrolase, xylanases, endoglucanases, β-glucosidases and glucoamylases, are used to depolymerize the pretreated cellulose and hemicellulose (Verardi et al. 2012). Examples of commercially available enzyme cocktails containing cellulose and hemicellulose degrading enzymes are Accellerase 1500, GC 220 and CTEC 2/3, produced by Novozymes and Dupont respectively. After hydrolysis of the lignocellulosic material with enzymes for 48-72h,

most sugars are monomerized and can be converted in a fermentation process to products such as lactic acid or ethanol.

Different bacteria, fungi and yeasts are capable of fermenting sugars to lactic acid (Abdel-Rahman et al. 2011, Hofvendahl and Hahn-Hägerdal 2000). In this thesis, several bacillus, lactobacillus and geobacillus strains were evaluated for their potential as lactic acid producer. For 1 litre scale fermentation experiments, *Bacillus coagulans* DSM2314 was chosen as the production strain. This strain has several benefits when compared to other lactic acid producers:

- *B. coagulans* produces lactic acid in a homo-fermentative fashion, both from glucose and xylose, the two most abundant sugars found in pretreated lignocellulose. Therefore, a high lactic acid production yield on both sugars can be achieved. Furthermore, the produced lactic acid is optically pure L(+)-lactic acid (Otto 2004). Optical purity of lactic acid is important for polymerization of lactic acid to PLA.
- *B. coagulans* has an optimal growth rate at temperatures around 50°C, where most (lacto) bacilli show optimal growth between 30°C and 37°C (Abdel-Rahman et al. 2011, Hofvendahl and Hahn-Hägerdal 2000, Maas et al. 2008). After chemical treatment, the pretreated lignocellulose has a temperature close to 100°C. Cooling of pretreated lignocellulose is a costly and time-consuming operation at larger scale. Moreover, most enzyme cocktails used to hydrolyse lignocellulose show highest activities at temperatures between 40°C and 60°C. When *B. coagulans* is used as production organism a combined process of enzymatic hydrolysis and fermentation may be applied.

In this thesis, it was also found that *B. coagulans* was able to produce lactic acid at a maximum productivity above 5 g/l/h, simultaneously conversion yields of glucose and xylose to lactic acid above 90% W/W were observed. These productivities and conversion yield are high when compared to results obtained for other other micro-organisms (Abdel-Rahman et al. 2011, Hofvendahl and Hahn-Hägerdal 2000).

SEPARATE HYDROLYSIS AND FERMENTATION (SHF) VERSUS SIMULTANEOUS SACCHARIFICATION AND FERMENTATION (SSF)

In most studies, the production of lactic acid from lignocellulose was performed in three phases. In the first phase, a thermo-chemical pretreatment is performed on lignocellulose-rich feedstock, increasing the accessibility of the cellulose. This pretreatment is followed by an enzymatic hydrolysis, in which the sugars are released as monomers. The sugars are then fermented into lactic acid. This process is called separate hydrolysis and fermentation (SHF).

Instead of applying enzymatic hydrolysis and fermentation separately, these process steps may be combined in a simultaneous saccharification and fermentation (SSF) process.

When separate processes are used, enzymatic hydrolysis alone takes between 48 and 72 hours, and the enzymes are inhibited in activity by the high concentration of monomeric sug-

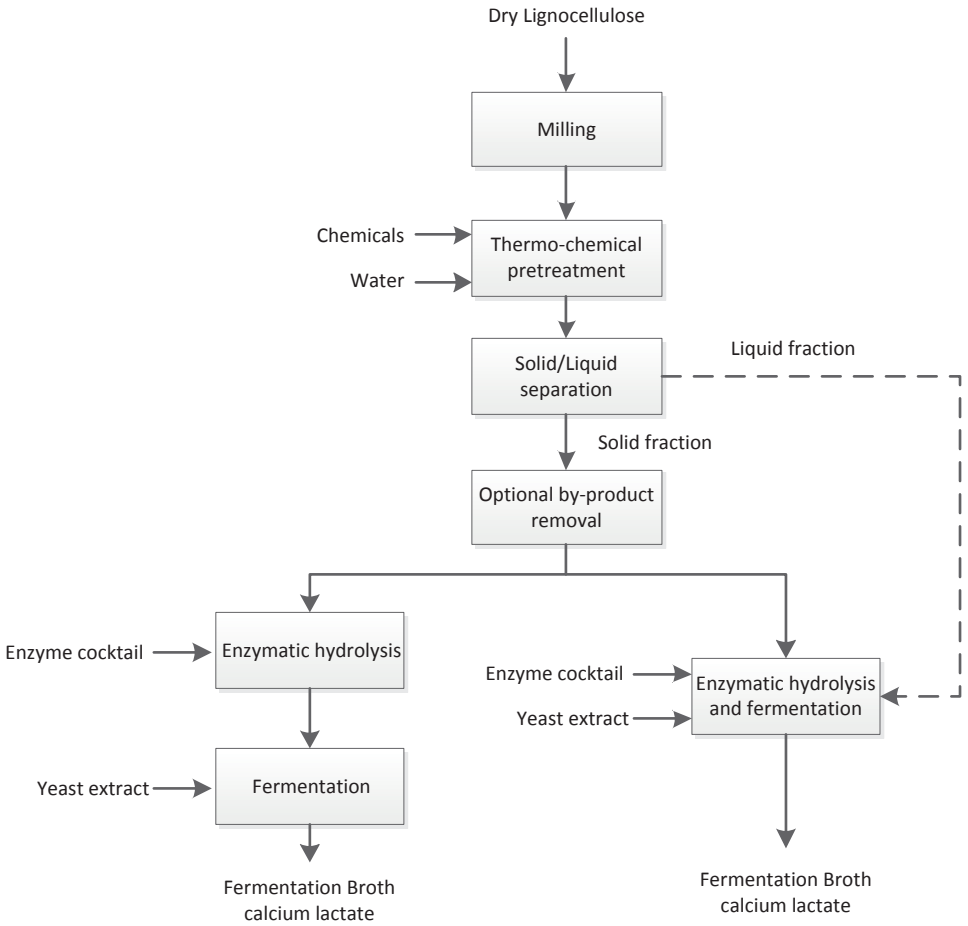


Figure 2: *An overview of the production process of lactic acid from sugarcane bagasse lignocellulose. The bagasse is pretreated using acid pretreatment at pilot plant scale. After a solid-liquid separation, the solid fraction is enzymatically hydrolysed and fermented, either separately (SHF) or simultaneously (SSF). The liquid fraction, containing part of the hemicellulosic sugars, may be added during the SSF process.*

ars formed (Lee et al 2004, Ouyang et al. 2013). When the enzymatic hydrolysis and fermentation are combined in an SSF process, the monomerized sugars can be directly consumed by the micro-organism, which should allow a much faster enzymatic hydrolysis, since product inhibition be reduced. Process time in an SSF process can therefore be strongly reduced. The potential success of combining both steps depends on the pH and temperature optimum of both processes. *B. coagulans* has temperature and pH optima close to that of enzyme cocktails like GC220 or CTeC 2/3, making a combined process feasible.

THESIS OUTLINE

The aim of this thesis is to identify and quantify inhibitory by-products resulting from thermo-chemical pretreatment of lignocellulose. The inhibitory effects of both single and combined by-products on microbial growth were determined. The obtained data were used to develop a process in which lactic acid is produced from acid pretreated sugarcane bagasse lignocellulose, reducing inhibitory effects of by products to a minimum.

In **Chapter 2**, a literature study is presented on lignocellulosic by-products. In this review, the composition of lignocellulose is described, and an overview is given on the formation of by-products using different pretreatment methods and lignocellulose sources. Furthermore, inhibitory mechanisms of lignocellulosic by-products are described, and strategies are proposed to reduce the inhibitory effect of these by-products.

Three of the main thermo-chemical pretreatment methods applicable on a large scale are acid pretreatment, alkaline pretreatment and autohydrolysis. In **Chapter 3**, these pretreatment methods are compared in terms of sugar monomerization and by-product formation. Via HPLC, LC-MS/MS and HPAEC analysis, the composition of the pretreated lignocellulose was analysed. By-products were identified which were present at high concentrations after thermo-chemical pretreatment.

In **Chapter 4**, a method was developed to test the toxicity of by-products identified and quantified in chapter 3. In a small-scale screening performed in 48-well plates, the concentration at which a by-product becomes a growth inhibitor was determined. Both inhibition of individual and combinations of by-products were studied.

Bacillus coagulans DSM2314 can be strongly inhibited by by-products found in acid pretreated lignocellulose. In **Chapter 5**, a method was provided to reduce inhibitory effects of by-products. Addition of furfural to precultures of *B. coagulans* results in a significant improvement of fermentation processes performed on media rich in lignocellulosic by-products. When these results were compared to fermentations inoculated with control precultures, which did not contain furfural, it was found that addition of furfural to the preculture strongly reduces total fermentation time, while it improves lactic acid productivity and conversion yields of sugars to lactic acid.

In **Chapter 6**, two efficient processes, batch SSF and two-stage SSF, are developed to enzymatically hydrolyse sugar polymers, and ferment the depolymerised sugars simultaneously to lactic acid with *B. coagulans*. Precultures to which furfural was added were used as inoculum for the SSF process. This resulted in a reduced lag phase compared to using control precultures, and led to an increased lactic acid titre, lactic acid productivity and conversion yield of sugar to lactic acid.

Lignocellulosic by-products can play an important role during fermentation of pretreated lignocellulose to lactic acid. In **Chapter 7**, the role of these by-products is discussed, together with solutions proposed in this thesis which can be used to overcome inhibition caused by

these by-products. Furthermore, both batch SSF and two stage SSF are evaluated, and are shown to be promising processes for an economically attractive production of lactic acid from lignocellulose. By-products present after fermentation may not only affect the fermentation, but may also influence the downstream processing of the lactic acid. This thesis is concluded by evaluating the economic potential of lactic acid production using lignocellulose as feedstock.

REFERENCES

Abdel-Rahman MA, Tashiro Y, Sonomoto K (2011) Lactic acid production from lignocellulose-derived sugars using lactic acid bacteria: Overview and limits, J Biotechnol 156:286-301 doi: 10.1016/j.jbiotec.2011.06.017

Allen S, Clark W, McCaffery JM, Cai Z, Lanctot A, Slininger P, Liu ZL (2010) Furfural induces reactive oxygen species accumulation and cellular damage in Saccharomyces cerevisiae, Biotechnology for Biofuels 3:2

Axe DD, Bailey JE (1995) Transport of lactate and acetate through the energized cytoplasmic membrane of Escherichia coli, Biotechnol Bioeng 47:8-19 doi:10.1002/bit.260470103

Bayer Material Science (2013) From Oil; http://materialscience.bayer.com/ media/special/backgrounders/plastic-production.aspx, (accessed 08/ 2014)

Bielaczyc P, Woodburn J, Klimkiewicz D, Pajdowski D, Szczotka A (2013) An examination of the effect of ethanol–gasoline blends' physicochemical properties on emissions from a light-duty spark ignition engine, Fuel Process Technol 107:50-63 doi: 10.1016/j.fuproc.2012.07.030

Bustos G, Moldes AB, Cruz JM, Domínguez JM (2004) Production of fermentable media from vine-trimming wastes and bioconversion into lactic acid by Lactobacillus pentosus J Sci Food Agr 84:2105-2112 doi: 10.1002/jsfa.1922

Christensen CH, Rass-Hansen J, Marsden CC, Taarning E, Egeblad K (2008) The renewable chemicals industry, Chem Sus Chem 1:283-289 doi: 10.1002/cssc.200700168

Claassen PAM, van Lier JB, Lopez Contreras AM, van Niel EWJ, Sijtsma L, Stams AJM, de Vries SS, Weusthuis RA (1999) Utilisation of biomass for the supply of energy carriers, Appl Microbiol Biotechnol 52:741-755 doi: 10.1007/s002530051586

Czernik S, Bridgwater AV (2004) Overview of applications of biomass fast pyrolysis oil, Energy Fuels 18:590-598 doi: 10.1021/ef034067u

Dusselier M, Wouwe P van, Dewaele A, Makshina E, Sels BF (2013) Lactic acid as a platform chemical in the biobased economy: the role of chemocatalysis, Energy Environ Sci 6:1415-1442 doi: 10.1039/c3ee00069a

E4tech, RE-CORD and WUR (2015) "From the Sugar Platform to biofuels and biochemicals". Final report for the European Commission, contract No. ENER/C2/423-2012/SI2.673791

Eggeman T, Elander RT (2005) Process and economic analysis of pretreatment technologies, Bioresource Technol 96:2019-2025 doi: 10.1016/j.biortech.2005.01.017

EU report (2013) Renewable energy statistics; http://ec.europa.eu/eurostat/statistics-explained/index.php/Renewable_energy_statistics (accessed 11-2015)

Fengel D, Wegener G (1983) Wood: Chemistry, ultrastructure, reactions De Gruyter, Berlin

Feron VJ, Til HP, Vrijer F de, Woutersen RA, Cassee FR, Bladeren PJ van (1991) Aldehydes: occurrence, carcinogenic potential, mechanism of action and risk assessmen,t Mutat Res-Genet Tox 259:363-385 doi: 10.1016/0165-1218(91)90128-9

Fitzgerald DJ, Stratford M, Gasson MJ, Ueckert J, Bos A, Narbad A (2004) Mode of antimicrobial action of vanillin against Escherichia coli, Lactobacillus plantarum and Listeria innocua, J Appl Microbiol 97:104-113 doi:10.1111/j.1365-2672.2004.02275.x

Garlotta D (2001) A Literature Review of Poly(Lactic Acid) J Polym Environ 9:63-84 doi: 10.1023/a:1020200822435

Ghantani VC, Lomate ST, Dongare MK, Umbarkar SB (2013) Catalytic dehydration of lactic acid to acrylic acid using calcium hydroxyapatite catalyst, Green Chem 15:1211-1217

Gosselink, RJA, Teunissen W, Dam JEG van, Jong E de, Gellerstedt G, Scott EL, Sanders JPM (2012) Lignin depolymerisation in supercritical carbon dioxide/acetone/water fluid for the production of aromatic chemicals, Bioresource Technol 106:173-177 doi: 10.1016/j.biortech.2011.11.121

Gray KA, Zhao L, Emptage M (2006) Bioethanol, Current Opinion in Chemical Biology 10:141-146

Haveren J van, Scott EL, Sanders J (2008) Bulk chemicals from biomass, Biofuels bioprod Bioref 2:41-57 doi: 10.1002/bbb.43

Heaton EA, Dohleman FG, Long SP (2008) Meeting US biofuel goals with less land: the potential of Miscanthus, Glob Change Biol 14:2000-2014 doi: 10.1111/j.1365-2486.2008.01662.x

Hendriks ATWM, Zeeman G (2009) Pretreatments to enhance the digestibility of lignocellulosic biomass, Bioresource Technol 100:10-18 doi: 10.1016/j.biortech.2008.05.027

Hofvendahl K, Hahn-Hägerdal B (2000) Factors affecting the fermentative lactic acid production from renewable resources, Enzyme Microb Technol 26:87-107 doi: 10.1016/S0141-0229(99)00155-6

Hoskinson RL, Karlen DL, Birrell SJ, Radtke CW, Wilhelm WW (2007) Engineering, nutrient removal, and feedstock conversion evaluations of four corn stover harvest scenarios, Biomass Bioenergy 31:126-136 doi: 10.1016/j.biombioe.2006.07.006

Klemm D, Heublein B, Fink H-P, Bohn A (2005) Cellulose: Fascinating Biopolymer and Sustainable Raw Material, Angew Chem Int Ed 44:3358-3393 doi: 10.1002/anie.200460587

Lashof DA, Ahuja DR (1990) Relative contributions of greenhouse gas emissions to global warming, Nature 344:529-531 doi: 10.1038/344529a0

Lee S-M, Koo Y-M, Lin J (2004) Production of Lactic Acid from Paper Sludge by Simultaneous Saccharification and Fermentation, Adv Biochem Engin 87:173-194 doi: 10.1007/b94365

Maas RHW, Bakker RR, Jansen MLA, Visser D, Jong E de, Eggink G, Weusthuis RA (2008) Lactic acid production from lime-treated wheat straw by Bacillus coagulans: neutralization of acid by fed-batch addition of alkaline substrate, Appl Microbiol Biotechnol 78:751-758 doi: 10.1007/s00253-008-1361-1

Modig T, Lidén G, Taherzadeh MJ (2002) Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase, Biochem J 363:769-776

Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M (2005) Features of promising technologies for pretreatment of lignocellulosic biomass, Bioresource Technol 96:673-686 doi: 10.1016/j.biortech.2004.06.025

Orij R, Brul S, Smits GJ (2011) Intracellular pH is a tightly controlled signal in yeast, BBA- Gen Subjects 1810:933-944

Otto R (2004) Preparation of lactic acid from a pentose-containing substrate, Patent WO 2004/063382 A2 1-19, US 10/755,392

Ouyang J, Ma R, Zheng Z, Cai C, Zhang M, Jiang T (2013) Open fermentative production of l-lactic acid by Bacillus sp. strain NL01 using lignocellulosic hydrolyzates as low-cost raw material, Bioresource Technol 135:475-480 doi: 10.1016/j.biortech.2012.09.096

Palmqvist E, Hahn-Hägerdal B (2000) Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition, Bioresource Technol 74:25-33 doi: 10.1016/S0960-8524(99)00161-3

Pol EC van der, Bakker RR, Baets P, Eggink G (2014) By-products resulting from lignocellulose pretreatment and their inhibitory effect on fermentations for (bio)chemicals and fuels, Appl Microbiol Biotechnol 98:9579-9593 doi: 10.1007/s00253-014-6158-9

Pol EC van der, Bakker RR, Zeeland ANT, Sanchez Garcia D, Punt A, Eggink G (2015) Analysis of by-product formation and sugar monomerization in sugarcane bagasse pretreated at pilot plant scale: Differences between autohydrolysis, alkaline and acid pretreatment, Bioresource Technol 181:114-123 doi: 10.1016/j.biortech.2015.01.033

Rohan M (2015) global acrylic acid market and its derivatives; http://www.marketsandmarkets.com/PressReleases/acrylic-acid.asp *(accessed 11-2015)*

Saha B (2003) Hemicellulose bioconversion, J Ind Microbiol Biot 30:279-291 doi: 10.1007/s10295-003-0049-x

Srinivasan S (2009) The food v. fuel debate: A nuanced view of incentive structures, Renew Energ 34:950-954 doi: 10.1016/j.renene.2008.08.015

Taherzadeh MJ, Niklasson C, Lidén G (1997) Acetic acid—friend or foe in anaerobic batch conversion of glucose to ethanol by Saccharomyces cerevisiae?, Chem Eng Sci 52:2653-2659

Tiwari R (2014) Lactic acid market derivatives 2016 forecast; http://www.prnewswire.com/news-releases/lactic-acid-market-and-derivatives-2016-forecasts-global-china-in-new-research-report-279286152.html *(accessed 11-2015)*

Toledano A, García A, Mondragon I, Labidi J (2010) Lignin separation and fractionation by ultrafiltration, Sep Purif Technol 71:38-43 doi: 10.1016/j.seppur.2009.10.024

Vennestrøm PNR, Osmundsen CM, Christensen CH, Taarning E (2011) Beyond Petrochemicals: The Renewable Chemicals Industry Angew Chem Int Ed 50:10502-10509 doi: 10.1002/anie.201102117

Verardi A, Bari I de, Ricca E, Calabro V (2012) Hydrolysis of Lignocellulosic Biomass: Current Status of Processes and Technologies and Future Perspectives, Bioethanol, Intech, ISBN:978-953-51-0008-9, doi 10.5772/23987

Verduyn C, Postma E, Scheffers WA, Van Dijken JP (1992) Effect of benzoic acid on metabolic fluxes in yeasts: A continuous-culture study on the regulation of respiration and alcoholic fermentation, Yeast 8:501-517 doi: 10.1002/yea.320080703

Zhao K, Qiao Q, Chu D, Gu H, Dao TH, Zhang J et al (2013) Simultaneous saccharification and high titer lactic acid fermentation of corn stover using a newly isolated lactic acid bacterium Pediococcus acidilactici DQ2, Bioresour Technol 135:481–489

CHAPTER 2

By-products resulting from lignocellulose pretreatment and their inhibitory effect on fermentations for (bio)chemicals and fuels

This chapter is published as: van der Pol E.C., Bakker R.R., Baets P., Eggink G. (2014) *Appl Microbiol Biotechnol* 98:9579-9593 doi: 10.1007/s00253-014-6158-9

ABSTRACT

Lignocellulose might become an important feedstock for the future development of the biobased economy. Although up to 75% of the lignocellulose dry weight consists of sugar, it is present in a polymerized state and cannot be used directly in most fermentations processes for the production of chemicals and fuels. Several methods have been developed to depolymerise the sugars present in lignocellulose, making the sugars available for fermentation. In this review, we describe five different pretreatment methods and their effect on the sugar and non-sugar fraction of lignocellulose. For several pretreatment methods and different types of lignocellulosic biomass, an overview is given of by-products formed. Most unwanted by-products present after pretreatment are dehydrated sugar monomers (furans), degraded lignin polymers (phenols), and small organic acids. Qualitative and quantitative effects of these by-products on fermentation processes have been studied. We conclude this review by giving an overview of techniques and methods to decrease inhibitory effects of unwanted by-products.

INTRODUCTION

Crude oil is an important feedstock for the production of both fuels and chemicals. Due to a depletion of crude oil reserves, as well as the environmental impact due to crude oil use such as greenhouse gas emission, alternatives for crude oil use have to be found (Lashof and Ahuja 1990). Two biobased alternatives for crude oil products have often been proposed in literature. As a replacement for fossil fuels, (Bio)ethanol produced by (recombinant) yeast or bacteria can be used (Gray et al. 2006). A biobased substitute for the plastic polyethylene terephthalate (PET) and polystyrene is poly-lactic acid (PLA) (Vennestrøm et al. 2011). Lactic acid, the building block of this polymer, can be produced by bacteria or fungi (Garlotta 2001).

Lactic acid and ethanol can be produced from either first generation or second generation sugars. First generation sugars are directly obtained from plant crops such as sugar canes, sugar beets, or from easily accessible starch sources such as corn or cassava which can also be used for food or feed. Using first generation sugars increases the competition on the raw sugar market, resulting in higher sugar prices, thus decreasing competiveness of the processes. Besides, the use of food for chemicals is socially debatable due to the conversion of food into chemicals (Srinivasan 2009).

Instead of using easily accessible sugars or starch present in sugar crops, second generation polymerised sugars can be used which are present in lignocellulosic plant material. Lignocellulose can be found in nearly all plants as part of the secondary cell wall, and makes up for 60-97% of the plant cell dry weight (Fengel D and G 1983). Lignocellulose can either be acquired from wood or agricultural residues such as corn stover, sugar cane bagasse or straw, or land can be directly used to cultivate lignocellulose-rich plants such as *Miscanthus* (Heaton et al. 2008; Hoskinson et al. 2007). Lignocellulose is the most abundant renewable biomass source and accounts for 50% of the world's biomass production, making it an interesting source to use as bulk feedstock (Claassen et al. 1999).

Decomposition of lignocellulose to acquire monomeric sugars results in the formation of a large amount of by-products (Klinke et al 2004). This review describes these lignocellulosic by-products, from their origin and formation during pretreatment and the concentrations they are present in to their effect on fermentation. At the end of this review, potential solutions to overcome inhibitory effects of these by-products are shown.

STRUCTURE OF LIGNOCELLULOSE

Lignocellulose is not a single defined molecule, but a structure consisting of cellulose, hemicellulose (also known as polyose) and lignin (Fengel D and G 1983). The amount of lignin, cellulose, hemicellulose and inorganic solid fraction (ash) which can be found in the plant material varies significantly (Table 1). Differences do not only occur due to the crop species

Table 1: *Composition of dry lignocellulose for different crops types on a w/w dry weight basis. Significant variances can be observed both between crop species as well as within one species.*

	Monocots			*Gymnosperm*	*Angiosperm*
Composition (%)	Bagasse	Wheat straw	Corn stover	Spruce	Poplar
Cellulose	36-45	38-48	36-41	40-44	43-49
hemicellulose	25-28	23-29	26-30	19-21	18-24
Lignin	17-20	13-19	16-21	25-29	23-29
Ash	1-3	5-9	2-6	0.1-0.5	1-2

and subvariaties used, but also due to growth conditions such as climate, seasonal variations and crop handling by the farmer (Öhgren et al. 2007; Zhang et al. 2007).

Cellulose molecules are well-defined polymers. The polymers are build up solely from D-glucose molecules, which are β-1-4 linked (Klemm et al. 2005). Cellulose polymers are rigid and long, with chain lengths over 10 000 molecules. Cellulose polymers are interacting with each other forming crystalline sheets (Fengel D and G 1983; Teeri 1997). Cellulose is of great interest for the biobased economy since it is the most abundant polysaccharide on earth, while depolymerisation of pure cellulose is relatively easy (Klemm et al. 2005; Teeri 1997).

Hemicellulose is a non-homogeneous polymer with significant variances in composition between crop types (Saha 2003). Hemicellulose polymers can consist of different 5- and 6 carbon sugars, mainly xylose, mannose, arabinose, glucose and galactose. Xylose is one of the main sugars in monocot (grass) hemicellulose, while mannose is one of the main sugars in gymnosperm (softwood) hemicellulose (Martín et al. 2007; Shafiei et al. 2010). Not only sugars, but also uronic acids, ferulates and coumarins can be part of the hemicellulose structure. Furthermore, up to 70% of the xylose molecules present in the hemicellulose backbone are acetylated (Fengel D and G 1983; Hatfield et al. 1999; Saha 2003).

Lignin is a complex structure mainly build up from phenolic compounds, with sizes ranging from 1 to 100 kDa (Toledano et al. 2010). In a simplified model of lignin, three types of building blocks can be distinguished (figure 1) (Boerjan et al. 2003; Whetten and Sederoff 1995). The simplest building block is p-hydroxyphenyl (H). It consists of a benzene molecule with 4' alcohol group and a 1' side group, which can vary between different p-hydroxyphenyls. Main 1' side groups found after monomerisation are acids, alcohols and aldehydes. The second building block is guaiacyl (G), which is a p-hydroxyphenyl with a 3' methyl ether. The third building block is syringyl (S), which has both a 3' and 5' methyl ether. P-hydroxyphenyl is the most abundant phenolic in monocot (grass) lignin, while guaiacyl can mainly be found in gymnosperm (softwood) and angiosperm (hardwood) lignin, and syringyl is mainly found in angiosperm lignin. Even though these building blocks describe lignin to some extent, the exact composition of each lignin molecule is more complicated and still partially unknown (Boerjan et al. 2003; Vanholme et al. 2010)

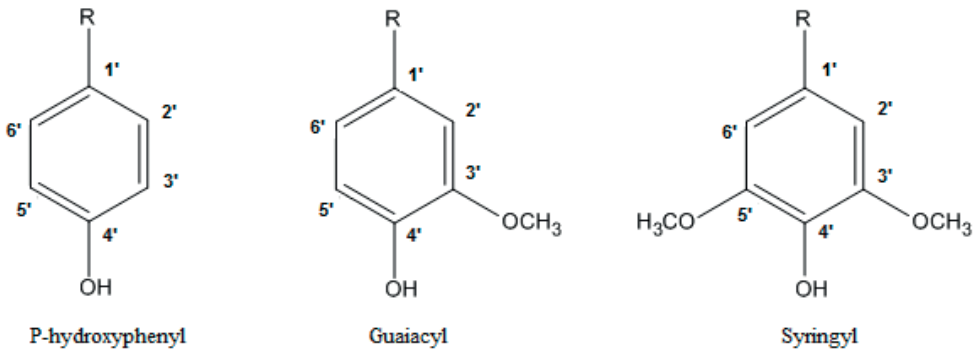


Figure 1: *The three building blocks of lignin in a simplified model. P-hydroxyphenyl is the most abundant phenolic in monocot lignin, guaiacyl is found in gymnosperm and angiosperm lignin, and syringyl is mainly found in angiosperm lignin. The 1' side group (R) can vary, from a hydroxide group to for example an ethylaldehyde.*

PRETREATMENT METHODS FOR MONOMERISATION OF LIGNOCELLULOSIC SUGAR POLYMERS

Lignocellulose conversion to chemicals often include a pretreatment, enzyme treatment, fermentation and downstream (DSP) processing step (figure 2). The pretreatment step is required to increase the accessibility of the carbohydrate polymers by enzymes. After this pretreatment, enzymes are used to depolymerise sugar polymers present in the lignocellulose, making them available for fermentation. During fermentation, the desired compound is produced using micro-organisms. A DSP is used to acquire the chemicals or fuels at desired purities. In this chapter, a selection of different methods to perform the initial chemical pretreatment are described, based on the scalability of the pretreatment methods and the availability of these pretreatment methods on a pilot plant scale, while taking into account economics and effectiveness of the pretreatment. An overview based on the current situation

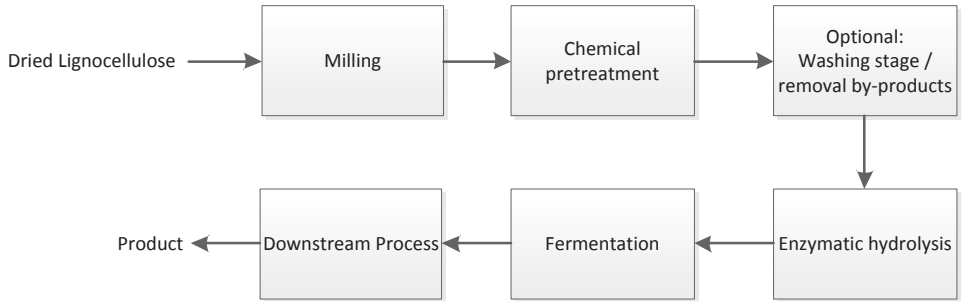


Figure 2: *Overview of a process to convert lignocellulose into biochemicals, as often proposed in literature. Lignocellulose-rich material is milled, pretreated chemically and enzymatically to depolymerize the sugars. The sugars are fermented to the desired product, which is purified in a downstream process.*

stated that lime (alkaline) pretreatment, AFEX and acid pretreatment had the most potential from an economic point of view, while an overview based on the production of ethanol from pretreated material has shown wet oxidation, sodium hydroxide (alkaline) and steam explosion as methods with a high potential (Eggeman and Elander 2005; McMillan et al. 2006). It should be noted that there are many alternative pretreatment methods including ultrasound, microwave, supercritical water treatment, organosolve, white-rot fungi (Hendriks and Zeeman 2009); (Mosier et al. 2005).

Dilute Acid hydrolysis

Acid hydrolysis is an often proposed thermo-chemical pretreatment method for the decomposition of lignocellulose. A combination of low pH and a high temperature (partially) hydrolyses the hemicellulose, increasing the enzymatic accessibility of cellulose and therefore the conversion to monomeric glucose. Not only hemicellulose is affected, lignin and cellulose can also be (partially) degraded (Larsson et al. 1999a). Commonly used conditions for an optimal enzyme digestibility of cellulose and hemicellulose polymers are 0.5-4% W/DW (sulphuric) acid at 120-210°C for 5-30 minutes.

Dilute acid pretreatment can hydrolyse 80 to 90% of the xylan to xylose (Karimi et al. 2006; Kootstra et al. 2009; Sanchez et al. 2004), also on a larger scale of 150 liter (Roberto et al. 2003). Furthermore, 10-25 % of the glucans are monomerized to glucose using acid pretreatment only (Karimi et al. 2006; Sanchez et al. 2004). In combination with an enzyme hydrolysis stage, glucan to glucose conversion efficiencies up to 85% can be achieved (Kootstra et al. 2009). Acid hydrolysis is most efficient for lignocellulose with low lignin contents such as monocot-type plants, due to the very moderate removal of lignin during pretreatment.

Steam explosion

The concept of steam explosion (SE) is based on explosive decompression (Foody 1984). In a steam explosion process, lignocellulosic material is heated up to 150-250°C by the addition of pressurized steam while being kept at an overpressure of 5-50 bar for 1-15 minutes. Acid can be added as a catalyst, resulting in (partial) degradation of hemicellulose via the same mechanisms as acid hydrolysis (Kaar et al. 1998; Tucker et al. 2003). After the stationary phase, the pressure is released within (milli) seconds, causing water to vaporize and/or become gaseous. The force of expansion caused by the water leads to explosive decompression, which combined with a degradation of hemicellulose in the stationary phase opens the lignocellulose structure. Steam explosion can lead to a 5-fold increase in enzymatic glucose conversion of lignocellulose (Kaar et al. 1998). The use of steam explosion alone results in an enzyme digestibility of cellulose of 65%, which is significantly higher than untreated material (García-Aparicio et al. 2006).

Ammonium fibre explosion (AFEX)

Ammonium fibre explosion (AFEX) is also based on an explosive effect. AFEX is generally performed at a more moderate temperature up to 110°C, at a 1:1 loading of liquid ammonia and biomass, with a residence time of 15 min to 1 hour (Dale et al. 1996; Holtzapple et al. 1991). AFEX leads to an increase in surface area of the lignocellulose and delignifies the lignocellulose. It also alters the lignin structure, making the polycarbonates more accessible for enzymes (Hendriks and Zeeman 2009). Using switchgrass as feedstock, a combination of AFEX and enzyme treatment resulted in a 90% conversion of hemicellulose and cellulose polymers to monomeric sugars. The overall sugar yield of AFEX and enzyme treatment is 5 times higher than enzyme treatment only. The ammonia used in the process can be almost fully recovered, saving in process costs (Dale et al. 1996). AFEX treated rice straw combined with enzyme treatment showed a glucan depolymerisation yield of 81%, and a xylan depolymerisation yield of 90% (Zhong et al. 2009). For poplar, which has a higher lignin content, an AFEX pretreatment at 180°C with a 2:1 ammonium:biomass load, followed by enzymatic conversion led to a glucan conversion to glucose monomers of 93% and a xylan conversion to xylose monomers of 65% (Balan et al. 2009).

Alkaline hydrolysis

Alkaline pretreatment has the potential to disrupt the lignocellulose by (partially) dissolving hemicellulose, lignin and silica while swelling the structure due to deacetylation, making cellulose and hemicellulose polymers more accessible for enzymes. (Chang and Holtzapple 2000; Jackson 1977). Alkaline pretreatment mechanisms are based on saponification of intermolecular ester bonds cross-linking xylan hemicellulose and other compounds such as lignin (Sun and Cheng 2002).

Different conditions have been proposed for an optimal sugar accessibility by enzymes. 2% Sodium hydroxide at 121°C for 60 min have resulted in a 5.6 times higher monomeric sugar yield after enzyme treatment compared to a pretreatment at 60°C without alkali (McIntosh and Vancov 2010). Using sodium hydroxide at a concentration of 12% V/W with an incubation time of 4 hours at 70°C resulted in a 77% delignification with a cellulose yield of more than 95% and a hemicellulose hydrolysis of 44% (de Vrije et al. 2002).

Wet oxidation

Wet oxidation uses oxygen at an overpressure of 5-20 bar, with the addition of sodium hydroxide, hydrogen peroxide or sodium carbonate at concentrations of 5-10 g/l. The pretreatment is performed at a temperature of 150-200°C for 10-20 minutes (Klinke et al. 2002). Wet oxidation dissolves the hemicellulose, making the hemicellulose and cellulose more accessible to enzymes. Simultaneously it can solubilize a large fraction of the lignin.

Wet oxidation can lead to a 40-50% solubilisation of lignin, with an 85-90% solubilisation of the hemicellulose (Martín et al. 2007). A 85% conversion of cellulose to glucose can be

reached in combination with an enzyme pretreatment (Bjerre et al. 1996). A 96% cellulose recovery with a 67% convertibility has been seen shown (Klinke et al. 2002). In a pilot plant scale experiment, the addition of hydrogen peroxide resulted in the highest ethanol production, with 208 kg ethanol per tonnes of straw, compared to 152 kg ethanol per tonnes of straw without the addition of chemicals (McMillan et al. 2006).

Economic evaluation of pretreatment methods

Several studies have been previously performed to define the economic potential of pretreatment methods in production processes towards bioethanol.

According to an NREL study performed in 2011, the cost for pretreatment using dilute-acid hydrolysis are 0.26$ per gallon of bioethanol. In total, the production of bioethanol from lignocellulose costs 2.15$ per gallon including feedstock, CAPEX and OPEX, making this process competitive with first generation bioethanol production (Humbird et al. 2011).

AFEX pretreatment in combination with enzyme hydrolysis and fermentation can potentially produce ethanol at 0.81$ per gallon, while current status should allow production at 1.41$ per gallon (Sendich et al. 2008). It should be noted that most data is based on laboratory experiments, since scaling up of the equipment has not yet been performed. Futhermore, prices used for lignocellulose feedstock are much lower than estimated in the NREL acid pretreatment study (0.50$ versus 0.74$ per gallon of ethanol), and enzyme and wastewater treatment costs are not clearly indicated.

A large study has been performed to compare different pretreatment methods. In this study, alkaline hydrolysis using lime as alkaline resulted in a bioethanol production price of 1.62$ per gallon, AFEX pretreatment resulted in bioethanol at a production price of 1.44$ per gallon, and dilute acid hydrolysis resulted in a bioethanol production price of 1.38$ per gallon (Eggeman and Elander 2005).

EFFECT OF PRETREATMENT ON FORMATION OF LIGNOCELLULOSE BY-PRODUCTS

During chemical pretreatment, numerous different by-products are formed. Differences in by-product formation occur either due to the source of lignocellulose, or due to the pretreatment method used to decompose the lignocellulose (Table 2). Three main groups of by-products formed are furans, organic acids and phenolics (Palmqvist and Hahn-Hägerdal 2000).

The presence of monomeric sugars, in combination with a high temperature and low pH, leads to the formation of furans (Kabel et al. 2007). Sugar monomers with 6 carbon atoms can be dehydrated to 5-hydroxymethylfurfural (5-HMF), sugar monomers with 5 carbon atoms can be dehydrated to furfural. In general, hemicellulose from a softwood source contains a large amount of mannose, while hemicellulose from a monocot source contains mainly

Table 2: *By product formation after initial chemical pretreatment, as measured in the liquid phase. All amounts shown are in gram per kg initial dry weight material. Sources: 1: (Du et al. 2010), 2: (Chundawat et al. 2010), 3: (Chen et al. 2006), 4: (Klinke et al. 2002), 5: (García-Aparicio et al. 2006), 6: (Cantarella et al. 2004), 7:(Larsson et al. 1999b)*

lignocellulose Source and pretreatment method Compound (g/L)	Corn Stover Acid hydrolysis (1)	Corn Stover Acid hydolysis (2)	Corn Stover Acid hydrolysis (3)	Corn Stover Wet oxidation (1)	Corn Stover AFEX (2)	Wheat straw Wet oxidation (4)	Wheat straw Wet oxidation (4)	Barley straw Steam explosion (5)	Pine Wet oxidation (1)	Poplar Steam explosion (6)	Spruce Acid hydrolysis (7)
5-HMF	4.4	15.70	0.88	0.28	0.64	0.00	0.16	0.8	0.06	2.6	29.5
furfural	22	7.94	17.97	0.65	0.00	0.00	1.46	2.8	0.19	5.9	5
furoic acid	0.24	0.16		0.12	0.01	0.00	0.17		0.09		
Total furans	**26.64**	**23.64**	**18.85**	**1.05**	**0.65**	**0**	**1.79**	**3.6**	**0.35**	**8.5**	**34.5**
Levulinic acid	4.1	3.65	1.74	0.19	0.02				0.05	0.79	13
acetic Acid	17	34.77	15.37	5.8	4.61	20.00	24.61		2.4	27.8	12
formic acid	12	3.17	1.29	7.9	0.91	23.69	69.86		6.6	11.2	8
glycolic acid						5.27	12.59				
lactic acid	2.0	1.55	36.93	2.4	0.32	4.61			1.8		
malic acid						1.10	2.44				
citric acid						0.78	0.79				
oxalic acid		0.26			0.60	0.29	0.14				
succinic acid	0.29	0.02		0.52	0.09	8.89	4.47		0.18		
malonic acid	0.15	0.02		0.11	0.00				0.17		
maleic acid	0.13	0.04		0.31	0.90				0.14		
cis-aconitic acid	0.16	0.62		0.09	2.90				0.11		
glutaric acid	0.06	0.58		0.07	0.02				0.03		
itaconic acid	0.72	0.04		0.21	0.36				0.02		
fumaric acid	0.37	0.00		0.18	0.00				0.03		
total organic acids	**37.01**	**44.74**	**55.34**	**17.80**	**10.73**	**64.63**	**114.90**	**-**	**11.54**	**39.79**	**33**

Table 2: *By product formation after initial chemical pretreatment, as measured in the liquid phase. All amounts shown are in gram per kg initial dry weight material. Sources: 1: (Du et al. 2010), 2: (Chundawat et al. 2010), 3: (Chen et al. 2006), 4: (Klinke et al. 2002), 5: (García-Aparicio et al. 2006), 6: (Cantarella et al. 2004), 7:(Larsson et al. 1999b) (continued)*

lignocellulose Source and pretreatment method Compound (g/L)	Corn Stover Acid hydrolysis (1)	Corn Stover Acid hydolysis (2)	Corn Stover Acid hydrolysis (3)	Corn Stover Wet oxidation (1)	Corn Stover AFEX (2)	Wheat straw Wet oxidation (4)	Wheat straw Wet oxidation (4)	Barley straw Steam explosion (5)	Pine Wet oxidation (1)	Poplar Steam explosion (6)	Spruce Acid hydrolysis (7)
Vanillin	0.4	0.28	0.09	0.67	0.20	0.08	0.96	0.25	0.71	0.35	0.6
di-H-coniferyl-OH											0.49
coniferyl aldehyde											0.18
vanillic acid	0.33	0.12	0.06	0.43	0.05	0.04	0.84	0.04	0.48		0.17
Hydroquinone											0.09
Catechol								0.17			0.05
4-H-benzoic Acid	0.00	0.00	0.03	0.01	0.00	0.01	0.12	0.02	0.01		0.03
guaiacol						0.18	0.08				
syringol						0.17	0.06				
4-H-benzaldehyde	0.36	0.09	0.09	0.44	0.09	0.12	0.59	0.03	0.19	0.31	
syringaldehyde	0.18	0.15	0.14	0.22	0.01	0.01	0.75	0.13	0.08	0.24	
4-H-acetophenone	0.08	0.01		0.05	0.02	0.02	0.09		0.01		
acetovanillone						0.07	0.15				0.04
acetosyringone						0.49	0.71				
syringic acid	0.2	0.12	0.07	0.22	0.05	0.06	0.5	0.02	0.06		
p-coumaric acid	0.56	1.84		1.10	1.08	0.13	0.13	0.18	0.00		
ferulic acid	0.66	1.31	0.30	0.01	0.10	0.09	0.15	0.1	0.01		
3,4-di-H-benzoic acid	0.24	0.03		0.00	0.01				0.07		
3,5-di-H-benzoic acid	0.04	0.00		0.00	0.00				0.00		
salicylic acid	0.19	0.05		0.20	0.03				0.07		
benzoic acid	0.15	0.01		0.03	0.01				0.17		
para-toluic acid	0.06	0.02		0.10	0.02				0.06		
total phenolics	**3.55**	**4.12**	**0.94**	**3.60**	**1.68**	**1.49**	**5.15**	**0.94**	**2.00**	**0.90**	**1.65**

xylose (Martín et al. 2007; Shafiei et al. 2010). Therefore, furfural is the most abundant furan pretreated monocot lignocellulose, while 5-HMF is found in high concentrations in pretreated softwood lignocellulose.

Alkali hydrolysis, wet oxidation, and AFEX, pretreatment methods are performed at high pH and result in relatively low furan formation under 2 gram per kg initial dry weight (DW) (Table 2). Steam explosion results in significant furan formation, although quantities are still relatively low around 4 grams per kg initial DW. Acid pretreatment of monocot lignocellulose leads to a furan formation of 8-25 gram per kg initial DW, while the use of softwood lignocellulose in combination with acid pretreatment can lead to 35 grams per kg initial DW of furans. Not only do these quantities of furans result in potential inhibition of fermentation, furan formation also represents a large loss in sugar yields.

Hemicellulose contains a significant non-sugar fraction within its structure. Acetyl and uronic acid groups can be present at the 2' and 3' position of the sugar in the backbone (Sun et al. 2004). These acids can be liberated when the polymers are hydrolysed (Saha 2003). Acetic acid is therefore the most abundant small organic acid in most cases. In wet oxidation, monomeric sugar molecules can be oxidized to formic acid and acetic acid (Klinke et al. 2002). In some cases, large amounts of up to 95 gram per kg initial DW of formic acid and acetic acid were formed.

Another organic acid found in higher quantities is lactic acid. Small amounts of lactic acid are naturally present inside the lignocellulosic structure. Lactic acid can also be formed due to contamination of the pretreated material with lactic acid producing organisms. In one report, lactic acid concentrations found exceeded 35 grams per kg initial DW (Chen et al. 2006).

Dehydration of furans results in the formation of furoic acid, formic acid and levulinic acid (Lewis Liu and Blaschek 2010). In alkaline hydrolysis, Wet oxidation and AFEX furoic acid and levulinic acid are (nearly) absent, while the formic acid present has a different origin. Acid pretreatment can yield between 2 and 8 gram levulinic acid per kg initial DW.

A large variety of phenolic compounds can be formed during pretreatment. Depending on the origin of the lignocellulose, either p-hydroxybenzyls, syringyls or guaiacyls are more abundant (figure 1). In monocot lignocellulose, hemicellulose and lignin are linked via coumarins and ferulates (Hatfield et al. 1999). Degradation of these bonds is relatively easy and leads to the formation of p-coumaric acid and ferulic acid. In softwood lignocellulose, these compounds are nearly absent. However, in contrary to what is expected, the levels of guaiacyls such as vanillin and vanillic acid is not elevated, resulting in a much lower overall monomeric phenol concentration. Overall, the concentrations of phenolic monomers are much lower than the concentrations of acids and, in case of acid pretreatment, the concentrations of furans.

When AFEX pretreatment is used, several amide containing by-products are formed. Up to 25 gram per kg initial DW acetamide, 14 gram per kg initial DW phenolic amides, 1 gram per kg initial DW pyrazines and imidazole, and nearly 3 gram per kg initial dry weight other nitrogen species are formed during AFEX pretreatment (Chundawat et al. 2010).

EFFECT OF LIGNOCELLULOSE BY-PRODUCTS ON FERMENTATION PROCESSES

Furans

Furans can inhibit fermentations processes by reducing the specific growth rate and productivity of cells. In *Saccharomyces cerevisiae*, furfural inhibits the glycolysis by inhibiting dehydrogenase enzymes, as well as inhibiting alcohol, aldehyde and pyruvate dehydrogenase (Banerjee et al. 1981; Modig et al. 2002). 5-hydroxymethyl furfural (5-HMF) also inhibits dehydrogenase enzymes, but to a lesser extent than furfural (Modig et al. 2002). Furthermore, furfural can inhibit the assimilation of the sulphur containing amino acids cysteine and methionine (Miller et al. 2009). Furfural is also linked to an increase in Reactive Oxygen Species (ROS), due to the large dipole moment of the aldehyde group (Allen et al. 2010; Feron et al. 1991). ROS can cause damage to the mitochondria and vacuole membranes, the cytoskeleton and nuclear chromatin.

