Microbial Conversion of Lignocellulose-Derived Carbohydrates into Bioethanol and Lactic Acid

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Dit onderzoek is uitgevoerd binnen de onderzoekschool VLAG.

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. M.J. Kropff,
in het openbaar te verdedigen
op woensdag 16 januari 2008
des namiddags te half twee in de Aula.

Maas, Ronald H.W.

Microbial conversion of lignocellulose-derived carbohydrates into bioethanol and lactic acid

Thesis Wageningen University, the Netherlands, 2008 – with summary in Dutch and English

ISBN: 978-90-8504-871-8

Voor mijn ouders

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CHAPTER 1

General Introduction

BACKGROUND

The limited availability and supply of fossil fuel and the worldwide rising energy demands result in increasing prices of fossil fuel derived energy and chemicals. Moreover, issues about air pollution and global warming effects associated with fossil fuel utilization received lots of attention throughout the foregoing years. The Intergovernmental Panel on Climate Change (IPCC) anticipates different scenarios varying between two-fold and four-fold increases of global energy consumption within the next 50 years (SRES, 2000). The majority of the globally used energy and chemicals are derived from fossil fuels such as oil, coal and gas. Factors such as limited availability of fossil fuel sources and import of oil from social and political unstable regions of the world resulted in price fluctuations during the last few decades. Data from the Energy Information Administration (EIA) showed that in the period between 1990 and 2004 the world crude oil prices were fluctuating between 9 and 32 US$ per barrel (Fig. 1). Since the beginning of 2004 the prices of crude oil went rapidly up to 55 US$ per barrel in January 2006. At the end of September 2007, the prices of crude oil has been raised further to 76 US$ per barrel.

Another important aspect confronting the world is the major ecological effect of large-scale fossil fuel utilization. Combustion of fossil fuels (*e.g.* gasoline, diesel and kerosine) or fossil fuel derived products (*e.g.* polyethylene terephthalate (PET), polyolephines and polystyrenes) generate besides energy also greenhouse gasses such as carbon dioxide (CO_2). Yet, the biomass fossilization process takes millions of years whereas the preserved carbon is released in a relatively short period of time by combustion resulting eventually in an accumulation of CO_2 in the atmosphere (McKendry, 2002a). Data from the EIA show a continuous rise of energy-related CO_2 emission throughout the past decades (EIA, 2007).

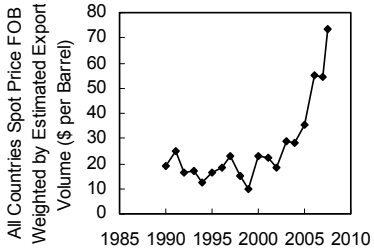

Figure 1. Development of the world crude oil prices per barrel (one barrel equals 158.99 liter) between 1990 and 2007 (data presented are values in the first week of January) (EIA, 2007).

CO_2 is besides aerosols and non-CO_2 greenhouse gasses such as methane (CH_4) and nitrous oxide (N_2O) presumably one of the main anthropogenic contributors to the global warming effect (Hansen et al., 2000). Although the effects of these climate changes on the long term are still uncertain, the level of greenhouse gasses into the atmosphere should be stabilized. Therefore, many of the industrialized nations signed in 1997 the Kyoto protocol which target is to reduce greenhouse gas emissions by 2010 with ~5% below 1990 levels (Bolin, 1998). The importance and urgency of this topic was subscribed by the Nobel Prize Committee in 2007 by rewarding Al Gore and the IPCC the Nobel Peace Prize for their contribution to enhance the public awareness for anthropogenic global climate changes. One major strategy to achieve a reduction of net greenhouse emissions is by large-scale substitution of petrochemical fuels by CO_2-neutral renewable energy sources (Reith et al., 2002).

BIOMASS AS RENEWABLE RESOURCE FOR PRODUCTION OF BIOENERGY AND CHEMICALS

For many centuries, mankind has used renewable energy sources such as wind and water (*e.g.* wind mills, sailing boats and waterwheels). In the twentieth century the use of bio-based energy sources shifted to large-scale utilization of fossil fuel energy sources. The limited nature of fossil fuel reserves, fluctuating and high energy prices, but, also the anticipated climate changes presumably caused by greenhouse gasses, are nowadays stimulating reasons for finding alternative energy sources (Sanders et al., 2007). Prospects are showing a shift from fossil fuel derived energy to the use of alternative sources. Renewable resources such as wind, solar, water, biomass and geothermal energy can be used for the generation of electricity, heat and biofuels.

Of these renewable energy sources, biomass is one of the main contributors to provide the global energy demand in the near future. Furthermore, biomass derived energy can play an important role in the reduction of greenhouse gas emission since the CO_2, arisen from biomass combustion, can be re-adsorbed by growing plants. In comparison to the CO_2-cycle of fossil fuel utilization, the short CO_2-cycle of biomass utilization results in less accumulation of CO_2 in the atmosphere. Biomass can be generically defined as a group of highly diverse organic products. The biomass is produced in green photosynthetic organisms (*e.g.* plants and algae) converting CO_2 and water (H_2O), with sunlight as energy source. The energy of sunlight is stored in chemical bonds between mainly carbon (C), hydrogen (H) and oxygen (O) molecules. The biomass can be converted in thermal energy or gaseous and liquid energy carriers.