Some micro-organisms, including the yeasts *S. cerevisiae* and *Pichia stipitis*, and the bacteria *Escherichia coli* and *Klebsiella oxytoca*, have the ability to reduce furfural into furfuryl alcohol (2-furanmethanol) and reduce 5-HMF to 5-hydroxymethyl furfuryl alcohol (2,5-Bis-hydroxymethylfuran, furan-2,5-dimethanol) (Gutiérrez et al. 2002; Laadan et al. 2008; Lewis Liu et al. 2009; Taherzadeh et al. 1999). Where furfuryl alcohol and 5-hydroxymethyl furfuryl alcohol are less inhibitory than furfural and 5-HMF for most micro-organisms, for instance for *S. cerevisiae* (Palmqvist et al. 1999), the inhibitory effect is still significant for other organisms such as *Rhodosporidium toruloides Y4* (Hu et al. 2009). The conversion of furans to furan alcohols in *S. cerevisiae* is performed by alcohol and aldehyde dehydrogenases such as *ARI1*, *ADH6*, *ADH7*, *ALD4* and *ALD7* (Almario et al. 2013; Lewis Liu et al. 2009; Park et al. 2011). Upregulation of the alcohol and aldehyde dehydrogenases decreased growth lag phases in the presence of furans, and increased furan tolerance (Lewis Liu et al. 2009). Most of these enzymes are regulated by *YAP1* in *S. cerevisiae*, which is increased a 2-3 fold during lag phases induced by HMF. Furthermore strains with a deletion of *YAP1* showed a 10 fold extension in lag phase, making *YAP1* an interesting target for genetic engineering (Ma and Liu 2010).

The ADH and ALD enzymes use NAD(P)H as a cofactor, where they prefer NADH over NADPH (Heer et al. 2009). Increasing the regeneration of NAD(P)H is a strategy to increase furan tolerance. In more tolerant yeast strains, genes involved in NAD(P)H regeneration like *GND1*, *GND2*, *TDH1* were found to be upregulated (Lewis Liu et al. 2009). Furthermore serine, arginine and lysine production can be downregulated, which is linked to an increased ATP and NADH regeneration in the TCA cycle, thereby increasing flux if NADH towards *ADH* and *ALD* enzymes (Almario et al. 2013).

Some organisms such as the yeast *Trichosporon fermentans* and the fungi *Coniochaeta lignaria* can oxidize furfural to 2-furoic acid, which is considered to be less toxic than furfural and furfuryl alcohol (Huang et al. 2012; Nichols et al. 2008). 5-HMF can also be oxidized to 2-furoic

acid via 2,5-furandicarboxylic acid (Koopman et al. 2010; Nichols et al. 2008). The oxidation to furoic acid has also been observed in aerobic chemostat cultures of *S. cerevisiae* (Sárvári Horváth et al. 2003). The bacteria *Pseudomonas putida* and *Cupriavidus basilensis* are also able to oxidize the furfural to furoic acid. These bacteria can metabolize the furoic acid further to 2-oxoglutaric acid, which is a metabolite in the TCA cycle. Therefore, these micro-organism can maintain themselves and grow on furfural, without the requirement of another carbon source (Koopman et al. 2010).

Several other targets were identified to be beneficial for furan tolerance. Overexpression of *GRE2* and *GRE3*, genes which are normally expressed under stress condition and linked to DNA replication stress, results in an increase in furan tolerance in yeast (Almario et al. 2013; Moon and Liu 2012). By increasing fluxes through the pentose phosphate pathway (PPP), *S. cerevisiae* was able to grow on furfural and HMF concentration at which the wild type was unable to grow. Especially the upregulation of ZWF1 increased the concentration of furans at which the yeast could grow by at least 20% (Gorsich et al. 2006). In *E. coli*, the expression of an NADH dependant oxidorectase *FucO*, combined with the deletion of an NADPH dependant *YqhD*, leads to an increase in furfural reduction (Wang et al. 2011; Wang et al. 2013).

Small organic acids

Pretreated lignocellulose can contain large amounts of organic acids, including acetic acid, formic acid, levulinic acid and lactic acid (table 2). These organic acids can inhibit cell growth and productivities (Larsson et al. 1999a).

Most small undissociated acids are able to passively diffuse through the cell wall and cell membrane into the intracellular cytosol, while the dissociated acids cannot passively access the cell in large amounts, even when present at higher concentrations (Axe and Bailey 1995). With a decrease in extracellular pH, the balance will move towards the undissociated form of the molecule, increasing the diffusion. Diffusion of undissociated acids leads to a drop in intracellular pH. Keeping the pH constant inside the cytosol is of great importance to many functions of the cell, such as signalling and optimal enzyme conditions (Orij et al. 2011; Pampulha and Loureiro-Dias 1989).

Active transport can help to remove the (dissociated) acids, while ATPase can remove the free protons which are responsible for the decrease in intracellular pH (Verduyn et al. 1992). The active transport and ATPase is at the expense of ATP, limiting other energy requiring processes such as growth and maintenance of the cells, leading to (partial) inhibition and eventually cell death (Pampulha and Loureiro-Dias 1989). If the diffusion is higher than the active transport can compensate due to ATP limitation, the pH will drop permanently and the cellular processes will stop, causing cell death.

To overcome inhibition, ATPase fluxes can be increased. In *S. cerevisiae* strains adapted to acetic acid in high concentrations, upregulation of ATPases such as *ATP5*, *PA2*, and vacuolar

ATPases *VPH1* and *VMA3* is observed (Almario et al. 2013; P Morsomme et al. 1996). *SPI1*, known for an increase in weak acid resistance, is also upregulated (Almario et al. 2013).

In the presence of acetate, accumulation of pentose phosphate pathway (PPP) metabolites occurs, while glycerol production is decreased. Upregulation of transketolase or transaldolase, involved in the PPP, resulted in higher ethanol productivities (Hasunuma et al. 2011).

Phenolic compounds

A large variety of phenolic compounds is formed during pretreatment, all different molecular weights, polarity and side-groups (Table 2). The number of side groups, position of side-groups and structure of the side group can play a major role in the inhibitory effect of the phenolic (Ando et al. 1986; Larsson et al. 2000; Nishikawa et al. 1988).

Phenol, p-cresol and potentially other phenolic compounds can increase the fluidity of cell membranes in *E. coli* and *Lactobacillus plantarum*, allowing more diffusion through the membrane (Fitzgerald et al. 2004). Due to this membrane change, intracellular potassium levels drop significantly (Fitzgerald et al. 2004). To compensate for this effect, *E. coli* can change the fatty acid composition in the membrane towards more saturated fatty acids (Keweloh et al. 1991). Phenolics also have the potential to inhibit the activity of some enzymes within the cell, inactivating pathways such as with the xylitol pathway in *Candida athensensis* (Zhang et al. 2012). Phenolic aldehydes have the potential to cause DNA damage due to ROS formation and the large positive charge at one side of the aldehyde group, especially if this group is linked to the next carbon with a double bond (Feron et al. 1991). Vanillin, and possibly other phenolic compounds, are linked to a repression of the translation in yeast cells (Iwaki et al. 2013).

As a defence mechanisms against phenolic aldehydes, *S. cerevisiae* can convert the phenolic aldehydes into phenolic alcohol under oxygen limited conditions via the same mechanism as furans (Larsson et al. 2000; Sundström et al. 2010). The phenolic alcohols are considerably less toxic to the yeast cells (Larsson et al. 2000). Several fungi, including the white-rot fungi *Pleurotus erygnii*, are able to secrete laccase enzymes. These enzymes degrade phenolic compounds, allowing the fungi to grow on lignin (Muñoz et al. 1997). *S. cerevisiae* which expressed laccase genes shows an increased phenolic tolerance, being able to grow in the presence of 1.5 mM coniferyl aldehyde, where the wild type strain shows no growth within 50 hours (Larsson et al. 2001).

The ergosterol synthesis pathway is a potential target for vanillin tolerance, where the downregulation of the pathway is suggested to cause a lower tolerance towards vanillin in *S. cerevisiae* (Endo et al. 2008). For coniferyl aldehyde, ferulic acid and isoeugenol, *S. cerevisiae* mutants lacking the genes *YAP1*, *ATR1*, and *FLR1* have been shown to have an increased sensitivity towards these phenolic compounds, making it a potential target for genetic engineering (Sundström et al. 2010).

INHIBITORY CONCENTRATIONS OF LIGNOCELLULOSE BY-PRODUCTS

To determine the toxicity of a pretreated substrate, both the amount of potential inhibitory compounds present in the substrate as well as the toxicity of those compounds for micro-organisms is of importance. The presence of potential inhibitory compounds has been shown in table 2.

An overview of toxicity studies of several lignocellulose derived by-products is given in table 3. Large differences are seen between microbial species, but also between different subspecies. In general, growth is inhibited slightly stronger than productivity. Furfural concentrations above 5 g/l and HMF concentrations above 8 g/l are inhibitory for all strains, while some strains are already severely inhibited at 1-2 g/l of furans. In most cases, concentrations

Table 3: *Inhibitory effect of lignocellulose by-products on different micro-organisms in model studies. Inhibition is displayed as relative inhibition compared to growth and ethanol productivity without any added by-products.*

compound	Concentration (g/L)	Relative ethanol productivity	Relative growth rate	Micro-organism	Reference
furfural	2.53	52%	50%	*K. marxianus*	Oliva et al. 2003
	5.56	0%		*I. orientalis*	Kwon et al. 2011
	1.6		50%	*Z. mobilis*	Franden et al. 2009
	5		0%	*Z. mobilis*	Franden et al. 2009
	4	44%	11%	*S. cerevisiae*	Taherzadeh et al. 2000
	3.4		50%	*E. coli*	Boopathy et al. 1993
	0.5	57%	53%	*S. cerevisiae*	Delgenes et al. 1996
	1	20%	19%	*S. cerevisiae*	Delgenes et al. 1996
	1	82%	81%	*Z. mobilis*	Delgenes et al. 1996
	2	56%	44%	*Z. mobilis*	Delgenes et al. 1996
5-HMF	4.01	53%	50%	*K. marxianus*	Oliva et al. 2003
	7.81	0%		*I. orientalis*	Kwon et al. 2011
	2.8		50%	*Z. mobilis*	Franden et al. 2009
	8		0%	*Z. mobilis*	Franden et al. 2009
	2	81%	60%	*S. cerevisiae*	Taherzadeh et al. 2000
	4	59%	29%	*S. cerevisiae*	Taherzadeh et al. 2000
	2.7		50%	*E. coli*	Boopathy et al. 1993
	1	29%	35%	*S. cerevisiae*	Delgenes et al. 1996
	3	17%	17%	*S. cerevisiae*	Delgenes et al. 1996

Table 3 *(continued)*

compound	Concentration (g/L)	Relative ethanol productivity	Relative growth rate	Micro-organism	Reference
	3	87%	69%	*Z. mobilis*	Delgenes et al. 1996
	5 (pH)	47%	33%	*Z. mobilis*	Delgenes et al. 1996
Acetic acid	3.5 (5)		0%	*P. pastoris*	Bellido et al. 2011
	5 (5.6)	99%	79%	*S. cerevisiae*	Delgenes et al. 1996
	15 (5.6)	62%	56%	*S. cerevisiae*	Delgenes et al. 1996
	5 (5.6)	90%	76%	*Z. mobilis*	Delgenes et al. 1996
	15 (5.6)	83%	26%	*Z. mobilis*	Delgenes et al. 1996
	12.6 (5.8)		50%	*Z. mobilis*	Franden et al. 2013
	21.6 (5.8)		0%	*Z. mobilis*	Franden et al. 2013
	3.5 (3.5)		66%	*S. cerevisiae*	Taherzadeh et al. 1997
	9 (5)		66%	*S. cerevisiae*	Taherzadeh et al. 1997
	15 (7)		79%	*E. coli*	Zaldivar and Ingram 1999
	30 (7)		31%	*E. coli*	Zaldivar and Ingram 1999
Formic acid	1.67 (6)		84%	*Z. mobilis*	Dong et al. 2013
	6.51 (6)		31%	*Z. mobilis*	Dong et al. 2013
	3.91 (5.8)		50%	*Z. mobilis*	Franden et al. 2013
	11.04 (5.8)		0%	*Z. mobilis*	Franden et al. 2013
Syringaldehyde	2.86	61%	50%	*K. marxianus*	Oliva et al. 2003
	0.2	74%	100%	*S. cerevisiae*	Delgenes et al. 1996
	1.5	33%	19%	*S. cerevisiae*	Delgenes et al. 1996
	0.75	101%	72%	*Z. mobilis*	Delgenes et al. 1996
	1.5	83%	60%	*Z. mobilis*	Delgenes et al. 1996
4-hydroxy benzaldehyde	1.02	61%	50%	*K. marxianus*	Oliva et al. 2003
	0.5	97%	75%	*S. cerevisiae*	Delgenes et al. 1996
	1.5	25%	13%	*S. cerevisiae*	Delgenes et al. 1996
	0.5	21%	16%	*Z. mobilis*	Delgenes et al. 1996
	0.75	14%	8%	*Z. mobilis*	Delgenes et al. 1996
vanillin	2.55	52%	50%	*K. marxianus*	Oliva et al. 2003
	3.17	0%		*I. orientalis*	Kwon et al. 2011
	0.5	70%	49%	*S. cerevisiae*	Delgenes et al. 1996
	1	17%	14%	*S. cerevisiae*	Delgenes et al. 1996
	0.5	86%	62%	*Z. mobilis*	Delgenes et al. 1996
	2	20%	12%	*Z. mobilis*	Delgenes et al. 1996

of 10 g/l of formic acid and 15 g/l of acetic acid severely inhibit growth rates, while no severe inhibition in productivity was observed at these concentrations. It should be noted that the pH is always an important factor when measuring the toxicity of acids (See chapter 5.2). At pH 3.5, the same inhibitory effect is reached with 3.5 g/l acetic acid, as with 9 g/l of acetic acid at pH 5 (Taherzadeh et al. 1997). The most toxic phenol tested was 4-hydroxybenzaldehyde, which can already cause significant inhibition at concentrations below 1 g/l.

Although research performed with pure compounds gives an indication of the inhibitory effect of lignocellulose by-products, combined effects should be taken into account when identifying the toxicity of lignocellulose substrates as a whole. Some inhibitory effects can be increased by synergy between different compounds. In *Kluyveromyces marxianus* fermentation, a combination of two compounds were added in concentrations which normally induce 25% inhibition in biomass production after 24 hours as a single inhibitor. The combinations of catechol and 4-hydroxybenzaldehyde, and catechol and furfural showed little synergy. However, vanillin and 4-HB, catechol and vanillin, and furfural and vanillin showed a large synergy, slowing down ethanol production by 80-95% (Oliva et al. 2004). Acetic acid and furfural were shown to interact both on growth rate inhibition and yields on biomass and ethanol, however not on ethanol production rates (Palmqvist et al. 1999). HMF and furfural can have a strong synergistic effect, a combination of 2 g/l HMF and 2 g/l furfural was shown to be more toxic than either 4 g/l furfural or 4 g/l HMF (Taherzadeh et al. 2000). E. coli was shown to be sensitive to synergies between furfural and HMF or furfural and vanillin (Zaldivar and Ingram 1999).

ACTIVE REMOVAL OF INHIBITORY COMPOUNDS

To decrease the effect of inhibitory by-products during fermentation using lignocellulose derived substrates, an extra upstream processing step can be added. In this step, (part of) the inhibiting by-products is removed, resulting in better fermentability.

Alkaline treatment, such as overliming, removes up to 40% of the furaldehydes, although it might enhance degradation of oligomeric phenolic compounds to monomers (Nilvebrant et al. 2003; Persson et al. 2002). The use of alkali at elevated temperatures might lead to a substantial loss of sugar due to degradation of sugars to aliphatic acids (Nilvebrant et al. 2003). Combining overliming with activated charcoal, up to 95% of the lignin derived phenolics can be removed (Converti et al. 1999).

A pre-fermentation with inhibitory by-product consuming micro-organisms can be used to remove by-products from pretreated lignocellulose. *C. basilensis* is able to consume furfural present in concentrations up to 1.2 g/l, with full conversion of 0.34 g/l furfural to furfuryl alcohol and furoic acid in 7 hours. Further conversion of furfuryl alcohol and acid to 2,5-furandicarboxylic acid is possible in 12 hours. *C. basilensis* is unable to consume the sugars present in the

media. *C. basilensis* can also consume organic acids like acetic acid, and aromatic compounds such as vanillin, guaiacol and 4-hydroxybenzaldehyde (Koopman et al. 2010).

C. ligniaria is another micro-organism which is able to fully metabolize furfural and HMF in the absence of sugars, however it does consume small amounts of sugar when sugars are present in the media. It also has the potential to metabolize aromatic acids, aliphatic acids and aldehydes (Nichols et al. 2005; Nichols et al. 2008).

Resins can also be used to remove the inhibitors from the medium by binding the inhibitors. The amino polyelectrolyte polyethyleneamine has shown the potential to remove up to 89% of the acetic acid, 59% of the 5-HMF and 82 % of the furfural in pretreated lignocellulose (Carter et al. 2011). Using the anion resin AG 1-X8 on pretreated lignocellulose can increase ethanol productivities to 1.71 g/l/h, compared to 0.34 g/l/h for untreated lignocellulose media. The ethanol productivity on lignocellulose media pretreated with non-charged resin XAD-8 also increased substantially to 0.9 g/l/h (Nilvebrant et al. 2001). Anion exchange resin Amberlite IRA-400 in combination with calcium hydroxide has been shown to fully resolve inhibitory effects of lignocellulose by-products on 2,3-butanediol producing *Klebsiella pneumoniae*, with yields over 90% of the theoretical maximum, while untreated material resulted in an overall yield of only 20% of the theoretical maximum (Frazer and McCaskey 1989).

Instead of expressing laccase in micro-organisms, the enzymes can also be added directly to the pretreated lignocellulose. Hibbert ketones can be decreased by 80% in laccase pretreated material (Larsson et al. 1999b). Using 0.5 U/ml laccase from *Trametes versicolor*, most phenolic monomers were polymerized within 24 hours, only acetophenone and 4-hydroxybenzaldehyde were not affected (Kolb et al. 2012). The addition of 1 µM laccase decreases the amount of investigated phenolic compounds from 2.56 to 0.17 g/l , increasing ethanol productivity of yeast on pretreated lignocellulose from 0.8 to 2.7 g/l/h (Jönsson et al. 1998).

Electrodialysis can be efficient to remove small organic acids. Almost all acetic acid can be removed with this method. However, electrodialysis has little influence on the furan concentration (Lee et al. 2013).

In a study different inhibitor removal methods, namely activated charcoal, anion resin (DIAION HPA 25), neutralization, overliming and laccase treatment, were compared. It was found that the anion resin had the best furan removal, while laccase treatment and the anion resin both had a good phenolic compound removal. The highest ethanol productivity and yield was achieved with the anion resin, followed by activated charcoal (Chandel et al. 2007). Another comparison study also shows the best fermentability of S. cerevisae is achieved with anion exchange (AG 1-X8), followed by calcium hydroxide treatment and laccase treatment. Anion exchange removed 70% of the furans, and more than 90% of the phenolic compounds. Ethanol productivity on anion exchange pretreated media was comparable to the reference with pure sugars with productivities up to 1.5 g/l/h after 6 hours, while untreated biomass showed a productivity of 0.04 g/l/h after 6 hours (Larsson et al. 1999b).

LIGNOCELLULOSE BY-PRODUCTS: ALWAYS A BURDEN?

In this review, the inhibitory effect of compounds present after pretreatment are shown. Sometimes, these compounds can also have a positive effect on yields and productivity. Some inhibitors can reduce the growth substantially, while the productivity is (almost) unaltered. This can result in a higher yield of product per amount of carbon source (Herbert et al. 1956; Pirt 1965).

S. cerevisiae fermentations with up to 9 g/l of acetic acid or up to 2 g/l of furfural showed increased ethanol yields (Palmqvist et al. 1999). Concentrations of undissociated acetic acid up to 3.3 g/l have been reported to enhance ethanol yields by 20% (Taherzadeh et al. 1997).

In steady state fermentations, an increase in ethanol yield (g ethanol/g glucose) by *S. cerevisiae* has been observed when the furfural concentration was increased up to 5.8 g/l in the inlet medium. This resulted in a furfuryl alcohol concentration of 4.2 g/l (Horváth et al. 2001).

Different phenolics at a concentration of 10 mM, including acetovanillone and 4-hydroxybenzaldehyde, increased the ethanol yield of *S. cerevisiae* compared to a reference fermentation by up to 10%, however, at the cost of a lower productivity (Klinke et al. 2003).

CONCLUDING REMARKS AND FUTURE PERSPECTIVE

In every pretreatment method, large amounts of by-products are formed (Table 2). Three major categories of by-products have been distinguished: Phenolic compounds, furans and small organic acids. These by-products can inhibit the fermentations towards biochemicals, decreasing productivity, growth and in some cases the yield of micro-organisms in these fermentations, based on quantities present and inhibitory effects. Small organic acids and furans are most likely to be inhibitory during fermentation. However, synergy among phenolic compounds and between furans and phenolic compounds still makes the phenolic compounds important to investigate. Two strategies have been reviewed to reduce the effect of by-products: Increasing the tolerance of micro-organisms, or actively remove the inhibitors. In both cases, it has been reported that significant improvements have been made in the micro-organisms productivity under inhibitory stress conditions. Using targeted approaches, it might be possible to limit inhibitory effects of some by-products in the (near) future.

ACKNOWLEDGEMENT

The writers of this article would like to thank the BE-Basic consortium, Corbion and DSM, for funding this research.

REFERENCES

Allen S, Clark W, McCaffery JM, Cai Z, Lanctot A, Slininger P, Liu ZL (2010) Furfural induces reactive oxygen species accumulation and cellular damage in Saccharomyces cerevisiae Biotechnol Biofuels 3:2

Almario MP, Reyes LH, Kao KC (2013) Evolutionary engineering of Saccharomyces cerevisiae for enhanced tolerance to hydrolysates of lignocellulosic biomass Biotechnol Bioeng 110:2616-2623 doi:10.1002/bit.24938

Ando S, Arai I, Kiyoto K, Hanai S (1986) Identification of aromatic monomers in steam-exploded poplar and their influences on ethanol fermentation by Saccharomyces cerevisiae J of Ferment Technol 64:567-570

Axe DD, Bailey JE (1995) Transport of lactate and acetate through the energized cytoplasmic membrane of Escherichia coli Biotechnol Bioeng 47:8-19 doi:10.1002/bit.260470103

Balan V, Sousa LdC, Chundawat SPS, Marshall D, Sharma LN, Chambliss CK, Dale BE (2009) Enzymatic digestibility and pretreatment degradation products of AFEX-treated hardwoods (Populus nigra) Biotechnol Progress 25:365-375 doi:10.1002/btpr.160

Banerjee N, Bhatnagar R, Viswanathan L (1981) Inhibition of glycolysis by furfural in Saccharomyces cerevisiae Appl Microbiol Biotechnol 11:226-228 doi:10.1007/bf00505872

Bellido C, Bolado S, Coca M, Lucas S, González-Benito G, García-Cubero MT (2011) Effect of inhibitors formed during wheat straw pretreatment on ethanol fermentation by Pichia stipitis Bioresource Technol 102:10868-10874 doi:10.1016/j.biortech.2011.08.128

Bjerre AB, Olesen AB, Fernqvist T, Plöger A, Schmidt AS (1996) Pretreatment of wheat straw using combined wet oxidation and alkaline hydrolysis resulting in convertible cellulose and hemicellulose Biotechnol Bioeng 49:568-577 doi:10.1002

Boerjan W, Ralph J, Baucher M (2003) LIGNIN BIOSYNTHESIS Annu Rev Plant Biol 54:519-546 doi:10.1146/annurev.arplant.54.031902.134938

Boopathy R, Bokang H, Daniels L (1993) Biotransformation of furfural and 5-hydroxymethyl furfural by enteric bacteria J of Ind Microbiol 11:147-150 doi:10.1007/bf01583715

Cantarella M, Cantarella L, Gallifuoco A, Spera A, Alfani F (2004) Effect of Inhibitors Released during Steam-Explosion Treatment of Poplar Wood on Subsequent Enzymatic Hydrolysis and SSF Biotechnol Progress 20:200-206 doi:10.1021/bp0257978

Carter B, Gilcrease PC, Menkhaus TJ (2011) Removal and recovery of furfural, 5-hydroxymethylfurfural, and acetic acid from aqueous solutions using a soluble polyelectrolyte Biotechnol Bioeng 108:2046-2052 doi:10.1002/bit.23153

Chandel AK, Kapoor RK, Singh A, Kuhad RC (2007) Detoxification of sugarcane bagasse hydrolysate improves ethanol production by Candida shehatae NCIM 3501 Bioresource Technol 98:1947-1950

Chang V, Holtzapple M (2000) Fundamental factors affecting biomass enzymatic reactivity Appl Biochem Biotech 84-86:5-37 doi:10.1385/abab:84-86:1-9:5

Chen S-F, Mowery RA, Castleberry VA, Walsum GPv, Chambliss CK (2006) High-performance liquid chromatography method for simultaneous determination of aliphatic acid, aromatic acid and neutral degradation products in biomass pretreatment hydrolysates J Chromatogr A 1104:54-61

Chundawat SPS, Vismeh R, Sharma LN, Humpala JF, da Costa Sousa L, Jones AD, Balan V, Dale BE(2010) Multifaceted characterization of cell wall decomposition products formed during ammonia fiber expansion (AFEX) and dilute acid based pretreatments Bioresource Technol 101:8429-8438

Claassen PAM, van Lier JB, Lopez Contreras AM, van Niel EWJ, Sijtsma L, Stams AJM, de Vries SS, Weusthuis RA (1999) Utilisation of biomass for the supply of energy carriers Appl Microbiol Biotechnol 52:741-755 doi:10.1007/s002530051586

Converti A, Perego P, Domínguez J (1999) Xylitol production from hardwood hemicellulose hydrolysates by Pachysolen tannophilus, Debaryomyces hansenii, and Candida guilliermondii Applied Biochem Biotech 82:141-151 doi:10.1385/abab:82:2:141

Dale BE, Leong CK, Pham TK, Esquivel VM, Rios I, Latimer VM (1996) Hydrolysis of lignocellulosics at low enzyme levels: Application of the AFEX process Bioresource Technol 56:111-116

de Vrije T, de Haas GG, Tan GB, Keijsers ERP, Claassen PAM (2002) Pretreatment of Miscanthus for hydrogen production by Thermotoga elfii Int J Hydrogen Energ 27:1381-1390 doi:10.1016/S0360-3199(02)00124-6

Delgenes JP, Moletta R, Navarro JM (1996) Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by Saccharomyces cerevisiae, Zymomonas mobilis, Pichia stipitis, and Candida shehatae Enzyme Microb Tech 19:220-225 doi:10.1016/0141-0229(95)00237-5

Dong H-W, Fan L-Q, Luo Z, Zhong J-J, Ryu DDY, Bao J (2013) Improvement of ethanol productivity and energy efficiency by degradation of inhibitors using recombinant Zymomonas mobilis (pHW20a-fdh) Biotechnol Bioeng 110:2395-2404 doi:10.1002/bit.24897

Du B, Sharma LN, Becker C, Chen S-F, Mowery RA, van Walsum GP, Chambliss CK (2010) Effect of varying feedstock–pretreatment chemistry combinations on the formation and accumulation of potentially inhibitory degradation products in biomass hydrolysates Biotechnol Bioeng 107:430-440 doi:10.1002/bit.22829

Eggeman T, Elander RT (2005) Process and economic analysis of pretreatment technologies Bioresource Technol 96:2019-2025 doi:10.1016/j.biortech.2005.01.017

Endo A, Nakamura T, Ando A, Tokuyasu K, Shima J (2008) Genome-wide screening of the genes required for tolerance to vanillin, which is a potential inhibitor of bioethanol fermentation, in Saccharomyces cerevisiae Biotechnol Biofuels 1:3 doi:10.1186/1754-6834-1-3

Fengel D, Wegener G (1983) Wood: Chemistry, ultrastructure, reactions De Gruyter, Berlin

Feron VJ, Til HP, de Vrijer F, Woutersen RA, Cassee FR, van Bladeren PJ (1991) Aldehydes: occurrence, carcinogenic potential, mechanism of action and risk assessment Mutat Res-Genet Tox 259:363-385 doi: 10.1016/0165-1218(91)90128-9

Fitzgerald DJ, Stratford M, Gasson MJ, Ueckert J, Bos A, Narbad A (2004) Mode of antimicrobial action of vanillin against Escherichia coli, Lactobacillus plantarum and Listeria innocua J Appl Microbiol 97:104-113 doi:10.1111/j.1365-2672.2004.02275.x

Foody P (1984) Method for increasing the accessibility of cellulose in lignocellulosic materials, particularly hardwoods agricultural residues and the like Google Patents http://www.google.nl/patents?hl=nl&lr=&vid=USPAT4461648&id=pJx1AAAAEBAJ&oi=fnd&dq=steam+AND+lignocellulose+AND+explosive+AND+decompression+AND+novel&printsec=abstract#v=onepage&q&f=false Accessed 28 Oktober 2013

Franden MA, Pienkos PT, Zhang M (2009) Development of a high-throughput method to evaluate the impact of inhibitory compounds from lignocellulosic hydrolysates on the growth of Zymomonas mobilis J Biotechnol 144:259-267 doi:10.1016/j.jbiotec.2009.08.006

Franden MA, Pilath H, Mohagheghi A, Pienkos P, Zhang M (2013) Inhibition of growth of Zymomonas mobilis by model compounds found in lignocellulosic hydrolysates Biotechnol Biofuels 6:99 doi:10.1186/1754-6834-6-99

Frazer FR, McCaskey TA (1989) Wood hydrolyzate treatments for improved fermentation of wood sugars to 2,3-butanediol Biomass 18:31-42 doi:http://dx.doi.org/10.1016/0144-4565(89)90079-6

García-Aparicio MAP, Ballesteros I, González A, Oliva JM, Ballesteros M, Negro MAJ (2006) Effect of Inhibitors Released During Steam-Explosion Pretreatment of Barley Straw on Enzymatic Hydrolysis Appl Biochem Biotech 129:278-288. doi:10.1385/ABAB:129:1:278

Garlotta D (2001) A Literature Review of Poly(Lactic Acid) J Polym Environ 9:63-84 doi:10.1023/a:1020200822435

Gorsich SW, Dien BS, Nichols NN, Slininger PJ, Liu ZL, Skory CD (2006) Tolerance to furfural-induced stress is associated with pentose phosphate pathway genes ZWF1, GND1, RPE1, and TKL1 in Saccharomyces cerevisiae Appl Microbiol Biotechnol 71:339-349 doi:10.1007/s00253-005-0142-3

Gray KA, Zhao L, Emptage M (2006) Bioethanol Curr Opin Chem Biol 10:141-146

Gutiérrez T, Buszko M, Ingram L, Preston J (2002) Reduction of Furfural to Furfuryl Alcohol by Ethanologenic Strains of Bacteria and Its Effect on Ethanol Production from Xylose Appl Biochem Biotechn 98-100:327-340 doi:10.1007/978-1-4612-0119-9_27

Hasunuma T, Sanda T, Yamada R, Yoshimura K, Ishii J, Kondo A (2011) Metabolic pathway engineering based on metabolomics confers acetic and formic acid tolerance to a recombinant xylose-fermenting strain of Saccharomyces cerevisiae Microb Cell Fact 10:2

Hatfield RD, Ralph J, Grabber JH (1999) Cell wall cross-linking by ferulates and diferulates in grasses J Sci Food Agr 79:403-407

Heaton EA, Dohleman FG, Long SP (2008) Meeting US biofuel goals with less land: the potential of Miscanthus Glob Change Biol 14:2000-2014 doi:10.1111/j.1365-2486.2008.01662.x

Heer D, Heine D, Sauer U, (2009) Resistance of Saccharomyces cerevisiae to High Concentrations of Furfural Is Based on NADPH-Dependent Reduction by at Least Two Oxireductases Appl Environ Microbiol 75:7631-7638 doi:10.1128/AEM.01649-09

Hendriks ATWM, Zeeman G (2009) Pretreatments to enhance the digestibility of lignocellulosic biomass Bioresource Technol 100:10-18 doi:10.1016/j.biortech.2008.05.027

Herbert D, Elsworth R, Telling RC, (1956) The Continuous Culture of Bacteria; a Theoretical and Experimental Study J Gen Microbiol 14:601-622 doi: 10.1099/00221287-14-3-601

Holtzapple M, Jun J-H, Ashok G, Patibandla S, Dale B (1991) The ammonia freeze explosion (AFEX) process Appl Biochem Biotech 28-29:59-74 doi:10.1007/bf02922589

Horváth IS, Taherzadeh MJ, Niklasson C, Lidén G (2001) Effects of furfural on anaerobic continuous cultivation of Saccharomyces cerevisiae Biotechnol Bioeng 75:540-549 doi:10.1002/bit.10090

Hoskinson RL, Karlen DL, Birrell SJ, Radtke CW, Wilhelm WW (2007) Engineering, nutrient removal, and feedstock conversion evaluations of four corn stover harvest scenarios Biomass Bioenergy 31:126-136 doi:10.1016/j.biombioe.2006.07.006

Hu C, Zhao X, Zhao J, Wu S, Zhao ZK (2009) Effects of biomass hydrolysis by-products on oleaginous yeast Rhodosporidium toruloides Bioresource Technol 100:4843-4847

Huang C, Wu H, Smith T, Liu Z-j, Lou W-Y, Zong M-h (2012) In vivo detoxification of furfural during lipid production by the oleaginous yeast Trichosporon fermentans Biotechnol Let 34:1637-1642 doi:10.1007/s10529-012-0948-x

Humbird D, Davis R, Tao L, Kinchin C, Hsu D, Aden A, Schoen P, Lukas J, Olthof B, Worley M, Sexton D, Dudgeon D (2011) Process design and economics for biochemical conversion of lignocellulosic biomass to ethanol NREL http://www.researchgate.net/publication/229843699_Process_Design_and_Economics_for_Biochemical_Conversion_of_Lignocellulosic_Biomass_to_Ethanol_Dilute-Acid_Pretreatment_and_Enzymatic_Hydrolysis_of_Corn_Stover/file/9fcfd5011638d5a2af.pdf Accessed 27 may 2013

Iwaki A, Ohnuki S, Suga Y, Izawa S, Ohya Y (2013) Vanillin Inhibits Translation and Induces Messenger Ribonucleoprotein (mRNP) Granule Formation in Saccharomyces cerevisiae: Application and Validation of High-Content, Image-Based Profiling PLoS ONE 8:e61748

Jackson MG (1977) Review article: The alkali treatment of straws Anim Feed Sci Tech 2:105-130 Doi: 10.1016/0377-8401(77)90013-X

Jönsson LJ, Palmqvist E, Nilvebrant NO, Hahn-Hägerdal B (1998) Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus Trametes versicolor Appl Microbiol Biotechnol 49:691-697 doi:10.1007/s002530051233

Kaar WE, Gutierrez CV, Kinoshita CM (1998) Steam explosion of sugarcane bagasse as a pretreatment for conversion to ethanol Biomass Bioenergy 14:277-287

Kabel MA, Bos G, Zeevalking J, Voragen AGJ, Schols HA (2007) Effect of pretreatment severity on xylan solubility and enzymatic breakdown of the remaining cellulose from wheat straw Bioresource Technol 98:2034-2042

Karimi K, Kheradmandinia S, Taherzadeh MJ (2006) Conversion of rice straw to sugars by dilute-acid hydrolysis Biomass Bioenergy 30:247-253

Keweloh H, Diefenbach R, Rehm H-J (1991) Increase of phenol tolerance of Escherichia coli by alterations of the fatty acid composition of the membrane lipids Arch Microbiol 157:49-53 doi:10.1007/bf00245334

Klemm D, Heublein B, Fink HP, Bohn A (2005) Cellulose: Fascinating Biopolymer and Sustainable Raw Material Angew Chem Int Ed 44:3358-3393 doi:10.1002/anie.200460587

Klinke HB, Ahring BK, Schmidt AS, Thomsen AB (2002) Characterization of degradation products from alkaline wet oxidation of wheat straw Bioresource Technol 82:15-26 doi:10.1016/S0960-8524(01)00152-3

Klinke HB, Olsson L, Thomsen AB, Ahring BK (2003) Potential inhibitors from wet oxidation of wheat straw and their effect on ethanol production of Saccharomyces cerevisiae: Wet oxidation and fermentation by yeast Biotechnol Bioeng 81:738-747 doi:10.1002/bit.10523

Kolb M, Sieber V, Amann M, Faulstich M, Schieder D (2012) Removal of monomer delignification products by laccase from Trametes versicolor Bioresource Technol 104:298-304

Koopman F, Wierckx N, De Winde JH, Ruijssenaars HJ (2010) Identification and characterization of the furfural and 5-(hydroxymethyl) furfural degradation pathways of Cupriavidus basilensis HMF14 Proc Natl Acad Sci 107:4919

Kootstra AM, Beeftink H, Scott E, Sanders J (2009) Optimization of the dilute maleic acid pretreatment of wheat straw Biotechnol Biofuels 2:31 doi:10.1186/1754-6834-2-31

Kwon Y-J, Ma A-Z, Li Q, Wang F, Zhuang G-Q, Liu C-Z (2011) Effect of lignocellulosic inhibitory compounds on growth and ethanol fermentation of newly-isolated thermotolerant Issatchenkia orientalis Bioresource Technol 102:8099-8104 doi:10.1016/j.biortech.2011.06.035

Laadan B, Almeida JRM, Rådström P, Hahn-Hägerdal B, Gorwa-Grauslund M (2008) Identification of an NADH-dependent 5-hydroxymethylfurfural-reducing alcohol dehydrogenase in Saccharomyces cerevisiae Yeast 25:191-198 doi:10.1002/yea.1578

Larsson S, Cassland P, Jönsson LJ (2001) Development of a Saccharomyces cerevisiae Strain with Enhanced Resistance to Phenolic Fermentation Inhibitors in Lignocellulose Hydrolysates by Heterologous Expression of Laccase Appl Environ Microbiol 67:1163-1170 doi:10.1128/AEM.67.3.1163-1170.2001

Larsson S, Palmqvist E, Hahn-Hägerdal B, Tengborg C, Stenberg K, Zacchi G, Nilvebrant N-O (1999a) The generation of fermentation inhibitors during dilute acid hydrolysis of softwood Enzyme Microb Technol 24:151-159

Larsson S, Quintana-Sáinz A, Reimann A, Nilvebrant N-O, Jönsson L (2000) Influence of lignocellulose-derived aromatic compounds on oxygen-limited growth and ethanolic fermentation by Saccharomyces cerevisiae Appl Biochem Biotechnol 84-86:617-632 doi:10.1385/abab:84-86:1-9:617

Larsson S, Reimann A, Nilvebrant N-O, Jönsson L (1999b) Comparison of different methods for the detoxification of lignocellulose hydrolyzates of spruce Applied Biochem Biotechnol 77:91-103 doi:10.1385/abab:77:1-3:91

Lashof DA, Ahuja DR (1990) Relative contributions of greenhouse gas emissions to global warming Nature 344:529-531 doi: 10.1038/344529a0

Lee H-J, Lim W-S, Lee J-W (2013) Improvement of ethanol fermentation from lignocellulosic hydrolysates by the removal of inhibitors J Ind Eng Chem 19:2010-2015 doi:10.1016/j.jiec.2013.03.014

Lewis Liu Z, Blaschek HP (2010) Biomass Conversion Inhibitors and In Situ Detoxification Biomass to Biofuels: Strategies for Global Industries John Wiley& sons Ltd, United Kingdom

Lewis Liu Z, Ma M, Song M (2009) Evolutionarily engineered ethanologenic yeast detoxifies lignocellulosic biomass conversion inhibitors by reprogrammed pathways Mol Genet Genomics 282:233-244 doi:10.1007/s00438-009-0461-7

Ma M, Liu ZL (2010) Comparative transcriptome profiling analyses during the lag phase uncover YAP1, PDR1, PDR3, RPN4, and HSF1 as key regulatory genes in genomic adaptation to the lignocellulose derived inhibitor HMF for Saccharomyces cerevisiae BMC Genomics 11:660

Martín C, Klinke HB, Thomsen AB (2007) Wet oxidation as a pretreatment method for enhancing the enzymatic convertibility of sugarcane bagasse Enzyme Microb Technol 40:426-432 doi: 10.1016/j.enzmictec.2006.07.015

McIntosh S, Vancov T (2010) Enhanced enzyme saccharification of Sorghum bicolor straw using dilute alkali pretreatment Bioresource Technol 101:6718-6727

McMillan J, Adney WS, Mielenz JR, Klasson KT, Thomsen MH, Thygesen A, Jørgensen H, Larsen J, Christensen BH, Thomsen AB (2006) Preliminary Results on Optimization of Pilot Scale Pretreatment of Wheat Straw Used in Coproduction of Bioethanol and Electricity Appl Biochem Biotech 130:448-460. doi:10.1385/ABAB:130:1:448

Miller EN, Jarboe LR, Turner PC, Pharkya P, Yomano LP, York SW, Nunn D, Shanmugam KT, Ingram LO (2009) Furfural inhibits growth by limiting sulfur assimilation in ethanologenic Escherichia coli strain LY180 Appl Environ Microbiol 75:6132-6141 doi:10.1128/AEM.01187-09

Modig T, Lidén G, Taherzadeh MJ (2002) Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase Biochem J 363:769

Moon J, Liu ZL (2012) Engineered NADH-dependent GRE2 from Saccharomyces cerevisiae by directed enzyme evolution enhances HMF reduction using additional cofactor NADPH Enzyme Microb Technol 50:115-120 doi:10.1016/j.enzmictec.2011.10.007

Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M (2005) Features of promising technologies for pretreatment of lignocellulosic biomass Bioresource Technol 96:673-686 doi:10.1016/j.biortech.2004.06.025

Muñoz C, Guillén F, Martínez AT, Martínez MJ (1997) Induction and Characterization of Laccase in the Ligninolytic Fungus Pleurotus eryngii Curr Microbiol 34:1-5 doi:10.1007/s002849900134

Nichols NN, Dien BS, Guisado GM, López MJ, Davison BH, Evans BR, Finkelstein M, McMillan JD(2005) Bioabatement to Remove Inhibitors from Biomass-Derived Sugar Hydrolysates Appl Biochem Biotech 121:379-390. doi:10.1385/ABAB:121:1-3:0379

Nichols NN, Sharma LN, Mowery RA, Chambliss CK, van Walsum GP, Dien BS, Iten LB (2008) Fungal metabolism of fermentation inhibitors present in corn stover dilute acid hydrolysate Enzyme Microb Technol 42:624-630

Nilvebrant N-O, Persson P, Reimann A, de Sousa F, Gorton L, Jönsson L (2003) Limits for alkaline detoxification of dilute-acid lignocellulose hydrolysates Appl Biochem and Biotechnol 107:615-628 doi:10.1385/abab:107:1-3:615

Nilvebrant N-O, Reimann A, Larsson S, Jönsson L (2001) Detoxification of lignocellulose hydrolysates with ion-exchange resins Appl Biochem and Biotechnol 91-93:35-49 doi:10.1385/abab:91-93:1-9:35

Nishikawa NK, Sutcliffe R, Saddler JN (1988) The influence of lignin degradation products on xylose fermentation by Klebsiella pneumoniae Appl Microbiol and Biotechnol 27:549-552 doi:10.1007/bf00451630

Öhgren K, Bura R, Saddler J, Zacchi G (2007) Effect of hemicellulose and lignin removal on enzymatic hydrolysis of steam pretreated corn stover Bioresource Technol 98:2503-2510

Oliva J, Sáez F, Ballesteros I, González A, Negro M, Manzanares P, Ballesteros M (2003) Effect of lignocellulosic degradation compounds from steam explosion pretreatment on ethanol fermentation by thermotolerant yeast Kluyveromyces marxianus Appl Biochem Biotechnol 105:141-153 doi:10.1385/abab:105:1-3:141

Oliva JM, Ballesteros I, Negro MJ, Manzanares P, Cabañas A, Ballesteros M (2004) Effect of Binary Combinations of Selected Toxic Compounds on Growth and Fermentation of Kluyveromyces marxianus Biotechnol Prog 20: 715-720 doi: 10.1021/bp034317p

Orij R, Brul S, Smits GJ (2011) Intracellular pH is a tightly controlled signal in yeast BBA-Gen Subjects 1810:933-944

Morsomme P,de Kerchove d'Exaerde A, De Meester S, Thines D, Goffeau A, Boutry M (1996) Single point mutations in various domains of a plant plasma membrane H(+)-ATPase expressed in Saccharomyces cerevisiae increase H(+)-pumping and permit yeast growth at low pH EMBO j 15:5513-5526

Palmqvist E, Grage H, Meinander NQ, Hahn-Hägerdal B (1999) Main and interaction effects of acetic acid, furfural, and p-hydroxybenzoic acid on growth and ethanol productivity of yeasts Biotechnol Bioeng 63:46-55 doi:10.1002

Palmqvist E, Hahn-Hägerdal B (2000) Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition Bioresource Technol 74:25-33 doi:10.1016/S0960-8524(99)00161-3

Pampulha ME, Loureiro-Dias MC (1989) Combined effect of acetic acid, pH and ethanol on intracellular pH of fermenting yeast Appl Microbiol Biotechnol 31:547-550 doi:10.1007/bf00270792

Park SE, Koo HM, Park YP, Park SM, Park JC, Lee OH, Park YC, Seo JH (2011) Expression of aldehyde dehydrogenase 6 reduces inhibitory effect of furan derivatives on cell growth and ethanol production in Saccharomyces cerevisiae Bioresource Technol 102:6033-6038

Persson P, Andersson J, Gorton L, Larsson S, Nilvebrant NO, Jönsson LJ (2002) Effect of Different Forms of Alkali Treatment on Specific Fermentation Inhibitors and on the Fermentability of Lignocellulose Hydrolysates for Production of Fuel Ethanol J Agric Food Chem 50:5318-5325 doi:10.1021/jf025565o

Pirt SJ (1965) The Maintenance Energy of Bacteria in Growing Cultures Proc R Soc B 163:224-231

Roberto IC, Mussatto SI, Rodrigues RCLB (2003) Dilute-acid hydrolysis for optimization of xylose recovery from rice straw in a semi-pilot reactor Ind Crop Prod 17:171-176 Doi: 10.1016/S0926-6690(02)00095-X

Saha BC (2003) Hemicellulose bioconversion J Ind Microbiol Biot 30:279-291 doi:10.1007/s10295-003-0049-x

Sanchez G, Pilcher L, Roslander C, Modig T, Galbe M, Liden G (2004) Dilute-acid hydrolysis for fermentation of the Bolivian straw material Paja Brava Bioresource Technol 93:249-256

Sárvári Horváth I, Franzén CJ, Taherzadeh MJ, Niklasson C, Lidén G (2003) Effects of Furfural on the Respiratory Metabolism of Saccharomyces cerevisiae in Glucose-Limited Chemostats App Environ Microbiol 69:4076-4086 doi:10.1128/aem.69.7.4076-4086.2003

Sendich E, Laser M, Kim S, Alizadeh H, Laureano-Perez L, Dale B, Lynd L (2008) Recent process improvements for the ammonia fiber expansion (AFEX) process and resulting reductions in minimum ethanol selling price Bioresource Technol 99:8429-8435 doi:10.1016/j.biortech.2008.02.059

Shafiei M, Karimi K, Taherzadeh MJ (2010) Pretreatment of spruce and oak by N-methylmorpholine-N-oxide (NMMO) for efficient conversion of their cellulose to ethanol Bioresource Technol 101:4914-4918 doi:10.1016/j.biortech.2009.08.100

Srinivasan S (2009) The food v. fuel debate: A nuanced view of incentive structures Renew Energ 34:950-954 doi:10.1016/j.renene.2008.08.015

Sun JX, Sun XF, Sun RC, Su YQ (2004) Fractional extraction and structural characterization of sugarcane bagasse hemicelluloses Carbohyd Polym 56:195-204 doi:10.1016/j.carbpol.2004.02.002

Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review Bioresource Technol 83:1-11 doi:10.1016/S0960-8524(01)00212-7

Sundström L, Larsson S, Jönsson L (2010) Identification of Saccharomyces cerevisiae Genes Involved in the Resistance to Phenolic Fermentation Inhibitors Appl Biochem Biotechnol 161:106-115 doi:10.1007/s12010-009-8811-9

Taherzadeh MJ, Gustafsson L, Niklasson C, Lidén G (1999) Conversion of furfural in aerobic and anaerobic batch fermentation of glucose by Saccharomyces cerevisiae J Biosci Bioeng 87:169-174

Taherzadeh MJ, Gustafsson L, Niklasson C, Lidén G (2000) Physiological effects of 5-hydroxymethylfurfural on Saccharomyces cerevisiae Appl Microbiol Biotechnol 53:701-708 doi:10.1007/s002530000328

Taherzadeh MJ, Niklasson C, Lidén G (1997) Acetic acid—friend or foe in anaerobic batch conversion of glucose to ethanol by Saccharomyces cerevisiae? Chem Eng Sci 52:2653-2659

Teeri TT (1997) Crystalline cellulose degradation: new insight into the function of cellobiohydrolases Trends Biotechnol 15:160-167

Toledano A, García A, Mondragon I, Labidi J (2010) Lignin separation and fractionation by ultrafiltration Sep Purif Technol 71:38-43 doi:http://dx.doi.org/10.1016/j.seppur.2009.10.024

Tucker M, Kim K, Newman M, Nguyen Q (2003) Effects of temperature and moisture on dilute-acid steam explosion pretreatment of corn stover and cellulase enzyme digestibility Appl Biochem Biotechnol 105:165-177 doi:10.1385/abab:105:1-3:165

Vanholme R, Demedts B, Morreel K, Ralph J, Boerjan W (2010) Lignin Biosynthesis and Structure Plant Physiol 153:895-905 doi:10.1104/pp.110.155119

Vennestrøm PNR, Osmundsen CM, Christensen CH, Taarning E (2011) Beyond Petrochemicals: The Renewable Chemicals Industry Angew Chem Int Ed 50:10502-10509 doi:10.1002/anie.201102117

Verduyn C, Postma E, Scheffers WA, Van Dijken JP (1992) Effect of benzoic acid on metabolic fluxes in yeasts: A continuous-culture study on the regulation of respiration and alcoholic fermentation Yeast 8:501-517 doi:10.1002/yea.320080703

Wang X, Miller EM, Yomano L, Zhang X, Shanmugam KT, Ingram LO (2011) Overexpression of NADH-dependent oxidoreductase fucO increases furfural tolerance in Escherichia coli strains engineered for the production of ethanol and lactate Appl Environ Microbiol 77:5132-5140 doi: 10.1128/AEM.05008-11

Wang X, Miller EM, Yomano LP, Zhang X, Shanmugam KT, Ingram LO (2013) Engineering furfural tolerance in Escherichia coli improves the fermentation of lignocellulosic sugars into renewable chemicals proc natl acad sci 110:4021-4026 doi:10.1073/pnas.1217958110

Whetten R, Sederoff R (1995) Lignin Biosynthesis Plant Physiol Vol. 7:1001-1013

Zaldivar J, Ingram LO (1999) Effect of organic acids on the growth and fermentation of ethanologenic Escherichia coli LY01 Biotechnol Bioeng 66:203-210 doi:10.1002/(sici)1097-0290(1999)66:4<203::aid-bit1>3.0.co;2-#

Zhang J, Geng A, Yao C, Lu Y, Li Q (2012) Effects of lignin-derived phenolic compounds on xylitol production and key enzyme activities by a xylose utilizing yeast Candida athensensis SB18 Bioresource Technol 121:369-378

Zhang YHP, Ding SY, Mielenz JR, Cui JB, Elander RT, Laser M, Himmel ME, McMilland JR, Lynd LR (2007) Fractionating recalcitrant lignocellulose at modest reaction conditions Biotechnol Bioeng 97:214-223 doi:10.1002/bit.21386

Zhong C, Lau M, Balan V, Dale B, Yuan Y-J (2009) Optimization of enzymatic hydrolysis and ethanol fermentation from AFEX-treated rice straw Appl Microbiol Biotechnol 84:667-676 doi:10.1007/s00253-009-2001-0

CHAPTER 3

Analysis of by-product formation and sugar monomerization in sugarcane bagasse pretreated at pilot plant scale: differences between autohydrolysis, alkaline and acid pretreatment

This chapter is published as: van der Pol E.C., Bakker R.R., Zeeland A.N.T., Sanchez Garcia D. Punt A., Eggink G. (2015) *Bioresource Technol* 181:114-123
doi: 10.1016/j.biortech.2015.01.033

ABSTRACT

Sugarcane bagasse is an interesting feedstock for the biobased economy, since a large fraction is polymerised sugars. Autohydrolysis, alkaline and acid pretreatment conditions combined with enzyme hydrolysis were used on lignocellulose rich bagasse to acquire monomeric sugars. By-products found after pretreatment included acetic, glycolyic and coumaric acid in concentrations up to 40, 21 and 2.5 g/kg dry weight bagasse respectively. Alkaline pretreated material contained up to 45 g/kg bagasse DW of sodium. Acid and autohydrolysis pretreatment results in a furan formation of 14 g/kg and 25 g/kg DW bagasse respectively. Monomerization efficiencies of pretreated solid material by enzymes after 72h were 81% for acid pretreatment, 77% for autohydrolysis and 57% for alkaline pretreatment. Solid material was washed with superheated water to decrease the amount of by-products. Washing decreased organic acid, phenol and furan concentrations in solid material by at least 60%, without a major sugar loss.