Thermo-chemical conversion of biomass

Biomass can be used as solid fuel in a gasifier via thermo-chemical routes such as pyrolyis (heating in absence of O_2), combustion (burning with O_2) and gasification yielding energy. Gasification of biomass results in synthesis gas (syngas) containing carbon monoxide (CO), carbon dioxide (CO_2) and hydrogen (H_2) as an intermediate for generating power or liquid fuel. In power generation application the syngas is directed to either drive a turbine or fuel a boiler. The gasses CO and H_2 can also be converted further to a synthetic petroleum substitute via the so-called Fisher-Tropsch process or function as feedstock for the production of chemicals. Other techniques producing bio-oils are hydro thermal upgrading (HTU) and liquefaction (McKendry, 2002b).

Seeds from crops such as Rape, Palm and Jatropha can be extracted mechanically followed by an esterification yielding liquid oils (bio-diesel), containing fatty acid methyl or ethyl esters and can be used as liquid replacement of diesel in engines or in generators producing electricity (Ramadhas et al., 2004; Barnwal and Sharma, 2005).

Bio-chemical conversion of biomass

Biomass can also serve as carbohydrate substrate for biological conversion via fermentation processes. In general, the saccharides present in biomass resources can be classified in three categories: (1) simple monomeric and dimeric sugars (*e.g.* glucose, fructose and sucrose); (2) storage carbohydrates (*e.g.* starch, inuline and glycogen); and (3) structural carbohydrates (*e.g.* hemicelluloses and celluloses). These sugars all occur abundantly in nature and can be distinguished from each other based on their molecular structure.

Simple sugars and storage carbohydrates

The simple sugars (from *e.g.* sugarcane, sugar beet and fruits) such as glucose and sucrose (a disaccharide of glucose and fructose) can be directly converted by micro-organisms. The storage carbohydrates such as starch (from corn, wheat, cassava, potatoes and root crops) and inuline (from roots of plants such as dandelion and artichoke) are polymers build up of glucose and fructose units, respectively, and require depolymerization prior to be taken up as carbohydrate source by fermenting micro-organisms. Some micro-organisms (*e.g.* fungal species) are able to produce extracellular hydrolytic enzymes and utilize the resulting monomeric sugars simultaneously. This microbial activity is also known as direct microbial conversion (DMC). If the fermenting micro-organism is unable to produce these enzymes itself, the polysaccharide need to be thermically (140-180°C) and enzymatically (α-amylase, glucoamylase) hydrolyzed prior to fermentation.

The simple sugars and/or starch – derived from crops and agro-industrial residues – are often used as substrate for industrial microbial processes producing the so-called first

generation biofuels. Sugar and starch containing crops are produced all over the world. The local geography and climate, however, will dictate which crops can be grown efficiently (Claassen et al., 1999). Nevertheless, an increase of energy production from biomass such as corn will compete for the limited land against corn-based food production. Currently, the competing claims for substrates lead to increasing prices of agricultural raw materials and therefore food and feed. So, crops containing simple sugars and starch might be less attractive feedstocks for large scale bioprocesses producing biofuels and bulk chemicals.

Structural carbohydrates

The so-called second generation biofuels are produced from structural polysaccharides such as (hemi-)celluloses present in lignocellulosic biomass (*e.g.* wood and wheat straw). Several benefits of using lignocellulose as substrate material can be listed: it is (1) widely available; (2) relatively inexpensive; (3) not directly competing with food applications; and (4) sustainable in terms of CO_2 emissions. Furthermore, the use of low-costs agro-industrial residues (*e.g.* wheat straw) in bioprocesses provides alternative substrates and helps also to solve their disposal problem (Lin and Tanaka, 2006).

A well known process is the conversion of organic matter to the biogas methane (CH_4) in anaerobic digesters (Chynoweth et al., 2001). In these reactors, a consortium of hundreds to thousands of bacterial species are active; producing hydrolytic enzymes, necessary for hydrolysis of polysaccharides to fermentable monosaccharides, and fermenting these sugars and other organic compounds eventually to CH_4 and CO_2. The biogas can be stored or directly burned in order to generate electricity. The solid residues (non-fermentable fraction) may be settled and/or dewatered and used as compost.

In other processes, the (hemi-)celluloses in biomass can function as substrate for microbial fermentation producing transportation fuels like ethanol, butanol or hydrogen (Claassen et al., 1999) or other bulk chemicals such as organic acids. But, in comparison to the conversion of simple sugars and storage carbohydrates containing crops, the lignocellulose-to-product conversion is more complex facing several techno-economic challenges (Galbe et al., 2007).

TABLE 1. Top ten product volumes by microbial fermentation processes (Dechema, 2004).