INTRODUCTION

Crude oil reserves are depleting fast, while the use of crude oil is linked to the greenhouse gas effect (Lashof and Ahuja, 1990). Therefore, oil use should be limited. Two major oil-derived products, plastics and fuels, can be produced using a biobased process. Bioethanol can be used to fuel modern cars, while the poly-lactic acid (PLA) can replace the petrochemically produced polyethylene (PE) and polystyrene (PS) (Bünger, 2010).

The most abundant biopolymer on earth is lignocellulose, making it an interesting feedstock for the biobased economy (Klemm et al., 2005; Teeri, 1997). Lignocellulose is a polymer consisting of three substructures; cellulose, hemicellulose and lignin. Cellulose consists solely of β-linked glucose molecules. Hemicellulose composition can vary significantly, it can contain many different sugars, including glucose, galactose, mannose, arabinose and xylose, while it can also contain different acids, and has phenolic interlinks with lignin (Hatfield and Grabber, 1999). Lignin is a non-sugar containing polymer mainly build up from phenolic compounds (Fengel and Wegener, 1984).

An interesting source of lignocellulose is sugarcane bagasse. Bagasse is the leftover of sugarcane stalks after sugar extraction. Bagasse has the following composition: 35-45% cellulose, 20-27% hemicellulose, 17-25% lignin and 10-15% other compounds (Kurakake et al., 2001; Neureiter et al., 2002; Martín et al., 2007). The amount of polymerised sugars in bagasse is between 60% and 70% on a dry weight basis.

Sugars present in lignocellulose can be used in fermentation processes towards bioethanol and lactic acid, the building block of PLA. To make the sugars available for fermentation, a pretreatment is required. A commonly used method for pretreatment requires the lignocellulose material to be milled and soaked in water. The mixture is heated to 160-180ºC for 15-45 minutes, in which the lignocellulose is partially decomposed and/or degraded. A low pH can enhance the monomerization of hemicellulose (acid pretreatment), while a high pH can enhance the deacetylation of hemicellulose, while the lignin can also be (partially) solubilized (alkaline pretreatment). The pretreated bagasse can be converted to monomeric sugars using enzymatic hydrolysis.

Acid and alkaline pretreatment are well studied and common methods to decompose lignocellulose, which have been shown to be cost efficient and applicable on a large scale (Mcmillan et al., 2006; Humbird et al., 2011). Autohydrolysis is very interesting as an alternative method due to the absence of chemical addition, saving on operating costs.

Many studies have been performed on the hydrolysis of bagasse using acid and alkaline pretreatment. However, no extensive study has been performed on the formation of by-products during the pretreatment of sugarcane bagasse. Knowledge of the formation of by-products from lignocellulosic material is very beneficial when the decomposed lignocellulose is used in a fermentation process. The by-products can result in problems further downstream, since they can inhibit the growth and production of micro-organisms during fermentation (van

der Pol et al, 2014). Knowledge on the inhibitory compounds can provide strategies towards efficient fermentation processes.

In this paper, by-product formation will be analysed in a process which uses a chemical pretreatment, steam explosion and enzyme treatment. By-products are also measured in the solid fraction after thermo-chemical pretreatment, a fraction which is often overlooked with regard to by-products. Sugar monomerization was taken into account to compare the efficiencies of the methods, showing that the pretreatment was performed at industrial relevant conditions. The experiments were performed on a pilot plant scale, using equipment which is comparable to large scale pretreatment facilities. A washing step was introduced as a cheap method to remove part of the by-products in the solid phase.

MATERIAL AND METHODS

Chemicals and feedstock

All chemicals used during analysis were ordered at Sigma Aldrich (Missouri, USA) and had a purity of at least 98%, with the exception of formic acid (purity of 95%). The enzyme Accellerase 1500 was obtained from Genencor (Denmark). Sugarcane grown in the Queensland area in Australia was harvested, milled and sugars were extracted. The bagasse material was stored dry outside until used.

Chemical pretreatment and Washing

Pretreatment took place in the Mackay Renewable Biocommodities Pilot Plant which is owned and operated by Queensland University, and which has been previously described (Wong et al, 2011). The pilot plant facility consist of a first stage horizontal pre-hydrolysis reactor capable of handling up to 22kg of biomass feedstock, solid-liquid separation via piston pressing, and a second stage vertical reactor capable of performing steam explosion at a maximum of 26 bars.

In this research, 20 kg of bagasse with a dry weight of ~50% was loaded in the horizontal reactor, which was pre-heated to 5ºC above reaction temperature for 20 min with 50 kg of demineralized water (Table 1 and figure 1). After the addition of bagasse and water, chemicals were added when required. In case of acid pretreatment, sulphuric acid (H2SO4) was added to a final concentration of 0.76% W/DW bagasse. In case of alkaline pretreatment, sodium hydroxide (NaOH) was added to a concentration of 10% W/DW bagasse. Autohydrolysis did not involve the addition of chemicals. For acid pretreatment, the reaction took place at 170ºC for 15 minutes. For alkaline pretreatment, the reaction took place at 160 ºC for 45 minutes. Autohydrolysis was performed at 170ºC for 45 minutes. The reaction was performed in triplicate. After pretreatment, a solid liquid separation took place by pressing the solids with a large piston press. The liquid fraction was stored at -20ºC. The solid fraction from one of the

Table 1: *Overview of the conditions used for the pretreatment of sugarcane bagasse. In total, 9 runs were performed (see figure 1)*

	Acid	Acid + Wash	Autohydrolysis	Autohydrolysis + Wash	Alkaline + Wash
Run	1,2	3	4	5,6	7,8,9
Bagasse used (kg DW)	10	10	10	10	10
DW in reactor (% W/W)	16.7	16.7	16.7	16.7	16.7
Chemical used	H_2SO_4	H_2SO_4	none	none	NaOH
Chemical used (% W/DW)	0.76	0.76	-	-	10
Temperature (°C)	170	170	170	170	160
Pretreatment time (min)	15	15	45	45	45
Wash water (L)	none	10	none	10	10
Washing time (min)	-	5	-	5	5
temperature washing (°C)	-	150	-	150	150

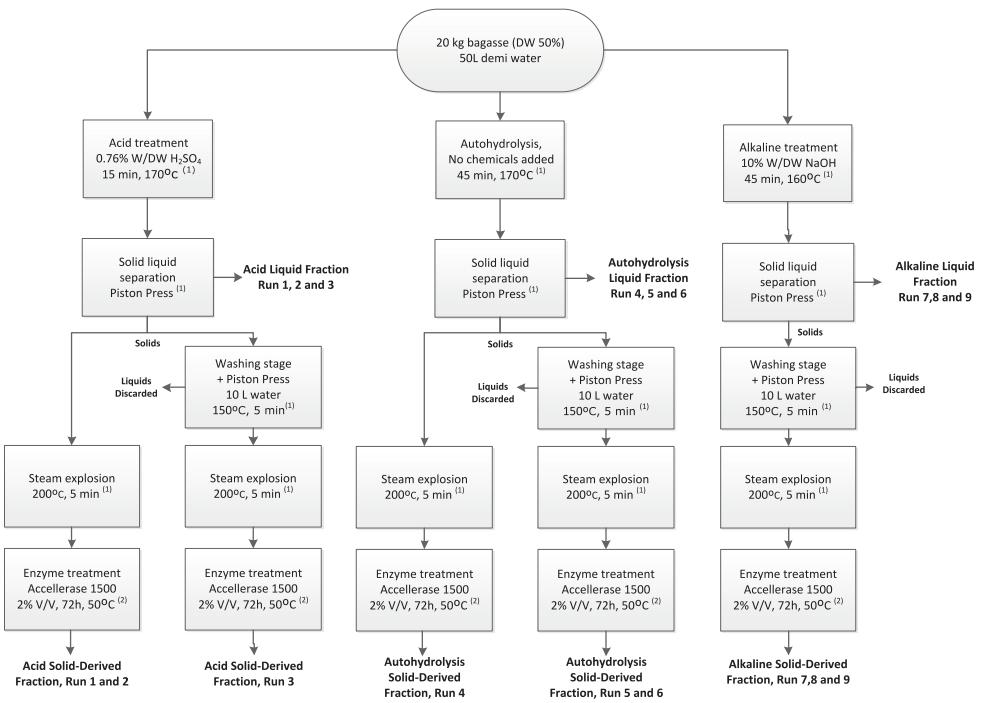


Figure 1: *Process scheme for the pretreatment of sugarcane bagasse. The pretreatment consists of a primary thermo-chemical pretreatment, an optional washing step, steam explosion and an enzymatic hydrolysis (more details in table 1). (1): pretreatment on 60 litre scale. (2): treatment on 50 ml scale.*

three runs performed using acid pretreatment (Run 3), the solid fraction from two of the three runs performed using autohydrolysis (Run 5 and 6) and all solid alkaline fractions (Run 7,8 and 9) were washed with 10 litre superheated water at a temperature of 150ºC for 5 minutes. A washing step was always combined with a new solid liquid separation, where the liquid fraction, mainly consisting of wash water, was discarded.

Steam explosion

A vertical pressure reactor was pre-heated for 5 minutes at 200ºC. The 9 different solid fractions, acquired after chemical pretreatment or washing, with an estimated dry weight of 20-30%, were placed without any additions in the reactor and heated for 5 minutes at 200ºC. After 5 minutes, the blow valve was immediately opened, which expelled the material with explosive force into the solid residue blow tank. Solid samples were stored at 4ºC (acid and autohydrolysis) or at -20ºC (alkaline).

Enzyme treatment at laboratory scale

Solid-derived material of all 9 runs acquired after steam explosion were brought to room temperature prior to use. 50 ml greiner tubes were filled with 10% w/w solids and 90% w/w water. For alkaline pretreated solid samples, 7.5% w/w DW solids was used due to swelling of the material. 1 ml of Accellerase 1500 (Genencor, Denmark) was added to each tube. Samples were kept at a temperature of 55ºC for 72 hours. After 24 and 48 hours, the pH was re-adjusted to pH 5, and 0.5 ml of Accellerase 1500 was added to ensure a maximal sugar yield was reached. In total, 4% V/V Accellerase 1500 was added. The enzyme treated solid-derived fraction was stored at -20ºC until analysis.

HPAEC-PAD method for sugar analysis

Samples were brought to room temperature prior to use. Samples were centrifuged for 10 minutes at 4000 RPM and 20-200 µl of supernatant was transferred to a 15 ml greiner tube. The supernatant was diluted 20-200 times to ensure a measurement within calibration curve. To 4 ml of diluted sample, 40 µl of fucose was added as an internal standard. 10 µl of bromine-phenolblue was used to check whether the pH of the sample was above 4. If the pH below 4, the pH was adjusted with bariumcarbonate until the solution is clear-blue. Samples were filtered prior to analysis (0.2 µm Spartan, Whatman GmbH, Dassel, Germany).

A HPAEC-PAD system was used to for analysis, using 4 different eluents (Dionex, California, USA). Eluent A consisted of milliQ water, eluent B contained milliQ water with 150 mM NaOH, Eluent C contained milliQ water with 600 mM NaAc, and Eluent D contained milliQ water with 500 mM NaOH. The flow of the eluent mix was set at 1 ml/min. Over time, the following eluent mixtures were used in gradient: t=0 min: 83% A 17% B -> t= 2-30 min: 100% A -> T=33-42 min: 30% A 35% B 35% C -> T=45-50 min: 100% B. Eluent D was used as a postcolumn flow. The column used was a Dionex CarboPac PA1 (length 250 mm – I.D. 4 mm) with precolumn.

Column oven temperature was set at 35ºC. Measurements were compared to standard runs, and corrected based on internal standard. Duplo measurements showed a standard deviation of less than 5 percent for all samples. Results were verified using a different sugar analysis machine and method (Eylen et al, 2011), no major irregularities were seen, and an average deviation between methods of 6% was observed.

HPLC method for organic acids and furan analysis

Samples were brought to room temperature prior to use. Samples were centrifuged for 7 minutes at 10000 RPM. The supernatant was diluted 5 times to ensure measurements within the calibration curve, and filtered using a 0.2 µm filter (Spartan, Whatman GmbH, Dassel, Germany). To 400 µl diluted sample, 400 µl internal standard consisting of 0.5 g/l phthalic acid was added. Samples were measured using HPLC equipment. The HPLC set-up consisted of a Waters 717plus autosampler, a Waters 1515 isocratic pump and column oven, a Waters 486 UV detector, and a waters 996 PDA (Milford, USA). Two columns were used to acquire sufficient separation. The first column was a Grace Preveal organic acid 5µm column (Length 250 mm – I.D. 4.6 mm), which was followed by a Shodex RSpak KC-811 ion exchange column (Length 300 mm – I.D. 8 mm). Acids and furans were determined via spectrometry at a wavelength of 210 nm, where PDA analysis at a range of 210-350nm was used to check for purity of components. As eluent, milliQ water supplemented with 250 µl/l 85% phosphoric acid (H3PO4) (3.7 mM) was used. The oven temperature was set at 60ºC, the flowrate was set at 1 ml/min. Samples were compared to standards to determine the concentration. Sample standard deviation in duplo measurements seen with this method did not exceed 7%.

Values for acetic acid were cross-checked using a different column in the same system. Using a Biorad aminex HPX-87H column (Length 300 mm – I.D. 7.8 mm), eluent 0.004M H2SO4 in milli-Q water, flowrate 0.6 ml/min, average differences in measurements between this method and double column method were less than 4%. Values for furfural and HMF were cross checked with the same Biorad aminex column on a different wavelength (280 nm), as well as on a different system (UPLC Dionex-3000) which was equipped with an Acquity UPLC BEH C18 RP column (length 50 mm – I.D. 2.1 mm) using an eluent of milliQ water with 2 ml/litre formic acid. The maximal difference observed between these three methods was 8%.

Enzymatic determination of formic acid

Due to the inability of the HPLC method to separate formic acid and pyruvic acid using HPLC, a formic acid determination enzyme kit manufactured by R-biopharm using UV (Roche, Switzerland) was used to determine the concentration of formic acid present in the different samples. Samples were diluted 10 times to ensure a measurement within the calibration curve.

ULC/MS method to determine phenolics

Prior to use, samples were brought to room temperature, centrifuged for 10 minutes at 4000 RPM, and supernatant was transferred to a clean eppendorf tube. These samples were diluted 10 times and filtered using a 0.2µm filter (Spartan, Whatman GmbH, Dassel, Germany). To determine the concentration of phenolic compounds, 100 µl of this 10x diluted sample was added to 900 µl milliQ water and 100 µl internal standard. ULC/MS analysis where performed on a Dionex RSLC system with an UltiMate 3000 Rapid Separation pump and auto-sampler. Phenolic compounds were detected at 280 nm with a Dionex Ultimate 3000 RS Diode Array Detector in combination with a Thermo ScientificTM LCQ Fleet Ion Trap Mass spectrometer. Phenolic compounds were separated using an Waters Acquity UPLC BEH C18 reversed phase column (length 150 mm – I.D. 2.1 mm, 1.7 µm particle size) with a sample loop of 100 µL. The guard column was a Waters VanGuard Acquity UPLC BEH C18 guard column (Length 5 mm – I.D. 2.1 mm, 1,7 µm particle size). The column temperature was maintained at 40°C. Elution was performed at a flow rate of 0.35 mL/min, using the following gradient (expressed as solvent B): initial composition: 4.0 % B, 0.0–1.0 min: 4.0 % B; 1.0–17.0 min: 56.0 % B; 17.0–18.0 min: 70.0 % B; 18.0–20.0 min: 70.0 % B; 20.0–21.0 min: 100 % B; 21.0–24.0 min: 100 % B; 24.0–25.0 min: 4.0 % B; 25.0–30.0 min: 4.0 % B. Eluent A consisted of Biosolve ULC/MS grade water with 2 mL/L acetic acid (MS positive modus) or Biosolve ULC/MS grade water with 50 mM ammonium acetate buffer (MS negative modus). Eluent B consisted of Biosolve ULC/MS grade acetonitrile. Heated electrospray ionization (HESI) mass spectrometry was performed in both positive and negative modes. The LCQ mass spectrometer was operated with the HESI set on 100°C and the capillary temperature at 130°C, sheath gas at 70 (arbitrary units), the auxiliary gas at 15 (arbitrary units) and the sweep gas at 2 (arbitrary units). The electrospray voltage was set to 3.5 kV. In the positive modus the capillary voltage was set at -22 V, and the tube lens offset at –89.89 V. In the negative modus the capillary voltage was set at 19 V, and the tube lens offset at 75 V. The injection time was 200 ms. Mass spectra were recorded from m/z 80–500 at unit mass resolution without in-source fragmentation. For sequential MS/MS experiments, the normalized collision energy was 35%, with wideband activation turned off. Several representative chromatograms are shown in the supplementary data. Concentrations were calculated based on standards with known concentrations.

Element analysis

30 different elements, including AL, Ca, Fe, K, Mg, Na, P, S and Si, were analysed using ICP (Varian vista PRO, Palo Alto, California, USA). Prior to analysis, the samples were centrifuged for 5 minutes at 8000 RPM. 1 gram supernatant was dissolved in 2 ml 70% nitric acid and 97 ml milliQ water. Concentrations were calculated based on standards with a known concentration.

RESULTS AND DISCUSSION

Bagasse has been pretreated to monomerize lignocellulosic sugars. The first step of this pretreatment is a thermo-chemical pretreatment. This pretreatment is performed either with the addition of acid, alkali, or without the addition of any chemicals. After this first pretreatment, the whole reactor was piston-pressed to acquire a solid-liquid separation. 3 types of liquid fractions can be distinguished (figure 1): liquid samples obtained after acid pretreatment (hence called acid liquid fraction), liquid samples acquired after alkaline pretreatment (alkaline liquid fraction), and liquid samples resulting from an autohydrolysis pretreatment (autohydrolysis liquid fraction).

The solid fraction, acquired after the solid-liquid separation, underwent several more processing steps. First, an (optional) washing stage was performed. Second, the solids were steam exploded. Afterwards, on a smaller scale, the solids were treated with enzymes to monomerize all sugars. The whole process acquired 5 different samples after enzyme treatment (figure 1): solid-derived samples which were initially pretreated with acid, and were washed (hence called washed solid-derived acid fraction) or unwashed (solid-derived acid fraction), solid derived samples which were initially pretreated without chemicals, and were washed (washed solid-derived autohydrolysed fraction) or unwashed (solid-derived autohydrolysed fraction), and solid-derived samples which were initially pretreated with alkali and washed (washed solid-derived alkaline fraction). Both the solid-derived and liquid fraction can be added up to form one total value of the amount of by-product formed (hence called total fraction).

Analysis were performed to determine the concentration of abundant acids, furans, phenolics and ions, in combination with the amounts of sugars present in each type of sample (Table 2). All values were recalculated to grams per kilogram initial dry weight bagasse, since concentrations are very subjective to the amount of initial biomass used.

Quantification of sugars in different fractions of pretreated bagasse

Analysis was performed to determine the amount of sugars present in both the liquid and solid-derived fraction (Table 2A). In the liquid fraction, acquired directly after chemical pretreatment, both monomeric sugars and oligomeric sugar polymers are present. In all three types of chemical pretreatment, the amount of polymeric sugar which could be depolymerized by enzymes in the liquid fraction was roughly 25 g/kg DW bagasse. The monomeric sugar content showed large differences. Very small amounts of monomeric sugars were found in the alkaline liquid fraction. On the other hand, a large amount of sugar monomers were found in the acid liquid fraction, with over 125 grams of sugars per kg of DW found after chemical pretreatment. The majority of these sugars was xylose, showing that a large monomerization of the hemicellulose occured during acid pretreatment. In the autohydrolysed liquid fraction, 56 grams per kg DW of sugars were found.

Table 2: *Presence of different compounds after pretreatment of bagasse, expressed in gram of compound per kilogram dry weight. Liquid fraction is acquired after the initial thermo-chemical pretreatment, Solid-derived fraction is acquired after enzyme treatment. N.D.: Not determined.* [1)] *sugar monomers are sugars present as monomers before enzymatic treatment, depolymerized sugars are sugars depolymerized during enzymatic treatment*

Average of run(s)	1,2,3	1,2	3	4,5,6	4	5,6	7,8,9	7,8,9
	Acid pretreated			**Autohydrolysis**			**Alkaline pretreated**	
A: Sugar monomers[1)]	Liquid Fraction	Solid derived fraction	Washed solid derived fraction	Liquid Fraction	Solid derived fraction	Washed solid derived fraction	Liquid Fraction	Washed solid derived fraction
Galactose	2.82	N.D.	N.D.	1.12	N.D.	N.D.	0.00	N.D.
Glucose	7.27	N.D.	N.D.	3.36	N.D.	N.D.	0.00	N.D.
Xylose	79.38	N.D.	N.D.	19.87	N.D.	N.D.	0.16	N.D.
Mannose	0.52	N.D.	N.D.	0.00	N.D.	N.D.	0.00	N.D.
Arabinose	9.70	N.D.	N.D.	2.63	N.D.	N.D.	0.00	N.D.
Depolymerized sugars[1)]								
Galactose	0.00	1.18	0.66	0.32	0.63	0.55	0.04	0.70
Glucose	6.98	438.09	417.41	6.23	401.26	435.35	4.46	337.09
Xylose	18.33	41.90	25.74	22.60	30.07	13.36	18.41	111.23
Mannose	0.90	0.58	1.12	0.74	0.82	0.90	0.35	0.17
Arabinose	0.00	2.48	1.45	0.00	1.25	1.05	0.00	1.62
Total sugars	***125.90***	***484.22***	***446.37***	***56.85***	***434.02***	***451.21***	***23.42***	***450.82***
B: Furans								
Furfural	7.73	4.33	0.18	18.87	2.79	0.09	0.07	0.00
HMF	0.98	1.30	0.00	2.61	1.41	0.00	0.00	0.17
Furoic acid	0.05	0.06	1.90	0.22	0.13	1.63	0.02	0.02
Total furans	***8.76***	***5.69***	***2.08***	***21.70***	***4.34***	***1.72***	***0.08***	***0.19***
C: Acids								
Acetic acid	13.93	10.17	3.01	20.17	13.98	3.57	33.01	7.12
Glutaric acid	0.52	0.60	0.15	0.30	0.38	0.13	0.11	0.79
Glycolic acid	4.54	8.28	6.47	12.42	9.94	8.39	7.53	7.31
Levulinic acid	8.37	13.02	0.69	38.82	18.24	1.16	4.97	1.65
Oxalic acid	0.75	0.29	0.13	0.66	0.28	0.16	1.27	0.24
Formic acid	1.22	2.08	0.21	3.08	1.69	0.11	7.12	0.19
Total acids	***29.33***	***34.45***	***10.66***	***75.44***	***44.51***	***13.52***	***54.00***	***17.31***

Table 2: *(continued)*

Average of run(s)	1,2,3	1,2	3	4,5,6	4	5,6	7,8,9	7,8,9
	Acid pretreated			**Autohydrolysis**			**Alkaline pretreated**	
	Liquid Fraction	Solid derived fraction	Washed solid derived fraction	Liquid Fraction	Solid derived fraction	Washed solid derived fraction	Liquid Fraction	Washed solid derived fraction
D: Phenols								
Coumaric acid	1.579	1.059	0.051	0.810	0.490	0.069	2.108	0.022
ferulic acid	0.189	0.152	0.024	0.107	0.067	0.018	0.106	0.005
4-hydroxybenzaldehyde	0.203	0.157	0.010	0.322	0.115	0.011	0.479	0.006
vanillin	0.386	0.135	0.020	0.212	0.088	0.015	0.094	0.002
4-hydroxyacetophenone	0.038	0.019	0.004	0.034	0.020	0.007	0.062	0.016
isovanillin	0.011	0.003	0.002	0.009	0.001	0.000	0.017	0.004
acetovanillone	0.000	0.000	0.000	0.000	0.000	0.000	0.052	0.012
acetosyringone	0.000	0.000	0.000	0.021	0.000	0.000	0.269	0.057
4-HB-Acid	0.100	0.045	0.000	0.054	0.036	0.012	0.050	0.113
Syringic Acid	0.063	0.074	0.160	0.065	0.054	0.123	0.096	0.060
Syringaldehyde	0.183	0.147	0.052	0.147	0.114	0.048	0.035	0.014
Total phenols	***2.753***	***1.792***	***0.322***	***1.782***	***0.986***	***0.302***	***3.367***	***0.310***
E: Ions								
Aluminium (Al)	0.06	0.04	0.03	0.03	0.05	0.03	0.00	0.17
Calcium (Ca)	0.54	0.18	0.23	0.34	0.18	0.19	0.09	1.00
Iron (Fe)	0.41	0.08	0.03	0.33	0.09	0.05	0.03	0.37
Potassium (K)	1.20	1.60	0.92	1.10	1.00	1.02	1.64	1.30
Magnesium (Mg)	0.42	0.15	0.10	0.36	0.18	0.15	0.03	0.69
Sodium (Na)	0.20	9.08	7.11	0.18	7.47	5.41	30.76	13.45
Phosphor (P)	0.20	0.73	0.70	0.19	0.69	0.68	0.13	0.89
Sulfur (S)	2.37	1.93	1.50	0.14	1.26	1.28	0.16	1.62
Silicon (Si)	0.53	0.28	0.24	1.00	0.29	0.27	0.21	0.60
Total ions	***5.93***	***14.04***	***10.86***	***3.68***	***11.21***	***9.08***	***33.05***	***20.11***

The most abundant sugar in the solid-derived fraction is glucose. In the solid-derived alkaline fraction, the xylose fraction is much larger than in the solid-derived autohydrolysed and acid fraction. The amount of xylose found in the solid-derived alkaline fraction was 110 g/kg DW bagasse, while the solid-derived autohydrolysis and acid fraction contained 30-40 g/kg DW bagasse. It can be concluded that were most hemicellulose in acid and autohydrolysis

pretreatment is solubilized, the hemicellulose largely remains part of the solid structure in alkaline pretreatment. All pretreatment methods yielded around 450 gram of monomerisable sugars per kg DW in the solid-derived fraction.

Using acid pretreatment on bagasse, combined with enzymatic hydrolysis, can result in a sugar monomerisation yield 600 gram sugars per kg DW bagasse, where alkaline and autohydrolysis pretreatment yield around 500 gram of monomeric sugars per kg DW bagasse (figure 2 and table 3A). According to literature, the sugar fraction in bagasse is 62-73% on a dry weight basis (Kurakake et al., 2001; Neureiter et al., 2002; Martín et al., 2007). When this data was used, it can be calculated that a combination of acid pretreatment, steam explosion and enzyme treatment resulted in a sugar monomerization of at least 82%. The main sugar loss in acid pretreated bagasse is in hemicellulose sugars. Using autohydrolysis and alkaline pretreatment, at least 65% of the sugars were monomerized during the whole pretreatment process. The main sugar loss in alkaline pretreatment is both in hemicellulose sugars as well as in glucans, in autohydrolysis lower sugar concentration is mainly due to a loss of hemicellulose sugars. The addition of a washing step led to a sugar content reduction of 8% in acid pretreated bagasse, while in autohydolysed bagasse the sugar content with a washing step was slightly higher, but not significantly with a difference of 3%.

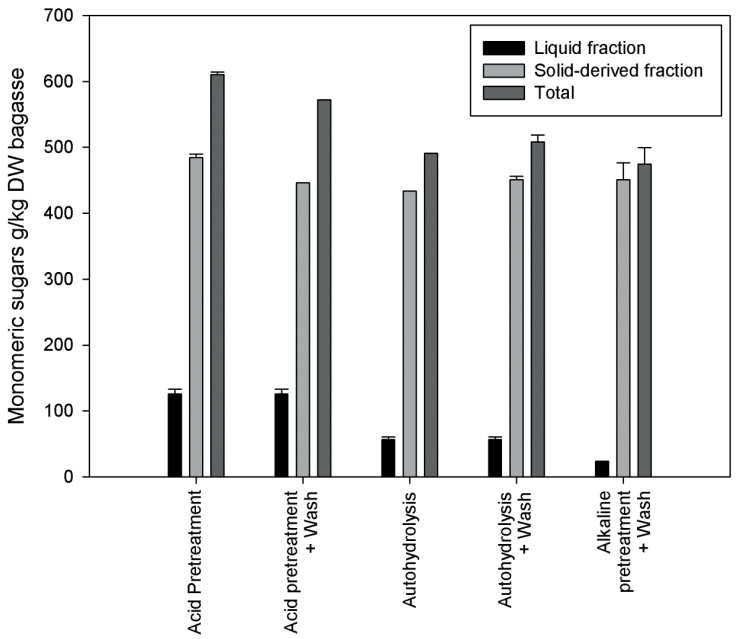


Figure 2: *The amount of monomeric sugars found after pretreatment in the solid-derived fraction and liquid fraction in grams of sugar per kg initial DW bagasse. The pretreatment consisted of a combination of chemical pretreatment, steam explosion and enzyme treatment. Standard deviations are based on multiple runs as shown in table 1.*

Table 3: *Sugar monomerization efficiencies of acid, autohydrolysis and alkaline pretreated material. A) monomeric sugars measured based on initial bagasse dry weight, including monomerization efficiency based on estimated polymerized sugars initially present in bagasse. B) Amount of sugars present in solid fraction based on chemical analysis, and enzymatic hydrolysis efficiency of these polymerized sugars.*

Table 3A (total fraction)	acid pretreatment	Autohydrolysis	Alkaline pretreatment
total sugars (g/kg initial DW)	597	502	474
Liquid fraction (g/kg initial DW)	126	57	23
Solid fraction (g/kg initial DW)	471	445	451
monomerized sugars (% initial polymerized sugars)	82-96	69-81	65-76
Table 3B (solid fraction)	**acid pretreatment**	**Autohydrolysis**	**Alkaline pretreatment**
Glucans present (g/kg DW)	480	480	520
% glucans monomerized	*81*	*79*	*59*
Xylans present (g/kg DW)	40	30	190
% Xylans monomerized	*80*	*66*	*52*
Total sugars (g/kg DW)	520	510	710
% total sugars monomerized	*81*	*77*	*56*

The solid material acquired after steam explosion was also analysed for polymeric sugar content using compositional analysis (Table 3B). Acid and autohydrolysis solid fraction contained 48% glucan and 3-4% xylan, while alkaline solid fraction contains 52% glucan and 19% xylan. Overall, the enzyme monomerization efficiency after 72h of acid pretreated material was 81% taking into account the weight of water required for hydrolysis, autohydrolysis has an enzyme monomerization efficiency of 77%, while the enzyme monomerization efficiency of alkaline pretreated material was much lower with 57%.

Presence of furans in pretreated bagasse

Furans are formed by when monomeric sugar molecules are dehydrated. The dehydration is stimulated in environments with a low pH and a high temperature (Dunlop, 1948). Furans can only be formed when monomeric sugars are released during the pretreatment.

After alkaline pretreatment of bagasse, only small amounts of furans are observed due to an absence of monomeric sugars, combined with the use of a high pH (Table 2B). In autohydrolysed and acid pretreatment, furans are found in a much larger quantity. Furfural and 5-Hydroxymethylfurfural (5-HMF), the two main furanic compounds, have different origins. Where furfural is a dehydrolysed pentose sugar, 5-HMF is a degraded hexose sugar. In bagasse, furfural is more abundant than 5-HMF, mainly due to the higher presence of monomeric pentoses such as xylose during the initial pretreatment. In the acid liquid fraction, 10% of the monomeric sugars are hexoses, while 90% of the monomeric sugars are pentoses.

In total, autohydrolysis results in a furfural formation of more than 20 gram per kg of DW bagasse, with most furfural being present in the liquid fraction. The presence of furfural in the acid solid-derived and liquid fraction is lower, but still considerable with 4.3 and 7.7 gram per kg DW bagasse respectively. Alkaline pretreatment did not result in significant furfural formation.

The furfural present in the solid-derived fraction after thermo-chemical pretreatment can be removed relatively easily by a washing step, reducing the amount of furans in solid-derived fraction by over 95%. Apart from furfural, smaller quantities of 5-HMF are formed, up to 2 gram per kg DW in acid pretreated and up to 4 gram per kg DW in autohydrolysis pretreated samples.

Furfural can be a main inhibitory by-product during fermentation. 1.6 gram per litre of furfural has been shown to decrease the growth rate of *Zymomonas mobilis* by 50%, while 1 gram per litre can severely inhibit some *Saccharomyces cerevisiae* strains (Delgenes et al., 1996; Franden et al., 2009). In fermentations performed at higher sugar concentrations acquired from acid pretreated or autohydrolysed bagasse, furfural can be a major inhibiting compound, and can therefore be seen as a major pitfall of acid and autohydrolysis pretreated material. Alkaline pretreated material did not contain any furfural, which can be an advantage during fermentation.

Presence of small organic acids in pretreated bagasse

Small organic acids can originate from different sources. The acids can already be present in the structure. An example is acetic acid, which is linked to the backbone of hemicellulosic xylans and arabinans (Sun et al., 2004). Thermo-chemical pretreatment can release these acids. The acids can also be formed during pretreatment. Examples are levulinic acid and formic acid, which are formed when furans are further dehydrated. Furthermore, micro-organisms can be present as contaminants in the substrate, producing a range of different acids, for example lactic acid.

Table 2C shows the presence of small organic acids in pretreated bagasse. The most abundant organic acid in acid and alkaline pretreated material is acetic acid. Where up to 23 grams of acetic acid per kg of DW bagasse can be present in total after acid pretreatment, over 40 grams per kg DW of acetic acid can be present in total after alkaline pretreatment. This equals an amount of up to 10 gram of acetic acid per 100 grams of sugar after alkaline pretreatment, while acid pretreated material contained around 4 gram of acetic acid per 100 grams of sugar. At low pH fermentations, 3.5 gram per litre of acetic acid can reduce growth rates by 33% in *S. cerevisiae*, while at higher pH 9 gram per litre of acetic acid has the same effect (Taherzadeh et al., 1997). It has been reported earlier that alkaline pretreatment enhances the deacetylation of hemicellulose (Kumar et al., 2009). High acetic acid concentrations can be a potential pitfall for fermentation of alkaline pretreated material feedstocks.

In autohydrolysis pretreated bagasse and to a lesser extent in acid pretreated bagasse, large amounts of furans are present, as shown in table 2B. In an environment with a low pH and high temperature, 5-HMF can dehydrate further to levulinic acid and formic acid. Autohydrolysis results in more than 55 grams of levulinic acid per kg DW bagasse. Acid pretreatment, which was slightly less severe, yielded 21 grams of levulinic acid per kg DW bagasse. In alkaline pretreated material, where no furans were formed, levulinic acid was found in an amount of 6 grams per kg DW bagasse.

Another small organic acid formed in large quantities is glycolic acid. It was found in concentrations up to 20 grams per kg DW after autohydrolysis. In acid and alkaline pretreated material, the formation was slightly lower with amounts measured of 12 and 15 gram per kg DW respectively. The presence of glycolic acid in substantial concentrations is typical for sugar-rich plants like sugarcane or sugar beets.

Lactic acid was not present in high concentrations. The presence of lactic acid was below the detection limit of 0.5 gram per kg DW bagasse.

In total, more 12% of the initial dry weight ended up as small organic acids in the case of autohydrolysis pretreatment. In alkaline pretreatment with a washing stage, 7% W/W of the initial dry weight ended up as small organic acids. Since alkaline pretreated material cannot be used without washing the material due to high sodium hydroxide addition, alkaline pretreatment was not performed without washing. However, when the washing efficiency of acid pretreated and autohydrolysed bagasse was used, it can be estimated that the total amount of acids in alkaline pretreatment without washing should be just below 10% W/W. Acid pretreated material yielded the lowest small acidic compound formation, only 6% W/W of the initial dry weight ended up as small organic acidic compounds.

Effect of sugar degradation on autohydrolysis yields

Due to large amounts of acids released during autohydrolysis pretreatment, the behaviour of the autohydrolysis pretreatment is expected to be similar to acid pretreatment. When comparing autohydrolysis and acid pretreatment, a large part of the xylose is missing in the autohydrolysed samples. The total amount of xylose found in alkaline pretreatment was 130 g/kg DW bagasse, in acid pretreatment 140 g/kg DW bagasse, while autohydrolysed bagasse contained only 70 g/kg DW bagasse. A lower hemicellulose monomerization efficiency and accessibility might occur due to the slightly higher pH compared to the acid pretreatment. However, higher xylose presence has not been observed in the solid fraction (Table 3), thus it is unlikely that this is the main reason for the missing xylose.

Degradation of sugars can also have an effect on the observed sugar concentrations. In an environment with elevated temperatures and a low pH, sugars which are monomerized can be dehydrated to form furfural and 5-HMF, and further dehydrated to levulinic acid, furoic acid and formic acid. In total, more than 80 gram per kg DW of furfural, HMF and levulinic acid is present in autohydrolysed material. The formation of these compounds is significantly lower

in acid pretreated material, where around 20 gram per kg DW of furans and levulinic acid are formed. The difference in furan and levulinic acid formation can explain the differences in xylose concentrations found between autohydrolysis and acid pretreatment. Although autohydrolysis is a cheaper method to perform due to absence of chemical addition, the degradation of hemicellulose sugars, and the formation of large amounts of potential inhibiting by-products make this method less interesting to use as pretreatment method for lignocellulose on a large scale.

Quantification and qualification of phenols in pretreated bagasse

Phenols are in quantity the smallest fraction of by-products formed during the pretreatment. However, they are in most cases more toxic for micro-organisms than for instance small organic acids (Klinke et al., 2003), and are therefore still important by-products of which the formation should be limited.

Among the abundant phenols found after pretreatment are coumaric acid and ferulic acid in total amounts up to 2.5 and 0.3 gram per kg DW bagasse respectively (Table 2D). In monocot-type lignocellulose such as sugarcane bagasse, both coumaric acid and ferulic acid have important structural properties in maintaining bonds between lignin and hemicellulose (Hatfield and Grabber, 1999). The release of coumaric and ferulic acid is therefore an indication of the degradation of the lignocellulosic structure. Besides pure ferulic acid and coumaric acid, dimers and oligomers are formed which contain one or more ferulate and/or coumarin subunit (table 4). In acid pretreated samples, coumarin-malic and ferulate-malic esters are observed, in line with earlier reports (Harbaum et al., 2007). Furthermore, coumarin-pentose

Table 4: *Qualitative analysis of important phenolic compounds found after pretreatment in the liquid fraction mass spectrometry (MS) combined with liquid chromatography (UHPLC).*

MS modus	Retention time	Mass	fractionation pattern (MS/MS)	present in	Compound
Positive	8.5	279	165;147;119	Acid-auto	Coumarin-malic ester
	8.65	95	N.D.	Acid-auto	Phenol
	9.11	309	195;177;145;117	Acid-auto	Ferulate-malic ester
	9.58	255	237;155;140;123	alkali	unknown syringate
	10.78	373	249;217;173;145;117	alkali	unknown ferulate
	10.81	179	161;147;133;119	Acid-auto	methoxycinnamic acid
	10.99	209	191;177;145	Acid-auto	dimethoxycinnamic acid
	11.01	343	219;147	alkali	unknown coumarin
	12.85	387	368;351,325;263;219;193	alkali	Diferulic acid
Negative	5.75	385	313;193	alkali	Diferulic acid
	9.11	325	265;235;217;193;175;149;134	Acid-auto	ferulate-mono pentose ester

sugar esters and ferulate-pentose sugar esters are observed. The pentose sugars are most likely arabinose (Hatfield and Grabber, 1999). In alkali pretreated material, two non-defined diferulic acids were found. One diferulic acid is likely to be 8-O-4 diferulic acid, based on earlier MS work (Appeldoorn et al., 2010; Vismeh et al., 2013). Also, two unknown esters of coumarin and ferulate were found with mass 342 and 372. Combined with the amounts of coumaric and ferulic acids formed, these observations indicate a loss of structure in the lignin-hemicellulose interaction, thus increasing the accessibility of the cellulose.

Other phenols which were found are 4-hydroxybenzaldehyde, vanillin, 4-hydroxybenzoic acid and syringaldehyde. 4-hydroxybenzaldehyde and syringaldehyde are building blocks of the lignin. Although concentrations of phenolic compounds were relatively small, 4-hydroxybenzaldehyde was found in much higher concentrations in alkaline pretreated bagasse with 0.5 gram per kg DW bagasse, compared to 0.2 gram per kg DW bagasse in acid pretreated bagasse. Since bagasse contains around 17-25% W/W lignin compounds (Kurakake et al., 2001; Neureiter et al., 2002; Martín et al., 2007), and the measured amounts of 4-hydroxybenzaldehyde and syringaldehyde are less than 0.1% W/W, it can be concluded that lignin degradation towards monomers is relatively small.

Where autohydrolysis and acid pretreatment resulted in the formation of phenolic acids and aldehydes, a large amount of phenolic ketones was observed in alkaline pretreated lignocellulose. The effect of a different pretreatment has a large influence on syringates. Where syringates were mainly observed as syringic acid in autohydrolysis and acid pretreatment, they were found as acetosyringone in alkali pretreated material. Furthermore a larger syringate based structure was found in alkali pretreated material (table 4).