	Production (million tons/yr)	Price (€/kg)[c]
Bio-ethanol	>18.5	0,40
L-Glutamate	1.5	1,20
Citric acid	1.0	0,80
L-Lysine	0.7	2,00
Lactic acid[a]	0.15	1,80
Lactic acid[b]	0.14	2,25
Acetic acid	0.19	0,50
Gluconic acid	0.1	1,50
Vitamin C	0.08	8,00
Penicillin	0.045	300,00
Xanthan	0.040	8,40

[a]Used for food application, leather and textile; [b]Used for polylactic acid production; [c]Prices can change depending on place of production and time of year.

Renewable fermentation products

Presently, concentrated sugar streams such as molasses are often used in industrial processes for microbial production of fuels and bulk chemicals. Table 1 represents a list of chemicals which are produced via microbial processes ranked by their global production volumes. Chemicals including organic acids, amino acids, vitamin, antibiotic and polysaccharide occur in the list, however, the alcohol bio-ethanol is by far the chemical produced at largest scale via microbial fermentation processes.

Since the early 1970s, Brazil was the major ethanol producing country applying ethanol as automotive transportation fuel. The USA initiated the production on large-scale throughout the foregoing decades and increased the production volume significantly during the last few years (Fig. 2). Data from the Renewable Fuels Association (RFA) showed that the USA and Brazil are the major ethanol producing countries (Table 2) using sugars derived mainly from the feedstocks corn and sugarcane. During the foregoing years the production of ethanol from lignocellulosic biomass gained increasing attention. Technologies are being developed to derive fermentable sugars from lignocellulosic materials. This knowledge and experiences will provides a widely available renewable resource with an impressive economic potential for a broad range of microbial fermentation processes (Claassen et al., 1999; Tsao et al., 1999). Recently, the US Department of Energy (DOE) awarded six companies a total funding of $US 385 million for developing various approaches/plants for the pilot-scale production of cellulosic ethanol. With the private sector sharing at least 60% of project costs, the DOE's funding leverages nearly $US 1000 million in total capital investments (Koukoulas, 2007).

TABLE 2. Major bioethanol producing countries in 2006 (RFA, 2005).

Countries	Production volumes of ethanol (million tons)
USA	14.63
Brazil	13.53
China	3.06
India	1.51
Others	6.06

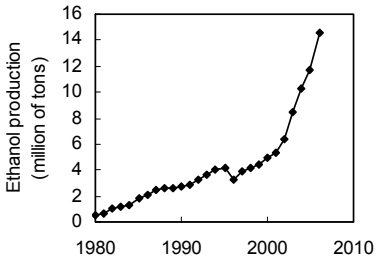

Figure 2. Bioethanol production in the USA in the period between 1980 and 2006 (RFA, 2005).

Ethanol can be used as transportation fuel in conventional vehicles; they can run on a mixture of 90% gasoline and 10% ethanol without adapting the engine. The so-called flexible fuel vehicles are able to use a blend of 0 to 85% ethanol in gasoline (E85) (Galbe and Zacchi, 2002). Today, all cars in Brazil run on at least 25% ethanol mixed with gasoline, with 60% of all cars being fuel-flexible (up to 100% ethanol). The USA serves gasoline as a blend with 10% ethanol and intends to increase this proportion in the near future.

The European Bioethanol Fuel Association (EBIO) estimated that the total European ethanol production in 2006 (Table 3) was 1.25 million tons (equivalent to 0.80 Million ton oil equivalent (Mtoe)) and corresponding to a growth of 71% with respect to 2005 (EurObserv'ER, 2007).

TABLE 3. Bioethanol production in the EU in 2005 and 2006 according the European Bioethanol Fuel Association (EurObserv'ER, 2007).

Countries	2005	2006
	Bioethanol (thousand tons)	Bioethanol (thousand tons)
Germany	131.4	343.2
Spain	241.2	320.1
France	114.7	199.0
Sweden	121.8	111.5
Italy	6.4	101.9
Poland	51.0	95.5
Hungary	27.9	27.1
Lithuania	6.4	14.3
Netherlands	6.4	11.9
Czech Republic	0	11.9
Latvia	9.6	9.6
Finland	10.4	0
Total	*726.9*	*1246.0*

The utilization of ethanol in the European Union was much higher namely approx. 3 Mtoe in 2005 and approx. 5.4 Mtoe in 2006 corresponding to a growth of 79% and 1.8% share of the total consumption of fuels devoted to transport.

Among the chemicals listed in Table 1, several organic acids (*e.g.* citric acid, lactic acid and acetic acid) occur. The total volume of lactic acid is divided over two almost equal parts; one which is applied in food as preservative, in leather and textile, and the other part for the production of poly-lactic acid (PLA). Recently, the application of biodegradable PLA, produced 100% from renewable substrates, gained increasing attention. PLA is produced by polymerization of optically pure lactic acid molecules and can be used as an environmental friendly replacement of petrochemically derived plastics (Datta et al., 1995; Drumright et al., 2000; Vaidya et al., 2005). PLA exhibits many characteristics that are comparable or even better than petroleum-derived plastics and makes it suitable for various applications such as bags, bottles and packaging materials.