Overall, autohydrolysis yielded the lowest amount of phenols, with a total of 2.7 grams phenolics per kg DW. Acid pretreated yielded in total around 4.5 gram per kg DW of phenols. Alkaline pretreatment yielded the highest amount of phenols in the liquid fraction, nearly twice as much as the liquid autohydrolysed fraction, and had a similar phenol concentration in the washed solid-derived fraction.

Metal and ion analysis in pretreated bagasse

Metals and ions are often overlooked as potential inhibitory by-product during fermentation processes. Plant material can contain large amounts of metals, especially when grown in an area with metal-rich soil. The thermo-chemical pretreatment also adds to the amounts of sulphur and sodium ions present in the bagasse after pretreatment.

In autohydrolysed material, a total of 15 gram of metals and ions per kg DW bagasse was found (table 2E). The main compound observed were sodium ions. Part of these sodium ion were added to ensure an optimal pH during enzyme treatment. Other metals and ions found in significant concentrations are sulphur, silicon and potassium.

Sulphuric acid was added in acid pretreatment. Therefore, enhanced concentrations of 2.4 gram sulphur ions per kg DW bagasse wer found in the liquid acid fraction. However, even

without washing the concentration sulphur ions in the solid-derived acid fraction was not elevated.

In alkaline pretreatment sodium hydroxide is added in large quantities. The presence of sodium ions in the liquid alkaline fraction is more than 30 gram per kg DW bagasse, which is much higher than the 0.2 gram per kg DW bagasse observed in the other two pretreatment methods. Even with a washing step, the amount of sodium ions is almost twice as high in the solid-derived alkaline fraction compared to the autohydrolysis and acid pretreatment, while much less sodium hydroxide was added during the enzymatic hydrolysis for pH control.

Effect of washing step on autohydrolysis and acid pretreated solid-derived fraction

In this research, the addition of a washing step was shown to be a good method to lower the amount of by-products present in the solid-derived fraction (figure 3). The concentration of most compounds in the solid material was significantly reduced by a washing step with superheated water. Furthermore the glucose yield compared to non-washed material was only not significantly decreased. The concentration of hemicellulose sugars in the solid-derived

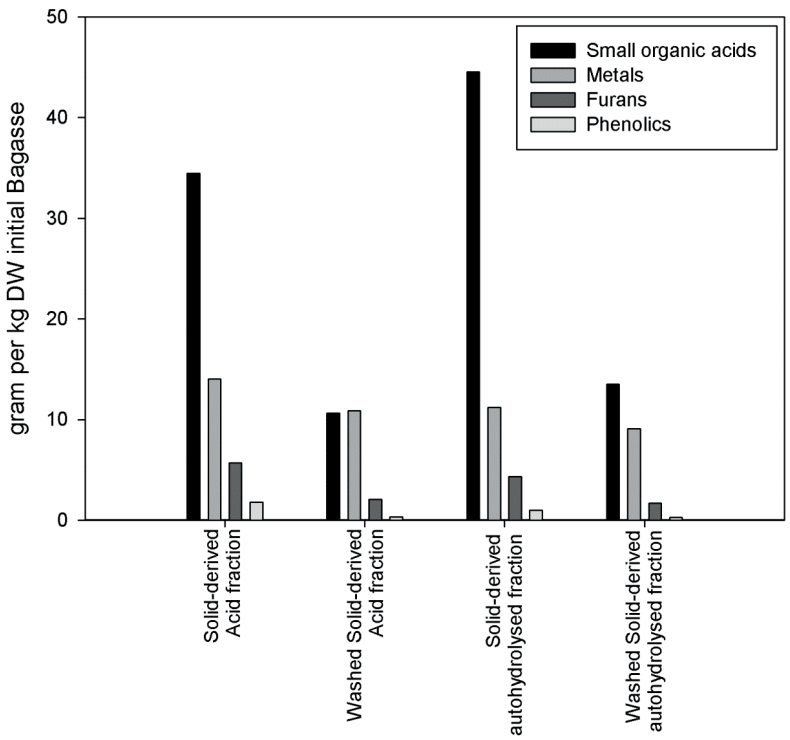


Figure 3: *Effect of washing with superheated water on the amount of by-products present in the solid-derived fractions.*

autohydrolysed and acid fraction after enzyme treatment was slightly lower when a washing step was introduced. Since most hemicellulose sugars are already present in the liquid fraction, this did not result in a major loss of sugar in these samples.

The presence of most compounds was significantly reduced in presence after the solid material was washed. These compounds include furfural, HMF, levulinic acid, acetic acid and all phenols. However, the presence of some compounds was not significantly reduced by a washing step, for instance glycolic acid and sodium. When samples were washed, furoic acid and syringic acid were present in higher concentration than in the non-washed samples. Either they are formed during the washing step, but most likely they are formed during the steam explosion stage of the pretreatment, which occurs after washing. The washing step is likely to cause a more neutral pH, and in combination with an elevated temperature furfural can be auto-oxidized to furoic acid (Dunlop 1948). This has earlier been observed for corn stover, pretreatment in a buffer of pH 7 with wet oxidation yielded high amounts of syringic acid yield (Du et al., 2010).

Overall, around 70% of the acids were removed. The amount of furans was reduced by 60%, the amount of phenols was reduced by 70 to 80%. On the other hand, only 20% of the ions were removed. In solid-derived acid fraction, washing resulted in a sugar loss of 8%, which was mainly xylose. In the autohydrolysis pretreated samples, a large fraction of the xylose was also removed, but the glucose yield was enhanced slightly.

CONCLUSIONS

This paper has given an overview of the strengths and weaknesses of different pretreatment methods. When looking at sugar monomerization efficiencies, acid pretreatment is the best choice. Acid pretreatment also shows the lowest formation of small organic acids. Where autohydrolysis and acid pretreatment have a high furan formation, alkaline pretreatment does not result in furan formation. Autohydrolysis has the highest acid and furan formation, but the lowest phenol and ion formation. A washing step is beneficial. It can lower the presence of organic acids, furans and phenols by 60-80%, while the loss of sugar is relatively small.

ACKNOWLEDGEMENT

The authors of this paper would like to thank the whole BE-Basic consortium for funding this project, with special thanks for Corbion and DSM. Furthermore, the authors would like to acknowledge the laboratory of Food chemistry, Wageningen UR, for performing several analysis performed on the pretreated bagasse.

SUPPLEMENTARY DATA

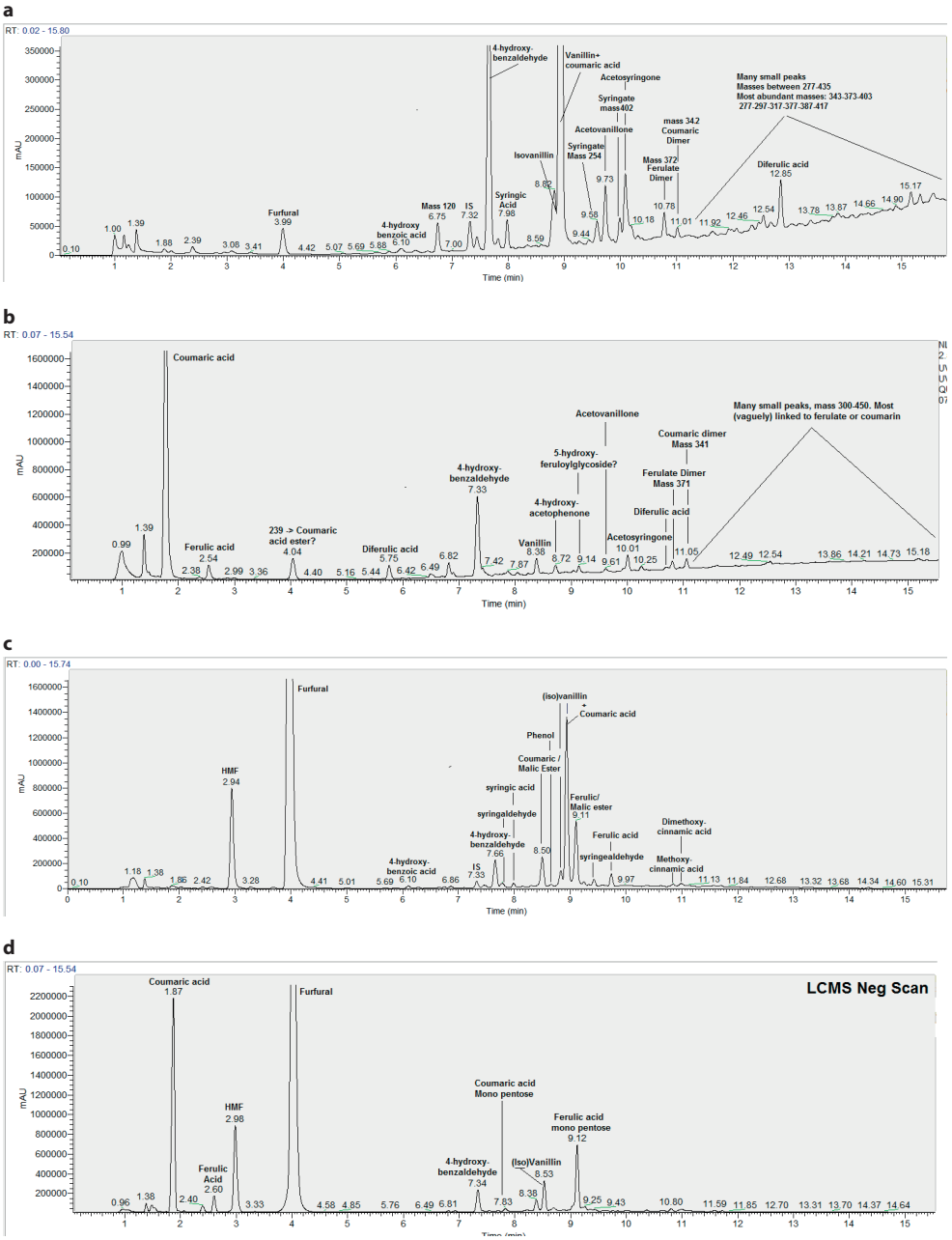


Figure A: *Representative ULC/MS graphs of acid and alkaline pretreated samples in positive and negative mode. a: alkaline pretreated material, positive scan, b: alkaline pretreated material, negative scan, c: acid pretreated material, positive scan, d: acid pretreated material, negative scan*

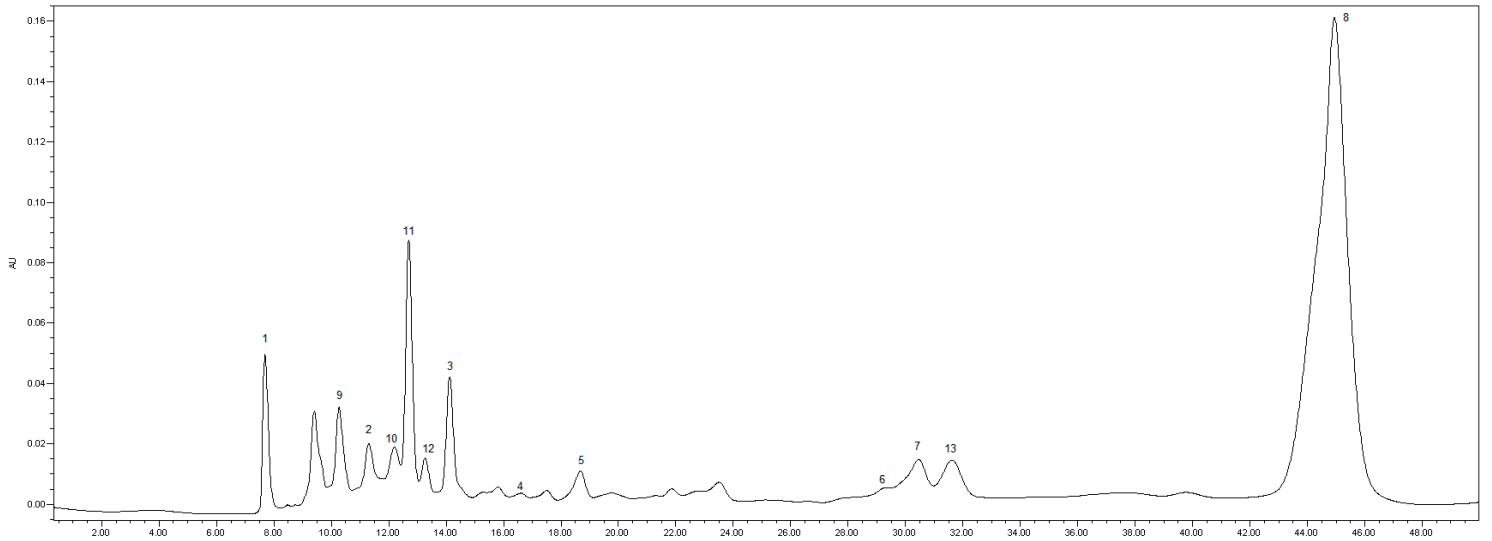


Figure B: *Representative HPLC chromatogram of the liquid stream of bagasse treated with 0.76% sulphuric acid. The following peaks are identified: 1: oxalic acid, 2: glycolic acid, 3: acetic acid, 4: glutaric acid, 5: levulinic acid, 6: Furoic acid, 7: 5-hydroxymethylfurfural, 8: furfural. Several peaks were identified, of which data was not used due to different circumstances: 9: citric acid and galacturonic acid, 10: formic acid, 11: Lactic acid, 12: succinic acid, 13: 4-hydroxybenzaldehyde.*

REFERENCES

Appeldoorn, M.M., Kabel, M.A., Van Eylen, D., Gruppen, H., Schols, H.A. 2010. Characterization of Oligomeric Xylan Structures from Corn Fiber Resistant to Pretreatment and Simultaneous Saccharification and Fermentation. J. Agric. Food Chem. 58, 11294-11301.

Chen S-F., Mowery R.A., Castleberry V.A., van Walsum G.P., Chambliss C.K. 2006. High-performance liquid chromatography method for simultaneous determination of aliphatic acid, aromatic acid and neutral degradation products in biomass pretreatment hydrolysates. J. Chromatogr. A 1104, 54-61.

Du, B., Sharma, L.N., Becker, C., Chen, S.-F., Mowery, R.A., van Walsum, G.P., Chambliss, C.K. 2010. Effect of varying feedstock–pretreatment chemistry combinations on the formation and accumulation of potentially inhibitory degradation products in biomass hydrolysates. Biotechnol. Bioeng. 107, 430-440.

Dunlop, A.P. 1948. Furfural formation and behavior. Ind. Eng. Chem. 40, 204-209.

Eylen, D. van, Dongen, F. van, Kabel, M, Bont, J. de. Corn fiber, cobs and stover: Enzyme-aided saccharification and co-fermentation after dilute acid pretreatment. Bioresource Technol. 102, 5995-6004.

Fengel, D., Wegener, G. 1984. Wood: Chemistry, ultrastructure, reactions, first ed. De Gruyter, Berlin.

Franden M.A., Pienkos P.T., Zhang M. 2009. Development of a high-throughput method to evaluate the impact of inhibitory compounds from lignocellulosic hydrolysates on the growth of Zymomonas mobilis. J. Biotechnol. 144, 259-267.

Harbaum, B., Hubbermann, E.M., Wolff, C., Herges, R., Zhu, Z., Schwarz, K. 2007. Identification of Flavonoids and Hydroxycinnamic Acids in Pak Choi Varieties (Brassica campestris L. ssp. chinensis var. communis) by HPLC–ESI-MS n and NMR and Their Quantification by HPLC–DAD. J. Agric. Food Chem. 55, 8251-8260.

Hatfield, R.D.R., John., Grabber, J.H. 1999. Cell wall cross-linking by ferulates and diferulates in grasses. J. Sci. Food Agr. 79, 403-407.

Humbird D., Davis R., Tao L., Kinchin C., Hsu D., Aden A., Schoen P., Lukas J., Olthof B., Worley M., Sexton D., Dudgeon D. 2011. Process design and economics for biochemical conversion of lignocellulosic biomass to ethanol. NREL http://www.researchgate.net/publication/229843699_Process_Design_and_Economics_for_Biochemical_Conversion_of_Lignocellulosic_Biomass_to_Ethanol_Dilute-Acid_Pretreatment_and_Enzymatic_Hydrolysis_of_Corn_Stover/file/9fcfd5011638d5a2af.pdf Accessed 12 Dec 2014.

Klemm, D., Heublein, B., Fink, H.-P., Bohn, A. 2005. Cellulose: Fascinating Biopolymer and Sustainable Raw Material. Angew. Chem. Int. Ed. 44, 3358-3393.

Klinke H.B., Ahring B.K., Schmidt A.S., Thomsen A.B. 2002. Characterization of degradation products from alkaline wet oxidation of wheat straw. Bioresource Technol. 82, 15-26.

Klinke, H.B., Olsson, L., Thomsen, A.B., Ahring, B.K. 2003. Potential inhibitors from wet oxidation of wheat straw and their effect on ethanol production of Saccharomyces cerevisiae: Wet oxidation and fermentation by yeast. Biotechnol. Bioeng. 81, 738-747.

Kumar, P., Barrett, D.M., Delwiche, M.J., Stroeve, P. 2009. Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production. Ind. Eng. Chem. Res. 48, 3713-3729.

Kurakake, M., Kisaka, W., Ouchi, K., Komaki, T. 2001. Pretreatment with ammonia water for enzymatic hydrolysis of corn husk, bagasse, and switchgrass. Appl. Biochem. Biotechnol. 90, 251-259.

Lashof, D.A., Ahuja, D.R. 1990. Relative contributions of greenhouse gas emissions to global warming. Nature 344, 529-531.

Martín, C., Klinke, H.B., Thomsen, A.B. 2007. Wet oxidation as a pretreatment method for enhancing the enzymatic convertibility of sugarcane bagasse. Enzyme Microb. Technol. 40, 426-432.

McMillan J., Adney W.S., Mielenz J.R., Klasson K.T., Thomsen M.H., Thygesen A., Jørgensen H., Larsen J., Christensen B.H., Thomsen A.B. 2006. Preliminary Results on Optimization of Pilot Scale Pretreatment of Wheat Straw Used in Coproduction of Bioethanol and Electricity. Appl. Biochem. Biotech. 130, 448-460. doi:10.1385/ABAB:130:1:448.

Negro, M.J., Manzanares, P., Oliva, J.M., Ballesteros, I., Ballesteros, M. 2003. Changes in various physical/chemical parameters of Pinus pinaster wood after steam explosion pretreatment. Biomass Bioenergy. 25, 301-308.

Neureiter, M., Danner, H., Thomasser, C., Saidi, B., Braun, R. 2002. Dilute-acid hydrolysis of sugarcane bagasse at varying conditions. Appl Biochem Biotechnol. 98-100, 49-58.

Sun, J.X., Sun, X.F., Sun, R.C., Su, Y.Q. 2004. Fractional extraction and structural characterization of sugarcane bagasse hemicelluloses. Carbohydr. Polym. 56, 195-204.

Taherzadeh M.J., Niklasson C., Lidén G. 1997. Acetic acid—friend or foe in anaerobic batch conversion of glucose to ethanol by Saccharomyces cerevisiae? Chem. Eng. Sci. 52, 2653-2659.

Teeri, T.T. 1997. Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. Trends Biotechnol. 15, 160-167.

Van der Pol E.C., Bakker R.R., Baets P., Eggink G. 2014. By-products resulting from lignocellulose pretreatment and their inhibitory effect on fermentations for (bio)chemicals and fuels. Appl. Microbiol. Biotechnol. 98, 9579-9593.

Vismeh, R., Lu, F., Chundawat, S.P.S., Humpula, J.F., Azarpira, A., Balan, V., Dale, B.E., Ralph, J., Jones, A.D. 2013. Profiling of diferulates (plant cell wall cross-linkers) using ultrahigh-performance liquid chromatography-tandem mass spectrometry. Analyst, 138, 6683-6692.

Wong H.H., Albertson P., O'Hara I.M. 2011. An update on the Mackay renewable biocommodities pilot plant: Preliminary trial results.Proc Aust Soc Sugar Cane Technol 1-11. http://eprints.qut.edu.au/56547/ Accessed: 15-12-2014

Wu, C-S, 2009. Renewable resource-based composites of recycled natural fibers and maleated polylactide bioplastic: Characterization and biodegradability. Polym. Degrad. Stabil. 94, 1076-1084.

CHAPTER 4

Identifying inhibitory effects of lignocellulosic by-products on growth of lactic acid producing micro-organisms using a rapid small scale screening method

This chapter has been submitted for publication as:
van der Pol E.C., Vaessen E., Weusthuis R.A., Eggink G.

ABSTRACT

Sugars obtained from pretreated lignocellulose are interesting as substrate for the production of lactic acid in fermentation processes. However, by-products formed during pretreatment of lignocellulose can inhibit microbial growth. In this study, a small-scale rapid screening method was used to identify inhibitory effects of single and combined by-products on growth of lactic acid producing micro-organisms. The small-scale screening was performed in 48-well plates using 5 bacterial species and 12 by-products. Large differences were observed in inhibitory effects of by-products between different species. Predictions can be made for growth behaviour of different micro-organisms on acid pretreated or alkaline pretreated bagasse substrates using data from the small-scale screening. Both individual and combined inhibition effects were shown to be important parameters to predict growth. Synergy between coumaric acid, formic acid and acetic acid is a key inhibitory parameter in alkaline pretreated lignocellulose, while furfural is a key inhibitor in acid pretreated lignocellulose.

INTRODUCTION

Poly-lactic acid (PLA) is a renewable alternative for petrochemically derived plastics such as polyethylene (PE) and polystyrene (PS) (Garlotta et al., 2001). Lignocellulose is an interesting renewable carbon source, which can be used as feedstock in lactic acid producing fermentation processes (van der Pol et al., 2014).

Thermo-chemical pretreatment and enzymatic hydrolysis are required to depolymerize lignocellulose to fermentable monomeric sugars. Conditions used during chemical pretreatment are often severe, using temperatures up to 200ºC in combination with the presence of chemicals such as sulphuric acid or sodium hydroxide (Hendriks and Zeeman, 2009). Although thermo-chemical pretreatment is an efficient method to increase accessibility of lignocellulosic sugars, unwanted by-products can be formed (van der Pol et al., 2015). These by-products can be divided in three main categories: furans, phenols and organic acids. Furans such as furfural, 5-hydroxymethylfurfural (HMF) and furoic acid are formed when monomeric sugars are exposed to high temperatures in an acidic environment (Kabel et al., 2007). Phenols, ferulates and coumarins are formed when lignin is degraded, or when the cross-links between hemicellulose and lignin are broken (Hatfield et al., 1999; Boerjan et al., 2003). Several organic acids are present in the hemicellulosic structure that are released when hemicellulose is depolymerized, e.g. acetic acid (Sun et al., 2004). Organic acids can be also formed when furans are dehydrated to levulinic acid and formic acid. Furthermore, oxidation of sugars can lead to the formation of formic acid and acetic acid during alkaline pretreatment in the presence of oxygen (Klinke et al., 2003). The type and quantity of by-products found in pretreated lignocellulose is mainly influenced by the pretreatment method and/or the lignocellulose composition of the crop species (van der Pol et al., 2014).

By-products can have an inhibitory effect on micro-organisms in fermentation processes, negatively effecting overall process performance. Currently, most studies on toxicity of by-products focus on ethanol producing yeast, using a limited amount of by-products in only one or a few concentrations (Delgenes et al., 1996; Larsson et al., 2000; Klinke et al., 2003). Combinations of different by-products can have synergistic inhibitory effects on growth of yeast strains (Taherzadeh et al., 1999; Oliva et al., 2004).

The toxicity of by-products on lactic acid producing strains has been investigated to a limited extent (Bisschof et al., 2010, Guo et al., 2010). More information is required to assess the suitability of lactic acid producing strains for the conversion of lignocellulosic hydrolysates. A complete evaluation is however not possible, since it requires toxicity analysis of a dozen by-products, at several concentrations, in several combinations on a number of strains, requiring billions of experiments.

In this research, a rapid screening method was used to identify inhibition effects of 12 lignocellulosic by-products, both individually and combined. Inhibition effects of individual by-products were evaluated for 5 lactic acid producing strains. This screening was used to

identify differences between microbial strains in their initial response towards the presence of by-products. Two parameters were determined during this screening. First, concentrations were determined, where by-products become inhibitory for the micro-organism. Furthermore, when all results for individual inhibition were combined, an overall sensitivity of micro-organisms towards by-products can be determined. Based on the overall sensitivity, 3 lactic acid producing strains, showing the highest potential in the individual screening, were assessed for synergistic inhibitory effect between combinations of by-products. The concentrations used for this synergy experiment were determined in the individual screening, and are the concentrations at which the by-products become inhibitory.

MATERIALS AND METHODS

Chemicals

All chemicals were ordered at Sigma-Aldrich (St. Louis, USA), and had a purity of at least 98% with the exception of formic acid, which was 95% pure. Medium components such as peptone, glucose, yeast extract and BIS-Tris were ordered at Duchefa (The Netherlands). Premixed MRS medium was ordered at Merck chemicals (Germany). All compounds tested for their inhibitory effect are shown in table 1.

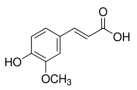
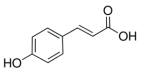
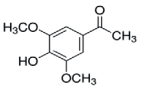
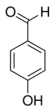
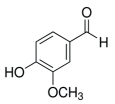
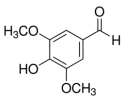
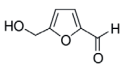
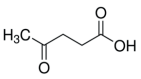
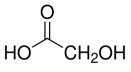
Micro-organisms

Lactobacillus casei DSM20011, *Lactobacillus delbrueckii* DSM20073, *Lactococcus lactis* DSM20481, *Bacillus coagulans* DSM2314 and *Bacillus smithii* DSM4216 were obtained as freeze dried stocks from the German collection of micro-organisms and cell cultures (DSMZ, Germany). Strain selection criteria used to choose these 5 strains were maximum productivities of lactic acid of at least 2 gram per litre per hour, capable of producing optically pure lactic acid, and converting glucose to lactic acid with a yield of at least 80 percent on a weight basis (Akerberg et al., 1998; Hofvendahl et al., 1999; González-vara et al., 1999; Michelson et al., 2006; Maas et al., 2008). Cells were suspended for 30 minutes in 5 ml PYPD medium, consisting of 5 g/l yeast extract, 10 g/l peptone, 20 g/l glucose and 10 g/l BIS-Tris. After 30 min pre-incubation, cells were transferred to 50 ml anaerobic flasks containing 45 ml fresh PYPD medium, sealed with a rubber cap, and incubated in these flasks for 16 hours to an optical density at 660 nm of around 2. After addition of glycerol to reach a concentration of 15% v/v in the sample, cells were stored in 1.5 ml aliquots in cryovials at -80ºC until used.

Media and solutions

L. delbrueckii was grown on MRS medium (de Man et al., 1960). All other strains were grown on PYPD medium. Both media were autoclaved for 20 minutes at 121ºC prior to use.

Table 1: *By-products often observed in higher concentrations in pretreated lignocellulosic material, as measured in a previous study (van der Pol et al., 2015). These by-products are used in the small-scale screening experiment.*

Compound	Molecular weight (g/mol)	Molecule type	Structure
Ferulic Acid	194	Phenol, acid	
p-Coumaric acid	164	Phenol, acid	
Acetosyringone	196	Phenol	
4-Hydroxybenzalde-hyde	122	Phenol	
Vanillin	152	Phenol	
Syringaldehyde	182	Phenol	
5-Hydroxymethylfurfural	126	Furan	
Furfural	96	Furan	
Levulinic acid	116	Acid	
Formic acid	46	Acid	
Acetic acid	60	Acid	
Glycolic acid	76	Acid	

All pure lignocellulosic by-products were dissolved in milliQ water. Stock solutions to test individual inhibitory effects were made in the following concentrations; phenols were dissolved in concentrations of 5 g/l, furans in concentrations of 10 g/l and small organic acids in 20 g/l. Stock solutions used to test synergistic effects had the following concentrations: Ferulic acid and coumaric acid 6 g/l, other phenols and furans 10 g/l, small organic acids 40 g/l. Acidic chemicals were pH adjusted to pH 6 using 4M KOH prior to use. All stock solutions were heated for 1 hour at 85ºC instead of being autoclaved to prevent thermal degradation.

Experiments in 48-well plates: cultivation and analysis

Cultivation was performed in Costar 48-well plates (Corning, New York, USA) with a working volume of 1 ml. 500 µl of 2x concentrated sterile PYPD medium was added to each well. Stocks solutions of pure lignocellulosic by-products were added to obtain the right concentration of by-product in each well, and sterile milliQ water was added to reach a final working volume of 1 ml.

Cultivations were performed in a Bactron II anaerobic chamber (Sheldon, Oregon, USA) for 16-18 hours, using pressurized gas with 4% v/v H2, 15% v/v CO2 and 81% v/v N2 to create an anaerobic chamber. To keep evaporation in the wells within 10% v/v, humidity was increased by placing beakers with water in the incubation room of the anaerobic chamber. The 4 corner wells experienced more than 10% v/v evaporation, and were therefore not used in the experiment. Each 48-well plate contained 4 negative control wells (PYPD without micro-organism), and 4 reference wells (PYPD with micro-organisms and without lignocellulosic by-products). The micro-organisms in the 48-well plates were incubated at temperature optima for the micro-organism based on literature (González-vara et al., 1999; Michelson et al., 2006; Akerberg et al., 1998; Hofvendahl et al., 1999; Maas et al., 2008) and cultivation times based on when the reference wells reached an OD660 of 1. The following cultivation conditions were chosen: *L. casei* was grown at 37ºC for 18h, *L. delbrueckii* was grown at 45ºC for 16h, *L. lactis* was grown at 30ºC for 17h, *B. coagulans* was grown at 50 ºC for 16h, and *B. smithii* was grown at 50ºC for 17h. Each concentration of lignocellulosic by-product was tested in triplicate, thus 12 different experiments can be performed per 48-well plate. Average standard deviations observed between triplicate experiments were 7%.

Inoculation occurred with 0.5% v/v cell stock coming directly from thawed -80ºC cryovials, acquiring an initial cell optical density at 660 nm (OD660) around 0.01. The OD660 of the 48-well plates was measured using a plate reader (Tecan, Switzerland) before and after fermentation. Relative growth was obtained with the following formula:

$$relative\ growth\ (\%) = \frac{(A_{ferm,t=end} - A_{ferm,t=end})}{(A_{ref,t=end} - A_{ref,t=end})}$$

Where A is the measured OD at 660nm, t=end is time at the end of the cultivation, t=0 is the time at the start of the cultivation, ref is the average of four reference wells, and ferm is the average of triplicate wells with a certain concentration of a pure lignocellulosic by-product.

Anaerobic flasks: cultivation and analysis

Experiments were performed in 60 ml glass anaerobic flasks with a working volume of 50 ml. The flasks were sealed off by rubber stoppers, which was kept in place with an aluminium crimp cap. The cultivation conditions used in the flask experiments were similar to the 48-well plates with regard to temperature, medium and inoculum size.

All glass flasks and rubber stoppers were autoclaved at 121ºC for 20 minutes prior to the cultivation. Simultaneously, a 2x concentrated medium and milliQ water was autoclaved. The pure lignocellulose by-product stock solutions were heated at 85ºC for 1 hour.

In table 2, 4 mixtures used to simulate alkaline or acid pretreated lignocellulose, alk-75, alk-150, acid-75 and acid-150, are shown. Every 50 ml anaerobic flask was filled with 25 of 2x concentrated medium, 25 ml of 2x concentrated alk-75,alk-150, acid-75 or acid-150 mixture, and inoculated with 250 µl of freezer stock (0.5% v/v).

Sampling was performed through the rubber stopper using sterile syringes and needles. Of every sample of 1.5 ml, 1 ml was used for determining the OD660 using a Ultrospec 200 spectrophotometer (Pharmacia Biotech, Sweden) and 0.5 ml was used to determine lactic

Table 2: *Mixtures of by-products as used in 50 ml cultivation. The composition of the mixtures resemble alkaline and acid pretreated sugarcane bagasse lignocellulose, in either 75 g/l sugar equivalent (Alkali-75 and Acid-75) or 150 g/l sugar equivalent (Alkali-150 and Acid-150) as measured previously (van der Pol et al., 2015).*

	Concentration in mixture (g/L)			
Lignocellulose by-product	Alkali-75	Alkali-150	Acid-75	Acid-150
Acetic acid	6.75	13.5	2.34	4.68
Glycolic acid	2.49	4.98	1.61	3.21
Levulinic acid	1.11	2.22	1.55	3.09
Formic acid	1.24	2.48	0.214	0.428
Furfural	0	0	1.22	2.45
HMF	0	0	0.142	0.284
Coumaric acid	0.354	0.708	0.215	0.431
Ferulic acid	0.018	0.036	0.031	0.062
4-hydroxybenzaldehyde	0.081	0.162	0.030	0.060
Vanillin	0.021	0.042	0.051	0.102
Syringaldehyde	0.023	0.045	0.031	0.062

acid concentrations using a Waters 717 HPLC system equipped with a Shodex RS-PAK KC-811 column as previously described (van der Pol et al., 2015).

RESULTS AND DISCUSSION

Experimental set up

To determine the individual and combined effects of 12 by-products in 6 different concentrations in triplicate for 5 strains, billions of fermentation experiments are required. Clearly, this asks for a simplified approach.

When only individual effects of by-products would be studied at 6 different concentrations in triplicate for 5 strains, 1080 experiments would still need to be performed. Preparing and sampling over 1000 anaerobic flasks will require extensive labour. As a solution, a rapid screening for initial growth in 48-well plates can be performed. Effects of individual by-products for 5 different strains in triplicate can be identified by using only 30 48-well plates. Therefore, this can be seen as a suitable first step in the screening of inhibitory effects of by-products. The concentrations at which individual by-products become inhibitory for a micro-organism were determined during the first screening. These concentrations can be used in synergy experiments, where inhibitory effects between combinations of by-products can be found using only 5.5 48-well plates per strain.

Determination of lactic acid concentrations may be difficult in screening experiments, since some by-products can interfere with enzyme assays used to determine lactic acid. Moreover, determining lactic acid for thousands of samples by other methods such as HPLC will require too much time. For ethanol producing micro-organisms, a correlation between by-product inhibition on growth and on ethanol production was observed (van der Pol et al., 2014). A similar correlation between inhibition of growth and inhibition of lactic acid production was found for *B. coagulans* DSM2314 and *B. smithii* DSM4216 in this research (See supplementary data). Therefore, determining only growth during a screening experiment provides a good indication of the overall process performance. Analysis of optical density in 48-well plates is simple and can be operated at high throughput rates. The effect of by-products on lactic acid producing micro-organisms was therefore determined by assessing growth rates using OD660 measurements.

Growth of lactic acid producing micro-organisms in presence of single by-products

In total, 12 different by-products were tested in the screening experiment. Six of the lignocellulosic by-products tested were phenolics, of which one was a phenolic ketone, two were phenolic acids, and three were phenolic aldehydes (table 1). Although syringaldehyde, vanillin and 4-hydroxybenzaldehyde are very similar in structure, their inhibitory potential differed

significantly (Table 3). 4-hydroxybenzaldehyde was observed to be more inhibitory than vanillin and syringealdehyde. The average growth inhibition for the 5 lactic acid producing micro-organisms in the presence of 1 g/l 4-hydroxybenzaldehyde, vanillin and syringaldehyde was

Table 3: *Growth of L. casei, L delbrueckii, L. lactis, B. coagulans and B. smithii in 48-well plates in the presence of 6 concentrations of 12 lignocellulosic by-products. The percentage shown is the relative growth compared to a fully-grown reference culture in the absence of lignocellulosic by-products.*

Lignocellulose by-product (g/l)		*Relative growth relation to reference in %*				
Ferulic Acid		*L. casei*	*L. delbrueckii*	*L. lactis*	*B. coagulans*	*B. smithii*
	0.1	100%	101%	103%	102%	89%
	0.25	100%	108%	109%	121%	87%
	0.5	97%	101%	92%	126%	67%
	0.75	93%	85%	100%	117%	27%
	1	82%	15%	75%	109%	19%
	2.5	42%	2%	7%	18%	-3%
Coumaric Acid		*L. casei*	*L. delbrueckii*	*L. lactis*	*B. coagulans*	*B. smithii*
	0.1	104%	108%	109%	110%	97%
	0.25	109%	106%	114%	123%	95%
	0.5	105%	91%	110%	127%	78%
	0.75	96%	77%	52%	115%	46%
	1	91%	66%	22%	116%	26%
	2.5	24%	5%	2%	0%	5%
4-Hydroxybenzaldehyde		*L. casei*	*L. delbrueckii*	*L. lactis*	*B. coagulans*	*B. smithii*
	0.1	94%	95%	115%	106%	104%
	0.25	93%	76%	104%	114%	100%
	0.5	73%	44%	111%	98%	79%
	0.75	68%	18%	118%	7%	34%
	1	55%	7%	129%	0%	7%
	2.5	1%	3%	1%	-1%	9%
Vanillin		*L. casei*	*L. delbrueckii*	*L. lactis*	*B. coagulans*	*B. smithii*
	0.1	107%	107%	118%	116%	97%
	0.25	107%	109%	117%	117%	96%
	0.5	98%	68%	121%	113%	89%
	0.75	84%	21%	118%	101%	68%
	1	64%	8%	121%	94%	43%
	2.5	11%	4%	69%	27%	6%

Table 3: *Growth of L. casei, L delbrueckii, L. lactis, B. coagulans and B. smithii in 48-well plates in the presence of 6 concentrations of 12 lignocellulosic by-products. The percentage shown is the relative growth compared to a fully-grown reference culture in the absence of lignocellulosic by-products. (continued)*

Lignocellulose by-product (g/l)		*Relative growth relation to reference in %*				
Syringaldehyde		*L. casei*	*L. delbrueckii*	*L. lactis*	*B. coagulans*	*B. smithii*
	0.1	97%	111%	107%	99%	103%
	0.25	103%	111%	101%	101%	106%
	0.5	94%	104%	99%	93%	104%
	0.75	89%	91%	99%	88%	100%
	1	85%	85%	97%	81%	99%
	2.5	57%	49%	74%	57%	24%
Acetosyringone		*L. casei*	*L. delbrueckii*	*L. lactis*	*B. coagulans*	*B. smithii*
	0.1	95%	99%	107%	108%	105%
	0.25	97%	107%	111%	101%	103%
	0.5	87%	111%	107%	105%	102%
	0.75	83%	105%	105%	108%	97%
	1	71%	94%	110%	109%	92%
	2.5	40%	62%	77%	103%	16%
5-HMF		*L. casei*	*L. delbrueckii*	*L. lactis*	*B. coagulans*	*B. smithii*
	0.25	95%	102%	105%	115%	89%
	0.5	96%	109%	103%	115%	84%
	0.75	93%	93%	89%	118%	70%
	1	81%	96%	91%	111%	48%
	2.5	41%	12%	39%	29%	-1%
	5	7%	5%	4%	3%	1%
Furfural		*L. casei*	*L. delbrueckii*	*L. lactis*	*B. coagulans*	*B. smithii*
	0.25	102%	115%	115%	103%	106%
	0.5	99%	110%	113%	94%	107%
	0.75	97%	103%	94%	20%	102%
	1	84%	72%	80%	7%	47%
	2.5	57%	6%	37%	-1%	1%
	5	11%	4%	7%	0%	2%

Table 3: *Growth of L. casei, L delbrueckii, L. lactis, B. coagulans and B. smithii in 48-well plates in the presence of 6 concentrations of 12 lignocellulosic by-products. The percentage shown is the relative growth compared to a fully-grown reference culture in the absence of lignocellulosic by-products. (continued)*

Lignocellulose by-product (g/l)		*Relative growth relation to reference in %*				
Formic acid		*L. casei*	*L. delbrueckii*	*L. lactis*	*B. coagulans*	*B. smithii*
	0.5	105%	133%	103%	92%	99%
	1	92%	129%	100%	94%	95%
	2.5	88%	101%	90%	71%	77%
	5	75%	86%	75%	65%	31%
	7.5	68%	74%	71%	57%	5%
	10	51%	68%	60%	49%	5%
Acetic acid		*L. casei*	*L. delbrueckii*	*L. lactis*	*B. coagulans*	*B. smithii*
	0.5	101%	109%	124%	117%	104%
	1	102%	97%	132%	107%	100%
	2.5	103%	97%	124%	109%	86%
	5	106%	89%	122%	91%	55%
	7.5	99%	102%	108%	73%	19%
	10	77%	115%	74%	74%	-1%
Glycolic acid		*L. casei*	*L. delbrueckii*	*L. lactis*	*B. coagulans*	*B. smithii*
	0.5	71%	108%	102%	100%	77%
	1	133%	105%	85%	96%	76%
	2.5	92%	104%	83%	79%	66%
	5	44%	95%	84%	53%	16%
	7.5	24%	126%	71%	34%	7%
	10	12%	109%	70%	35%	8%
Levulinic acid		*L. casei*	*L. delbrueckii*	*L. lactis*	*B. coagulans*	*B. smithii*
	0.5	103%	87%	117%	104%	110%
	1	108%	86%	125%	111%	108%
	2.5	92%	83%	129%	116%	108%
	5	93%	67%	108%	109%	104%
	7.5	88%	94%	117%	100%	92%
	10	89%	98%	74%	91%	52%

60%, 34% and 11% respectively. A higher toxicity of 4-hydroxybenzaldehyde in comparison to vanillin and syringealdehyde has also been previously observed for ethanol producing yeast (Delgenes et al., 1996; Zaldivar and Ingram, 1999; Oliva et al., 2003), and for lactic acid producing bacteria (Bisschof et al., 2010).

Large differences between micro-organisms were observed for the inhibitory effect of phenolic aldehydes. Where *L. delbrueckii* was significantly inhibited by vanillin concentrations of 0.75 g/l, *L. lactis* was still growing at vanillin concentrations of 2.5 g/l. Overall, *L. lactis* was least inhibited by phenolic aldehydes. Although phenolic aldehydes can severely inhibit cell growth, their individual presence in most pretreated lignocellulosic feedstocks is below 0.2 g/l (van der Pol et al 2014, van der Pol et al 2015). At these concentrations, phenolic aldehydes are not likely to induce significant inhibition as individual compound (table 3).

Phenolic acids such as coumaric acid and ferulic acid were significantly inhibiting growth of most lactic acid producing micro-organisms in concentrations above 1 g/l, only *L. casei* is not fully inhibited at a phenolic acid concentration of 2.5 g/l. Coumaric acid can be present in pretreated lignocellulose in concentrations up to 0.75 g/l, which can cause significant inhibition of growth for *L. lactis* and *B. smithii* as individual compound (table 2) (van der Pol et al 2014, van der Pol et al 2015).

The concentration at which furans becomes inhibitory for growth differs significantly between the tested strains. The differences may be related to growth temperature, where strains grown at a higher growth temperature of 50°C (*B. coagulans* and *B. smithii*) or 45°C (*L. delbreuckii*) show a stronger growth inhibition in the presence of furans. In previous studies, furfural toxicity was linked to the formation of reactive oxygen species (ROS). A high temperature accelerates the formation of ROS (Feron et al., 1991; Allen et al., 2010). Strains growing at lower temperatures are therefore less exposed to ROS, thus it can be theorized that they may therefore tolerate higher concentrations of furans. In contrast to what was found in previous experiments with ethanol producing yeast strains (Delgenes et al 1996, Oliva et al 2003), most micro-organisms tested in this research did not show a significant difference in inhibitory effects between furfural and 5-HMF. *B. coagulans* was an exception, this micro-organism was less growth-inhibited by 5-HMF.

Levulinic acid is the least inhibitory by-product out of 12 different by-products tested in this study. At a concentration of 10 g/l, only *B. smithii* is significantly growth inhibited. Acetic acid results in a minor growth inhibition at a concentration of 10 g/l. Acetic acid has previously been described as only a minor growth inhibitor for *B. coagulans* (Walton et al., 2010). Only *B. smithii* was significantly growth inhibited at acetic acid concentrations of 5 g/l. Formic acid can result in significant growth inhibition, and has the most inhibitory effect of all organic acids tested. All strains tested show a significant inhibition of growth at formic acid concentrations of 7.5 g/l. Overall, *L. casei*, *L. delbrueckii*, *L. lactis* and *B. coagulans* show a similar response to different small organic acids when present as individual compound, while *B. smithii* is more sensitive towards these organic acids.

L. casei DSM20011, *L. lactis* DSM20481 and *B. coagulans* DSM2314 are in general less inhibited in growth by individual lignocellulosic by-products. *L. delbrueckii* DSM20073 shows significant inhibition towards vanillin and 4-hydroxybenzaldehyde at a concentration of 0.5 g/l, and towards furans at concentrations exceeding 1 g/l. *B. smithii* DSM4216 is significantly inhibited by organic acids at low concentrations, and was also growth inhibited at relatively low concentrations of phenolic acids and phenolic aldehydes.

Growth of lactic acid producing micro-organisms in presence of combinations of by-products

Screening for the effects of individual by-products on the growth of different lactic acid producing bacteria showed that *L. lactis*, *B. coagulans* and *L. casei* were overall least sensitive towards by-products. To further reduce the amount of experiments required, only the most promising strains based on individual by-product screening were evaluated for combined effects of by-products. During individual screening, concentrations were determined where the by-product start to become inhibitory for the micro-organism. Only these concentrations were used in combined by-products screening, reducing the amount of experiments required even further.

Combinations of the phenolic aldehydes vanillin, syringaldehyde and p-hydroxy-benzaldehyde showed a large synergistic growth inhibition effect for all micro-organisms tested (table 4). The large combined growth inhibition of these compounds may be explained by their similarity. The only difference between the phenolic aldehydes is the number of methyl ether groups on the benzene ring (table 1). Combined growth inhibition between the phenolic aldehydes has been observed previously for ethanol producing yeast (Oliva et al., 2004). A large synergistic growth inhibition effect between phenolic aldehydes and phenolic acids was observed for *B. coagulans* and *L. lactis*, while synergy between phenolic aldehydes and phenolic acids was not observed for *L. casei* (table 4). The observed synergistic growth inhibition effect show that identifying individual inhibition effects only is not sufficient to determine growth of micro-organisms on lignocellulose-derived substrates.

For all micro-organisms, combinations of formic acid and other by-products resulted in a large synergistic growth inhibition effect (table 4). For *L. lactis*, acetic acid also showed a large synergistic inhibition effect with other by-products, while for *L. casei* and *B. coagulans* most combinations involving acetic acid did not result in synergistic growth inhibition. Therefore, while a similar inhibition for all strains may be expected when looking at toxicity of acetic acid as individual compound, the inhibitory effect of acetic acid can be significantly different between micro-organisms in mixtures of by-products.

B. coagulans has a diverse response towards combinations of by-products. Cultivation of this strain in the presence of combinations of inhibitors, for instance including by-products such as 5-HMF and furfural, do not show additive growth inhibition effects towards any by-products except p-hydroxybenzaldehyde. However, other combinations of by-products can

Table 4: *Synergistic inhibition effects of lignocellulosic by-products on growth of L. casei, L. lactis and B. coagulans, observed in 48-well plates. Percentage shown is relative growth compared to a fully-grown reference culture in the absence lignocellulosic by-products.*

L. lactis		5-HMF	Furfural	Glycolic acid	Levulinic acid	Acetic acid	Formic acid	Ferulic acid	Coumaric acid	4-Hydroxy-benzaldehyde	Syringaldehyde	Vanillin
Lignocellulosic by-product (g/l)		1.5	1	5	10	10	7.5	1	0.5	1.5	2.5	2.5
5-HMF	1.5	-	76%	90%	84%	32%	47%	46%	83%	32%	37%	25%
Furfural	1	76%	-	88%	76%	35%	73%	42%	98%	29%	61%	31%
Glycolic acid	5	90%	88%	-	100%	44%	69%	21%	100%	62%	66%	40%
Levulinic acid	10	84%	76%	100%	-	3%	16%	39%	111%	17%	27%	10%
Acetic acid	10	32%	35%	44%	3%	-	3%	10%	36%	4%	5%	4%
Formic acid	7.5	47%	73%	69%	16%	3%	-	36%	74%	11%	17%	8%
Ferulic acid	1	46%	42%	21%	39%	10%	36%	-	13%	11%	25%	18%
Coumaric acid	0.5	83%	98%	100%	111%	36%	74%	13%	-	32%	24%	29%
4-H.benzaldehyde	1.5	32%	29%	62%	17%	4%	11%	11%	32%	-	4%	2%
Syringaldehyde	2.5	37%	61%	66%	27%	5%	17%	25%	24%	4%	-	4%
Vanillin	2.5	25%	31%	40%	10%	4%	8%	18%	29%	2%	4%	-

B. coagulans		5-HMF	Furfural	Glycolic acid	Levulinic acid	Acetic acid	Formic acid	Ferulic acid	Coumaric acid	4-Hydroxy-benzaldehyde	Syringaldehyde	Vanillin
Lignocellulosic by-product (g/l)		1.5	0.5	4	10	10	4	1.5	1.5	0.5	1.5	1.5
5-HMF	1.5	-	90%	70%	96%	84%	69%	91%	79%	2%	96%	80%
Furfural	0.5	90%	-	60%	89%	90%	46%	80%	17%	7%	82%	77%
Glycolic acid	4	70%	60%	-	71%	71%	45%	113%	92%	73%	87%	85%
Levulinic acid	10	96%	89%	71%	-	45%	54%	58%	106%	85%	80%	94%
Acetic acid	10	84%	90%	71%	45%	-	53%	20%	99%	49%	84%	107%
Formic acid	4	69%	46%	45%	54%	53%	-	68%	29%	41%	63%	63%
Ferulic acid	1.	91%	80%	113%	58%	20%	68%	-	14%	0%	24%	11%
Coumaric acid	1.5	79%	50%	92%	106%	99%	29%	14%	-	2%	19%	27%
4-H.benzaldehyde	0.5	2%	7%	73%	85%	49%	41%	0%	2%	-	5%	0%
Syringaldehyde	1.5	96%	82%	87%	80%	84%	63%	24%	19%	5%	-	4%
Vanillin	1.5	80%	77%	85%	94%	107%	63%	11%	27%	0%	4%	-

L. casei		5-HMF	Furfural	Glycolic acid	Levulinic acid	Acetic acid	Formic acid	Ferulic acid	Coumaric acid	4-Hydroxy-benzaldehyde	Syringaldehyde	Vanillin
Lignocellulosic by-product (g/l)		1.5	1.5	4	10	10	7.5	1.5	1.5	0.75	1.5	1
5-HMF	1.5	-	55%	72%	90%	75%	46%	68%	63%	60%	54%	80%
Furfural	1.5	55%	-	61%	54%	67%	33%	61%	65%	49%	49%	71%
Glycolic acid	4	72%	61%	-	71%	80%	14%	58%	66%	33%	49%	42%
Levulinic acid	10	90%	54%	71%	-	69%	43%	65%	68%	69%	80%	74%
Acetic acid	10	75%	67%	80%	69%	-	44%	54%	49%	52%	66%	61%
Formic acid	7.5	46%	33%	14%	43%	44%	-	29%	40%	9%	30%	19%
Ferulic acid	1.5	68%	61%	58%	65%	54%	29%	-	63%	58%	48%	63%
Coumaric acid	1.5	63%	65%	66%	68%	49%	40%	63%	-	55%	48%	52%
4-H.benzaldehyde	0.75	60%	49%	33%	69%	52%	9%	58%	55%	-	29%	34%
Syringaldehyde	1.5	54%	49%	49%	80%	66%	30%	48%	48%	29%	-	34%
Vanillin	1	80%	71%	42%	74%	61%	19%	63%	52%	34%	34%	-

result in a full inhibition in cell growth. For *L. casei,* no combination of by-products was found which fully inhibited cell growth, but nearly all combinations showed a minor synergistic effect. A large number of combinations of by-products caused synergistic growth inhibition for *L. lactis*.

Growth of lactic acid producing bacteria in mixtures of by-products resembling acid or alkaline pretreated lignocellulose

The behaviour of the lactic acid producing micro-organisms in mixtures of many different by-products was determined. To see whether growth can be predicted in these mixtures, and whether key inhibitors can be identified using data from the small-scale screening, *L. lactis*, *L. casei*, *B. coagulans* and *B. smithii* have been cultivated at 50 ml scale with simulated alkaline or acid pretreated substrates at two different concentrations (figure 1). The lower Alkali-75 and acid-75 mixtures resemble the amount of by-products which are found in lignocellulosic substrates containing 75 gram per litre of lignocellulosic sugar. The Alkali-150 and Acid-150 mixtures resemble the amount of by-products which are found in lignocellulosic substrates containing 150 gram per litre of lignocellulosic sugars (table 2). Figure 1 shows growth of the four strains on the four media in comparison to a reference grown without any by-products.

Alkaline pretreated material contains high concentrations of organic acids, with acetic acid, glycolic, levulinic, formic acid and coumaric acid being present in a concentration of 6.8, 2.5, 1.1, 1.2 and 0.35 g/l respectively in alkali-75. In the small-scale screening, *B. smithii* showed significant growth inhibition at a concentration of 7.5 gram per litre of acetic acid, with acetic acid alone causing an significant inhibition of growth. *B. smithii* was significantly growth inhibited in the presence of alkaline-75 mixture, this could be largely contributed to the presence of acetic acid.

Large synergistic growth inhibition effects between different acids, and between acetic acid and phenolic acids and aldehydes were seen for *L. lactis* during small-scale screening. None of the individual by-products was present at a concentration in which it could cause individual growth inhibition. However, the combination of acetic acid with formic acid, glycolic acid, coumaric acid and phenolics causes major growth inhibition for *L. lactis* on the alkali-75 mixture.

For *B. coagulans* and *L. casei*, none of the individual by-products were present in a concentration that can significantly inhibit growth in the alkali-75 mixture. Acetic acid, glycolic, levulinic, formic acid and coumaric acid did not show large synergistic growth inhibition for these micro-organisms in the small-scale screening. As a result, only a small inhibition was observed for *B. coagulans* and *L. casei* when grown on the alkali-75 mixture.

In alkali-150, *L. casei* was expected to be strongly, but not fully, growth inhibited based on the small-scale screening. Strong growth inhibition was indeed observed for *L. casei*, but some growth was still observed. Growth of *B. coagulans* on the alkali-150 mixture was the only experiment not accurately predicted using data from the small-scale screening. Rapid

screening predicted that *B. coagulans* would to be able to grow on the alkali-150 mixture, although strongly inhibited. However, in the shake flask experiment it was observed that *B. coagulans* was fully inhibited by the alkali-150 mixture.

In acid pretreated lignocellulose, one by-product found in larger quantities is furfural, with a concentration of 1.2 g/l of furfural being present in the acid-75 mixture. Small-scale screening of individual by-products showed that 1.2 g/l of furfural is fully inhibiting growth of *B. coagulans*, and significantly inhibiting *B. smithii*. On the other hand, *L. casei* and *L. lactis* were only mildly inhibited by the acid-75 mixture. All other by-products in acid-75 were present in concentrations that did not result in individual growth inhibition for these micro-organisms, and furfural did not show large combined growth inhibition with most other by-products. Growth for all strains observed on the acid-75 mixture is therefore very similar to growth observed in small-scale experiments for furfural concentrations of 1.2 g/l.

Only *L. casei* and *L. lactis* were not fully growth inhibited in acid-150 mixtures, containing 2.5 g/l of furfural. The relative growth rates found for each strain in acid-150 mixtures are very similar to the growth rates observed during the small-scale screening in the presence of 2.5 g/l furfural as individual by-product. Therefore, it can be concluded that furfural is the key inhibitor in acid-pretreated lignocellulose.

CONCLUSION

In this study, the effects of lignocellulosic by-products on growth of lactic acid producing micro-organisms were evaluated using a small-scale screening method. Both individual and combined growth inhibition effects between different by-products were studied. Large differences were observed between micro-organisms with respect to the concentration where by-products become inhibitory. Results from the rapid screening method can predict growth in mixtures of different by-products, and can identify inhibitory by-product(s) in the mixture. This rapid screening method can therefore help to select the most appropriate micro-organism for a chosen combination of pretreatment method and lignocellulosic feedstock.

ACKNOWLEDGEMENT

The authors would like to thank BE-Basic, DSM and Corbion, for funding this research. Furthermore, the authors would like to thank Jorrit Knoef for his help with laboratory experiments.

SUPPLEMENTARY DATA

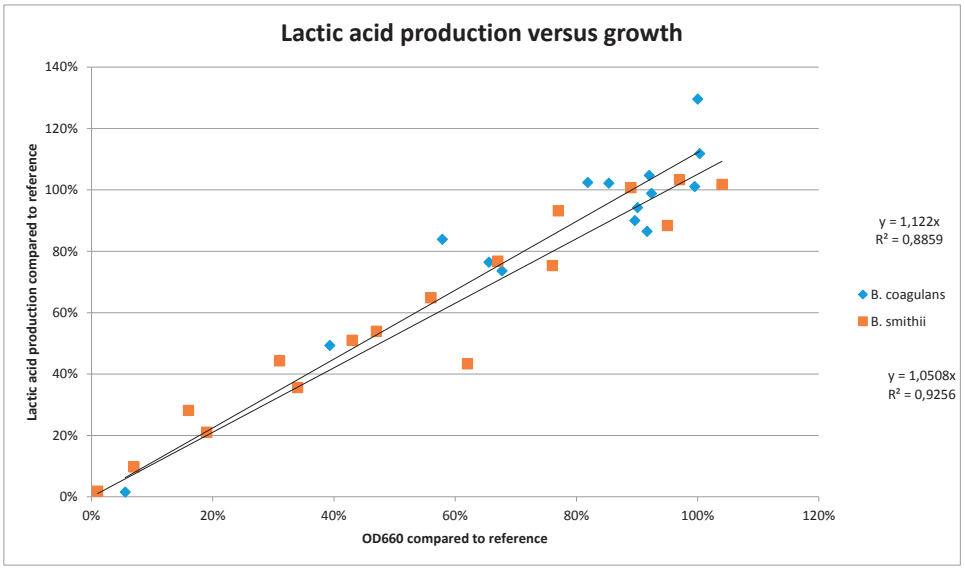


Figure A: *Correlation between OD measurements and lactic acid production in 50 ml anaerobic flasks. Correlations were measured for different organic acids, furans and phenolics, for both B. coagulans DSM2314 and B. smithii DSM4216.*

REFERENCES

Akerberg, C., Hofvendahl, K., Zacchi, G., Hahn-Hägerdal, B. 1998. Modelling the influence of pH, temperature, glucose and lactic acid concentrations on the kinetics of lactic acid producting by *Lactococcus lactis* ssp. *lactis* ATCC 19435 in whole-weat flour. Appl. Microbiol. Biotechnol. 49, 682-690.

Allen S., Clark W., McCaffery J.M., Cai Z., Lanctot A., Slininger P., Liu Z.L. 2010. Furfural induces reactive oxygen species accumulation and cellular damage in Saccharomyces cerevisiae. Biotechnol. Biofuels. 3,2, 1-10.

Bisschof, K.M., Liu, S., Hughes, S.R., Rich, J.O. 2010. Fermentation of corn fiber hydrolysate to lactic acid by moderate thermophile *Bacillus coagulans*. Biotechnol. Lett. 32,823-828.

Boerjan, W., Ralph, J., Baucher, M. 2003. Lignin biosynthesis. Annu. Rev. Plant Biol. 54, 519-546.

De Man, J.C., Rogosa, M., Sharpe, M.E. 1960. A medium for the cultivation of lactobacilli. J. appl. Bact. 23, 130-135

Delgenes J.P., Moletta R., Navarro J.M. 1996. Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Pichia stipitis*. Enzyme Microb. Tech. 19, 220-225.

Feron V.J., Til H.P., de Vrijer F., Woutersen R.A., Cassee F.R., van Bladeren P.J. 1991. Aldehydes: occurrence, carcinogenic potential, mechanism of action and risk assessment. Mutat. Res-Genet. Tox. 259,363-38

Garlotta D. 2001. A Literature Review of Poly(Lactic Acid). J. Polym. Environ. 9, 63-84 .

González-Vara, A., Vaccari, G., Dosi, E., Trilli, A., Rossi, M., Matteuzzi, D. 1999. Ehanced production of L-(+)-Lactic Acid in Chemostat by *Lactobacillus casei* DSM 20011 using Ion-Exchange Resins and Cross-Flow Filtration in a Fully Automated Pilot Plant Controlled via NIR. Biotechnol. Bioeng. 67, 147-156

Guo W., Jia W., Chen S. 2010. Performances of Lactobacillus brevis for producing lactic acid from hydrolysate of lignocellulosics. Appl. Biochem. Biotechnol. 161, 124-136.

Hatfield, R.D.R., John., Grabber, J.H. 1999. Cell wall cross-linking by ferulates and diferulates in grasses. J. Sci. Food Agr. 79, 403-407.

Hendriks, A.T.W.M., Zeeman, G. 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresource Technol. 100, 10-18.

Hofvendahl, K., Akerberg, C., Zacchi, G., Hahn-Hägerdal, B. 1999. Simultaneous enzymatic wheat starch saccharification and fermentation to lactic acid by *Lactococcus lactis.* Appl. Microbiol. Biotechnol. 52, 163-169.

Kabel, M.A., Bos, G., Zeevalking, J., Voragen, A.G.J., Schols, H.A. 2007. Effect of pretreatment severity on xylan solubility and enzymatic breakdown of the remaining cellulose from wheat straw. Bioresource Technol. 98, 2034-2042.

Klinke, H.B., Olsson, L., Thomsen, A.B., Ahring, B.K. 2003. Potential inhibitors from wet oxidation of wheat straw and their effect on ethanol production of Saccharomyces cerevisiae: Wet oxidation and fermentation by yeast. Biotechnol. Bioeng. 81, 738-747.

Larsson S., Quintana-Sáinz A., Reimann A., Nilvebrant N.-O., Jönsson L. 2000. Influence of lignocellulose-derived aromatic compounds on oxygen-limited growth and ethanolic fermentation by Saccharomyces cerevisiae. Appl. Biochem. Biotechnol. 84-86, 617-632.

Maas, R.H.W., Bakker, R.R., Jansen, M.L.A., Visser, D., de Jong, E., Eggink, G., Weusthuis, R.A. 2008. Lactic acid production from lime treated wheat straw by *Bacillus coagulans*: neutralization of acid by fed-batch addition of alkaline substrate. Appl. Microbiol. Biotechnol. 78, 751-758.

Michelson, T., Kask, K., Jõgi, K., Talpsep, E., Suitso, I., Nurk, A. 2006. L(+)-lactic acid producer *Bacillus coagulans* SIM-7 DSM 14043 and its comparison with *Lactobacillus delbrueckii* spp. *Lactis* DSM20073. Enzyme microb. Technol. 39, 861-867.

Oliva J., Sáez F., Ballesteros I., González A., Negro M., Manzanares P., Ballesteros M. 2003. Effect of lignocellulosic degradation compounds from steam explosion pretreatment on ethanol fermentation by thermotolerant yeast Kluyveromyces marxianus. Appl. Biochem. Biotechnol. 105, 141-153.

Oliva, J.M., Ballesteros, I., Negro, M.J., Manzanares, P., Cabañas, A., Ballesteros, M. 2004. Effect of binary combinations of selected toxic compounds on growth and fermentation of *Kluyveromyces marxianus.* Biotechnol. Prog. 20, 715-720.

Sun, J.X., Sun, X.F., Sun, R.C., Su, Y.Q. 2004. Fractional extraction and structural characterization of sugarcane bagasse hemicelluloses. Carbohydr. Polym. 56, 195-204.

Taherzadeh M.J., Gustafsson L., Niklasson C., Lidén G. 1999. Conversion of furfural in aerobic and anaerobic batch fermentation of glucose by Saccharomyces cerevisiae. J. Biosci. Bioeng. 87, 169-174.

Van der Pol, E.C., Bakker, R.R., Baets, P., Eggink, G. 2014. By-products resulting from lignocellulose pretreatment and their inhibitory effect on fermentations for (bio)chemicals and fuels. Appl. Microbiol. Biotechnol. 98, 9579-9593.

Van der Pol, E.C., Bakker, R.R., van Zeeland, A.N.T., Sanchez Garcia, D., Punt, A., Eggink, G. 2015. Analysis of by-product formation and sugar monomerization in sugarcane bagasse pretreated at pilot plant scale: Differences between autohydrolysis, alkaline and acid pretreatment. Bioresource Technol. 181, 114-123.

Walton, S.L., Bisschoff, K.M., van Heiningen, A.R., van Walsum, G.P. 2010. Production of lactic acid from hemicellulose extracts by *Bacillus coagulans* MXL-9. J. Ind. Microbiol. Biotechnol. 37, 823-830.

Zaldivar J., Ingram L.O. 1999. Effect of organic acids on the growth and fermentation of ethanologenic *Escherichia coli* LY01. Biotechnol. Bioeng. 6, 203-210.

CHAPTER 5

Precultivation of *Bacillus coagulans* DSM2314 in presence of furfural decreases inhibitory effects of lignocellulosic by-products during L(+)-lactic acid fermentation

This chapter will be submitted as:
van der Pol E.C., Springer J., Vriesendorp B., Weusthuis R.A., Eggink G.

ABSTRACT

By-products resulting from thermo-chemical pretreatment of lignocellulose can inhibit fermentation of lignocellulosic sugars to lactic acid. Furfural is such a by-product, which is formed during acid pretreatment. pH controlled fermentations with 1 litre starting volume, containing YP medium and a mixture of lignocellulosic by-products, were inoculated with precultures of *B. coagulans* DSM2314 to which 1 g/l furfural was added. The addition of furfural resulted in an increase in L(+)-lactic acid productivity by a factor 2 to 1.39 g/l/h, increase in lactic acid production from 54 to 71 gram, and increase in conversion yields of sugar to lactic acid from 68% to 88% W/W. The improved performance was not caused by furfural consumption or conversion, indicating that the cells acquired a higher tolerance to furfural. This adaptation coincided with a significant elongation of *B. coagulans* cells, and upregulation of pathways involved in the synthesis of cell wall components such as bacillosamine, peptidoglycan and spermidine. Furthermore, an upregulation was observed for *SigB,* and genes promoted by *SigB* such as *NhaX* and *YsnF*. These genes are involved in stress responses in bacilli.

INTRODUCTION

Lignocellulose is the most abundant biomaterial on earth. It consists for 60-75% W/W of sugars, which can be used in fermentation processes to produce biobased chemicals such as lactic acid (van der Pol et al 2014). Polymerized lactic acid (PLA) can be moulded into bioplastics, which may be a suitable alternative to oil-derived plastics such as polystyrene (PS) and polyethylene (PE) (Garlotta 2001).

Lignocellulosic sugars are polymerized, strongly condensed and covered by lignin, making it difficult for lactic acid producing bacteria to directly consume these sugars (Fengel and Wegener 1983). A thermo-chemical pretreatment process combined with enzymatic hydrolysis releases the sugars as fermentable monomers or oligomers (Hendriks and Zeeman 2009). However, thermo-chemical pretreatment also leads to the formation of unwanted by-products such as phenolic aldehydes, organic acids and furans, which by-products can inhibit growth and product formation of micro-organisms during fermentation processes (Palmqvist and Hahn-Hägerdal 2000a, Palmqvist and Hahn-Hägerdal 2000b, van der Pol et al. 2014). The presence of different by-products in pretreated lignocellulose depends on both the type of thermo-chemical pretreatment used, as well as the source of lignocellulose (van der Pol et al. 2014, van der Pol et al. 2015). Furfural is such a by-product, formed by dehydration of xylose during pretreatment at low pH and high temperature. The presence of furfural can generate reactive oxygen species (ROS), which can damage DNA and membranes (Allen et al. 2010, Feron et al. 1991).

Bacillus coagulans DSM2314 has been studied as microbial cell factory for the production of lactic acid (Maas et al. 2008). It is a moderate thermophilic bacterium able to grow in slightly acidic environments. *B. coagulans* can consume both glucose and xylose homofermentatively, with a conversion yield of glucose and xylose to lactic acid of over 90% on a weight basis and a high lactic acid productivity up to 5 g/l/h (Maas et al. 2008). Although *B. coagulans* may be a suitable candidate for the production of lactic acid from lignocellulosic sugars, earlier experiments have shown that *B. coagulans* is relatively sensitive towards lignocellulosic by-products (van der Pol et al 2016, Walton et al 2010).

Bacilli like *B. coagulans* are able to adapt to different environmental conditions (Wiegeshoff et al. 2006). Sigma factors play an important role in this adaptation. One of the sigma factors involved in responses towards stress is *SigB*. This gene is upregulated when cells are exposed to stress conditions, and tightly regulates expression of around 150 genes (Hecker et al. 2007, Price et al. 2001). Adaptation has not only been observed in environments with suboptimal pH and temperature, but has also been observed to play a role in resistance towards toxic compounds (Price et al. 2001).

In this research, we examined whether *B. coagulans* can adapt to environments rich in potentially inhibiting lignocellulosic by-products. Adaptation was accomplished by addition of non-lethal amounts of lignocellulosic by-products to precultures. These precultures were

used as inoculum for fermentation processes with medium resembling acid pretreated sugarcane bagasse, rich in furfural, phenolics and small organic acids.

MATERIAL AND METHODS

Chemicals

Glucose, xylose, galactose were ordered at Duchefa (The Netherlands), and had a purity of at least 99% W/W. yeast extract, peptone and BIS-Tris were also ordered at Duchefa (The Netherlands). Other chemicals were ordered at Sigma-Aldrich (United States), and had a purity of at least 98% W/W, with the exception of formic acid, which was 95% W/W pure.

Micro-organism

Bacillus coagulans DSM2314 was acquired as freeze dried stock from the German collection of micro-organisms and cell cultures (DSMZ, Germany). Cells were suspended for 30 minutes in 5 ml PYPD medium, consisting of 5 g/l yeast extract, 10 g/l peptone, 20 g/l glucose and 10 g/l BIS-Tris, which was pre-sterilized for 20 minutes at 121°C. After 30 minutes of pre-incubation, the cell suspension was transferred to 50 ml anaerobic flasks containing 45 ml fresh PYPD medium, sealed with a rubber cap, and incubated for 16 hours at 50°C to an optical density at 660 nm of around 2. After addition of 15% v/v glycerol, cells were stored in 1.5 ml aliquots in cryovials at -80°C until used.

Cultivation in anaerobic flasks at 50 ml scale

Cultivation was performed in 50 ml glass anaerobic flasks, sealed with a rubber stopper and aluminium crimp cap, in an incubator set at 50°C without shaking, starting at a pH of 7.2.

All glass flasks and rubber stoppers, 2x concentrated PYPD medium and milliQ water were autoclaved at 121ºC for 20 minutes prior to cultivation. Lignocellulosic by-product mixture solutions and single by-product solutions were heated at 85ºC for 1 hour. An acid-150 lignocellulosic by-product mixture was used in this experiment, which resembles an acid pretreated bagasse lignocellulose substrate containing 150 g/l monomeric lignocellulosic sugars (table 1) (van der Pol et al 2015). Not only by-product mixtures were tested, but also single by-products examined in 50 ml shake flasks. Individual by-products tested were furfural, vanillin, syringaldehyde, acetic acid and formic acid.

The initial flasks used to adapt the micro-organism were inoculated with 250 µl of freezer stock to obtain a starting OD660 of 0.01, and contained 25 ml of 2x concentrated PYPD medium, to which 25 MilliQ water, a 2x concentrated single by-product solution, or a 2x concentrated acid-150 mixture was added. New flasks, which evaluated adaptation effects, contained 25 ml 2x concentrated PYPD medium and 25 ml of either 2x concentrated single by-product solution, 2x concentrated acid-150 mixture or 6 g/l furfural . These flasks were

Table 1: *Composition of acid-100 and acid-150 by-product mixtures, as measured after acid pretreatment of bagasse lignocellulose in a previous study (van der Pol et al 2015). The compositions were used to prepare the model substrate resembling acid pretreated sugarcane bagasse.*

Amount (g/l)	Acid-100	Acid-150
Acetic acid	3.13	4.68
glycolic acid	2.15	3.21
levulinic acid	2.06	3.08
formic acid	0.29	0.43
furfural	1.63	2.44
5-HMF	0.19	0.28
coumaric acid	0.29	0.43
ferulic acid	0.04	0.06
4-hydroxybenzaldehyde	0.04	0.06
vanillin	0.07	0.10
syringealdehyde	0.04	0.06

inoculated with culture taken from previous anaerobic shake flasks, using amounts between 200 and 500 µl to obtain a starting OD660 in the new flask of 0.01.

Sterile syringes and needles were used to take samples through the rubber stopper of the anaerobic flasks. Growth was monitored using a spectrophotometer (Ultrospec 2000, Pharmacia Biotech, Sweden) at an optical density wavelength of 660nm. Furthermore, at different time points microscope images were taken to follow morphological changes. The microscope (Axioplan, Carl Zeiss, Germany) was equipped with a camera (Axiocam ICc3, Zeiss, Germany), and a Zeiss plan Neofluar 40x lens (Zeiss, Germany) was used.

Preculture cultivation in 50 ml anaerobic flasks, used as inoculum for fermentations

50 ml sterile anaerobic flasks were filled with 25 ml 2x concentrated PYPD medium, and 25 ml of either milliQ water (reference preculture) or 2 g/l furfural dissolved in milliQ water (furfural containing preculture), resulting in a final furfural concentration of 1 g/l in the anaerobic flask. The anaerobic flasks were inoculated with 250 µl *B. coagulans* freezer stock to obtain a starting OD at 660 nm of 0.01, and cultivated in an incubator at 50°C without shaking. When the preculture reached an OD660 of 1, which occurs around 15 hours for reference precultures, and around 24 hours for furfural containing precultures, the preculture was used as inoculum for 1 litre pH controlled fermentations.

Cultivation in 1 litre pH controlled fermentations

Fermentations were performed in 1.5 litre Multifors reactors (Infors, Switzerland). Each fermentation started with 100 g/l of sugar mixture, containing of 3.3 g/l galactose, 24.2 g/l xylose and 72.5 g/l glucose, unless stated otherwise. Fermentations performed using peptone medium contain 10 g/l yeast extract and 10 or 20 g/l peptone as nitrogen source (YP10 / YP20 medium). Fermentations performed using ammonium medium contain 10 g/l yeast extract, 2 g/l $(NH_4)_2PO_4$ and 3.5 g/l $(NH_4)_2PO_4$ as nitrogen source (YA medium). The medium components were dissolved in 500 ml of milliQ water in a pre-sterilised fermentation vessel, the pH was set below 5.5 with citric acid to reduce Maillard reactions, and the fermenter including 500 ml medium was sterilized for 12 minutes at 121°C. After sterilization, 500 ml of either sterile MilliQ water or 500 ml of 2x concentrated by-product mixture, which was pre-heated at 85ºC for 1 hour (table 1), was added to the fermenter. The fermenter was inoculated with 50 ml (5% V/V) of a preculture to which 1 g/l of furfural was added, or a reference preculture without furfural addition, which served as a control. The temperature was controlled at 50°C, the pH was maintained at pH 6 with 4N KOH, and stirring was controlled at 100 RPM. No active aeration was applied during fermentation. At regular intervals during fermentation, samples of 10 ml were taken, the OD at 660 nm was determined, and the remainder of sample was frozen at -20°C for HPLC analysis.

Analysis of lactic acid, monomeric sugars and furans

Analysis of lactic acid, sugars and other fermentation products of *B. coagulans* which may occur such as ethanol and acetic acid was performed using a Waters e2695 HPLC system (Milford USA) equipped with Waters RI2414 and Waters UV/Vis 2489 (measuring at 210 nm) detectors. The column used was a Shodex RS pak KC-811 ion exchange column (length 300 mm – I.D. 8 mm), controlled at 65°C. As eluent, 3mM H_2SO_4 in milliQ water was used. The flow used was 1 ml/min. Samples obtained during fermentation were de-frozen prior to analysis. 250 µl of this sample was mixed with 250 µl of internal standard, containing 0.25 g/l phthalic acid, and 500 µl of milliQ water. Samples were filtered using 0.2 µm Spartan filters, and supernatants were measured using HPLC.

To determine furan concentrations, UPLC-MS/MS measurements were performed using a Dionex Ultimate 3000 RSLC system, equipped with a Waters Acquity BEH C18 RP column, in combination with a Thermo ScientificTM LCQ Fleet Ion Trap Mass spectrometer, as previously described (van der Pol et al. 2015).

Isolation of RNA and DNA

4 reference and 4 furfural-containing cultures were prepared in 50 ml anaerobic flasks as described previously for precultivation. When the cultures reached an OD of 1, the cultures were transferred to 50 ml Greiner tubes and directly frozen using liquid nitrogen. After overnight storage at -80°C, samples were thawed and vortexed for 5 seconds. 10 ml sample

of each culture was placed in 15 ml Greiner tubes, together with one extra sample from a reference culture for DNA extraction. The 9 tubes were centrifuged at 4°C for 15 minutes at 4700 RPM. After centrifugation, the tubes were kept on ice whenever possible. Supernatant was removed, cells were resuspended in 0.5 ml ice cold TE buffer (pH 8) and transferred to 2 ml tubes containing specialized Lysing matrix E beads (Lysing matrix E, MP Biomedicals, Ohio, USA). After addition of 500 µl extraction buffer (0.6% SDS and 0.2M sodium acetate (pH 5.2)), samples were vigorously shaken using a Precellys 24 homogenizer (Bertin Technology, France) for 2x 30 seconds at 5500 RPM, with a 60 second interval. 500 µl of phenol-choloroform (50%-50% V/V) was added, tubes were vortexed for 5 seconds, and kept for 10 minutes on ice. Tubes were then centrifuged for 5 minutes at 10000 rpm and 4°C. 400 µl of the liquid fraction was transferred to a fresh RNA-free Eppendorf vial, 400 µl of chloroform was added, and samples were centrifuged for 3 minutes at 12000 RPM and 4°C.

After washing with chloroform, either a RNA or DNA kit was used for the purification RNA and gDNA respectively. For RNA, 300 µl of the water phase was transferred to a clean Eppendorf vial, and 300 µl of lysis buffer was added from an RNA extraction kit. The protocol of the extraction kit (Roche High Pure RNA isolation kit v12, Roche, Switzerland) was followed, with the exception that a DNAse incubation time of 45 minutes was used. For DNA purification, a Sigma-Aldrich Bacterial gDNA kit was used according to protocol (NA2110-1KT, Sigma-Aldrich, United States). The extraction resulted in 4 purified RNA samples containing biological replicates of the reference culture, 4 purified RNA samples containing biological replicates of the furfural containing culture, and 1 purified gDNA sample.

DNA analysis and assembly

gDNA quantities in the purified gDNA sample was estimated using a Nanodrop Lite system (Thermo Scientific, DE, USA). The integrity of the DNA was observed using gel electrophoresis. Samples were sent to Baseclear (The Netherlands), which sequenced the gDNA via paired-end sequencing using an Illumina HiSeq 2500 system. The obtained FASTQ file was filtered for reads containing adapters and/or PhiX control signals, and for reads that did not pass the Illumina Chastity filtering. A second filtering was performed using FASTQC quality control tool version 0.10.0.

For DNA assembly, the raw Illumina data, containing paired ends of 126 bp reads, was used as input for de-novo assembly via IDBA (idba-1.1.1, Peng et al. 2012) using a K-mer range from 20 to 120, with steps of 3, and via Ray (Ray 2.3.1, Boisvert et al. 2010) with K-mers ranging from 15 to 81, selecting for low scaffold runs. Both runs gave comparable results, while the output from Ray with a K-mer of 61 was chosen for final assembly, since it contained less contigs, and protein prediction was of slightly higher quality compared to IDBA run. The final assembly contained 3 569 489 bp, and a GC content of 46.3%. Genes were annotated using PROKKA v1.11 (Seemann 2014) which predicted a total of 3402 coding sequences, 11 rRNAs and 60 tRNAs.

RNA-seq analysis and assembly

RNA quantities in the purified RNA samples was estimated using a Nanodrop Lite system (Thermo Scientific, DE, USA). The integrity of the RNA was observed using gel electrophoresis. Samples were sent to Baseclear (The Netherlands), which analysed the RNA samples using deep sequencing on an Illumina HiSeq 2500 system. This resulted in raw sequence data containing single-end reads of 50 bp. A FASTQ file was generated using Illumina Casava v1.8.3. This file was filtered by Illumina chastity filtering, removal of adapters, and quality assessment based in FASTQC quality control tool v0.10.0.

RNA sequences were mapped to the reference sequence using BWA (Burrows-Wheeler Aligner) (Li 2013) run with default settings. RNA-seq analysis was performed using DESeq method (Mortazavi et al. 2008), and was used to compute differential expression and estimate p-values (Anders and Huber 2010). p-Values were adjusted for multiple testing with Benjamini and Hochberg (1995) approach for adjusting the false discovery rate (FDR). A cut-off p-value of 0.05 was used.

RESULTS

Effect of pre-cultivation in presence of lignocellulosic by-products on growth of *B. coagulans* in 50 ml anaerobic flasks

B. coagulans was precultivated in the presence of either organic acids (formic acid, acetic acid), phenolic aldehydes (vanillin, syringaldehyde), or furfural. These precultures were used to inoculate 50 ml anaerobic flasks containing the same by-products (table 2). Pre-cultivation in the presence of organic acids increased fermentation time required to reach maximum OD by 20-40%. Pre-cultivation of *B. coagulans* in the presence of vanillin did not have an effect on fermentation time. The presence of syringaldehyde resulted in a small reduction of fermentation time by 6 hours (a reduction of 23 %) (Table 2). When the cells were pre-cultivated in the presence of furfural, a reduction of approximately 50% in fermentation time was observed, mainly due to a reduction in lag phase (figure 1a). By-product concentrations did not change significantly during the anaerobic flask experiments (data not shown), indicating that they were not consumed or converted by *B. coagulans*. Cells grown in the presence of furfural showed a change in morphology: the cell length increased drastically (figure 2). When these cells were used as inoculum for a new 50 ml flask which did not contain by-products, cell morphology changed to a non-elongated state within 7 generations, a morphology silimar to what is observed in a reference preculture.

To optimize adaptation, different furfural concentrations were tested during precultivation. These precultures were used as inocula for flasks containing either 3 g/l furfural, or acid-150 mixture of by-products (table 1). For both situations, it was found that fastest growth occurred

Table 2: *Effect of presence of lignocellulosic by-products during precultivation on growth of B. coagulans in presence of the same by-product. Batch time of cultivation was the time required to reach maximum OD.*

	By-product concentration during precultivation	By-product concentration During cultivation	Batch time of cultivation
	g/l	g/l	h
Control	0	0	16
Furfural	0	2	40
	1	2	23
	2	2	25
	0	3	60
	1	3	27
	2	3	32
Vanillin	0	4	20
	2	4	19
	3	4	20
Syringaldehyde	0	5	26
	2.5	5	20
Acetic acid	0	20	23
	10	20	28
Formic acid	0	7.5	16
	7.5	7.5	23

when 1 g/l furfural was added to the preculture (figure 1). Therefore, a furfural concentration of 1 g/l was used during pre-cultivation in the next experiments.

Although sufficient substrate was still available at the end of these fermentations, the reduction of pH due to the production of lactic acid stopped growth in the anaerobic flasks. To accurately assess lactic acid productivity, yield and titre, pH controlled fermenter experiments were performed.

Studies in pH-controlled fermentations

pH-controlled fermenters with a start volume of 1 litre were either inoculated with a furfural containing preculture, or a reference preculture to which no by-products were added. Two different media were used. The first medium was similar to the medium used at 50 ml scale, and contained 100 g/l sugars (72.6% glucose, 24.2% xylose, 3.2% galactose), yeast extract and 10 or 20 g/l peptone (YP10/YP20 medium). In the second medium, peptone was replaced by ammonium salts (YA medium).

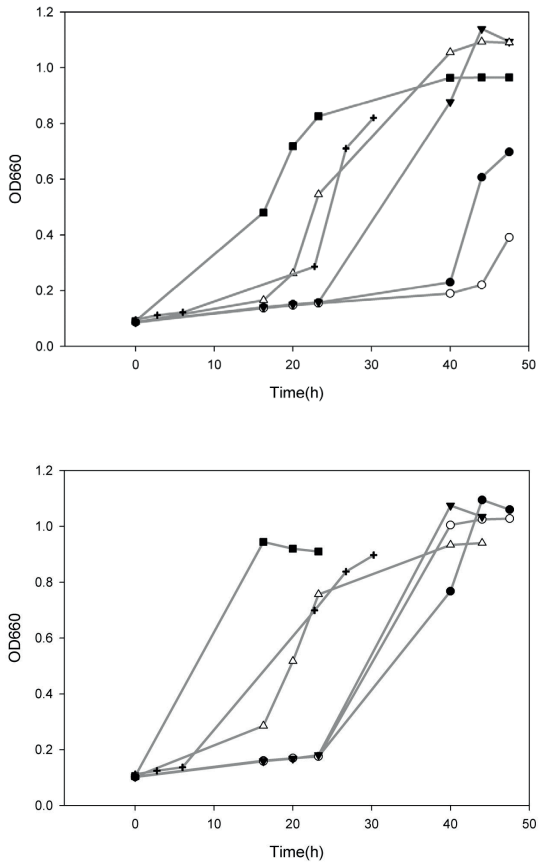


Figure 1: *Effect of furfural presence and concentration during adaptational growth on the growth of Bacillus coagulans DSM2314 in a second 50 ml anaerobic flasks containing (a) 3 g/l furfural, (b) acid-150 mixture of by-products. The following furfural concentrations were tested during precultivation: ●: 0 g/l; , ○: 0.1 g/l , ▼: 0.25 g/l △: 0.5 g/l, ■: 1 g/l, ✚: 1.5 g/l.*

Addition of furfural to precultures, used as inocula in fermenters containing YP10 medium and acid-100 mixture of by-products, reduced the total fermentation time by 50% (table 3, figure 3a). This resulted in an increase in average lactic acid productivity from 0.56 g/l/h to 1.39 g/l/h. Furthermore, total lactic acid production increased from 54.0 gram to 71.5 gram.

A similar result was obtained in fermenters containing YP20 medium and acid-150 by-product mixture. Addition of furfural to precultures increased the lactic acid productivity from 0.42 g/l/h to 0.93 g/l/h, and the yield from 68% (w/w) to 81% (w/w). The total amount of lactic acid produced increased by 25% to 65.3 g (table 3,figure 3b).

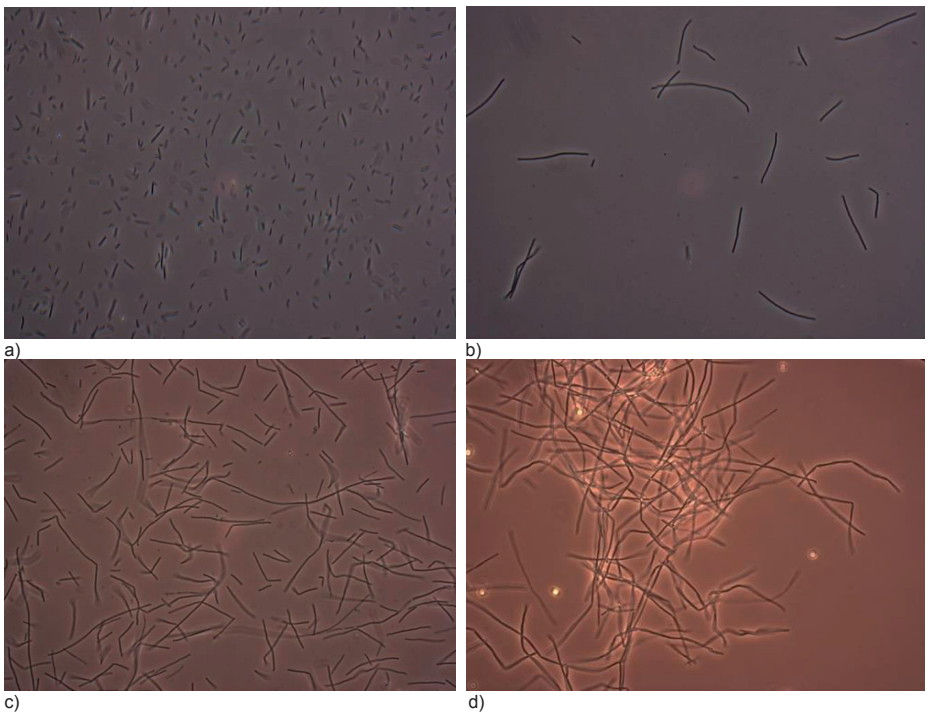


Figure 2: *Effect of growth in the presence of by-products on the morphology of B. coagulans. a: reference preculture, no by-products present; b:furfural-containing preculture, 1 g/l furfural present; c: Fermentation containing YA medium and acid-100 mixture, cultivated for 24h, inoculated with furfural-containing preculture. D: Fermentation containing YP20 medium and acid-150 mixture, cultivated for 40h, inoculated with furfural containing preculture.*

The morphological changes that occurred in anaerobic flasks as a response to precultivation in presence of furfural were also observed in these fermenter experiments: In the late-exponential phase of the fermentation, all cells were strongly elongated (figure 2).

Using furfural-containing inocula instead of reference inocula in fermenters containing YA medium and acid-100 by product mixture did not result in an increased lactic acid titre (table 3, figure 4). However, the total fermentation time was reduced by 60%, resulting in an increase in lactic acid productivity from 0.8 g/l/h to 2.1 g/l/h.

In fermentations containing lignocellulosic by-products, xylose was only partially consumed (figure 5). At the start of the fermentation 24.2 gram of xylose was present. In both reference fermentations using YA medium and reference fermentations using YP20 medium, a final residual xylose amount of around 3 gram was measured. On the other hand, a final residual xylose amount of 21 gram was observed in acid-100 containing fermentation processes on YA medium, and 16.6 gram of xylose was residual when YP10 medium was used. Addition of

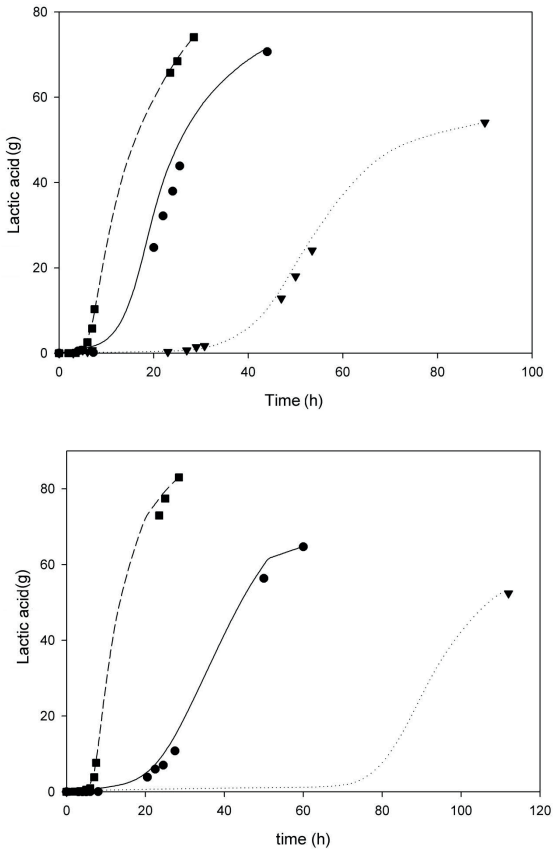


Figure 3: *Effect of precultivation in the presence of furfural on lactic acid production during fermentation by B. coagulans growing in the presence of a mixture of by-products. Either YP10 medium (a) or YP20 medium (b) was used during fermentation. Lactic acid production was measured by HPLC (symbols) or calculated based on KOH addition (lines).* ■ *and (-----): reference preculture without addition of by-products,* ● *and (———): furfural-containing preculture, addition of by-product mixture acid-100 (a) or acid-150 (b),* ▼ *and (·····): reference preculture, addition of by-product mixture acid-100 (a) or acid-150 (b).*

furfural to the preculture did not influence the total xylose consumption in the subsequent fermentation process.

The lactic acid production potential on glucose was determined for *B. coagulans*, pregrown in the presence of furfural, in YA medium, containing 125 gram of glucose as carbon source (table 3, figure 4). These fermentations resulted in a total lactic acid production of 103 gram in 52 hours with an average productivity of 1.68 g/l/h, and a conversion yield of glucose to lactic acid of 91% W/W.