LIGNOCELLULOSIC BIOMASS

Lignocellulosic biomass resources can be classified in four categories: (1) wood residues; (2) municipal solid waste; (3) agricultural residues; and (4) dedicated energy crops (Lin and Tanaka, 2006). Wood residues are by far the largest source of lignocellulose biomass for energy production. Among the other biomass resources, short-rotation woody crops, herbaceous crops and dedicated energy crops such as switchgrass (*Panicum virgatum*) and miscanthus (*Miscanthus spp*) are promising resources due to numerous harvests from a single planting. Lignocellulosic biomass typically contains 55 to 75 % (*w/w*) carbohydrate polymers build up of five-carbon and six-carbon sugar units. The major structural components in lignocellulosic biomass are cellulose, hemicellulose and lignin, and non-structural components such as extractives, proteins and ash whole amounts can vary depending on the species, age and cultivation conditions (Table 4). Apart from cellulose, the hemicelluloses are composed of different pentose and hexose sugars. Nevertheless, these lignocellulosic sugars are often expressed as homo-polymeric fraction, for example xylan (polymer of xylose), arabinan (polymer of arabinose), galactan (polymer of galactose) and mannan (polymer of mannose). The two sugar fractions glucan and xylan are abundantly present in lignocellulose. Other polysaccharides such as arabinan, galactan and mannan are present in lower quantities.

TABLE 4. Compositions of wood and herbaceous species based on percent dry weight[a] (Wiselogel et al., 1996).

Feedstock	Glucan	Xylan	Galactan	Arabinan	Mannan	Lignin	Extractive	Ash
Pine	46.4	8.8	0	2.4	11.7	29.4	0	0.4
Poplar	49.9	17.4	1.2	1.8	4.7	18.1	0	0.5
White Oak	43.6	18.0	0.4	2.4	2.9	23.2	0	0.6
Corn stover	36.4	18.0	1.0	3.0	0.6	16.6	7.3	9.7
Bagasse[b]	40.2	21.1	0.5	1.9	0.3	25.2	4.4	4.0
Wheat straw	38.2	21.2	0.7	2.5	0.3	23.4	13.0	10.3
Switch Grass	31.0	20.4	0.9	2.8	0.3	17.6	17.0	5.8

[a]Analyzed by the National Renewable Energy Laboratory; [b]Residue of sugar cane after juice extraction.

Cellulose is an insoluble linear polymer of ß-1,4 linked glucose subunits (>10.000) and can appear as a highly crystalline material with an average molecular weight of around 100.000 (Fig. 3A). The polyglucan chains are composed parallel and bound by intra- and intermolecular hydrogen bonds. This fibrillar structure results in highly ordered crystalline structure interspersed with less ordered amorphous regions (Kögel-Knabner, 2002). Cellulose can vary in depolymerization degree (DP) and the level of cristallinity dependent on the location of the plant and species.

In contrast to cellulose, hemicellulose is a heterogenous branched polysaccharide composed of primarily xylose and other five-carbon sugars such as arabinose, but also six-carbon compounds such as glucose, galactose and mannose, methylglucoronic acid and galacturonic acid, with an average molecular weight of <30.000 (Fig. 3B). In hardwoods xylan is the major hemicellulose whereas in softwoods arabinoxylans and mannans are more abundant (see for overview hemicelluloses (Brigham et al., 1996)). Xylans are a widespread hemicellulose group, consisting of ß-1,4 linked xylosyl subunits with substitutions of arabinosyl, acetyl and glucuronosyl residues. The release of acetyl groups during pretreatment processes results in formation of acetic acid which can have detrimental effect on the fermentation activity and will be discussed later in more detail.

The (hemi-)celluloses are tightly bound to lignin by lignin-carbohydrate complexes (lcc). Lignin is a high molecular weight polymer and generally contains three aromatic alcohols coniferyl alcohol, sinapyl alcohol and *p*-coumaryl alcohol (Fig. 3C). In addition, grass and dicot lignin also contain large amounts of phenolic acids such as *p*-coumaric and ferulic acid, which are esterified to alcohol groups of each other and to other such as sinapyl and *p*-coumaryl alcohols (Howard et al., 2003). The enzymatic degradation of lignin, by e.g. white-rot fungi, is a complex and slow process involving many enzymatic reactions. Because no commercial lignin-degrading enzyme preparations are available, physical and chemical treatments are necessary to degrade or modify lignin. Furthermore, a part of the carbohydrates is bound stringently to lignin, tough to liberate and therefore not fermentable. In a growing plant, the presence of (hemi-)cellulose determines the strength

properties whereas lignin serves to protect cellulose against environmental exposure such as microbial attack (Kögel-Knabner, 2002), acting as a glue and hydrophobic agent.
The chemical composition and amount of polysaccharides are one of the factors determining the suitability of a feedstock for industrial processes. Lignocellulose is difficult to access due to its complex structure, the high cristallinity of cellulose and the presence of lignin. The lignocellulose-to-product process requires a physical and/or chemical pretreatment step, a chemical and/or enzymatic hydrolysis and a fermentation.

In this thesis, we focus on the feedstock wheat straw which is an abundantly available substrate in the Netherlands and the European Union. With wheat and barley production data from the FAOstat (FAOstat) we calculated, assuming an average ratio of straw/grain of 0.78 *w/w* (Edwards et al., 2005) and the variable productivity (tons/ha) of each country, that the Netherlands produced between 1999 and 2003 yearly 0.87 million tons wheat straw and 0.31 million tons barley straw. The EU25 produced yearly 89.5 million tons wheat straw and 4.2 million tons barley straw. This quantity of straw makes it a potential feedstock for the production of second generation biofuels and chemicals.

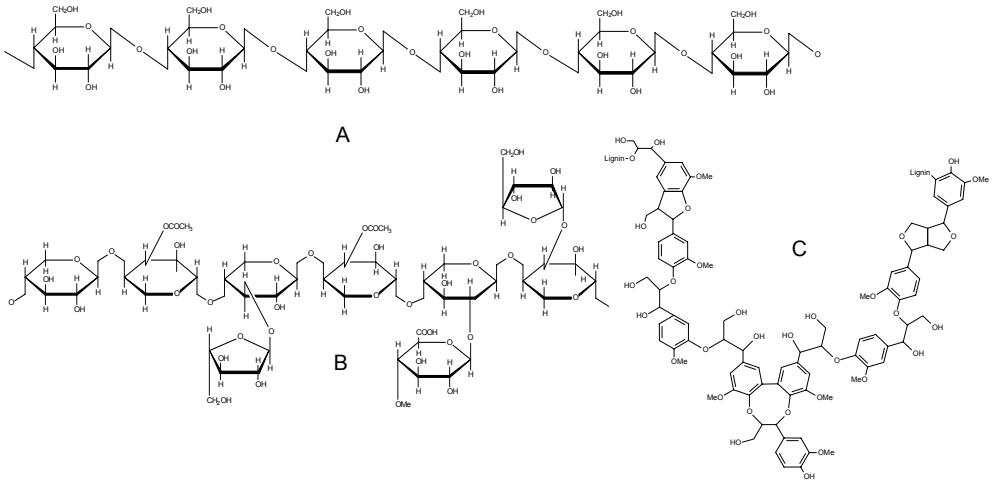

Figure 3. Structural composition of cellulose (*A*), hemicellulose (*B*) and lignin (*C*).

PRETREATMENT

In general, a pretreatment step aims to remove or modify lignin and hemicellulose, reduce cellulose cristallinity and increase the porosity of the lignocellulosic biomass (Fig. 4). Pretreatments can be classified in four groups: (1) physical; (2) chemical; (3) biological; and (4) combinations of these treatments.

Physical pretreatment

The goal of a physical treatment is to reduce particle size leading to easier handling of material during the subsequent processing steps, to enlarge the fiber surfaces and to reduce cellulose cristallinity. Physical pretreatments include comminution (chipping, grinding and milling), irridation, steam explosion and hydrothermolysis (Sun and Cheng, 2002; Mosier et al., 2005).

Chemical pretreatment

Chemical treatments can be defined as techniques involving chemical agents such as acid, alkaline, organic solvent, ammonia, SO_4, CO_2 or pH-controlled hydrothermolysis. A chemical treatment aims to increase the surface of the substrate by swelling of fibers and to modify or remove the hemicellulose and/or lignin in order to make the cellulose more accessible for enzymatic hydrolysis (Hsu, 1996; Sun and Cheng, 2002; Mosier et al., 2005). The alkaline extraction with lime ($Ca(OH)_2$) is a potential method showing high yields of fermentable sugars with low formation of inhibitors. Furthermore, lime at mild temperature also is a promising agent due to its low costs and safety (Kaar and Holtzapple, 2000; Chang et al., 2001). Different lime concentrations and reaction conditions have been studied with wheat straw, bagasse and corn stover as feedstocks (Chang et al., 1998; Kim and Holtzapple, 2005). In this thesis, we focus on mild-temperature (85°C) alkaline pre-treatment with $Ca(OH)_2$ as chemical agent (Chapter 2).

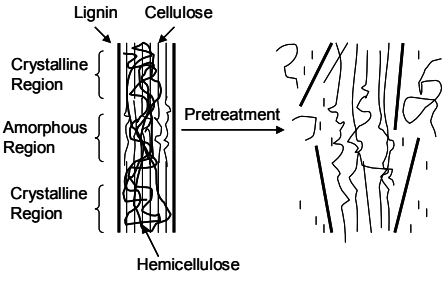

Figure 4. Effect of pretreatment on lignocellulose material (Mosier et al., 2005).

TABLE 5. Effect of various pretreatments on lignocellulosic biomass (Reith et al., 2002).