Table 3: *Effect of precultivation on pH controlled fermentations with a start volume of 1 litre using different media, with or without the addition of acid-100/acid-150 by-product mixture. As inoculum, 5% V/V B. coagulans culture was used, either precultivated in the presence of 1 g/l furfural (furfural preculture) or in the absence of furfural (reference preculture).*

Preculture	By-products	C_s (g/L)[A]	Medium	C_{La} (g/l)[B]	A_{La} (g)[C]	$Q_{v,av}$ (g/l/h)[D]	$Q_{v,max}$ (g/l/h)[E]	$Y_{s/La}$[F]	Time (h)[G]
Reference	None	100	YP10	52.9	74.2	2.23	4.6	90%	29
Furfural	Acid-100	100	YP10	50.4	71.5	1.39	3.0	88%	44
Reference	Acid-100	100	YP10	40.5	54.0	0.56	1.4	68%	85
Reference	None	100	YP20	59.3	83.0	2.50	5.1	92%	29
Furfural	Acid-150	100	YP20	46.6	65.3	0.93	1.6	81%	60
Reference	Acid-150	100	YP20	39.6	52.4	0.42	1.2	68%	110
Reference	None	100	YA	55.6	78.6	2.40	3.90	86%	28
Furfural	Acid-100	100	YA	47.4	68.4	2.06	2.96	84%	28
Reference	Acid-100	100	YA	47.3	66.4	0.81	1.43	76%	70
Furfural	Acid-100	125	YA	70.8	103.9	1.68	3.80	91%	52

A: C_s: concentration of sugar at the start of the fermentation, 100 g/l is a mix of 72.5% glucose, 24.2% xylose and 3.3% galactose, while medium with 125 g/l sugars is solely glucose
B: C_{La}: concentration of lactic acid at the end of the fermentation in g/l
C: A_{La}: Total lactic acid produced in g
D: $Q_{v,av}$: average volumetric lactic acid productivity in g/l/h
E: $Q_{v,max}$: maximum volumetric lactic acid productivity in g/l/h
F: $Y_{S/La}$: conversion yield of consumed sugars to lactic acid in W/W
G: total fermentation time, from inoculation to reaching final lactic acid concentration

RNA-seq analysis of *B. coagulans* grown in the presence or absence of furfural

RNA-seq analysis was performed to identify possible mechanisms behind the reduction in toxicity of by-products when cells are precultivated in the presence of furfural. mRNA expression levels in cultures grown in the presence and absence of furfural, with cultivation conditions similar to precultivation, were compared (Table 5). Suprisingly, genes involved in detoxification of ROS were downregulated 3 fold in the presence of furfural. Peptidoglycans and spermidine are part of the cell wall structure of bacillus strains. The pathway leading from peptidoglycan precursor UDP-N-Acetylglucosamine to more complicated peptidoglycan structures was upregulated by a factor 1.4-2.3 when furfural was present. The pathway also requires the presence of UDP-GlcNAc, another precursor necessary for the production of peptidoglycans. Genes involved in UDP-GlcNAc production were upregulated by 1.3-3.2 fold. Other genes involved in the production of more complicated peptidoglycans such as UDP-N-acetylgalactosamine-undecaprenyl-phosphate N-acetylgalactosaminephosphotransferase were upregulated 3.2-5.3 fold. Genes involved in spermidine production, another molecule

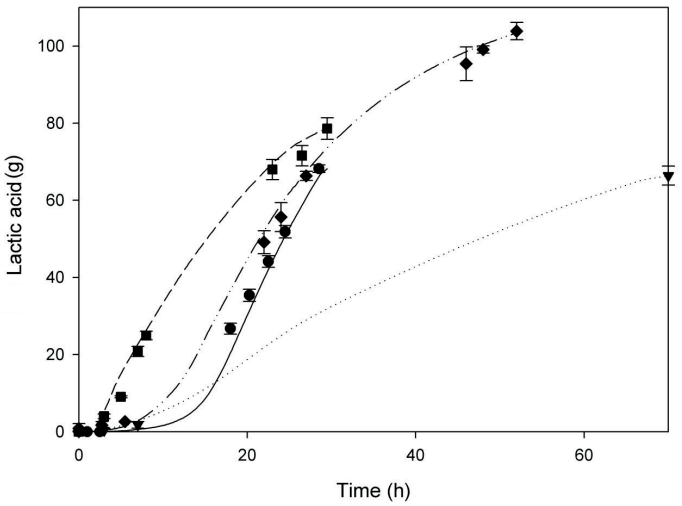


Figure 4: *Effect of precultivation in the presence of furfural on lactic acid production during fermentation by B. coagulans growing in the presence of a mixture of by-products. In the fermentation phase, YA medium was used. Lactic acid production was measured via HPLC (squares, circles, triangles and diamonds) or calculated based on KOH addition (Lines).* ■ *and (-----): reference preculture without addition of by-products, 100 g/l mixed sugars,* ● *and (———):furfural preculture in presence of acid-100 by-product mixture,100 g/l mixed sugars,* ▼ *and (·····): reference preculture in presence of acid-100 by-product mixture, 100 g/l mixed sugars,* ◆ *and (–··–··–): furfural preculture in presence of acid-100 by-product mixture, 125 g/l glucose.*

which can be part of the cell wall, were upregulated by 2.2-4.4 fold, while genes involved in the conversion of glutamate to ornithine, a precursor of spermidine, were upregulated 2.8-13.5 fold, and pathways leading from ornithine to proline were downregulated 2 fold.

Most genes in *B. coagulans* are tightly regulated by sigma factors (Price et al. 2001, Schmidt et al. 1990). Two sigma factors were observed to be upregulated in the presence of furfural, namely *SigF* by 4 fold, and *SigB* by 2.5 fold. For both *SigF* and *SigB*, anti-sigma factor antagonists, required for the activation of the sigma factors, were upregulated in the presence of furfural. *SigB* controls the expression of other genes such as *YsnF* and *NhaX*, which were found to be upregulated by 3.7-4.0 fold.

The pyrimidine synthesis pathway, leading from aspartate and carbomoyl phosphate to UDP, was downregulated by 20 to 50 fold in the presence of furfural, with actual expression values close to zero. An alternative in *B. coagulans* for the synthesis of UMP/UDP is forming them from compounds present in the rich YP medium used during cultivation. A 1.8-2.5 fold upregulation was observed for genes involved in the conversion of cytosine to UMP via cytidine, and from uracil to UMP, while a transporter involved in cytosine uptake was found to be upregulated 10-fold.

Table 4: *Differences in gene expression between reference precultures and furfural precultures of B. coagulans using RNA-Seq analysis. A foldchange above 1 relates to a higher expression of the gene in the furfural preculture, while a foldchange below 1 relates to a higher expression of the gene in the reference preculture. The P-value expresses the significance of the fold-change.*

			Amount of RNA measured			
	Gene name	Enzyme classification	Control	furfural	Foldchange	p-value
Uracil synthesis	Carbamoyl-phosphate synthase (large chain)	6.3.5.5	6947 ± 1543	172 ± 19	0.03	<0.001
	Carbamoyl-phosphate synthase (small chain)	6.3.5.5	1419 ± 223	47 ± 9	0.03	<0.001
	Aspartate Carbamoyltransferase	2.1.3.2	735 ± 134	43 ± 11	0.06	<0.001
	Dihydroorotase	3.5.2.3	1412 ± 207	45 ± 8	0.03	<0.001
	Dihydroorotate dehydrogenase B	1.3.1.14	1435 ± 292	58 ± 11	0.04	<0.001
	Orotate phosphoribosyltransferase	2.4.2.10	865 ± 215	19 ± 5	0.02	<0.001
	Orotidine 5′-phosphate decarboxylase	4.1.1.23	1443 ± 156	30 ± 5	0.02	<0.001
Alternative UDP production from cytosine	Cytosine permease		692 ± 87	6832 ± 1424	9.9	<0.001
	Pyrimidine-nucleoside phosphorylase	2.4.2.2	3118 ± 81	6193 ± 485	2.0	<0.001
	cytidine deaminase	3.5.4.5	1150 ± 82	1911 ± 106	1.7	<0.001
	Uracil phosphoribosyltransferase	2.4.2.9	5123 ± 888	11996 ± 2241	2.3	<0.001
	Uridylate kinase	2.7.4.22	3877 ± 426	5703 ± 543	1.5	<0.001
bacillosamine production	UDP-N-acetylglucosamine 1-carboxyvinyltransferase 1	2.5.1.7	52809 ± 5883	123575 ± 11825	2.3	<0.001
	D-alanyl-D-alanine carboxypeptidase DacA	3.4.16.4	18418 ± 2280	42332 ± 4796	2.3	<0.001
Production of UDP-GlcNAc	Glutamine--fructose-6-phosphate aminotransferase	2.6.1.16	27672 ± 4657	87570 ± 14507	3.2	<0.001
	Bifunctional protein GlmU	2.7.7.23	25201 ± 2539	54997 ± 4154	2.2	<0.001

Table 4: *Differences in gene expression between reference precultures and furfural precultures of B. coagulans using RNA-Seq analysis. A foldchange above 1 relates to a higher expression of the gene in the furfural preculture, while a foldchange below 1 relates to a higher expression of the gene in the reference preculture. The P-value expresses the significance of the fold-change. (continued)*

			Amount of RNA measured			
	Gene name	Enzyme classification	Control	furfural	Foldchange	p-value
other genes involved in cell wall peptidoglucan production	UDP-4-amino-4-deoxy-L-arabinose--oxoglutarate aminotransferase	2.6.1.87	2258 ± 156	7185 ± 2911	3.2	<0.001
	UDP-N-acetylgalactosamine-undecaprenyl-phosphate N-acetylgalactosaminephosphotransferase	2.7.8.40	565 ± 124	2467 ± 1134	4.4	<0.001
	UDP-N-acetyl-alpha-D-glucosamine C6 dehydratase	4.2.1.135	2194 ± 275	11681 ± 4131	5.3	<0.001
Spermidine synthesis and transportation	Carboxynorspermidine synthase	1.5.1.43	3390 ± 507	14834 ± 2472	4.4	<0.001
	Carboxynorspermidine/carboxyspermidine decarboxylase	4.1.1.96	3852 ± 659	12476 ± 1888	3.2	<0.001
	Spermidine/putrescine transport system permease protein PotB		593 ± 39	1145 ± 272	2.0	0.07
	Spermidine/putrescine import ATP-binding protein PotA	3.6.3.31	1296 ± 80	2576 ± 589	2.0	<0.001
	Spermidine/putrescine-binding periplasmic protein precursor		1159 ± 75	2367 ± 587	2.0	<0.001

Table 4: *Differences in gene expression between reference precultures and furfural precultures of B. coagulans using RNA-Seq analysis. A foldchange above 1 relates to a higher expression of the gene in the furfural preculture, while a foldchange below 1 relates to a higher expression of the gene in the reference preculture. The P-value expresses the significance of the fold-change. (continued)*

			Amount of RNA measured			
	Gene name	Enzyme classification	Control	furfural	Foldchange	p-value
Production of precursors for spermidine production such as ornithine	Arginine biosynthesis bifunctional protein ArgJ	2.3.1.35	233 ± 26	818 ± 112	3.5	<0.001
	Acetylglutamate kinase	2.7.2.8	80 ± 25	232 ± 67	2.9	0.005
	N-acetyl-gamma-glutamyl-phosphate reductase	1.2.1.38	239 ± 32	814 ± 134	3.4	<0.001
	Acetylornithine aminotransferase	2.6.1.11	118 ± 78	295 ±74	2.5	0.006
	Ornithine aminotransferase	2.6.1.13	354 ± 161	4785 ± 639	13.5	<0.001
	Pyrroline-5-carboxylate reductase	1.5.1.2	2536 ± 530	1250 ± 90	0.49	<0.001
	Gamma-glutamyl phosphate reductase	1.2.1.41	3646 ± 492	1494 ± 194	0.54	<0.001
	1-pyrroline-5-carboxylate dehydrogenase	1.2.1.88	165 ± 83	79 ± 17	0.48	0.04
Entner Doudoroff pathway	Altronate oxidoreductase	1.1.1.58	459 ± 101	3453 ± 294	7.5	<0.001
	Altronate dehydratase	4.2.1.7	241 ± 173	1428 ± 144	5.9	<0.001
	2-dehydro-3-deoxygluconokinase	2.7.1.45	953 ± 128	3422 ± 735	3.6	0.07
	KHG/KDPG aldolase	4.1.3.16	176 ± 49	733 ± 60	4.2	<0.001
Involved in degradation of reactive oxygen species	Superoxide Dismutase (Mn)	1.15.1.1	49363 ± 7333	13805 ± 2269	0.28	<0.001
	Thioredoxin reductase	1.8.1.9	30512 ± 3734	9465 ± 1700	0.31	<0.001
	Catalase	1.11.1.6	80961 ± 15194	26199 ± 8907	0.32	<0.001
Synthesis of pantoate	3-methyl-2-oxobutanoate hydroxymethyltransferase	2.1.2.11	5473 ± 1011	818 ± 35	0.15	<0.001
	Pantothenate synthetase	6.3.2.1	3896 ± 444	745 ± 63	0.19	<0.001

Table 4: *Differences in gene expression between reference precultures and furfural precultures of B. coagulans using RNA-Seq analysis. A foldchange above 1 relates to a higher expression of the gene in the furfural preculture, while a foldchange below 1 relates to a higher expression of the gene in the reference preculture. The P-value expresses the significance of the fold-change. (continued)*

			Amount of RNA measured			
	Gene name	Enzyme classification	Control	furfural	Foldchange	p-value
Sigma factor F	RNA polymerase sigma-F factor		462 ± 26	1797 ± 363	3.9	<0.001
	Anti-sigma F factor antagonist		207 ± 17	813 ± 143	3.9	<0.001
	Anti-sigma F factor		267 ± 17	1076 ± 143	4.0	<0.001
Sigma factor B	Anti-sigma-B factor antagonist		1242 ± 64	2344 ± 260	1.9	<0.001
	RNA polymerase sigma-B factor		2014 ± 192	4941 ± 920	2.5	<0.001
Sigma factor B regulated stress proteins	Stress response protein YsnF		346 ± 38	1296 ± 283	3.7	<0.001
	Stress response protein NhaX		190 ± 23	759 ± 132	4.0	<0.001
	General stress protein 13 (YugI)		15960 ± 1782	23581 ± 2314	1.5	<0.001
	Stress response protein CsbD		201 ± 97	396 ± 76	2	0.015
	General stress protein 20U (DPS)		2576 ± 285	6187 ± 1470	2.4	<0.001
	General stress protein 17M (YflT)		843 ± 124	2038 ± 272	2.4	<0.001
	General stress protein 30 (YxaB)		523 ± 69	1939 ± 190	3.7	<0.001
Pathway from pyruvate to formate and acetyl- CoA	Formate acetyltransferase	2.3.1.54	4916 ± 664	32408 ± 3242	6.6	<0.001
	Pyruvate formate-lyase-activating enzyme	1.97.1.4	1034 ± 142	6914 ± 731	6.7	<0.001
Sigma A	RNA polymerase sigma factor SigA		16055 ± 1485	14655 ± 779	0.91	0.37

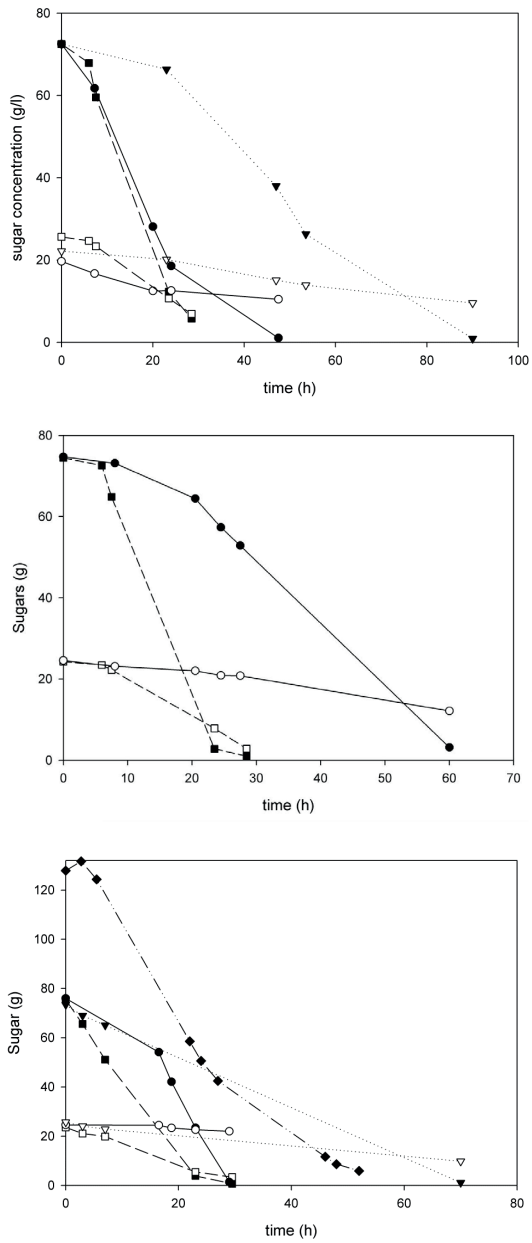


figure 5: *Glucose (closed symbols) and xylose (open symbols) amount present in 1 liter controlled fermentations. Either YP10 medium (a), YP20 medium (b) or YA medium (c) was used. ■ and (-----): Control with reference preculture, absence of by-products. ● and (————): Furfural preculture with addition of by-product mixture acid-100 (a, c) or acid-150 (b), ▼ and (·····): Reference preculture with addition of by-product mixture acid-100 (a, c) or acid-150 (b). ◆ and (–·–·–·–): Furfural preculture, 125 g/l glucose as only carbon source, addition of by-product mixture acid 100 (c).*

DISCUSSION

Addition of furfural during precultivation of *B. coagulans* DSM2314 results in an improved fermentation on substrates rich in lignocellulosic by-products. This effect has to our knowledge not been described before. Addition of other by-products during precultivation had no or a less significant impact.

This improvement was observed in lactic acid yield, titres and productivity, both on YP media and YA media. On YP medium, also a strong reduction of the lag phase was also observed. whereas The reason for this difference is unknown. Elongation of cells, which appeared during precultivation in the presence of furfural, seemed to be related to the improvement of fermentation using by-product rich substrates. This elongation was reversed when cells were transferred back to original medium, suggesting that the effects of precultivation are adaptation effects, and not based on changes in the genotype.

When reference precultures were used in by-product rich medium, the adaptation may also occur during the long lag phase. This adaptation most likely occurs since furfural was also present in the acid-100 and acid-150 mixture (table 1). However, since the fermentation medium also contains numerous other by-products, the adaptation takes 30h (acid-100) to 60h (acid-150) to occur. By allowing the cells to adapt during preculture, the adaptation can be accelerated. It was observed that the amount of furfural added had a significant impact on the performance during the actual fermentation (figure 1). Furthermore, it was observed that when the adaptation already occured during precultivation, it also had a positive effect on yield and maximum productivity.

Conversion of xylose to lactic acid was decreased in the presence of by-products, resulting in a high residual xylose concentration at the end of fermentation (figure 5). In reference fermentations, when cells switched from co-consumption of glucose and xylose to consumption of xylose as sole carbon source, lactic acid production immediately decreased from 1.9 g/l/h to 1.2 g/l/h. It may be speculated that capacity of pathways involved in xylose fermentation is too low to produce the amount of energy required for the high maintenance in media rich in by-products.

94% of the sugars present in acid-pretreated sugarcane bagasse fibres is glucose, while most hemicellulose sugars such as xylose are present in the liquid fraction (van der Pol et al. 2015). *B. coagulans* precultivated in the presence of furfural can be used to efficiently produce lactic acid from glucose present in bagasse fibres, as shown in figure 4. However, fermentation of the liquid fraction containing mostly xylose may not be efficient. A possible solution is to ferment this fraction in a separate process by a different micro-organism.

Micro-organisms are able to reduce furfural toxicity by either converting the furfural, or by increasing its tolerance to this inhibitor. *Escherichia coli*, *Cupriavidus basilensis*, *Saccharomyces cerevisiae* were previously shown to be able to convert furfural, either into furfuryl alcohol (Gutiérrez et al. 2002, Laadan et al. 2008) or into 2-furoic acid/2,5-furandicarboxylic acid

(Koopman et al. 2010, Nichols et al. 2008), compounds that are less toxic for these micro-organisms. No changes in furfural concentration or formation of furfuryl alcohol or furoic acid was observed during precultivation of *B. coagulans* in the presence of furfural. Both genomic data and RNA-seq data were screened for the presence of genes known to convert furfural to less toxic compounds, however no genes were found with similarities to known furfural converting genes. This makes it likely that the beneficial effect of furfural addition is caused by increased tolerance.

Previous studies suggested that furfural toxicity is mainly caused by the formation of Reactive Oxygen Species (ROS), which are created by the large dipole moment of the aldehyde group in furfural molecules (Allen et al. 2010, Feron et al. 1992). Several genes are present in *B. coagulans* which are known to decrease the toxicity of ROS, including superoxide dismutase, thioredoxin reductase and catalase (Cabiscol et al. 2000). However, all these genes were downregulated in cultures to which furfural was added. It is therefore unlikely that these enzymes are involved in the furfural adaptation.

The presence of furfural had the largest impact on genes involved in *de novo* pyrimidine pathway, used for the synthesis of uracil. Uracil can also be produced from compounds present in yeast extract like cytosine. It was found that a cytosine transporter gene was strongly upregulated in precultures grown in the presence of furfural, while genes involved in the conversion of cytosine to uracil and cytidine were slightly upregulated. Since significant uracil and cytosine concentrations are present in yeast extract, *de novo* pyrimidine synthesis may not be required. The relevance of these changes in increasing furfural tolerance is unclear.

Elongation of cells was observed when grown in the presence of furfural (figure 2). Their length increased up to a factor 3 during precultivation, and up to a factor 8 during fermentation. RNA-seq analysis showed that several pathways related to the production of cell wall compounds like peptidoglycans, bacillosamines and spermidines were found to be upregulated in the presence of furfural (Table 5). An earlier study has shown that heat stress resulted in a similar elongation of bacillus cells. This also led to a shift in cell wall peptidoglycan composition. As a result, the cells showing elongation during heat stress were less vulnerable for autolysis (Novitsky et al. 1974). Another study showed that peptidoglycans and spermidine can covalently bind in cell walls, stabilizing bacillus cells during osmotic stress (Wortham et al. 2007, Yokoyama et al. 1989). Apparently, elongated cell morphology and cell wall composition are related to stress response in bacilli and as such also play a role in the observed adaptation to furfural.

Sigma factors tightly regulate the expression of many genes in bacilli. One sigma factor found to be upregulated in the presence of furfural is *SigF* (Schmidt et al. 1990). *SigF* is believed to be involved in the first stage of sporulation. However, the culture used in RNA-seq analysis was in the (late) exponential growth phase, which is confirmed by high presence of *SigA*, a sigma factor only expressed during exponential growth (Qi and Doi. 1990). Expression of *SigE*, involved in the second stage of sporulation, was also not observed. It can be theorized

that *SigF* may have a role apart from sporulation, and may be involved in increasing cell wall strength normally required for the formation of forespores.

Another sigma factor that was upregulated in the presence of furfural is *SigB* (van Schaik et al. 2005). Previous research showed that *SigB* expression is induced by various stress factors, varying from temperature stress to acid stress to oxygen stress (Hecker et al. 2007, Price et al. 2001). *SigB* is estimated to control the expression of up to 150 genes (Price et al. 2001). However, the function of most of these genes is unknown (Price 2001). During RNA-Seq analysis, several genes were found to be upregulated which are promoted by *SigB*, such as *YsnF*, *NHaX*, *DPS/ general stress factor 20U*, the *APP/OPP* transport gene cluster, and *GsiC*.

According to Hecker et al (2007), inducing a mild stress to bacilli can activate *SigB*. When *SigB* is activated, a cross protection against different stress conditions which are otherwise lethal was observed. This cross protection may also be triggered in *B. coagulans* cells exposed to 1 g/l of furfural during preculture. Gaidenko and Price (1998) showed that a knockout of *SigB* in *Bacillus subtilis* decreased cellular tolerance against inhibitory conditions by 10-fold. It may be interesting to investigate whether upregulation of *SigB*, *YsnF* and/or *NhaX* via genetic engineering will increase the viability of *B. coagulans*, when grown directly in the presence of potentially inhibiting by-products.

REFERENCES

Allen SA, Clark W, McCaffery JM, Cai, Z, Lanctot A, Slininger PJ, Liu ZL (2010) Furfural induces reactive oxygen species accumulation and cellular damage in Saccharomyces cerevisiae Biotechnol Biofuels 3:1-10

Anders S, & Huber W (2010) Differential expression analysis for sequence count data Genome biol 11: R106.

Benjamini Y, & Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Series B Stat Methodol 57:289-300.

Boisvert S, Laviolette F, Corbeil J (2010) Ray: simultaneous assembly of reads from a mix of high-throughput sequencing technologies. J of Comp Biol, 17:1519-1533.

Cabiscol E, Tamarit J, Ros J (2000) Oxidative stress in bacteria and protein damage by reactive oxygen species, Int Microbiol 3:3-8

Fengel, D, Wegener, G. (1983). Wood: chemistry, ultrastructure, reactions. Walter de Gruyter, Berlin.

Feron VJ, Til HP, De Vrijer F, Woutersen RA, Cassee FR, Van Bladeren PJ (1991) Aldehydes: occurrence, carcinogenic potential, mechanism of action and risk assessment Mutat Res-Gen Tox 259:363-385.

Gaidenko TA, Price, CW (1998) General stress transcription factor ςB and sporulation transcription factor ςH each contribute to survival of Bacillus subtilis under extreme growth conditions J bacteriol 180, 3730-3733.

Gutiérrez T, Buszko ML, Ingram LO, Preston JF (2002) Reduction of furfural to furfuryl alcohol by ethanologenic strains of bacteria and its effect on ethanol production from xylose Appl Biochem biotechnol 98:327-340

Hecker M, Pané-Farré J, Uwe V (2007) SigB-dependent general stress response in Bacillus subtilis and related gram-positive bacteria Annu Rev Microbiol 61:215-236.

Hendriks, ATWM, Zeeman G (2009) Pretreatments to enhance the digestibility of lignocellulosic biomass Bioresource technol 100:10-18.

Koopman F, Wierckx N, de Winde JH, Ruijssenaars HJ (2010) Identification and characterization of the furfural and 5-(hydroxymethyl) furfural degradation pathways of Cupriavidus basilensis HMF14. Proc Natl Acad Sci 107:4919-4924.

Laadan B, Almeida JRM, Rådström P, Hahn-Hägerdal B, Gorwa-Grauslund M (2008) Identification of an NADH-dependent 5-hydroxymethylfurfural-reducing alcohol dehydrogenase in Saccharomyces cerevisiae Yeast 25:191-198

Li H (2013) Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM http://arxiv.org/abs/1303.3997 (accessed 11-2015)

Maas RHW, Bakker RR, Jansen MLA, Visser D, Jong E de, Eggink G, Weusthuis RA (2008) Lactic acid production from lime-treated wheat straw by Bacillus coagulans: neutralization of acid by fed-batch addition of alkaline substrate Appl Microbiol Biotechnol 78:751-758

Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq Nat methods 5:621-628.

Nichols NN, Sharma LN, Mowery RA, Chambliss CK, van Walsum GP, Dien BS, Iten LB (2008) Fungal metabolism of fermentation inhibitors present in corn stover dilute acid hydrolysate Enzyme Microb Technol 42:624-630

Novitsky TJ, Chan M, Himes RH, Akagi JM (1974) Effect of temperature on the growth and cell wall chemistry of a facultative thermophilic Bacillus J bacteriol 117:858-865.

Palmqvist E, Hahn-Hägerdal B (2000) Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition, Bioresource Technol 74:17-24

Palmqvist E, Hahn-Hägerdal B (2000) Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition, Bioresource Technol 74:25-33 doi: 10.1016/S0960-8524(99)00161-3

Peng Y, Leung HC, Yiu SM, Chin FY (2012) IDBA-UD: a de novo assembler for single-cell and metagenomic sequencing data with highly uneven depth Bioinformatics 28:1420-1428.

Price CW, Fawcett P, Ceremonie H, Su N, Murphy CK, Youngman P (2001) Genome-wide analysis of the general stress response in Bacillus subtilis. Mol microbiol 41:757-774.

Qi FX, Doi RH (1990) Localization of a second SigH promoter in the Bacillus subtilis sigA operon and regulation of dnaE expression by the promoter J bacteriol 172:5631-5636.

Schmidt R, Margolis P, Duncan L, Coppolecchia R, Moran CP, Losick, R (1990) Control of developmental transcription factor sigma F by sporulation regulatory proteins SpoIIAA and SpoIIAB in Bacillus subtilis. Proc Natl Acad Sci 87:9221-9225.

Seemann T (2014) Prokka: rapid prokaryotic genome annotation. Bioinformatics btu153:1-2

Van der Pol EC, Bakker RR, Baets P, Eggink G (2014) By-products resulting from lignocellulose pretreatment and their inhibitory effect on fermentations for (bio)chemicals and fuels Appl Microbiol Biotechnol 98:9579-9593

van der Pol EC, Bakker RR, Zeeland ANT, Sanchez Garcia D, Punt A, Eggink G (2015) Analysis of by-product formation and sugar monomerization in sugarcane bagasse pretreated at pilot plant scale: Differences between autohydrolysis, alkaline and acid pretreatment Bioresource Technol 181:114-123

van Schaik W, Tempelaars MH, Zwietering MH, de Vos WM, Abee T (2005) Analysis of the role of RsbV, RsbW, and RsbY in regulating σB activity in Bacillus cereus. J bacteriol 187:5846-5851.

Walton, SL, Bisschoff, KM, van Heiningen, AR, van Walsum, GP (2010 Production of lactic acid from hemicellulose extracts by Bacillus coagulans MXL-9. J. Ind. Microbiol. Biotechnol. 37:823-830.

Wiegeshoff F, Beckering CL, Debarbouille M, Marahiel MA (2006) Sigma L is important for cold shock adaptation of Bacillus subtilis J bacteriology 188:3130-3133.

Wortham BW, Oliveira MA, Patel CH (2007) Polyamines in Bacteria: Pleiotropic effects yet specific mechanisms Adv Exp Med Biol 603:106-115

Yokoyama K, Mizuguchi H, Araki Y, Kaya S, Ito E (1989) Biosynthesis of linkage units for teichoic acids in gram-positive bacteria: distribution of related enzymes and their specificities for UDP-sugars and lipid-linked intermediates J bacteriol 171:940-946.

CHAPTER 6

Production of L(+)-lactic acid from acid pretreated sugarcane bagasse using *Bacillus coagulans* DSM2314 in a Simultaneous Saccharification and Fermentation strategy

This chapter will be submitted as:
van der Pol E.C., Eggink G., Weusthuis R.A.

ABSTRACT

Sugars derived from lignocellulose-rich sugarcane bagasse can be used as feedstock for production of L(+)-lactic acid, a precursor for renewable bioplastics. In our research, acid pretreated bagasse was hydrolysed with the enzyme cocktail GC220 and fermented by the moderate thermophilic bacterium *Bacillus coagulans* DSM2314. Saccharification and fermentation were performed simultaneously (SSF), adding acid pretreated bagasse either in one batch or in two stages. SSF was performed at low enzyme dosages of 10.5-15.8 FPU/g DW bagasse.

The first batch SSF resulted in an average productivity of 0.78 g/l/h, which is not sufficient to compete with lactic acid production processes using high-grade sugars. Addition of 1 g/l furfural to precultures can increase *B. coagulans* resistance towards by-products present in pretreated lignocellulose. Using furfural-containing precultures increased productivities to 0.92 g/l/h, with a total lactic acid production of 91.7 gram in a reactor with 1 litre starting volume, containing 20% W/W DW bagasse. Bagasse in these processes was solubilised with a liquid fraction, obtained directly after acid pretreatment, to increase sugar concentrations. Solubilising the bagasse fibres with water increased the average productivity to 1.14 g/l/h, with a total lactic acid production of 84.2 gram in a reactor with 1 liter starting volume. Due to fast accumulation of lactic acid, enzyme activity was repressed during two-stage SSF. This resulted in a slightly lower total lactic acid production of 75.6 gram. However, the two-stage SSF resulted in an average lactic acid productivity of 1.81 g/l/h. The average productivity in the first 23h of 2.54 g/l/h was similar to productivities obtained in fermentations with high-grade sugars.

INTRODUCTION

Lactic acid is conventionally used as natural preservative in food and cosmetics. A relatively new application is its utilization as intermediate for the production of chemicals and polymers [1]. Polymerized lactic acid (PLA) can be used as bioplastic, serving as a biobased alternative to oil-derived plastics such as polyethylene and polystyrene [1]. Lactic acid can be further converted into acrylic acid, an intermediate used in the plastic and textile industry [2].

Lactic acid is commonly produced in bacterial fermentation processes, using high-grade sugar or sugar-rich resources such as molasses or starch as feedstock [3]. However, due to the limited availability of sugar-rich crops, exploration of alternative feedstocks is a main target of current research [4]. Lignocellulose, consisting of 60-75% polymerized sugar on weight basis, is an interesting alternative feedstock [5].

A combination of thermo-chemical pretreatment and enzymatic hydrolysis can be used to obtain fermentable monomeric sugars from lignocellulose [6,7]. However, thermo-chemical pretreatment also results in the formation of by-products such as organic acids, phenolics and furans. These compounds can negatively influence microbial growth and product formation during fermentation [6,8].

After thermo-chemical pretreatment, a solid liquid separation is performed (figure 1). This results in a solid fraction in which bagasse fibres are present consisting mainly of cellulose, whereas it can also contain part of the hemicellulose and lignin [5,7]. The liquid fraction contains some hemicellulose-derived oligomeric and monomeric sugars, but also a part of the lignin is present.

Pretreated bagasse fibres have a high water holding capacity, resulting in a viscous broth at high dry weight (DW) concentrations [7]. Therefore, a maximum of 20% can be added to the fermentation before the broth becomes too viscous. Since bagasse has a DW of 31% W/W after thermo-chemical pretreatment, it has to be diluted before it can be hydrolysed and fermented. Sugar concentrations can be increased by solubilising the bagasse fibres with the liquid fraction, resulting from solid-liquid separation of pretreated biomass (figure 1).

Separate Hydrolysis and Fermentation (SHF) is an often described process to monomerize (hemi)cellulosic sugars, and to ferment those sugars to lactic acid in two separate steps [9]. During enzymatic hydrolysis, increasing concentrations of sugars inhibit enzymatic activity, making it difficult to efficiently obtain high sugar concentrations [10]. In simultaneous saccharification and fermentation processes (SSF), enzymes and micro-organisms are simultaneously active in the same reactor. This reduces product inhibition on enzymatic activity, since released monomeric sugars are directly consumed by the micro-organism [11]. Conditions applied in SSF processes such as pH and temperature should be suitable for both the enzyme cocktail and micro-organism.

Bacillus coagulans is an interesting strain for the production of lactic acid from lignocellulose in an SSF process [11]. It can ferment both glucose and xylose homo-fermentatively with conver-

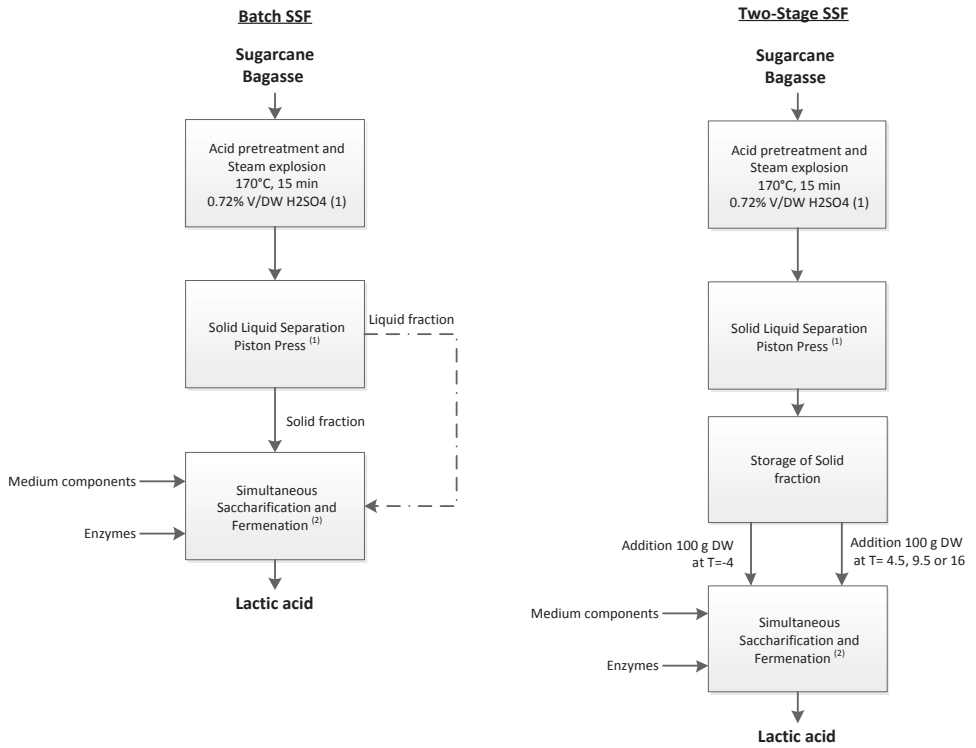


Figure 1: *Process overview for the production of lactic acid from sugarcane bagasse, either using a Batch or Two-Stage Simultaneous Saccharification and Fermentation (SSF). 1: acid pretreatment on pilot plant scale as shown in described research (van der Pol et al. 2015). 2: SSF of the solid pretreated bagasse fraction obtained after solid-liquid separation (This work). The liquid fraction can either be co-fermented during the SSF, or it can be used in a separate fermentation.*

sion efficiencies of over 90% W/W. Furthermore, *B. coagulans* produces lactic acid with a high productivity of 2.5-3 g/l/h [12,13,14]. It is able to grow in slightly acidic environments while it is also a moderate thermophile with an optimum growth temperature of approximately 50°C, similar to optimal conditions for commercial enzyme cocktails such as GC220 (Genencor, Denmark) and CTeC2 (Novozymes, Denmark). However, growth of *B. coagulans* can be inhibited by by-products present in pretreated lignocellulose [8]. A previous study has shown that the inhibitory effects of by-products can be reduced by adding low concentrations of furfural, one of the inhibitory by-products found in acid pretreated bagasse, to the preculture of *B. coagulans* [15]. Using *B. coagulans* pre-cultures to which 1 g/l furfural was added as inoculum increased lactic acid productivity, yield and titre in fermentation processes with substrates resembling the composition of acid pretreated bagasse [15]. In this paper, it is investigated whether furfural addition to precultures of *B. coagulans* also has a beneficial effect on fermentation processes using acid-pretreated bagasse as substrate. Furthermore, other challenges of the SSF process

such as viscosity are tackled in this study. To obtain an SSF process which is economically attractive, and which can compete with lactic acid production on high-grade sugars, lactic acid productivities and yields similar to fermentations on high-grade sugars should be reached.

MATERIAL AND METHODS

Chemicals, enzymes and biomass

All chemicals used as well as ammonium phosphate and calcium hydroxide were ordered at Sigma-Aldrich (St. Louis, USA), and had a purity of at least 98%. Yeast extract, peptone, glucose and BIS-Tris were ordered at Duchefa (The Netherlands).

The enzyme cocktail used in most processes was Genencor GC220, which had an activity of 105 FPU/ml (Genencor, Denmark) [16]. In one experiment, Novozymes CTeC 2 was used, which had an activity of 168 FPU/ml (Novozymes, Denmark).

Sugarcane was harvested in Queensland, Australia. The bagasse residue after sugar extraction was pretreated for 15 minutes at 170°C using 0.72% sulphuric acid, followed by steam explosion, as described previously [7]. The final solid material contained 47 % glucan and 3% xylan.

Micro-organism

Bacillus coagulans DSM 2314 was acquired as freeze dried stock at the German collection of micro-organisms and cell cultures (DSMZ, Germany). Cells were suspended for 30 minutes in 5 ml PYPD medium, consisting of 5 g/l yeast extract, 10 g/l peptone, 20 g/l glucose and 10 g/l BIS-Tris, pre-sterilized for 20 minutes at 121°C. Cells were transferred to 60 ml anaerobic flasks containing 50 ml PYPD medium, and grown for 16 hours to an optical density measured at 660 nm (OD660) of approximately 2. After addition of glycerol to reach a concentration of 15% v/v in the sample, cells were stored in 1.5 ml aliquots in cryovials at -80°C until used.

Preculture preparation

60 ml sterile anaerobic flasks were filled with either 50 ml PYPD medium for control precultures, or 25 ml 2x concentrated PYPD medium sterilized at 121°C for 20 minutes, and 25 ml of 2 g/l furfural dissolved in milliQ water, which was pasteurized for 1 hour at 85°C, for furfural containing precultures. The anaerobic flasks were inoculated with 250 µl *B. coagulans* freezer stock to obtain a starting OD660 of 0.01, and cultivated in an incubator at 50°C without shaking. When an OD660 of 1 was reached, which occurs after approximately 15 hours for control precultures, and after approximately 25 hours for furfural containing precultures, the preculture was used as inoculum for SSF.

Batch Simultaneous Saccharification and Fermentation (SSF)

Fermentations were performed in Multifors reactors (Infors, Switzerland), which were pre-sterilized empty for 20 minutes at 121°C. The SSF consisted of two phases, a pre-hydrolysis phase of 4-6 hours and a fermentation phase. During the pre-hydrolysis phase, 645 gram of pretreated solid bagasse (31% W/W dry weight), corresponding to 200 grams of dry weight, was added to the fermenter, together with 245 ml of sterile water or simulated liquid fraction, of which the composition was based on previous findings [7] (table 1). Also, 25% of the total amount of enzymes was added. The pH was set to 5.0 and controlled by addition of 4N $Ca(OH)_2$ (14.8% W/V). The temperature of the fermenter was set at 50°C, the stirrer speed was initially started at 40 RPM, and increased to 100 RPM during the first hour when the viscosity of the biomass was sufficiently decreased, and no active aeration was applied. After the pre-hydrolysis phase, 100 ml of 100 g/l of yeast extract was added and the fermenters were inoculated with 50 ml of preculture (5% V/V).

Table 1: *composition of the liquid fraction acquired after solid liquid separation of acid pretreated sugarcane bagasse, as determined in a previous study (van der Pol 2015). The presence of different compounds is shown in gram per 245 ml of liquid fraction.*

by-product	Presence (g)
Acetic acid	0.521
Formic acid	0.052
Glycolic acid	0.165
furfural	0.272
coumaric acid	0.064
vanillin	0.016
xylose	3.98
glucose	1.15

B. coagulans has an optimal growth at a temperature of 54°C and a pH of 6.5, but can grow at a broad temperature range between 30-58°C, and pH range of 4.8-7.5. Hydrolysis of pretreated bagasse was achieved by the enzyme cocktail Genencor GC220, which has a temperature optimum of 50°C and a pH optimum at 5.0. At pH 5.8 the conversion rate of glucan to glucose by GC220 is at 85% of the conversion rate at pH 5. As conditions for the SSF processes, 50°C and pH 5.8 were chosen.

During fermentation, the stirrer speed was set at 100 RPM, the temperature was controlled at 50°C and the pH was controlled at 5.8 by the addition of 4N $Ca(OH)_2$ (14.8% W/V). The total amount of GC220 enzyme cocktail added during SSF was 10% V/DW / 10.5 FPU per g DW material, 12.5% V/DW / 13.1 FPU per g DW material or 15 % V/DW / 15.8 FPU per g DW mate-

rial. At regular intervals during all SSF experiments, samples of 10 ml were taken and stored at -20°C until analysis was performed.

Two-stage Simultaneous Saccharification and Fermentation (SSF)

The pre-hydrolysis phase of two-stage SSF was started with the addition of 322.5 gram of pretreated bagasse, equivalent to 100 gram dry weight, 245 ml of 20.4 g/l $(NH_4)_3PO_4$ in sterile water, and 25% of the total enzymes used. The pH was initially set to pH 5 by addition of 4N H_2SO_4 or 4N $Ca(OH)_2$, and was controlled afterwards by addition of 4N $Ca(OH)_2$. Temperature was kept at 50°C, and stirring was kept at 100 RPM. The inoculation procedure after pre-hydrolysis was similar to the batch SSF. At 4.5h or 9.5h, 322.5 gram of additional pretreated bagasse was added gradually over the time span of 1.5 hours, while the stirring speed was increased to 150 RPM. In total, 15% V/DW enzymes was added, corresponding to 15.8 FPU/g DW pretreated bagasse.

Analysis of lactic acid, monomeric sugars and lignocellulosic by-products

Analysis of lactic acid and sugars was performed using a Waters e2695 HPLC system (Milford, USA) equipped with a Waters Refractive index RI2414 and a Waters 2489 UV/Vis spectrophotometer measuring at 210 nm. The column used was a Shodex RS pak KC-811 ion exchange column (length 300 mm – I.D. 8 mm), controlled at 65°C. As eluent, 3mM H_2SO_4 in milliQ water was used. The flow used was 1 ml/min.

Samples taken during SSF were thawed and vortexed. 1.5 ml of sample was transferred to a 2 ml eppendorf tube and placed in a heating block for 15 minutes at 70°C, ensuring all calcium lactate was dissolved. Samples were centrifuged for 4 minutes at 13,200 RPM. 250 µl of supernatant was mixed with 250 µl of water and 500 µl of 1M H_2SO_4/ 1mM phthalic acid (which was used as internal standard) in milliQ water. Samples were heated again to 70°C for 15 minutes, cooled down for 2 hours, filtered using 0.2 µm Spartan filters to ensure all calcium was precipitated and removed, and supernatants were measured using HPLC.

Glucan, xylan and uronic acid analysis

The neutral sugar content and composition was determined in duplicate according to Englyst and Cummings [17]. After pre-hydrolysis with 72% (w/w) H_2SO_4 for 1 h at 30°C, the samples were hydrolysed with 1 M H_2SO_4 at 100°C for 3 h. The monosaccharides were derivatised to their alditol acetates and analysed by gas chromatography (Focus-GC, Thermo Scientific, Waltham, MA, USA). Inositol was used as internal standard.

The uronic acid content was determined in duplicate according to the automated colorimetric m-hydroxydiphenyl assay [18], using an auto-analyser (Skalar Analytical B.V., Breda, The Netherlands). Galacturonic acid was used for calibration.

Effect of lactic acid concentration on enzymatic hydrolysis

150 ml shake flasks were filled with 6.5 grams of acid pretreated bagasse, corresponding to 2 gram of DW. To these shake flasks, 15 ml 0.1M citric acid and 23 ml 0.2 M $Na_2HPO4.12H_2O$ was added to obtain a buffer at pH 5.8. To these flasks, 0, 1.25, 2.5 and 5 ml of (D/L)-lactic acid solution (80% in H_2O, density 1.2 g/ml) was added. MilliQ water was used to set the final working volume at 50 ml. The pH was reset to 5.8 using KOH pellets when required. The shake flasks were incubated for 15 minutes at 50°C and 100 RPM. After addition of 100 µl of the enzyme cocktail GC220, the flasks ware shaken at 50°C and 200 RPM. After 1 and 2 hours, samples were taken, immediately heated to 98°C for 10 minutes to inactivate the enzymes, cooled on ice, filtered using 0.2 µm Spartan filters, and stored at 4°C. Glucose concentrations were measured using the previously described HPLC method.

Calculations determining lactic acid production, yields and sugar monomerization

Due to a large increase in volume during the SSF, values were recalculated to gram per batch instead of gram per litre to determine yields and total lactic acid produced. Volume of the reactor at time point t ($V_{R,t}$) was calculated based on the amount of base added in percentage of the total amount of base added at time point t (B_t), volumes of the reactor at T=0 in litre ($V_{F,0}$), determined volume of the reactor at the end of the SSF in litre ($V_{F,end}$), and the total volume of sample taken at time point t in litre (V_S). The amount of lactic acid in gram at time point t ($A_{LA,t}$) was calculated based on the concentration of lactic acid in gram per litre determined via HPLC at time point t ($C_{LA,t}$), the volume of the reactor at time point t($V_{R,t}$), and a correction factor for the amount of lactic acid taken out by sampling at sample n ($CF_{LA,n}$).

$$A_{LA,t}=C_{LA,t}*V_{R,t}+CF_{LA,n} \text{ with } V_{R,t}=V_{F,0}+\frac{B_t}{B_{end}}*(V_{F,end}-V_{F,0})-V_S$$

$$\text{and } CF_{LA,n}=\sum_{i=1}^{n-1} C_{LA,i}*V_{S,i}$$

The solid fraction contained 47% glucans and 3% xylans when added to the reactor. Due to the monomerization reaction being a hydrolysis reaction, the molecular weight increases from 162 gram per mol of subunit glucan to 180 gram/mol glucose, and from 132 gram per mol of subunit xylan to 150 g/mol xylose, thus the maximum amount of monomeric sugars which can be formed from 200 gram of dry weight solid material is 110.2 gram of sugars.

During earlier experiments [7], and by residual glucan/xylan analysis at the end of the SSF (table 3), it was observed that from 200 grams of pretreated solid biomass, 92 gram of sugars were monomerizable by the enzyme cocktail and were thus available for fermentation.

Lactic acid production can be estimated based on the amount of $Ca(OH)_2$ added to the reactor. Lactic acid concentration at a certain time ($A_{LA,t}$) was calculated by taken the amount of base added at a certain timepoint (B_t) divided by the total amount of base added ($B_{t=end}$),

and multiplied by the final lactic acid titre ($A_{LA,t=end}$) measured in triplo via HPLC. The overall calculation is:

$$A_{LA,t}=\frac{B_t}{B_{t=end}}A_{LA,t=end}$$

No difference in lactic acid production larger than 2 gram was observed between the calculations based on the $Ca(OH)_2$ addition, and HPLC analysis of samples taken at different time points during the SSF.