	Yield of fermentable sugars	Inhibitor production	Recycling of chemicals	Waste production	Investment costs
Weak acid	++	--	--	-	+/-
Strong acid	++	--	--	-	-
Steam explosion	+	--	++	+	-
Organosolv	++	++	--	+	--
Wet oxidation	+/-	+	++	+	+
Mechanical methods	-	++	++	++	+
Alkaline extraction	++/+	++	--	-	++
Carbonic acid	++	++	++	++	+

'+' Positive characteristic; '-' Negative characteristic; A '+' score implies: 'high yield of fermentable sugars', 'no or low inhibitor production', 'no requirement for chemicals recycling', no or low waste production' and 'low investment costs'.

Biological pretreatment

Biological treatments involve the use of lignin-degrading micro-organisms for enhancing enzymatic hydrolysis of cellulose. These treatments require no chemicals and a relatively low energy input, but, are slow processes and most lignin-solubilizing micro-organisms utilize also (hemi-)cellulose (Sun and Cheng, 2002) resulting in low yields of cellulose.

Combination pretreatments

The combination pretreatments consist of two or more pretreatment steps such as the Ammonia Fiber EXplosion (AFEX) where lignocellulose is exposed to liquid ammonia at high temperature (*e.g.* 200°C) and high pressure (*e.g.* 3 MPa) for a certain time followed by quick reduction of pressure causing the biomass to 'explode'. In general, the combination pretreatments are more effective than a single method, however, require often additional equipment and chemicals which will add costs (Hsu, 1996).

As shown in Table 5 pretreatments have variable modes of action on the lignocellulose. Several criteria can be listed determining an effective pretreatment: (1) improve the formation of sugars or the ability to subsequently form sugars by the enzymatic hydrolysis; (2) preserve the carbohydrate fraction; (3) limit the formation of degradation by-products inhibitory to the subsequent hydrolysis and fermentation; (4) minimize requirement of chemicals recycling; (5) low or zero waste production (*e.g.* gypsum); and (6) be cost-effective (Reith et al., 2002; Sun and Cheng, 2002; Mosier et al., 2005).

Inhibitor formation

Compounds inhibiting microbial activity can be categorized in two groups: (1) inhibitors originally present in the biomass; (2) inhibitors produced due to too severe pretreatment conditions. The inhibitors - present in lignocellulosic biomass - are liberated relatively easy during the pretreatment step. For instance, under relatively mild conditions, acetylated hemicellulose is hydrolyzed and the sugars xylose, arabinose, mannose, glucose, galactose and the organic acid acetic acid, a known inhibitor, are liberated.

The second group of inhibitors can be produced under too extreme pretreatment conditions. For instance, treatments involving too severe acidic conditions combined with high temperatures might lead to the formation of a range of compounds such as sugar degradation products, lignin degradation products and organic acids. Xylose degradation results into furfural whereas hexose sugars are degraded to 5-hydroxymethyl furfural (HMF) which are known inhibitors (Mussatto and Roberto, 2004). When these components are further degraded, formic acid and levulinic acid can be formed. Phenolic compounds such as 4-hydroxybenzoic acid, vanillin, syringaldehyde and catechol can be formed by partial breakdown of lignin (Palmqvist and Hahn-Hägerdal, 2000). The inhibitory effects of these compounds have been studied for the conversion of lignocellulosic hydrolysates to ethanol by yeast and bacteria (Klinke et al., 2003; Klinke et al., 2004).

A variety of biological, physical and chemical detoxification methods (*e.g.* precipitation, active charcoal, vacuum evaporation) can be applied in order to reduce the concentration of inhibitors prior to enzymatic hydrolysis and fermentation (Mussatto and Roberto, 2004). Another approach to avoid the formation of inhibitors is by a pretreatment consisting of a two-stage process. Throughout the first step of the process, the relatively easy hydrolysable hemicellulose is released under mild conditions followed by a second step where cellulose is treated under more severe conditions. By removing pentose and hexose sugars released from the hemicellulose prior to cellulose hydrolysis, no degradation products can be formed from these monosaccharides (Galbe and Zacchi, 2002).

ENZYMATIC HYDROLYSIS

The hydrolysis of polysaccharide material into fermentable sugars can be accomplished by physical/chemical (acid-based technology reviewed by (Taherzadeh and Karimi, 2007)) and enzymatic treatments. Because of the scope of this study, we focus on the hydrolysis of polymeric material by enzymes. The polysaccharides present in pretreated lignocellulose can be hydrolysed by enzymes which are produced by (an)aerobic and mesophilic or thermophilic bacteria and fungi. Bacteria belonging to the genera *Clostridium, Cellulomonas, Bacillus, Thermomonospora, Ruminococcus, Bacteriodes, Erwinia, Acetovibrio, Microbispora,* and *Streptomyces* and fungal species including members of the genera *Trichoderma*, *Aspergillus, Schizophyllum* and *Penicillum* are known producers of cellulases. Although bacteria produce cellulases with high specifity, they do not produce high enzyme concentrations (Galbe and Zacchi, 2002). Of all fungal species *Trichoderma reesei* is by far the most efficient cellulase-hydrolysing organism. Enzyme preparations produced by these organisms usually consist of a mixture of enzymes (for an overview see (Rabinovich et al., 2002)). Three main groups of cellulases can be defined: (A) endoglucanase which attacks randomly regions of low cristallinity cellulose; (B) exoglucanase or cellobiohydrolase releases cellobiose from the reducing ends or the non-reducing ends; and (C) β-glucosidase or cellobiase which hydrolyzes cellobiose to glucose units (Sun and Cheng, 2002). The enzymatic hydrolysis of cellulose is a complex process involving different combined enzymatic actions (Fig. 5). The synergetic effect of these enzymes results in more end-product glucose than each could produce separately (Galbe and Zacchi, 2002). The hydrolysis of hemicellulose (*e.g.* xylan) requires a large number of enzymes (for an overview see (Shallom and Shoham, 2003)). First, the substituents (branched sugars and acetyl groups) are chemically or enzymatically removed following by an efficient hydrolysis of the xylan backbone by endo-xylanases. Enzymes such as xylanase, β-xylosidase, arabinase, glucanase, mannase, glucuronidase, galactomannanase, glucomannanase and acetyl esterase are involved in the hydrolysis of hemicellulose (Brigham et al., 1996). Approximately 25 types of hemicellulolytic activity have been characterised so far.

Optimal reaction conditions for enzymatic hydrolytic activity are often temperatures around 50±5°C and a pH between 4.0 and 5.0. In laboratory studies, a frequently used cellulase dosage is 10 FPU/g cellulose because it provides a high yield of glucose in 48-72 hours at reasonable enzyme cost (Sun and Cheng, 2002).

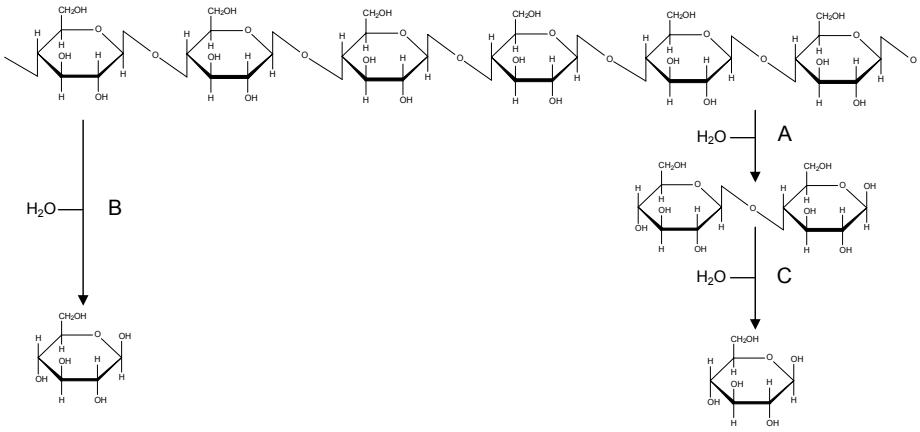

Figure 5. Schematic representation of enzymatic hydrolysis of cellulose to cellobiose by endoglucanase (*A*) and to glucose by exoglucanase (*B*). Cellobiose can be hydrolysed to glucose by β-glucosidase (*C*).

We have studied and modelled the enzymatic hydrolysis of pure cellulose (Avicel) with commercial available enzyme preparations (Drissen et al., 2007). Results from this research showed that the enzymatic hydrolysis of cellulose is influenced by: (1) the heterogeneity of cellulose (recalcitrance); (2) substrate limitation; (3) adsorption of enzymes onto substrate; (4) temperature dependency of reaction rates; (5) temperature inactivation; and (6) end-product inhibition.

In case of pretreated lignocellulosic substrates, the enzymatic hydrolysis of polymeric sugars is complex. The hydrolysis of cellulose in these complex matrices can be influenced by the presence of hemicellulose which covers the cellulose. Furthermore, it is reported that lignin can irreversible interact with proteins and causes deactivation of enzymes (Yang and Wyman, 2006). As the feedstock and enzymes represent a significant part of the total process costs, rapid and efficient conversion of all polysaccharides to fermentable sugars is required for an economical feasible process. Then, the lignocellulosic hydrolysates contain typically a mixture of pentose and hexose monosaccharides of mainly glucose and xylose, and small amounts of arabinose, mannose and galactose (Table 4). For a commercial lignocellulose-to-product bioconversion process, all monosaccharides, including the pentoses, need to be fermented efficiently (Hinman et al., 1989).

MICROBIAL FERMENTATION

A large variety of micro-organisms such as bacteria, yeast and fungi can convert lignocellulose derived sugars via (an)aerobic fermentation. In comparison to chemical processes producing fuels and chemicals from fossil fuel sources, microbial processes have several advantages: (1) the micro-organisms can use renewable carbohydrate feedstocks; (2) many complex reactions occur simultaneously in one reactor; and (3) compounds such as lactic acid are generally stereo-specific.
In this thesis, two microbial fermentative routes have been studied: (1) the production of ethanol; and (2) the formation of lactic acid.