RESULTS AND DISCUSSION

Batch SSF, solubilising bagasse fibres with liquid fraction

Since lactic acid is a bulk chemical with low added value, it requires an efficient conversion of lignocellulosic material into lactic acid with high productivities, yields and titres [19]. The dry matter content of bagasse fibres after pretreatment and solid liquid separation was 31% W/W. A bagasse fibre concentration over 20% (W/W, dry weight basis) results in too high viscosities in the fermenter. Therefore, the fibre fraction was diluted by adding 245 ml of liquid fraction, acquired during solid-liquid separation performed directly after thermo-chemical pretreatment.

In this batch SSF process, a total lactic acid production of 77.6 gram was obtained in a fermenter with 1 litre starting volume, with a final lactic acid concentration of 64.1 g/l. The average lactic acid productivity was 0.78 g/l/h, which is low compared to the 2.5-3 g/l/h observed for lactic acid fermentation processes using pure sugars[12,13,14] (table 2b). The conversion yield of lignocellulose to lactic acid was 74% W/W, whereas the conversion yield on monomerizable sugars was 80% W/W. Since both the average productivity and yield are not sufficient to compete with processes using pure sugars, the SSF process should be optimised to increase lactic acid productivity on the lignocellulosic material.

Batch SSF inoculated with *B. coagulans* preculture containing furfural

In earlier research, it was found that the addition of furfural to precultures of *B. coagulans* improved growth and lactic acid production on substrates rich in lignocellulosic by-products [15]. However, this research was performed using a model substrate. Although the composition of this substrate resembles acid pretreated bagasse with regard to presence of by-products, other process parameter such as increased viscosity during SSF, and the presence of compounds like lignin are not taken into account. The effect of furfural addition to precultures was therefore also investigated in an SSF set-up using bagasse fibres solubilised with liquid fraction.

Addition of 1 g/l furfural to the preculture, used as inoculum for the batch SSF experiment, reduced the initial lag phase by 20 hours (Table 2a, figure 2). Besides the reduction in lag

phase, the yield of lactic acid on total lignocellulosic sugars was 87% W/W when a furfural preculture was used, which is significantly higher than the 74% W/W observed when a normal preculture was used. Furfural addition increased total lactic acid production produced in the fermenter with 1 litre starting volume by 18% to 91.7 grams, while average lactic acid productivity increased with 0.14 g/l/h to 0.92 g/l/h. The maximum lactic acid productivity was

Table 2: *a: SSF experiments performed with 20% DW pretreated bagasse using the enzyme cocktail GC220 at different enzyme dosages to monomerize sugars, and B. coagulans DSM2314 for fermentation to lactic acid. Either liquid fraction acquired after acid pretreatment or MilliQ water is used to solibulise the solids. As inoculum, either a preculture to which furfural was added was used, or a control preculture was used which did not contain furfural. Addition of 20% DW bagasse fibres was either done as batch at the start of fermentation, or in two stages of 10% DW. b: different fermentation processes containing high-grade sugars, inoculated with B. coagulans. Different nitrogen sources were used: YE=yeast extract, PE= peptone*

2a

SSF	Biomass liquefied with	Enzyme added %V/DW	Pre-culture	C_{La} (g/l)[A]	A_{La} (g)[B]	$Q_{v,av}$ (g/l/h)[C]	$Q_{v,max}$ (g/l/h)[D]	$Y_{s/La}$[E] %	$Y_{Lc/La}$[F] %	Time (h)[G]
Batch	Liquid fraction	12.5	control	64.1	77.6	0.78	4.2	80	74	90
Batch	Liquid fraction	12.5	furfural	74.6	91.7	0.92	4.2	94	87	90
Batch	Water	10	furfural	70.6	84.2	0.92	4.4	92	83	84
Batch	Water	15	furfural	70.4	83.8	1.14	4.2	91	83	68
Two-stage 9.5h	Water	15	furfural	58.2	75.9	1.81	4.3	90	73	37

2b

Strain	Carbon source	C_s (g/l)	N-source (g/l)	C_{La} (g/l)[A]	A_{La} (g)[B]	$Q_{v,av}$ (g/l/h)[C]	$Q_{v,max}$ (g/l/h)[D]	$Y_{s/La}$[E] %	Time (h)[G]	Source
B. coagulans DSM2314	Glucose +xylose	72 +24	10 YE 20 PE	59.3	83	2.50	5.1	92	29	Van der Pol 2016 [15]
B. coagulans DSM2314	Glucose +xylose	72 +24	10 YE 2 NH_4PO_4 3 NH_4SO_4	55.6	78.6	2.40	3.9	86	28	Van der Pol 2016 [15]
B. coagulans SIM-7	glucose	126	25 YE	89.3	-	2.8	5.8	90	31	Michelson 2006 [24]

A: C_{La}: concentration of lactic acid at the end of the SSF in g/l
B: A_{La}: Total lactic acid production in g
C: $Q_{v,av}$: average volumetric lactic acid productivity in g/l/h
D: $Q_{v,max}$: maximum volumetric lactic acid productivity in g/l/h
E: $Y_{s/La}$: estimated conversion efficiency of sugar monomers to lactic acid in W/W
F: $Y_{Lc/La}$: lactic acid production yield on total lignocellulosic sugars in W/W
G: total fermentation time from inoculation of SSF to reaching final lactic acid concentration

4.2 g/l/h, which is to our knowledge not yet observed in SSF processes using acid pretreated lignocellulosic material.

It was found that precultivation in the presence of furfural was beneficial for fermentation processes using acid pretreated bagasse fibres in an SSF set-up. Therefore, all subsequent SSF experiments were inoculated with *B. coagulans* precultivated in the presence of furfural.

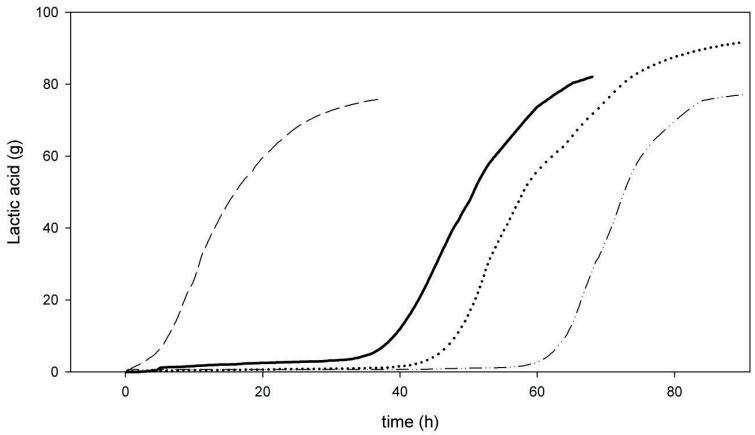


Figure 2: *Simultaneous Saccharification and Fermentation (SSF) experiments performed using 200 g/l acid pretreated bagasse DW and 10.5-15.8 FPU/g DW bagasse GC220 enzyme cocktail in pH controlled fermentations with a start volume of 1 litre. As inoculum, either furfural containing or reference preculture was used. The bagasse was either added in one batch or in two stages. Lactic acid production was followed by calcium hydroxide addition, and verified with HPLC measurements. The following experiments were performed: batch SSF solubilised with liquid fraction, inoculated with reference preculture (–···–···–), batch SSF solubilised with liquid fraction, inoculated with furfural preculture (·····), batch SSF solubilised with water, inoculated with furfural preculture (———), two-stage SSF solubilised with water, inoculated with furfural preculture (– – – –).*

Batch SSF, solubilising bagasse fibres with water

Although sufficient lactic acid production, and a high conversion yield of lignocellulose to lactic acid was observed in the batch SSF solubilised with liquid fraction, the average productivity was not yet competitive with the productivity reached on high-grade sugars. This is mainly caused by a long lag phase of approximately 40 hours. The liquid fraction contains approximately 20 gram of sugar per litre, but it also contains 2 g/l of acetic acid, 1 g/l gram of furfural and some other by-products in minor concentrations (Table 1). These by-products add up to those present in the pretreated bagasse fibres, and are one of the main challenges when using pretreated lignocellulose in fermentation processes [5]. While the addition of a small extra amount of sugars may be interesting to slightly increase total lactic acid production, there is a risk that the additional amount of inhibiting by-products result in a decrease in productivity.

Therefore, experiments were performed where the solid fraction was solubilised with milliQ water instead of liquid fraction.

Similar to results observed for batch SSF solubilised with liquid fraction, using furfural containing precultures as inoculum for batch SSF solubilised with water led to a lag phase reduction of 15 hours compared to using a control preculture (data not shown). Still, an extensive lag phase of approximately 32h was observed at the beginning of the batch SSF solubilised with water. The production phase however showed a high lactic acid productivity, with a maximum lactic acid productivity of 4.2 g/l/h, similar to batch SSF solubilised with liquid fraction.

Batch SSF using solid fraction solubilised with water was performed with two different enzyme concentrations, 7.5% V/DW and 15% V/DW (Figure 2 and table 2a). In both fermentations the sugar concentrations decreased during the production phase and reached levels below 5 g/l after 52 and 55 hours for the fermentation with 7.5% V/DW and 15% V/DW enzyme cocktail respectively. The fermenter in which 7.5% V/DW of enzymes was used showed a sharp decrease in lactic acid productivity after 52 hours to only 0.51 g/l/h, whereas the SSF containing 15% V/DW showed an average productivity of 1.44 g/l/h between 55 and 62 hours. At 66 hours, 5ml (2.5% V/DW) additional enzyme cocktail was administered to increase enzyme concentrations to 10% V/DW. Immediately, the glucose concentration increased, reaching a maximum concentration of 5 g/l, and lactic acid production resumed in a similar fashion as was observed for fermentations using 15% V/DW enzymes. This shows that enzymatic hydrolysis of sugars was the rate limiting step of the fermentation containing 7.5% V/DW enzyme cocktail, while 10% V/DW enzymes was a sufficiently high enzyme dosage.

After 68 hours, the process with the 15% V/DW enzyme cocktail reached a lactic acid concentration of 70.4 g/l, equivalent to a total lactic acid production of 83.8 gram. This corresponds to a lactic acid production yield on total lignocellulosic sugars of 83% W/W and a conversion efficiency for *B. coagulans* of monomeric sugar to lactic acid of 92% W/W. Overall, an average lactic acid productivity of 1.14 g/l/h was reached, which is significantly higher than the average productivity of 0.92 g/l/h observed for batch SSF solubilised with liquid fraction. However, average productivity during batch SSF solubilised with water was still low compared to lactic production on high-grade sugars.

Compared to SHF however, the process time of 68 hours during batch SSF is short. Where enzyme hydrolysis alone during SSF can take between 48-72hours, and the subsequent fermentation requires another 40-60 hours [13]. Therefore, although batch SSF cannot compete with high-grade sugar processes, it is already an interesting alternative to SHF. The enzyme dosage of 10.5-15.8 FPU/g DW that was administered to the batch SSF is relatively low, in most other studies an enzyme dosage of 20-40 FPU/g DW material was used [11,20,21] . Enzymatic hydrolysis is considered to be an expensive process step in the conversion of lignocellulose to chemicals due to the high costs of enzyme cocktails combined with high addition. In an earlier study, in which ethanol was produced from lignocellulose at commercial scale, it was

calculated that an enzyme dosage of 18 FPU/g DW material contributes to 19% of the total costs of the ethanol production [6].

Two-stage Simultaneous Saccharification and Fermentation (SSF)

Although the maximum productivity in batch SSF was in a similar range as observed for fermentations using high-grade sugars, the overall productivity was still low because of the long lag phase. The end of the lag phase coincided with a reduced viscosity of the culture due to enzymatic hydrolysis, suggesting that the long lag phase was caused by the high viscosity of the broth.

A decrease in viscosity can be achieved by adding the lignocellulosic material in two stages, instead of adding all lignocellulose as batch at the start of the SSF.

Using this approach, the micro-organisms directly started to grow without a visible lag phase after inoculation, and a significant lactic acid production was observed within 3 hours. After 37 hours, a total lactic acid production of 75.9 gram was reached with an average lactic acid productivity of 1.81 g/l/h, and a maximum productivity of 4.3 g/l/h (table 2a, figure 2). During the first 23 hours of the two-stage SSF, an average productivity of 2.54 g/l/h was observed. This productivity is close to the average productivity of 2.5-3 g/l/h [12,13,14] (table 2b) which is often seen during fermentation of *B. coagulans* using high-grade sugars as carbon source.

The average lactic acid in two-stage SSF is much higher than for batch SSF, but total lactic acid production and lactic acid production yields on lignocellulose were slightly lower for two-stage SSF experiments. In previous studies, it is suggested that activity of enzymes present in the cocktail can be repressed by increasing lactic acid concentrations [22,23]. The activity of GC220 was tested in the presence of high lactic acid concentrations. An experiment to determine initial enzyme activity showed that the presence of 50 g/l of lactic acid resulted in a 51% reduction in glucan monomerization by the enzyme cocktail GC220, whereas the presence of 100 g/l lactic acid results in a full inhibition of the enzymes.

It was also found that 11% more residual glucans and xylans were present at the end of the two-stage SSF compared to the end of the batch SSF (table 3). In the batch SSF, a total of 93.4 grams of sugar polymers were monomerized, while in the two-stage SSF, 83.2 grams of sugar polymers were monomerized (table 3). Since the batch SSF shows an extensive lag phase

Table 3: *Glucan and xylan monomerization during batch SSF and two-stage SSF, the average lactic acid produced in Batch SSF and Two-Stage SSF, and the conversion yield of lactic acid on monomerized sugars*

	Monomerized sugars (g) [A]	La (g)[B]	$Y_{g/La}$[C]
Batch SSF 15% V/DW enzymes	93.4	83.8	90.1%
two-stage SSF 2nd batch T=9.5	84.2	75.9	90.0%

A: sugars: total monomerization of glucan and xylan polymers during SSF.

B: La: lactic acid production at the end of the fermentation in g

C: $Y_{g/La}$: conversion efficiency of sugar monomers to lactic acid in W/W

where lactic acid concentrations are low, it can be suggested that the enzymes in batch SSF are less inhibited, leading to an increased monomerization compared to two-stage SSF. Both the batch SSF and the two-stage SSF resulted in similar conversion yield of monomeric sugars to lactic acid of approximately 90% W/W. Therefore the lower lactic acid titre observed in the two-stage SSF is probably caused by a decreased enzymatic hydrolysis of the lignocellulosic polymers.

CONCLUSION

Production of lactic acid from acid pretreated sugarcane bagasse was performed with two different processes, a batch SSF and a two-stage SSF. In these processes, enzyme hydrolysis and fermentation were combined for shorter process times compared to SHF. The objective of this study was to reach a lactic acid productivity in SSF experiments using lignocellulose similar to fermentations using high-grade sugars as feedstock.

It was shown that addition of furfural to the preculture reduced the initial growth lag phase and increased lactic acid production. In batch SSF, up to 91.7 gram of lactic acid could be produced, with a conversion yield of monomeric sugars to lactic acid of 94% W/W, and a lactic acid production yield on total sugars present in pretreated bagasse of 87% W/W. Productivities during batch SSF of 0.78-1.14 g/l/h still low compared to productivities of 2.5-3 g/l/h reached on high-grade sugars.

In two-stage SSF, viscosity of the fermentation broth was reduced. This results in an average productivity over the total process of 1.81 g/l/h. During the first 23 hours of fermentation, an average lactic acid productivity of 2.54 g/l/h was observed. This productivity is similar to the productivity obtained in fermentation process using high-grade sugars as feedstock.

It can be concluded that adequate production of lactic acid from lignocellulose was successfully accomplished by an SSF process which uses a combination of acid pretreated bagasse, *B. coagulans* as micro-organism and GC220 as enzyme cocktail. Furthermore, an improvement of enzymatic hydrolysis at high lactic acid concentrations will increase the competitiveness of the SSF process in comparison to using high-grade sugars.

ACKNOWLEDGEMENT

The authors of this paper want to acknowledge the laboratory of Food Chemistry of Wageningen UR, with special thanks to Arjen Punt for helping with glucan analysis. Furthermore, the authors would like to thank the BE-Basic consortium, Corbion-Purac and DSM for funding this research.

SUPPLEMENTARY DATA

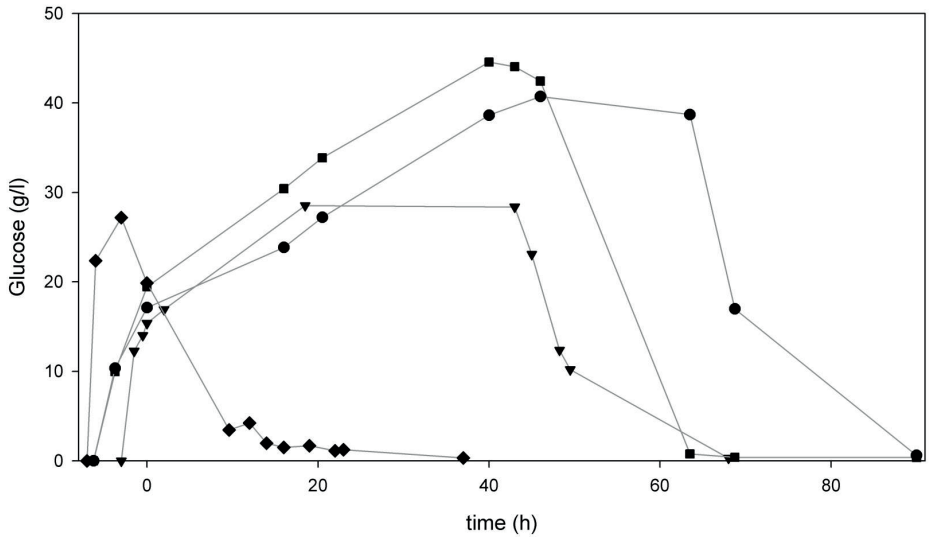


Figure A: *Simultaneous Saccharification and Fermentation (SSF) experiments performed using 200 g/l acid pretreated bagasse DW and 10.5-15.8 FPU/g DW bagasse GC220 enzyme cocktail in pH controlled fermentations with a start volume of 1 litre. As inoculum, either furfural containing or reference preculture was used. The bagasse was either added in one batch or in two stages. Glucose was measured using HPLC. The following experiments were performed: ●: batch SSF solubilised with liquid fraction, inoculated with reference preculture, ■: batch SSF solubilised with liquid fraction, inoculated with furfural preculture, ▼: batch SSF solubilised with water, inoculated with furfural preculture, ◆: two-stage SSF solubilised with water, inoculated with furfural preculture.*

REFERENCES

1 D. Garlotta, J *Polym Environ*, 2001, **9**, 63-84
2 V.C. Ghantani, S.T. Lomate, M.K. Dongare, S.B. Umbarkar, *Green Chem*, 2013, **15**, 1211-1217
3 F.A.C. Martinez, E.M. Balciunas, J.M. Salgado, J.M.D. González, A. Converti, R. P. de Souza Oliveira, *Trends Food Sci Tech*, 2013, **30**, 70-83
4 S. Srinivasan, *Renewable Energy*, 2009, **34**, 950-954
5 E.C. van der Pol, R.R. Bakker, P. Baets, G. Eggink, *Appl Microbiol Biotechnol,* 2014, **98**, 9579-9593
6 Humbird Et al 2011, NREL Technical report, https://www.researchgate.net/profile/Ling_Tao/publication/229843699_Process_Design_and_Economics_for_Biochemical_Conversion_of_Lignocellulosic_Biomass_to_Ethanol_Dilute-Acid_Pretreatment_and_Enzymatic_Hydrolysis_of_Corn_Stover/links/0fcfd5011638d5a2af000000.pdf (accessed July 2015)
7 E. van der Pol, R. Bakker, A. van Zeeland, D. Sanchez Garcia, A. Punt, G. Eggink, *Bioresource Technol*, 2015, **181**, 114-123
8 E.C. van der Pol, E. Vaessen, R.A. Weusthuis, G. Eggink. Chapter 4, To be published in 2016
9 J. Ouyang, R. Ma, Z. Zheng, C. Cai, M. Zhang, T. Jiang, *Bioresource Technol*, 2013, **135**, 475-480
10 S.-M. Lee, Y.-M. Koo, J. Lin, *Adv Biochem Eng/Biotechnol*, 2004, **87**, 173-194
11 R.H.W. Maas, R.R. Bakker, M.L.A. Jansen, D. Visser, E. de Jong, G. Eggink, R.A. Weusthuis, *Appl microbiol biotechnol*, 2008, **78**, 751-758
12 M. A. Abdel-Rahman, Y. Tashiro, K. Sonomoto, *J Biotechnol*, 2011, **156**, 286-301
13 K. Hofvendahl, B. Hahn-Hägerdal, *Enzyme Microb Technol*, 2000, **26**, 87-107
14 E.F. Bosma, J. van der Oost, W.M. de Vos, R. van Kranenburg, *Curr Biotechnol*, 2013, **2**, 360-379
15 E.C van der Pol, B. Vriesendorp, R.A. Weusthuis, G. Eggink, Chapter 5, to be published in 2016
16 M.A. Kabel, M.J.E.C. van der Maarel, G. Klip, A.G.J. Voragen, H.A. Schols, *Biotechnol Bioeng*, 2006, **93**, 56-63
17 H.N. Englyst, J.H. Cummings, *Analyst*, 1984, **109**, 937-942
18 J.-F. Thibault, *Lebensm Wiss Technol*, 1979, **21**, 247-251
19 R.P. John, K.M. Nampoothiri, A. Pandey, *Appl Microb Biotechnol,* 2007, **74**, 524-534
20 Hu, Z. Zhang, Y. Lin, S. Zhao, Y. Mei, Y. Liang, N. Peng, *Bioresource Technol*, 2015, **182**, 251-25
21 S. Hama, S. Mizuno, M. Kihara, T. Tanaka, C. Ogino, H. Noda, A. Kondo, *Bioresource Technol*, 2015, **187**, 167-172
22 P.V. Iyer, Y.Y. Lee, *Biotechnol Lett*, 1999, **21**, 371-373
23 A.B. Moldes, J. L. Alonso, J.C. Parajó, *J Chem Technol Biotechnol*, 2001, **76**, 279-284
24 T. Michelson, K. Kask, E. Jõgi, E. Talpsep, I. Suitso, A. Nurk, *Enzyme microb Technol*, 2006, **39**, 861-867

CHAPTER 7

General discussion

INTRODUCTION

Lignocellulose is the most abundant renewable biomaterial on earth, and its dry weight consists for up to 75% of polymerized sugars (Claassen et al. 1999). Therefore, lignocellulose is often proposed as potential feedstock for the production of chemicals and fuels. One such renewable chemical intermediate is lactic acid. Polymerized lactic acid (PLA) can be moulded into a bioplastic, which can be used as an alternative for petrochemically produced polystyrene (PS) and polyethylene (PE) (Garlotta 2001).

A pretreatment process is required to obtain monomeric sugars from lignocellulose, which can be used in fermentation processes for the production of lactic acid (van der Pol et al. 2014). Pretreatment of lignocellulose does not only result in formation of monomeric sugar, but also in the generation of potentially inhibiting by-products. In this thesis, by-products formed during pretreatment were identified, quantified, and their inhibitory effect on lactic acid producing bacteria was determined. This information was used to develop a process in which pretreated lignocellulose was efficiently fermented to lactic acid.

FORMATION OF BY-PRODUCTS DURING THERMO-CHEMICAL PRETREATMENT OF LIGNOCELLULOSE-RICH SUGARCANE BAGASSE

Thermo-chemical pretreatment is a process method which can be applied to increase the accessibility of polymeric sugars and/or (partially) monomerize sugar polymers present in lignocellulose-rich biomass (Hendriks and Zeeman 2009, Mosier et al. 2005). However, this pretreatment process can also result in the generation of unwanted by-products, most of them being furans, phenolics and organic acids (Larsson et al. 1999). The concentration of by-products found after thermo-chemical pretreatment is influenced by both the lignocellulose source used, and pretreatment method applied. **Chapter 2** summarizes differences in by-product concentrations found in pretreated lignocellulose originating from different sources and pretreated by different methods. For an efficient use of lignocellulose-rich biomass as feedstock for fermentation, the pretreatment has to be optimized to achieve both a high sugar monomerization and/or accessibility of the polymerised sugars, while minimizing the generation of by-products (Hendriks and Zeeman 2009, Mosier et al. 2005).

The formation of furans, phenols and some of the organic acids can be (partially) prevented by optimizing process conditions applied during pretreatment. Other by-products such as acetic acid, coumaric acid and ferulic acid are part of the hemicellulosic structure, thus depolymerization of hemicellulose will lead to the release of these by-products (Hatfield et al. 1999, Sun et al. 2004).

In **table 2 of chapter 2**, by-product concentrations are shown for lignocellulose originating from different sources and pretreated using various methods. Furthermore, **Chapter 3**

describes the pretreatment of lignocellulose-rich sugarcane bagasse in more detail, including differences between in by-product formation and sugar depolymerization between acid pretreatment, alkaline pretreatment and autohydrolysis. In chapter 2 and 3 it is shown that it can be difficult to reduce overall by-product formation by choosing different pretreatment methods. As an example, acid pretreatment and alkaline pretreatment can be compared. Acid pretreatment results in high furan concentrations, where furans can be considered as a key inhibitory by-product during fermentation of pretreated lignocellulose (Heer and Sauer 2008, Taherzadeh et al. 1999). Furans were not present after alkaline pretreatment, however a much higher concentration of acetic acid and phenolic compounds was found in alkaline pretreated lignocellulose. In **chapter 4**, it is shown that alkaline pretreated lignocellulose and acid pretreated lignocellulose have a similar inhibitory effect on growth of lactic acid producing bacteria, despite the absence of furans in alkaline pretreated lignocellulose.

The conditions chosen during thermo-chemical pretreatment can have a significant influence on the amount of by-products formed. In a study performed by Bustos et al in 2004 using experimental design software, it was shown that both acid pretreatment with 2% W/DW H_2SO_4 for 120 minutes and 5% W/DW H_2SO_4 for 15 minutes result in sugar concentrations of around 35 g/l (Bustos et al. 2004). However, whereas the first method resulted in a furfural concentration of 4.7 g/l, furfural formation in the second method was only 1.0 g/l (Bustos et al. 2004). Even while treatment with 2% H_2SO_4 during 120 minutes leads to a 3% higher sugar monomerization, treatment with 5% H_2SO_4 for 15 minutes may be the preferred process condition for pretreatment.

INHIBITORY EFFECT OF LIGNOCELLULOSIC BY-PRODUCTS ON THE GROWTH AND PRODUCTIVITY OF MICRO-ORGANISMS

By-products resulting from thermo-chemical pretreatment of lignocellulose may have an inhibitory effect on growth and productivity of micro-organisms. In **chapter 2**, the effects of different furans, acids and phenolics on ethanol producing micro-organisms is described. Most of the tested micro-organisms were yeasts strains like *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*, but ethanol producing bacteria such as *Escherichia coli* and *Zymomonas mobilis* were also evaluated. In general, by-products have a slightly larger inhibitory effect on growth than on ethanol productivity and yield. 4-Hydroxybenzaldehyde was found to be the most toxic of all evaluated by-products for ethanol producing micro-organisms, closely followed by furfural and vanillin.

Chapter 4 evaluates the inhibitory effect of lignocellulosic by-products on growth of 5 different lactic acid producing micro-organisms. This research showed that 4-hydroxybenzaldehyde was the most toxic by-product for *Lactobacillus casei*, *Lactobacillus delbrueckii* and

Bacillus coagulans. For *Bacillus smithii* and *Lactococcus lactis*, coumaric acid was the most toxic by-product.

Besides toxicity of a by-product, two other parameters should be taken into account when determining the level of inhibition: the by-product concentration after thermo-chemical pretreatment, and the synergistic inhibition effect between different by-products present. Since pretreated lignocellulose contains a mixture of by-products, synergistic effects between different by-products may play a major role in toxicity in these mixtures. Most studies previously performed on inhibitory effects of by-products on micro-organisms do not consider synergistic effects (Bisschof et al. 2010, Franden et al. 2009, Zaldivar and Ingram 1999,). Studies that do describe synergistic effects between by-products, for instance between phenolic compounds (Olivia et al. 2004), and between furans (Taherzadeh et al. 2000), show that synergy can play a major role in mixtures of by-products. However, the work executed on synergistic effects has only been performed with a limited number of by-products, and *S. cerevisiae* was the only micro-organism tested. The synergistic effect between different by-products on the growth of lactic acid producing micro-organisms was studied in chapter 4. When these micro-organism were grown on a medium resembling alkaline pretreated lignocellulose, no by-product is present in concentrations where it can inhibit growth based on individual effects. However, the synergistic inhibition effects between acetic acid, levulinic acid, glycolic acid, formic acid and coumaric acid resulted in a significant inhibition of growth for all strains. These observations, combined with measurements observed for synergistic effects of by-products on *S. cerevisiae* (Oliva et al. 2004, Taherzadeh et al. 2000) demonstrate the importance of synergistic inhibitory effects between different by-products, and show that studying individual effects is not sufficient to determine the toxicity of lignocellulosic by-products.

DECREASING INHIBITORY EFFECTS OF LIGNOCELLULOSIC BY-PRODUCTS

Several methods have been proposed in literature to decrease inhibitory effects of by-products during fermentation of pretreated lignocellulose. Two different methods can be distinguished to decrease this inhibitory effect; either the by-products can be removed (detoxification), or micro-organisms can be improved to withstand higher concentrations of by-products.

Detoxification can be done either chemically or biologically. Chemical detoxification methods that have been studied include addition of alkaline material, activated charcoal and anion resins. Although addition of an alkali can help to remove furans and phenols, sugar loss may occur due to degradation of sugars to aliphatic acids (Nilvebrant et al. 2003, Persson et al. 2002). Activated charcoal can bind up to 40% of the furans and 75% of the phenolics, thereby reducing their presence during fermentation (Chandel et al 2007). Anion resins can remove 90% of the acetic acid and 60-80% of the furans (Carter et al. 2011). Detoxification using anion

resins was shown to have a large beneficial effect on productivity during fermentation towards ethanol (Carter et al. 2011, Frazer and McCaskey 1989, Nilvebrant et al. 2001,). A comparison study showed that treating lignocellulose with anion resions resulted in the highest ethanol productivity during fermentation, closely followed by activated charcoal.

Biological detoxification involves a pre-fermentation of the pretreated lignocellulosic material with a micro-organism which is able to consume furans, organic acids and/or phenols, but does not consume any sugars. Koopman showed that *Cupriavidus basilensis* is able to metabolise furfural, organic acids and phenolics without consuming sugars present in de substrate (Koopman et al. 2010). *Coniochaeta ligniaria* and *Amorphotheca resinae* have similar detoxification capabilities, although these strains co-consume limited amounts of sugars (Nichols et al. 2005, Nichols et al 2008, Zhao et al. 2013).

Although detoxification can significantly increase productivity of micro-organisms on pretreated lignocellulose, its use is not preferred. The costs of detoxification can be substantial, both in operating costs such as addition of charcoal, replacing/cleaning ion resins, heating, mixing, as well as in capital costs. Furthermore, the extra process stage can result in a loss of sugars. Since lactic acid production is a process with little added value (John et al. 2007), process costs should be limited, thus detoxification should be avoided.

In *S. cerevisiae*, several targets have been found for genetic engineering, increasing resistance of the cells towards inhibitory effects of by-products. These targets are specific for yeast strains, and are not present in bacilli. Sigma factors regulate the expression of different genetic domains in bacilli, making genetic engineering more difficult (Price et al. 2001). Furthermore, genetic tools for thermophilic bacilli are still in their infancy (Bosma et al. 2015). It is therefore very challenging to apply genetic engineering on *B. coagulans* to increase its resistance towards inhibitory effects of by-products.

A solution is to adapt *B. coagulans* cells to lignocellulosic by-products during pre-cultivation. In **chapter 5**, it was shown that precultivation of *B. coagulans* in the presence of 1 g/l furfural improves productivity, titres and yield during a subsequent fermentation in the presence of a mixture of by-products. Furthermore, fermentation times could be decreased by over 50% when furfural was added during precultivation. A large benefit of this adaptation method is that it does not increase process costs as would occur when detoxification is used, and it does not require extensive laboratory experiments to identify and modify genetic expression levels in the micro-organism.

ENZYMATIC HYDROLYSIS AND FERMENTATION OF ACID PRETREATED SUGARCANE BAGASSE

Chapter 6 describes the optimization of an SSF process for the conversion of acid pretreated sugarcane bagasse into lactic acid. The objective was to develop a process, in which lactic

Table 1: *Production of lactic acid from pretreated lignocellulosic material using either a separate enzymatic hydrolysis and fermentation process (SHF), or a combined process (SSF). LF = Liquid fraction. RT = Room Temperature. EH=enzymatic hydrolysis. Acc = Accelerase. Cell = Cellulast. Novo = Novozymes. n.d. = not determined*

	Micro-organism	Feedstock	Chemical pretreatment	SSF/SHF	Detoxification	Enzymatic hydrolysis	EH[A] (h)
1	*P. Acidilatici DQ2*	Corn stover	2.5% H2SO4, 3m, 190C	SSF	Biological 5d	Acc 1000 15 FPU/g DW	8
2	*Bacillus sp. NL01*	Corn stover	3% H2SO4 2h RT, 5m 170C, SE	SHF	Centrifugation after EH	Cell 1.5L 15 FPU/ g DW + Novo 188 30 CBU/ g DW	48
3	*B. coagulans DSM2314*	Wheat straw	10% NaOH, 16h, 85C	SSF	none	GC220 14 FPU/ g DW	2
4	*E. faecalis RKY1*	Oak wood	0.5%H2SO4 24h RT, 5m 215C, SE	SHF	Centrifugation after EH	Cell 1.5L 15 FPU/ g DW + Novo 188 30 CBU/ g DW	48
5	*B. coagulans DSM2314*	Sugarcane bagasse + LF	0.72% H2SO4, 15m, 170C, SE	SSF	none	GC220 13.4 FPU/ g DW	5
6	*B. coagulans DSM2314*	Sugarcane bagasse + LF	0.72% H2SO4, 15m, 170C, SE	SSF	none	GC220 13.4 FPU/ g DW	5
7	*B. coagulans DSM2314*	Sugarcane bagasse	0.72% H2SO4, 15m, 170C, SE	SSF	none	GC220 15.2 FPU/ g DW	4
8	*B. coagulans DSM2314*	Sugarcane bagasse	0.72% H2SO4, 15m, 170C, SE	SSF	none	GC220 15.2 FPU/ g DW	7

	Micro-organism	Inoculum (% v/v)	C_{LA}[B] (g/l)	$Y_{Lc/La}$[C] (%)	$Y_{S/La}$[D] (%)	$Q_{LA,F}$[E] (g/l/h)	$Q_{LA,O}$[F] (g/l/h)	Source
1	*P. Acidilatici DQ2*	20	101.6	77.2	n.d.	1.06	0.45	Zhao et al 2013
2	*Bacillus sp. NL01*	8	61.3	n.d.	70	1.46	0.67	Ouyang et al 2013
3	*B. coagulans DSM2314*	20	40.7	43	81	0.74	0.56	Maas et al 2008
4	*E. faecalis RKY1*	?	93	n.d.	80	1.7	0.74	Wee et al 2004
5	*B. coagulans DSM2314*	5	64.1	74	80	0.78	0.72	**Chapter 6**
6	*B. coagulans DSM2314*	5	74.6	87	94	0.92	0.79	**Chapter 6**
7	*B. coagulans DSM2314*	5	70.4	83	90	1.14	0.98	**Chapter 6**
8	*B. coagulans DSM2314*	5	58.7	73	90	1.81	1.33	**Chapter 6**

A: Enzymatic hydrolysis time required during SHF, or pre-hydrolysis time prior to inoculation during SSF

B: C_{La}: concentration of lactic acid at the end of the SSF in g/l

C:$Y_{Lc/La}$: lactic acid production yield on total lignocellulosic sugars in W/W

D $Y_{s/La}$: estimated conversion efficiency of sugar monomers to lactic acid in W/W

E: $Q_{LA.F}$: average volumetric lactic acid productivity during fermentation phase in g/l/h

F: $Q_{LA.O}$: average volumetric lactic acid productivity over the total process in g/l/h

acid could be produced from bagasse with productivities similar to fermentations using first generation sugars such as glucose, sugarcane juice or starch. Whereas the production of lactic acid from lignocellulosic biomass has been shown technically feasible in previous studies, the overall lactic acid productivity was relatively low, and the processes were therefore not competitive with first generation fermentation processes (Table 1). Where an average lactic acid productivity of 2.5-3 g/l/h can be reached on first generation sugars (Hofvendahl et al. 2000, Abdel-Rahman et al. 2011), fermentations on pretreated lignocellulose resulted in an average lactic acid productivity of 0.45-0.75 g/l/h (Table 1).

In most studies pretreated lignocellulose is used in a separate hydrolysis and fermentation (SHF) process, in which enzymatic hydrolysis is performed prior to fermentation in a separate reactor. Although lactic acid concentrations of 90 g/l can be achieved using SHF at laboratory scale, the separate enzymatic hydrolysis process step can be disadvantageous for overall process productivity and yield. Accumulation of monomeric sugars can repress enzyme activity, resulting in long process times for enzymatic hydrolysis of 48-72 hours. (Wee et al. 2004, Ouyang et al. 2013).

Simultaneous Saccharification and Fermentation (SSF) is an alternative to SHF, in which enzymatic hydrolysis and fermentation is performed simultaneously in one reactor. Compared to SSF, SHF has a higher energy consumption due to the extra process time required for enzymatic hydrolysis, SHF uses larger equipment due to longer residence times, and SHF shows reduced activity of enzymes during later stages of the enzymatic hydrolysis (Ojeda et al. 2011). Maas et al used an SSF approach for fermentation towards lactic acid, but they observed a low lactic acid productivity over the total process of only 0.56 g/L/h, and a low final lactic acid concentration of 40.8 g/l (Maas et al. 2008). Zhoa et al achieved a high lactic acid concentration of 101.6 g/l, with a lactic acid productivity of 1.06 g/l/h during the SSF process (Zhao et al. 2013). However, the lactic acid productivity of the overall process was only 0.45 g/l/h when including the time required for a biodetoxification process step used prior to SSF was included. In this biodetoxification, the pretreated lignocellulosic material was fermented with fungi which consume small organic acids and furans, but do not convert sugars. This biodetoxification requires significant amounts of extra resources such as energy and equipment, and after the non-sterile biodetoxification, autoclaving of the substrate was needed to reduce the risk of infections (Zhao et al. 2013).

In **chapter 6**, two SSF processes, batch SSF and two-stage SSF, were used for the production of lactic acid from sugarcane bagasse. Reactors in these experiments were inoculated with *B. coagulans* DSM2314, which was pre-cultivated in the presence of furfural. Addition of furfural was shown to reduce the growth lag phase and increase lactic acid productivity, titres and production yields during SSF. A benchmark batch SSF process, using micro-organisms pre-cultivated without the presence of furfural, resulted in an average lactic acid productivity including pre-hydrolysis of 0.73 g/l/h, comparable to the productivity described for SSF in literature (table 1). When furfural precultures were used as inoculum, both batch SSF and

two-stage SSF show a higher lactic acid productivity over the total process of 0.98 g/l/h and 1.33 g/l/h respectively, which is 1.5-3 fold higher than overall lactic acid productivities in other studies (table 1). Batch SSF also reached a high yield on lignocellulosic sugars between 83 and 87% W/W, and a conversion of monomeric sugar to lactic acid of over 90% W/W. In two-stage SSF, a similarly high conversion of monomeric sugars to lactic acid was obtained. However, lactic acid production yield on lignocellulosic sugars was lower in two-stage SSF, although this yield was still comparable to yields described in literature (table 1). The lower yield on lignocellulosic sugars during two-stage SSF was found to be caused by a decreased depolymerization of (hemi)cellulose by the enzymatic cocktail, resulting in the presence of around 20 g/l residual glucan and xylan at the end of two-stage SSF (**Chapter 6**). Rapid accumulation of lactic acid during two-stage SSF could be the cause of the reduced enzyme activity (**Chapter 6**, Iyer et al 1999, Moldes et al 2001). Another advantage of batch SSF and two-stage SSF over the other processes described in table 1 is the inoculum size. Since precultures use high-grade sugars and rich medium components, a decrease of inoculum volume can significantly reduce costs of the fermentation process.

Further optimization of the proposed SSF processes may be realized by reducing the amount of yeast extract used, and/or by (partially) replacing this nitrogen source with a cheaper alternative such as corn steep liquor, cotton nitrogen or mineral salts. In conclusion, both batch SSF and two-stage SSF are good methods for the conversion of pretreated lignocellulose to lactic acid, with several distinct benefits over other processes performed in previous studies.

SCALE UP OF PRODUCTION PROCESSES FOR LACTIC ACID PRODUCTION FROM LIGNOCELLULOSE-RICH BIOMASS

Scale up of thermo-chemical pretreatment process

In **chapter 3 and 6**, the production of monomeric sugars from pretreated lignocellulosic biomass, and fermentation towards lactic acid, is demonstrated at laboratory and small pilot-plant scale. A further scale up of this process is required to produce adequate amounts of lactic acid.

DSM, in cooperation with Poët, is currently constructing and evaluating a pretreatment and fermentation plant, which produces bioethanol from acid pretreated corn stover. The thermo-chemical pretreatment process is followed by separate enzymatic hydrolysis and fermentation (Project liberty 2015). The production target of this plant is 80.000 tonnes of bioethanol per year, which means that at least 200.000 tonnes of DW corn stover has to be pretreated and processed. This process plant, which is now in operation, uses acid pretreatment on a large scale to increase the accessibility of cellulose and hemicellulose for enzymatic hydrolysis. Several other studies also report successful pilot-plant trials using acid pretreatment on lignocellulose-rich biomass. In this thesis, a batch process using 10 kg of dry weight biomass

was performed in the Queensland pilot plant facility. Over 80% of the sugars in this pretreated lignocellulose can be monomerized by enzymes, results similar to laboratory experiments performed at 100 g scale (Wong et al. 2011, **chapter 3**). In larger scale pretreatment facilities, a continuous process is preferred, since it can have a higher throughput of material due to an optimised heat transfer, and potentially lower operating cost can be achieved due to the more efficient heating and cooling of the material (Jørgensen et al. 2006, Kim et al. 2013,). Several studies have evaluated continuous pretreatment processes which can handle up to 1000 kg of lignocellulosic material per hour, equivalent to up to 7200 metric tonnes per year. (Jørgensen et al. 2006, Petersen et al. 2009, Thomsen et al. 2008). In continuous processes at larger scale, up to 85% of the hemicelluloses and up to 94% of the celluloses could be recovered, whereas a cellulose conversion efficiency to monomeric sugars of 91% was reached when the pretreatment was combined with enzymatic hydrolysis. It can be concluded that processes which combine a low pH and elevated temperature (up to 180°C) can be efficiently used in large-scale continuous pretreatment processes.

Scale up of enzymatic hydrolysis and fermentation process

The two-stage SSF process as proposed in **chapter 6** may be further improved by scaling up. Instead of adding additional pretreated lignocellulose in a second batch, it can be added as continuous feed at a larger scale. This can result in both an improved viscosity of the fermentation broth, as well as an increase in average residence time of the pretreated material in the fermentation reactor. With an extended residence time, the enzymatic hydrolysis of the pretreated lignocellulose may be increased. A screw feeder has been successfully used for continuous feeding of pretreated lignocellulose (Maas et al. 2008).

One of the challenges in large-scale fermentation using lignocellulosic material is mixing. The fermentation broth contains a high dry matter content to provide a sufficient amount of sugars. A high DM content results in an increased viscosity, thus a higher energy input is required for mixing of the broth. Increased mixing can also result in a significant amount of shear stress in the reactor, which can decrease the viability of the microbial culture. Zhang et al describe a SSF experiment at a high DM content of 30% using a Rushton impellor for mixing. Even when applying a high mixing power input of 40 W/kg DW lignocellulose, mixing was still a limiting factor during the SSF process (Zhang et al. 2009). To produce 1 kg of ethanol, an energy input of 6 kWh was required for mixing only using a Rushton impeller (Zhang et al. 2009). In the research of Zhang et al, a helical stirring system was shown to be a promising alternative to Rushton impellers for fermentation of pretreated lignocellulose at high dry matter content. Helical stirring required a much lower power input, while it results in higher mixing efficiencies in fermentation broths with high dry matter content. (Ouyang et al. 2013, Zhang et al. 2009).

DOWNSTREAM PROCESSING OF LACTIC ACID PRODUCED FROM LIGNOCELLULOSIC MATERIAL

A downstream process (DSP) is required to purify the lactic acid from the fermentation broth. Purification of lactic acid produced from lignocellulosic material is to our knowledge not well investigated, whereas the effectiveness of downstream processing is an important parameter for the feasibility of producing polymer-grade lactic acid from sugarcane bagasse.

When conventional sugar sources such as sugar beet syrup, sugarcane juice or cassava starch are used for the production of lactic acid, downstream processing involves calcium carbonate precipitation at high pH, filtration, and re-acidification with sulphuric acid towards lactic acid (Schopmeyer and Arnold 1944) (figure 1). Although this is an efficient method to purify lactic acid, the re-acidification results in the formation of a large quantity of gypsum (calcium sulphate). Where gypsum is traditionally used in small quantities in hospitals and similar niche markets, and may be used as material for the construction of buildings, it is expected that large surplus of gypsum will be formed when PLA will be produced on such a large scale. Therefore, gypsum is seen as an unwanted by-product (Datta and Henry 2006). Several studies have investigated the possibility of recycling gypsum towards elemental sulphur and calcium carbonate (de Beer et al. 2015, Song et al. 2012). However, the recycling process itself is costly, and additional chemical compounds such as hydrogen sulphide or ammonium carbonate are required.

As an alternative to calcium carbonate, magnesium hydroxide can be used in combination with organic amines to sediment lactic acid. Magnesium hydroxide has the benefit of being easier to recycle than calcium carbonate (van Krieken 2011).

Acquiring a high purity of lactic acid after DSP is important for PLA production, since impurities can deteriorate the properties of the PLA polymer (Inkinen et al. 2011). Examples of compounds present in fermentation broth when using conventional sugar sources are organic acids, alcohols, nutrients and cell debris. Besides these impurities, lignocellulosic biomass itself contains different impurities after pretreatment, such as organic acids, phenolics, furans, lignin, extractives, ions and sand (Inkinen et al. 2011) (Chapter 3). The impurities present in lignocellulose-rich biomass can add to the complexity of the downstream process to obtain an efficient purification of lactic acid, and thus for the production of a high quality PLA bioplastic. To our knowledge, it has not been evaluated whether high purities of lactic acid can be achieved when calcium precipitation or magnesium precipitation is used as downstream process method for lignocellulose derived fermentation broths.

Other alternative downstream processes for lactic acid purification use the isoelectric point of the acid group. One such process is electro dialysis (Madzingaidzo. et al 2002). Although this method is efficient in concentrating lactic acid and separating it from non-ionizable molecules, Madzingaidzo et al found that acetic acid, and potentially other acids or impurities, are also concentrated, and are present in the fraction containing lactic acid (Madzingaidzo et

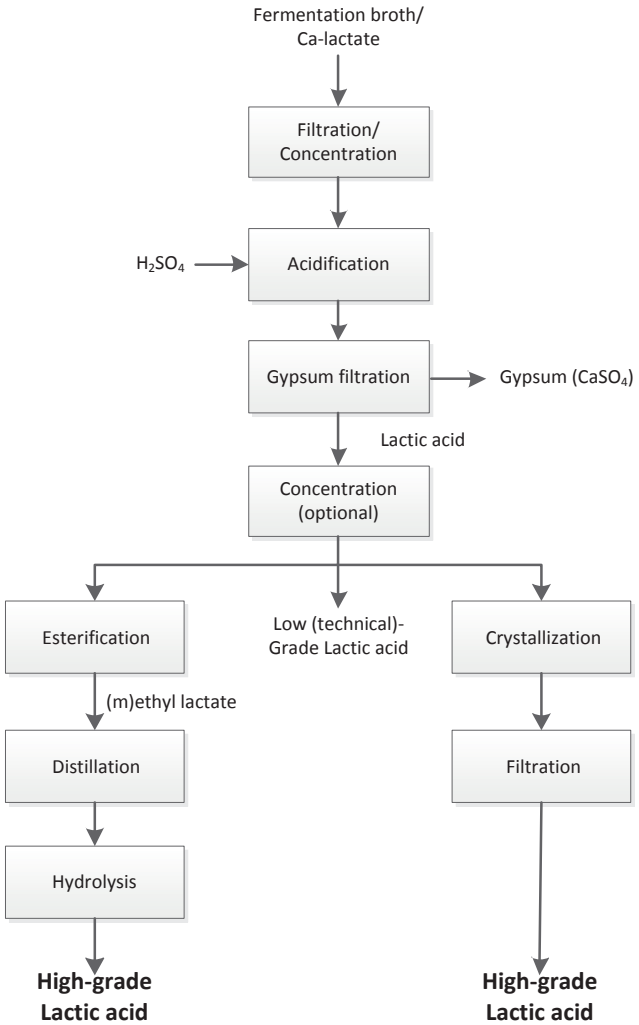


Figure 1: *Classical downstream processing method for the purification of lactic acid. Fermentation broth containing calcium lactate is acidified, and the gypsum formed is filtered out.*

al. 2002). Since the fermentation broth after SSF may contain up to 5 g/l of acetic acid, 3 g/l of levulinic acid and glycolic acid, and 1 g/l of formic acid, using an ion separation method is not likely to be efficient when lactic acid is produced from lignocellulosic material. Ion exchange resins such as PVP can also be used to recover lactic acid, however problems similar to electro dialysis may occur when separating lactic acid and other acids (Joglekar et al. 2006). Although these processes cannot purify lactic acid in a single stage, they may still be used when a combination of methods is applied. Also, ion separation methods may be used to remove organic acids before SSF, potentially simplifying the DSP. More experiments should be

performed to investigate the potential of these purification methods for lactic acid produced from pretreated lignocellulose.

AVAILABILITY OF LIGNOCELLULOSE-RICH BIOMASS

Lignocellulose-rich biomass is considered to be a nearly unlimited and unused source of carbon. However, some types of lignocellulose which are frequently proposed as potential feedstock for fermentation, for instance wheat straw, are already used for other purposes. Bakker et al estimated that more than 50% of the wheat straw produced worldwide is already used, mainly in cattle farming (Bakker 2013). With an increasing demand for meat, wheat straw availability is expected to decrease further in the (near) future. Increasing straw harvest is not recommended, since it can decrease soil nutrients such as nitrogen and phosphate (Scarlat et al. 2010).

A lignocellulose source that is largely unused is corn stover. Most corn fields in the United States are centralized around first generation ethanol plants, making transport distances to lignocellulose processing plants short and therefore cheaper. It is estimated that in the US alone, up to 100 Mt of corn stover can be collected in a sustainable and economically viable manner. Around 20 Mt of this corn stover may be required for other purposes, thus up to 80 Mt of corn stover can become available for fermentation processes (Kadam and McMillan 2003).

Sugarcane bagasse is another interesting source of lignocellulose-rich biomass. To produce sugarcane juice, the sugar canes are transported to sugar mills. Since sugarcane bagasse is the left-over of the sugar extraction process, the biomass is present at centralized locations, and is available without transportation costs. However, approximately 50% of this bagasse is currently used for heat and power generation. This amount may be reduced to 36% by more efficient modes of operation, such as more efficient heating boilers (Lavarack et al. 2002). Another solution to power the sugarcane mills while acquiring fermentable sugars present in the lignocellulose is by extracting the lignin from the lignocellulose. Lignin does not only have a high energy density, it also contains less water than the original biomass due to its hydrophobicity, and may therefore be burned more efficiently. The concept of sedimentation and burning lignin is already applied in the paper and pulp industry (Glasser and Sarkanen 1989).

Currently, 1500 Mt DW of sugarcane bagasse is produced by sugarcane mills on an annual basis (Cerqueira et al. 2007), which should be more than sufficient to produce the estimated 90 million tonnes of PE and PS which is produced in the petrochemical industry (Bakker 2013). Sugarcane bagasse can therefore be seen as a suitable feedstock for biobased production of chemicals. Besides sugarcane bagasse, sugar cane leaves and tops also represent a good

source of lignocellulose (Bakker 2013). This material is not yet collected, thus transportation costs should be considered when using this material.

Similar to sugarcane bagasse, empty fruit bunches (EFB), a leftover residue from palm oil production, is collected at the palm oil mills. In Malaysia, one of the main palm oil producing countries, 7Mt of DW EFB is not used for other purposes and thus available as feedstock (Bakker 2013). Although this is a relatively small amount of biomass compared to sugarcane bagasse, the presence in a centralised location makes EFB a potentially interesting feedstock for fermentation.

PRODUCTION OF LACTIC ACID VERSUS PRODUCTION OF ETHANOL FROM SUGARCANE BAGASSE

In **chapter 2**, it is estimated that 125 gram of monomeric sugars per kilogram dry weight bagasse can be found in the liquid fraction after acid pretreatment. Furthermore, 520 gram of sugar polymers per kilogram of initial dry weight can be found in the solid fraction after acid pretreatment.

When using the efficiencies as observed in **chapter 6**, 520 gram of polymers can be converted into 432 gram of lactic acid in the batch SSF, or to 380 gram of lactic acid in the two-stage SSF. When batch SSF is used with partial addition of the liquid fraction, which contains part of the hemicellulosic sugars, 470 grams of lactic acid can be produced from 1 kg of bagasse dry weight.

Ethanol fermentations using lignocellulosic biomass are reported to produce around 220-270 kg of ethanol per ton of dry weight biomass (Humbird et al. 2011). The low productivity per tonnes of dry weight in comparison to lactic acid is mainly due to the coproduction of CO_2, which results in a low maximum theoretical yield on a weight basis. A maximum theoretical yield of 1 gram of lactic acid per gram of glucose or xylose can be achieved when *B. coagulans* is used as production organism. However, a maximum theoretical yield of only 0.51 gram of ethanol per gram of glucose or xylose can be achieved by *S. cerevisiae* (Kuyper et al. 2004). Theoretically, a maximum ethanol production of 314 kg per tonnes of sugarcane bagasse can therefore be achieved.

Current ethanol selling prices are estimated at approximately 2.8 dollar per gallon, equivalent to 600 dollar per tonnes (Sánchez-Segado et al. 2012). Lactic acid selling price is estimated at 1300-5000 dollar per tonnes, depending on the quality and purity of the lactic acid (Cellulac 2015). It should be noted that downstream processing of lactic acid may be more expensive. Whereas bioethanol can be distilled from the broth, leaving most impurities in the water phase, lactic acid purification requires different process stages, and calcium carbonate precipitation leads to gypsum formation as by-products. If the purification of lactic acid can be

optimized, producing lactic acid from lignocellulosic material can be an economically more interesting process than the production of ethanol from lignocellulosic material.

CONCLUSIONS

In this thesis, a thermo-chemical pretreatment of sugarcane bagasse, using sulphuric acid as catalyst, was combined with a simultaneous saccharification and fermentation (SSF) process for the production of lactic acid. *Bacillus coagulans DSM2314* was used for the fermentation of lignocellulosic sugars to lactic acid, since its cultivation conditions are similar to conditions at which (hemi)cellulose degrading enzymes are active. Although the thermo-chemical pretreatment resulted in the formation of numerous by-products, inhibition of *B. coagulans* by these by-products could be overcome by pre-growing the micro-organism in the presence of furfural. During SSF, lignocellulosic material was added in two stages to decrease viscosity of the broth. The combination of two-stage SSF inoculated with a pre-culture grown in the presence of 1 g/l furfural resulted in an average productivity of 2.4 g/l/h during the first 23 hours of fermentation, similar to average productivities observed in fermentations using high-grade sugars. During SSF, conversion yields of monomeric sugars to lactic acid above 90% W/W were observed. These results show that an SSF process using acid pretreated sugarcane bagasse can be an economically attractive alternative to fermentation using high-grade sugars.

Scaling up of both the chemical pretreatment and SSF process is not expected to result in major challenges, while it may even allow for an optimized and thus more efficient process. Sugarcane bagasse is the most interesting agricultural waste residue to use as feedstock for the process, due to its large and centralized availability, while empty fruit bunches, corn stover and rice straw may also be suitable sources of lignocellulose. Optimizing the downstream process for the production of high-grade lactic acid from pretreated lignocellulose, required for the production of PLA with a high quality, should be investigated in more depth. When the downstream processing is optimised, a large scale lignocellulose based production process of lactic acid as bulk chemical can become economically attractive.

REFERENCES

Abdel-Rahman MA, Tashiro Y, Sonomoto K (2011) Lactic acid production from lignocellulose-derived sugars using lactic acid bacteria: Overview and limits, J Biotechnol 156:286-301 doi: 10.1016/j.jbiotec.2011.06.017

Bakker RRC (2013) Availability of lignocellulosic feedstocks for lactic acid production http://edepot.wur.nl/291817 (accessed 11-2015)

Bisschof, KM, Liu, S, Hughes, SR, Rich, JO (2010) Fermentation of corn fiber hydrolysate to lactic acid by moderate thermophile Bacillus coagulans Biotechnol Lett 32:823-828 doi: 10.1007/s10529-010-0222-z

Bustos G, Moldes AB, Cruz JM, Domínguez JM (2004) Production of fermentable media from vine-trimming wastes and bioconversion into lactic acid by Lactobacillus pentosus J Sci Food Agr 84:2105-2112 doi: 10.1002/jsfa.1922

Chandel AK, Kapoor RK, Singh A, Kuhad RC (2007) Detoxification of sugarcane bagasse hydrolysate improves ethanol production by Candida shehatae NCIM 3501 Bioresource Technol 98:1947-1950

Carter B, Gilcrease PC, Menkhaus TJ (2011) Removal and recovery of furfural, 5-hydroxymethylfurfural, and acetic acid from aqueous solutions using a soluble polyelectrolyte Biotechnol Bioeng 108:2046-2052 doi:10.1002/bit.23153

Cellulac (2015) poly-Lactic Acid market http://cellulac.co.uk/en/market/ (accessed 11-2015)

Cerqueira DA, Filho GR, da Silva Meireles C (2007) Optimization of sugarcane bagasse cellulose acetylation Carbohydr Polym 69:579-582 doi: 10.1016/j.carbpol.2007.01.010

Claassen PAM, van Lier JB, Lopez Contreras AM, van Niel EWJ, Sijtsma L, Stams AJM, de Vries SS, Weusthuis RA (1999) Utilisation of biomass for the supply of energy carriers, Appl Microbiol Biotechnol 52:741-755 doi: 10.1007/s002530051586

Datta R, Henry M (2006) Lactic acid: recent advances in products, process and technologies – a review Chem Technol Biotechnol 81:1119-1129 doi: 10.1002/jctb.1486

De Beer M., Doucet FJ, Maree JP, Lievenberg L (2015) Synthesis of high-purity precipitated calcium carbonate during the process of recovery of elemental sulphur from gypsum waste Waste Manage 46:619-627 doi: 10.1016/j.wasman.2015.08.023

Franden MA, Pienkos PT, Zhang M (2009) Development of a high-throughput method to evaluate the impact of inhibitory compounds from lignocellulosic hydrolysates on the growth of Zymomonas mobilis J Biotechnol 144:259-267 doi:10.1016/j.jbiotec.2009.08.006

Frazer FR, McCaskey TA (1989) Wood hydrolyzate treatments for improved fermentation of wood sugars to 2,3-butanediol Biomass 18:31-42 doi:http://dx.doi.org/10.1016/0144-4565(89)90079-6

Garlotta D (2001) A Literature Review of Poly(Lactic Acid) J Polym Environ 9:63-84 doi: 10.1023/a:1020200822435

Glasser WG, Sarkanen S (1989) Lignin, properties and materials http://www.osti.gov/scitech/biblio/6495223 (accessed 11-2015)

Hatfield RD, Ralph J, Grabber JH (1999) Cell wall cross-linking by ferulates and diferulates in grasses J Sci Food Agr 79:403-407

Heer D, Sauer U (2008) Identification of furfural as a key toxin in lignocellulosic hydrolysates and evolution of a tolerant yeast strain Microb Biotechnol 1:497-508 doi: 10.1111/j.1751-7915.2008.00050.x

Hendriks ATWM, Zeeman G (2009) Pretreatments to enhance the digestibility of lignocellulosic biomass, Bioresource Technol 100:10-18 doi: 10.1016/j.biortech.2008.05.027

Hofvendahl K, Hahn-Hägerdal B (2000) Factors affecting the fermentative lactic acid production from renewable resources, Enzyme Microb Technol 26:87-107 doi: 10.1016/S0141-0229(99)00155-6

Humbird D, Davis R, Tao L, Kinchin C, Hsu D, Aden A, Schoen P, Lukas J, Olthof B, Worley M, Sexton D, Dudgeon D (2011) Process design and economics for biochemical conversion of lignocellulosic biomass to ethanol NREL https://www.researchgate.net/publication/229843699_Process_Design_and_Economics_for_Biochemi-

cal_Conversion_of_Lignocellulosic_Biomass_to_Ethanol_Dilute-Acid_Pretreatment_and_Enzymatic_Hydrolysis_of_Corn_Stover?ev=prf_pub (Accessed 11-2015)

Inkinen S, Hakkarainen M, Albertsson A-C, Södergård A (2011) From lactic acid to Poly(lactic acid) (PLA): characterization and analysis of PLA and its precursors Biomacromolecules 12:523-532 doi: 10.1021/bm101302t

Iyer PV, Lee YY (1999) Product inhibition in simultaneous saccharification and fermentation of cellulose into lactic acid Biotechnol Lett 21:371-373 doi: 10.1023/A:1005435120978

John RP, Nampoothiri KM, Pandey A (2007) Fermentative production of lactic acid from biomass: an overview on process developments and future perspectives, Appl Microb Biotechnol 74:524-534

Jonglekar HG, Rahman I, Babu S, Kulkarni BD, Joshi A (2006) Comparative assessment of downstream processing options for lactic acid Sep Purif Technol 52:1-17

Jørgensen H, Vibe-Pedersen J, Larsen J, Felby C (2006) Liquefaction of lignocellulose at high-solid concentrations Biotechnol Bioeng 96:862-870 doi: 10.1002/bit.21115

Kadam KL, McMillan JD (2003) Availability of corn stover as a sustainable feedstock for bioethanol production Bioresource Technol 88:17-25 doi: 10.1016/S0960-8524(02)00269-9

Kim TH, Choi CH, Oh KK (2013) Bioconversion of sawdust into ethanol using dilute sulfuric acid-assisted continuous twin screw-driven reactor pretreatment and fed-batch simultaneous saccharification and fermentation Bioresource Technol 130:306-313 doi: 10.1016/j.biortech.2012.11.125

Koopman F, Wierckx N, De Winde JH, Ruijssenaars HJ (2010) Identification and characterization of the furfural and 5-(hydroxymethyl) furfural degradation pathways of Cupriavidus basilensis HMF14 Proc Natl Acad Sci 107:4919

Kuyper M, Winkler AA, van Dijken JP, Pronk JT (2004) Minimal metabolic engineering of Saccharomyces cerevisiae for efficient anaerobic xylose fermentation: a proof of principle FEMS Yeast Res 4:655-664 doi: 10.1016/j.femsyr.2004.01.003

Larsson S, Palmqvist E, Hahn-Hägerdal B, Tengborg C, Stenberg K, Zacchi G, Nilvebrant N-O (1999) The generation of fermentation inhibitors during dilute acid hydrolysis of softwood Enzyme Microb Technol 24:151-159

Laverack BP, Griffin GJ, Rodman D (2002) The acid hydrolysis of sugarcane bagasse hemicellulose to produce xylose, arabinose, glucose and other products Biomass Bioenergy 23:367-380 doi: 10.1016/S0961-9534(02)00066-1

Maas RHW, Bakker RR, Jansen MLA, Visser D, Jong E de, Eggink G, Weusthuis RA (2008) Lactic acid production from lime-treated wheat straw by Bacillus coagulans: neutralization of acid by fed-batch addition of alkaline substrate, Appl Microbiol Biotechnol 78:751-758 doi: 10.1007/s00253-008-1361-1

Madzingaidzo L, Danner H, Braun R (2002) Process development and optimisation of lactic acid purification using electrodialysis J Biotechniol 96:223-239 doi: 10.1016/S0168-1656(02)00049-4

Moldes AB, Alonso JL, Parajó JC (2001) Strategies to improve the bioconversion of processed wood into lactic acid by simultaneous saccharification and fermentation Chem Technol Biotechnol 76:279-284 doi: 10.1002/jctb.381

Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M (2005) Features of promising technologies for pretreatment of lignocellulosic biomass, Bioresource Technol 96:673-686 doi: 10.1016/j.biortech.2004.06.025

Nichols NN, Dien BS, Guisado GM, López MJ, Davison BH, Evans BR, Finkelstein M, McMillan JD(2005) Bioabatement to Remove Inhibitors from Biomass-Derived Sugar Hydrolysates Appl Biochem Biotech 121:379-390. doi:10.1385/ABAB:121:1-3:0379

Nichols NN, Sharma LN, Mowery RA, Chambliss CK, van Walsum GP, Dien BS, Iten LB (2008) Fungal metabolism of fermentation inhibitors present in corn stover dilute acid hydrolysate Enzyme Microb Technol 42:624-630

Nilvebrant N-O, Persson P, Reimann A, de Sousa F, Gorton L, Jönsson L (2003) Limits for alkaline detoxification of dilute-acid lignocellulose hydrolysates Appl Biochem and Biotechnol 107:615-628 doi:10.1385/abab:107:1-3:615

Nilvebrant N-O, Reimann A, Larsson S, Jönsson L (2001) Detoxification of lignocellulose hydrolysates with ion-exchange resins Appl Biochem and Biotechnol 91-93:35-49 doi:10.1385/abab:91-93:1-9:35

Ojeda K, Sánchez E, El-Halwage M, Kafarov V (2011) Exergy analysis and process integration of bioethanol production from acid pre-treated biomass: Comparison of SHF, SSF and SSCF pathways Chem Eng J 176-177:195-201 doi: 10.1016/j.cej.2011.06.083

Oliva, JM, Ballesteros, I, Negro, MJ, Manzanares, P, Cabañas, A, Ballesteros, M (2004) Effect of binary combinations of selected toxic compounds on growth and fermentation of Kluyveromyces marxianus Biotechnol Prog 20:715-720 doi: 10.1021/bp034317p

Ouyang J, Ma R, Zheng Z, Cai C, Zhang M, Jiang T (2013) Open fermentative production of l-lactic acid by Bacillus sp. strain NL01 using lignocellulosic hydrolyzates as low-cost raw material, Bioresource Technol 135:475-480 doi: 10.1016/j.biortech.2012.09.096

Petersen MØ, Larsen J, Thomsen MH (2009) Optimization of hydrothermal pretreatment of wheat straw for production of bioethanol at low water consumption without addition of chemicals Biomass Bioenerg 33:834-840 doi: 10.1016/j.biombioe.2009.01.004

Price, C. W., Fawcett, P., Ceremonie, H., Su, N., Murphy, C. K., & Youngman, P. (2001). Genome-wide analysis of the general stress response in Bacillus subtilis. Molecular microbiology, 41(4), 757-774.

Project Liberty (2015) http://poet-dsm.com/liberty (accessed 10-2015)

Sánchez-Segado S, Lozano LJ, de los Ríos AP, Hernández-Fernández FJ, Godínez C, Juan D (2012) Process design and economic analysis of a hypothetical bioethanol production plant using carob pod as feedstock Bioresource Technol 104:324-328 doi: 10.1016/j.biortech.2011.10.046

Scarlat N, Martinov M, Dallemand J-F (2010) Assessment of the availability of agricultural crop residues in the European Union: Potential and limitations for bioenergy use Waste Manage 30:1889-1897 doi: 10.1016/j.wasman.2010.04.016

Schopmeyer HH, Arnold CR (1944) Lactic acid purification, Patent US2350370A, http://google.com/patents/us2350370 (accessed 11-2015)

Song K, Jang Y-N, Kim W, Lee MG, Shin D, Bang J-H, Jeon CW, Chae SC (2012) precipitation of calcium carbonate during direct aqueous carbonation of flue gas desulfurization gypsum Chem Eng J 213:251-258 doi: 10.1016/j.cej.2012.10.010

Sun JX, Sun XF, Sun RC, Su YQ (2004) Fractional extraction and structural characterization of sugarcane bagasse hemicelluloses Carbohyd Polym 56:195-204 doi:10.1016/j.carbpol.2004.02.002

Taherzadeh MJ, Gustafsson L, Niklasson C, Lidén G (2000) Physiological effects of 5-hydroxymethylfurfural on Saccharomyces cerevisiae Appl Microbiol Biotechnol 53:701-708 doi:10.1007/s002530000328

Thomsen MH, Thygesen A, Thomsen AB (2008) Hydrothermal treatment of wheat straw at pilot plant scale using a three-step reactor system aiming at high hemicellulose recovery, high cellulose digestibility and low lignin hydrolysis Bioresource Technol 99:4221-4228 doi: 10.1016/j.biortech.2007.08.054

Van der Pol EC, Bakker RR, Zeeland ANT, Sanchez Garcia D, Punt A, Eggink G (2015) Analysis of by-product formation and sugar monomerization in sugarcane bagasse pretreated at pilot plant scale: Differences between autohydrolysis, alkaline and acid pretreatment, Bioresource Technol 181:114-123 doi: 10.1016/j.biortech.2015.01.033

Van der Pol EC, Bakker RR, Baets P, Eggink G (2014) By-products resulting from lignocellulose pretreatment and their inhibitory effect on fermentations for (bio)chemicals and fuels, Appl Microbiol Biotechnol 98:9579-9593 doi: 10.1007/s00253-014-6158-9

Van Krieken, J (2011) Method for preparing an organic amine-lactic acid complex, Patent US7867736 B2, https://www.google.com/patents/US7867736 (accessed 11-2015)

Wee Y-J, Yun J-S, Park D-H, Ryu H-W (2004) Biotechnological production of l(+)-lactic acid from wood hydrolyzate by batch fermentation of Enterococcus faecalis Biotechnol Lett 26:71-74 doi: 10.1023/B:BILE.0000009464.23026.e0

Wong HH, Albertson P, O'Hara IM (2011) An update on the Mackay renewable biocommodities pilot plant: Preliminary trial results Proc Aust Soc Sugar Cane Technol 1-11 http://eprints.qut.edu.au/56547/ Accessed: 15-12-2014

Zaldivar J, Ingram LO (1999) Effect of organic acids on the growth and fermentation of ethanologenic Escherichia coli LY01 Biotechnol Bioeng 6:203-210 doi: 10.1002/(SICI)1097-0290(1999)66:4<203::AID-BIT1>3.0.CO;2-#

Zhang J, Chu D, Huang J, Yu Z, Dai G, Bao J (2009) Simultaneous saccharification and ethanol fermentation at high corn stover solids loading in a helical stirring bioreactor Biotechnol Bioeng 105:718-728 doi: 10.1002/bit.22593

Zhao K, Qiao Q, Chu D, Gu H, Dao TH, Zhang J et al (2013) Simultaneous saccharification and high titer lactic acid fermentation of corn stover using a newly isolated lactic acid bacterium Pediococcus acidilactici DQ2, Bioresour Technol 135:481–489

SUMMARY

Lignocellulose is the most abundant biomaterial on earth, making it an interesting feedstock for the production of bulk chemicals. Lignocellulose consists for 60-75% W/W of sugars, which can be used in fermentation processes to produce chemicals such as lactic acid, a building block for PLA (Poly-Lactic acid). PLA can be moulded into a bioplastic, which is a suitable alternative to oil-derived plastics like polystyrene (PS) and polyethylene (PE). In this thesis, lignocellulose was thermo-chemically pretreated and enzymatically hydrolysed to monomeric sugars. These sugars are used as feedstock for lactic acid producing micro-organisms.

In **Chapter 2**, a literature study was performed which focusses on the formation of unwanted by-products, such as furans, phenols and organic acids, during the thermo-chemical pretreatment of lignocellulose. The effect of pretreatment and source of lignocellulose on the formation of by-products is evaluated. By-products formed during pretreatment are shown to inhibit growth and productivity of micro-organisms used for the production of biochemicals. In this chapter, strategies are provided which can help to reduce the inhibitory effect of lignocellulosic by-products.

In **chapter 3**, the effect of autohydrolysis, acid and alkaline pretreatment on sugarcane bagasse is described. It was found that acid pretreatment, combined with enzymatic hydrolysis of the pretreated bagasse, resulted in a high monomeric sugar formation of 610 gram per kg initial dry weight bagasse. This pretreatment also showed a limited formation of small organic acids of 6.4% W/DW, while a relatively high amount of furans (1.4% W/DW) was measured. Alkaline pretreatment did not result in the formation of furans, but compared to acid pretreatment, higher amounts of small organic acids (10% W/DW) and phenolics (0.47% W/DW) were found in the pretreated bagasse. Also, the sugar monomerization efficiency of alkaline pretreated bagasse was slightly lower. A high amount of both furans (2.6% W/DW) and small organic acids (12% W/DW) were found after autohydrolysis.

The inhibitory effect of by-products resulting from thermo-chemical pretreatment of lignocellulose was evaluated in **chapter 4**. Using a rapid small-scale screening method in 48-well plates, both individual and combined effects of by-products present in pretreated lignocellulose were determined. Large differences in the sensitivity for by-products were found for 5 different lactic acid producing bacteria tested in this study. Results from the rapid screening, combined with the quantification of by-products, can be used to predict whether by-products will be inhibitory for growth, Furthermore, the most inhibitory by-product can be identified. The rapid screening method can therefore help to select the most appropriate micro-organism for a chosen combination of pretreatment method and lignocellulosic feedstock.

Bacillus coagulans DSM2314 is an interesting micro-organism to ferment lignocellulosic material, since it can consume both glucose and xylose, and it can convert these to lactic acid

with a high productivity and yield. Furthermore, its optimal growth conditions are similar to optimal conditions of enzymes used to hydrolyse pretreated lignocellulose, allowing a simultaneous saccharification and fermentation process (SSF). However, *B. coagulans* was shown in chapter 4 to be sensitive for several by-products found after pretreatment.

In **chapter 5**, it was shown that the inhibitory effects of by-products *on B. coagulans* can be substantially reduced when it was precultivated in the presence of 1 g/l furfural. Inoculating fermenters rich in lignocellulosic by-products with furfural-containing precultures increased lactic acid yield, titre and productivity. The adaptation effect was not caused by consumption or conversion of furfural by the cells. Using RNA-Seq analysis, it was observed that genes involved in the production of cell wall components such as peptidoglycans, bacillosamine and spermidine were upregulated in the presence of furfural. Furthermore, the sigma factor *SigB* was found to be upregulated. *SigB* regulates gene expression of up to 150 genes, most of them being related to enhanced protection of the cells in stress environments.

In **chapter 6**, It was evaluated whether inoculation with furfural-containing precultures of *B. coagulans* also had a positive effect on simultaneous saccharification and fermentation of pretreated lignocellulosic material. When 1 litre pH controlled fermentations were inoculated with a reference preculture, a total lactic acid production of 77.6 gram was obtained, with an average productivity of 0.78 g/l/h. Using the same amount of lignocellulosic biomass, SSF experiments inoculated with a furfural-containing preculture showed a total lactic acid production of 91.7 gram, with a productivity of 0.92 g/l/h. When the lignocellulosic biomass was added in two stages, 75.9 grams of lactic acid was produced, while the average productivity was increased to 1.81 g/l/h. An average productivity of 2.54 g/l/h was observed during the first 23h of two-stage SSF. This productivity is similar to fermentations using high-grade sugars.

Lignocellulosic by-products play an important role during fermentation of pretreated lignocellulose to lactic acid. In **Chapter 7**, the work of this thesis is discussed, more specifically the role of by-products. Solutions are presented to reduce the inhibitory effects of these by-products. Overall, it was shown that both batch SSF and two-stage SSF, inoculated with furfural-containing preculture, are efficient processes for the production of lactic acid. It can be concluded that using lignocellulose as feedstock for the production of lactic acid as bulk chemical can become economically attractive.

SAMENVATTING

Lignocellulose is het meest voorkomende biomateriaal op aarde. Mede hierdoor is het een zeer interessante grondstof voor de productie van bulk chemicaliën. Lignocellulose bestaat voor 60-75% op gewichtsbasis uit suikers. Deze suikers kunnen gebruikt worden in fermentatie processen waarin chemicaliën zoals melkzuur, een bouwstof voor PLA (poly-melkzuur), worden geproduceerd. PLA kan verwerkt worden in bioplastics, welke een goed alternatief vormen voor uit aardolie geproduceerde plastics zoals polystyreen (PS) en polyethyleen (PE). In deze thesis wordt beschreven hoe lignocellulose thermo-chemisch kan worden voorbehandeld en enzymatisch gehydrolyseerd kan worden. Hierbij worden suikers als monomeren vrijgemaakt, die kunnen worden gebruikt als grondstof voor melkzuur fermentaties.

Allereerst is er een literatuurstudie uitgevoerd, deze is te vinden in **hoofdstuk 2**. Hierin wordt beschreven hoe de formatie van ongewenste bijproducten zoals furanen, phenolen en organische zuren plaats vind tijdens de thermo-chemische voorbehandeling van lignocellulose. Het effect van verschillende voorbehandelingsmethoden en lignocellulose bronnen op de formatie van ongewenste bijproducten is hierbij geëvalueerd. De gevormde bijproducten kunnen de groei en productiviteit remmen van micro-organismen die worden gebruikt voor de productie van bulk chemicaliën. In dit hoofdstuk worden strategieën beschreven die kunnen helpen om deze ongewenste effecten van de lignocellulose bijproducten te verminderen.

In **hoofdstuk 3** wordt beschreven welke bijproducten worden gevormd tijdens neutrale, zure en alkalische voorbehandeling van suikerriet bagasse. Daarnaast is er ook gekeken naar de effectiviteit van deze voorbehandelingen, in combinatie met enzymatische hydrolyse, in het vrijmaken van suikers als monomeren. Een zure voorbehandeling resulteerde in een hoge monomerische suiker opbrengst van 610 gram per kilogram initiële biomassa. Verder werden er ook organische zuren gevormd, hoewel dit in mindere mate was dan bij de andere voorbehandelingsmethoden (6.4% W/DW). Er werd echter wel een hoge concentratie aan furanen gemeten na de pretreatment (1.4% W/DW).

Tijdens alkalische voorbehandeling van bagasse werden geen furanen gevormd, maar werd na afloop wel een hoge concentratie organische zuren (10% W/DW) en phenolische verbindingen (0.47% W/DW) waargenomen. Met een monomerische suiker opbrengst van 475 gram per kilogram initiele droge biomassa was de opbrengst van alkalische voorbehandeling lager dan zure voorbehandeling. Na neutrale voorbehandeling werd zowel een hoge concentratie furanen (2.6% W/DW) als organische zuren (12% W/DW) gemeten.

De toxische effecten van bijproducten, welke zijn ontstaan na de thermo-chemische behandeling van lignocellulose, op lactaatproducerende micro-organismen zijn beschreven in **hoofdstuk 4**. Zowel individuele als gecombineerde effecten van bijproducten kunnen worden geïdentificeerd door gebruik te maken van een snelle kleine schaal screening methode.

Voor de 5 geteste stammen in deze studie zijn grote verschillen gevonden in sensitiviteit voor verschillende bijproducten. Deze resultaten kunnen, samen met de resultaten van de gecombineerde effecten, worden gebruikt om te voorspellen of bijproducten in voorbehandelde lignocellulose kunnen zorgen voor remming van de groei van micro-organismen. Daarnaast kunnen met deze resultaten de meest toxische bijproducten worden geïdentificeerd. Deze snelle screening methode kan daarom helpen om het meest geschikte micro-organisme te vinden voor een gekozen combinatie van voorbehandelingsmethode en lignocellulose bron.

B. coagulans DSM2314 is een interessant micro-organisme voor de fermentatie van lignocellulose suikers, omdat dit micro-organisme zowel glucose als xylose kan consumeren. Daarnaast kan dit micro-organisme met een hoge productiviteit en efficiëntie melkzuur produceren. De groeicondities van deze stam zijn vergelijkbaar met de ideale condities waarbij enzymatische hydrolyse van voorbehandelde lignocellulose plaats vind, waardoor hydrolyse en fermentatie tegelijk kan worden uitgevoerd (SSF proces). In **hoofdstuk** 4 is echter geobserveerd dat *B. coagulans* relatief gevoelig is voor lignocellulose bijproducten, wat mogelijk nadelig kan werken tijdens de fermentatie van lignocellulose suikers.

In **hoofdstuk 5** wordt aangetoond dat het toxische effect van bijproducten op de groei en productiviteit van *B. coagulans* sterk kan worden gereduceerd door 1 g/l furfural toe te voegen aan het begin van de voorcultuur. Om het effect van furfural toevoeging te zien zijn fermentatie experimenten uitgevoerd met een medium waaraan verschillende bijproducten zijn toegevoegd. Fermentoren, welke zijn geënt met voorcultures waaraan furfural is toegevoegd, laten een verbeterde melkzuur productie, productiviteit en yield zien. Het adaptatie effect van de cellen in aanwezigheid van furfural kon niet worden toegeschreven aan de consumptie of omzetting van furfural door de cellen. RNA-Seq analyse was uitgevoerd om te bekijken welke genen meer of minder tot expressie kwamen. Genen welke betrokken zijn bij het produceren van celwand componenten, zoals peptidogylcanen, bacillosamines en spermidines, kwamen meer tot expressie in de aanwezigheid van furfural. Ook *SigB* kwam meer tot expressie in de aanwezigheid van furfural. Dit gen reguleert de expressie van 150 genen die de cel beschermen tegen stress condities.

In **hoofdstuk 6** is onderzocht of de toevoeging van furfural aan de voorculturen van *B. coagulans* ook een positief effect heeft tijdens simultane saccharificatie en fermentatie (SSF proces) van voorbehandelde bagasse. In een 1 liter fermentor, geënt met een voorcultuur zonder furfural, kan een totale melkzuurproductie van 77.6 gram worden bereikt, met een productiesnelheid van 0.78 g/l/h. Wanneer dezelfde reactor wordt geënt met een voorcultuur waaraan furfural is toegevoegd, dan kan totale melkzuurproductie van 91.7 gram worden gehaald, met een productiesnelheid van 0.92 g/l/h. Wanneer de bagasse niet in een maar in twee delen wordt toegevoegd aan de reactor, dan kan een productiesnelheid van 1.81 g/l/h worden bereikt, met een productiesnelheid van 2.54 g/l/h over de eerste 23 uur. De productiviteit in de eerste 23 uur is vergelijkbaar met de productiviteit zoals wordt gemeten in fermentaties op pure suikers.

In **hoofdstuk 7** wordt het werk van deze thesis bediscussieerd, waarbij vooral aandacht wordt gegeven aan de rol die lignocellulose bijproducten tijdens fermentatie hebben. Verschillende oplossingen worden aangedragen om de effecten van bijproducten op fermentaties te minimaliseren. Er kan worden geconcludeerd dat zowel batch SSF als Fed-Batch SSF, welke worden geënt met furfural voorgegroeide cellen, interessante en efficiënte processen zijn om melkzuur te produceren. Het gebruik van lignocellulose als grondstof voor de productie van melkzuur als bulk chemicalie kan daarom een economisch interessant proces zijn.

DANKWOORD

Ik heb tijdens mijn PhD het genoegen gehad om met veel mensen samen te mogen werken, zowel binnen BioProcess Engineering, Food and Biobased Research als ook binnen het BE-Basic consortium.

Ik wil graag beginnen met het bedanken van mijn begeleiders. **Gerrit**, bedankt dat je mij de kans gaf om mijn PhD onder jou leiding te starten. Mede dankzij jou is het gelukt om mijn PhD vrijwel binnen de stelde 4 jaar succesvol af te ronden.

Ruud, bedankt dat je mij wilde helpen in het laatste deel van mijn thesis, en dat je veel van je vrije tijd wilde besteden om mijn thesis tot een succesvol einde te brengen. Ik waardeer deze inzet enorm! **Michel**, al was je officieel alleen aan het begin mijn begeleider, ik vond het erg fijn hoe je soms even langs kwam om te vragen hoe het ging, om tips te geven, en hoe je me aan het eind van mijn PhD hebt geholpen om een baan te vinden. En tot slot **Rob**, al vind ik het enorm jammer dat je ineens weg bent gegaan, je hebt mij in de eerste deel van mijn PhD goed geholpen. Daarvoor wil ik je graag hier bedanken!

De meeste tijd heb ik doorgebracht in het atrium (later axis), het gebouw van FBR. **Lenny**, ik vond het ontzettend fijn dat jij mijn kantoorgenoot was. Als ik met een probleem zat, dan wist je altijd de juiste raad te geven, maar daarnaast was het ook erg gezellig! En gelukkig had je ook een oog voor goede koffie ;). Ook wil ik je nogmaals bedanken dat je mij hebt geholpen met het leggen van laminaat J, zonder jou was dat nooit zo goed gelukt. Ik ben erg blij voor je dat je nu je plek hebt gevonden in Kopenhagen samen met Anne. **Vincent,** ik vond het erg leuk toen je later bij ons kwam als kamergenoot. Ik vond het dan ook erg jammer dat jij in het axis bent gebleven na de verhuizing van BPE.

Binnen FBR zijn er veel mensen die mijn PhD een super fijne en leerzame tijd hebben gemaakt. **Alniek**, jij kwam al vrij snel helpen met mijn project. Jij hebt mij veel geleerd, hoe ik nu hoe je een HPLC in en uit elkaar schroeft, en wat er allemaal bij analytische chemie komt kijken. Ook vond ik het altijd leuk en leerzaam om samen koffie te drinken, jij hebt mij zeker geholpen mij blik op de wereld te verbreden! **Truus**, ik vond het altijd erg leuk en leerzaam om met je te praten. **Emil**, jij maakte het altijd gezellig op het lab en in koffiepauzes. **Jan**, ik wil je enorm bedanken voor je hulp met mijn RNA sequencing! **Lonneke**, ik vond het altijd heel gezellig als jij er was, en ik vond het erg jammer dat je naar het Zodiac verhuisde. **Book**, it was very nice to meet you, I wish you all the best in Thailand! **Ellen**, eerst collega, later ook buurvrouw, dankje voor alle hulp en gezellige momenten! Verder wil ik alle andere mensen bedanken die mij hebben geholpen tijdens mijn PhD, en die mijn PhD een mooie periode van mijn leven

hebben gemaakt: **Paulien, Hans, Jacqueline, Jacinta, Steef, Hetty, Bwee, Astrid, Ana, Marc, Odette, Yvonne, Hadrien, Florent, Noon, Pang** en natuurlijk alle anderen!

Het laatste jaar ben ik verhuisd naar BPE in het Radix om mijn thesis the schrijven. **Ilse,** ik ben heel blij dat jij mijn kamergenoot was. Jij maakte het laatste jaar heel gezellig, en was bovendien altijd bereid the luisteren als ik weer eens schrijffrustratie had ;). **Mitsue**, it a great pleasure to be your officemate, I really enjoyed your presence and our conversations J! **Edgar**, although I nearly finished in the lab, it was nice to have you around in the office! **Marjon**, het was erg fijn om je als kantoorgenoot te hebben, en ik heb enorm veel bewondering voor je doorzettingsvermogen.

Gerard, je interesse in mij heeft mij erg goed gedaan! Daarnaast maakte je het tijdens de koffie en lunchpauzes altijd een stuk gezelliger met je aanwezigheid en mooie verhalen. **Kiira**, we had a lot of fun during the PhD-trip, I will never forget how people though we were a couple those days.

The coffee and breaks were always the best time of the day. **Rupali**, **Tim**, **Richard**, **Bregje**, **Wendy**, **Agi**, **Ward**, **Jorijn**, **Joao, Youri**, **Douwe**, **Miranda**, **Marina**, **Mark**, **Dirk**, **Marcel**, **Sebastiaan**, **Hans**, **Packo**, **Lenneke**, **Guido**, and of course all the others, thanks for making the breaks and my stay at BPE awesome!

Een van de mooie dingen aan een PhD is het mogen begeleiden van studenten. **Evelien**, dankzij jou scherpe en kritische blik, en uitmuntend inzicht, heb ik chapter 4 tot een goed mooi chapter weten te maken. De cum laude voor je bachelor had je dan ook volledig verdiend! **Jorrit**, ondanks dat ik je een eindeloze hoeveelheid flesjesexperimenten liet doen, bleef je altijd vrolijk en altijd doorgaan! Dank voor al je hulp!

Al vrij snel werd woensdagavond de 'mannenavond', samen met **Jan**, **Wolf** en **Stefan**. Deze avonden werden snel een van de hoogtepunten van de week, en ik ben heel blij dat we ze hebben kunnen voortzetten ondanks mijn verhuizing. Verder waren de gameweekenden en de trip naar Berlijn geweldig! Ook hebben jullie me enorm geholpen met ontspannen tijdens mijn PhD, en gesteund als ik het wat moeilijker had.

Marit, je was een van de eerste mensen die ik goed leerde kennen. Ik heb enorm genoten van alle gezellige avonden, aan het begin vaak met zn 2en, later vaak met **Jan** erbij. Ook heel erg bedankt voor al jullie gastvrijheid, als ik weer eens een experiment had wat tot laat duurde, of als ik een keer een gezellige avond wilde hebben, kon ik vaak bij jullie terecht. Hierdoor hebben jullie mijn verhuizing naar Zwolle een stuk makkelijker gemaakt, de waardering hiervoor is zo groot dat ik hem moeilijk kan verwoorden. I owe you!

Wolf, je humor maakte elke laboratiumdag bij FBR weer een stuk leuker! En petje af voor alle kookkunsten die je aan mij helpt laten zien. Ik ben heel blij dat jij mijn Paranimf wil zijn!

Lieve **Ilse**, Een jaar nadat ik was begonnen leerde ik je kennen toen ik actief begon te tennissen. Al vrij snel voelde het alsof je altijd in mijn leven bent geweest. Je vrolijkheid en gezelligheid maakte de dag altijd goed. Het is lastig in woorden te beschrijven hoe ontzettend veel je mijn leven verrijkt hebt. Jou steun heeft mij altijd fantastisch geholpen, ik hoop dat ik hetzelfde kan doen in tijdens jou PhD project. Sorry dat je soms mijn eindeloze gezeur hebt moeten aanhoren, maar mede dankzij jou is het gelukt ;)!

Inge, ik weet nog dat we elkaar vroeger nogal vaak in de haren vlogen. Wat ben ik blij dat dat nu anders is! Alle leuke dingen, of het nu een dagje Rotterdam is, of de reizen naar Boedapest en Antwerpen, heb ik enorm goede herinneringen aan! Enorm leuk dat je me nu wilt steunen als paranimf bij deze mijlpaal.

Pap en Mam, bedankt voor al jullie liefde en steun! Ik ben ontzettend trots dat jullie mijn ouders zijn. Jullie hebben altijd voor mij klaar gestaan, of het was voor verhuizing nummer 7, of om mij te verwennen als ik een weekendje thuis kwam! Zonder jullie goede opvoeding was ik waarschijnlijk nooit zo ver gekomen!

Age, Alberta, Nynke, Arianna, ik vind het fijn dat ik jullie heb leren kennen, en ik ben heel blij met alle steun die jullie mij geven! Ik vind het heel leuk dat jullie altijd interesse hebben gehad in mij en mijn PhD, en dat jullie aanwezig zijn op de defense.

-Edwin-

CURRICULUM VITAE

Edwin Christiaan van der Pol was born in Schiedam, the Netherlands on the 13th of October 1988. He went to the Stedelijk Gymnasium in Schiedam, where he received his VWO diploma in 2006.

In the same year, he started to study Life Science & Technology at both the University of Leiden and Delft University of Technology. The bachelor degree of this study was successfully obtained in 2010. In 2011 a Master thesis project was successfully performed at the Industrial Microbiology group of Delft University of Technology, focussing on 2nd generation biofuel production. After an Internship at DSM nutritional products in Switzerland, he obtained his MSc degree, specialization biochemical engineering, in 2011.

The work on 2nd generation biosubstrates was continued in his PhD project, which was performed within the Bioprocess Engineering group of Wageningen University. The project focussed on the production of lactic acid from lignocellulose-rich material. This project was performed in collaboration with BE-Basic. Currently, he is working as Concept Developer within AVEBE, a company which processes potatoes to starch and other valuable compounds.

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Disciplince specific activities

Courses

European Biorefining Training School 2014 (Climate-KIC, Budapest, Hungary)
Design of experiments for Bioprocess Optimization (MBI, London, UK)
Metabolic Engineering (VLAG, Wageningen, The Netherlands)
Food and Biorefinery enzymology (Vlag, Wageningen, The Netherlands)

Symposia and meetings

BE-Basic symposia and flagship meetings (BE-Basic, The Netherlands)
CLIB conference (CLIB2020, Germany)
Symposium on Biotechnology for Food and Chemicals (SIMB, California, USA)

General courses

Scientific publishing (WGS, Wageningen, The Netherlands)
Scientific writing (WGS, Wageningen, The Netherlands)
Teaching and supervising thesis students (DO, Wageningen, The Netherlands)
Project and time management (WGS, Wageningen, The Netherlands)
Networking course as introduction for PhD trip (Wageningen, The Netherlands)
Business awareness workshop (BE-BIC, Delft, The Netherlands)
VLAG PhD week (VLAG, Wageningen, The Netherlands)

Optionals

Preparation research proposal (Wageningen, The Netherlands)
PhD trip 2012 (Spain)
Team meetings BE-Basic (Gorinchem, The Netherlands)
Bioprocess Engineering Brainstorm (AiO) days (Wageningen, The Netherlands)

Layout: Ridderprint BV - www.ridderprint.nl
Printed by: Ridderprint BV - www.ridderprint.nl
Cover Design by: Jardine Media – Rotterdam

This study was carried out at the Bioprocess Engineering Group of Wageningen University, The Netherlands, in cooperation with Food and Biobased Research, Wageningen UR, The Netherlands. The project was supported by the BE-Basic foundation, Delft, The Netherlands, and co-funded by DSM and Corbion Purac. The author would like to thank DSM, Corbion Purac, the BE-Basic consortium, The Laboratory of Food Chemistry of Wageningen University, The Netherlands, and the Laboratory of Microbiology of Wageningen University, The Netherlands, for the collaboration and discussions.