Ethanol production

An ideal micro-organism for the biomass-to-ethanol conversion meets the following traits; (1) high ethanol yield; (2) high ethanol tolerance; (3) resistant to compounds present in hydrolysates; (4) no oxygen requirement; (5) low fermentation pH; and (6) broad substrate utilization range (Picataggio and Zhang, 1996). Many of these traits are present in bakers' yeast *Saccharomyces cerevisiae* which is therefore the most frequently used micro-organism for industrial ethanol production processes. *S. cerevisiae* produces mainly ethanol (85-90% of theoretical maximum) with minor by-product formation (glycerol and acetate), has a rapid fermentation (productivities of around 40 g ethanol/l/h with continuous in-situ product removal (Groot et al., 1992)), can ferment under acidic conditions and is resistant to lignocellulose hydrolysates (Olsson and Hahn-Hägerdal, 1993; Picataggio and Zhang, 1996). Furthermore, this micro-organism is tolerant to high sugar (up to 40%) and ethanol concentrations (up to 18%).

Under aerobic and carbon-limiting conditions, *S. cerevisiae* oxidizes glucose to CO_2 and H_2O yielding energy efficiently (28 ATP per hexose) and can be used for biosynthesis (growth). Under aerobic or oxygen limiting conditions combined with high sugar concentration, *S. cerevisiae* respires and ferments glucose simultaneously and is therefore so-called Crabtree positive, meaning alcoholic fermentation under aerobic conditions. In the absence of O_2, the yeast only ferments glucose to ethanol. As shown in Figure 6, *S. cerevisiae* converts glucose through the glycolysis pathway into pyruvate yielding two ATP per hexose. Then, pyruvate is decarboxylated to acetaldehyde accompanied with the release of CO_2. Acetaldehyde is converted to ethanol. Ethanol synthesis from glucose is a redox neutral conversion. Biomass production from glucose, however, results in the formation of surplus NADH. This is counterbalanced by the reductive conversion of glucose into glycerol.

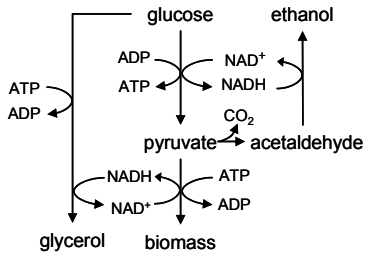

Figure 6. Glucose metabolism of *S. cerevisiae* under anaerobic conditions to ethanol, glycerol and biomass.

A major disadvantage of wild-type *S. cerevisiae* is the narrow substrate range, which cannot ferment pentoses such as xylose because of the lack of enzymes converting xylose to xylulose. Yet, the wild-type *S. cerevisiae* ferments xylulose (isomer of xylose) into ethanol. Therefore, an alternative approach called Simultaneous Fermentation and Isomeration of Xylose (SFIX) couples the use of xylose isomerase in order to convert xylose into xylulose and simultaneous fermentation of xylulose (Picataggio and Zhang, 1996). Because of the high costs of commercial xylose isomerase, instability of the enzyme, different optimal conditions of fermentation and enzymatic conversion, the SFIX process is limited in its application.

The bacterium *Zymomonas mobilis* meets many of the essential traits such as high ethanol yield and tolerance, low pH optimum and is robust in lignocellulose hydrolysates, however, it does not naturally ferment pentose sugars such as xylose (Lin and Tanaka, 2006). Alternatively, other micro-organisms having a broader range of sugar utilization are preferred. Several studies/screenings have been performed identifying other strains meeting the characteristics of ideal ethanol producers. Nevertheless, wild-type micro-organisms fermenting pentose sugars were often lacking one of the other traits as described before. Yeast species such as *Candida shehatae*, *Pachysolen tannophilus* and *Pichia stipitis* are capable to convert xylose to ethanol (Nigam, 2001), however, they require oxygen and show a lower ethanol production rate and a lower ethanol tolerance in comparison to *S. cerevisiae* (Claassen et al., 1999).

Two metabolic routes of xylose-to-xylulose conversion have been described (Fig. 7). First, the bacterial route via a one-step reaction with xylose isomerase. Secondly, the fungal route via a two-step pathway where xylose is first reduced by a NADPH-dependent xylose reductase to xylitol and which is subsequently oxidized to xylulose by a NAD-dependent xylitol dehydrogenase. Regeneration of these cofactors via other metabolic reactions is a necessity to avoid cofactor imbalance. Since no significant transhydrogenation from NADH to $NADP^+$ is likely to occur in most fungal species, the organism needs other ways to reduce $NADP^+$ and oxidize NADH.

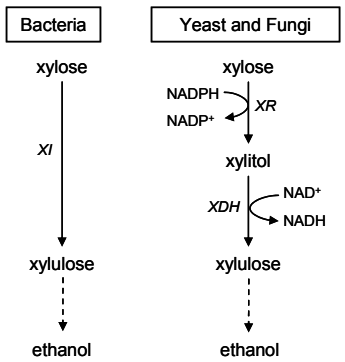

Figure 7. Bacteria and Yeast/Fungi generally employ different pathways for xylose conversion. *XI*: xylose isomerase, *XR*: xylose reductase and *XDH*: xylitol dehydrogenase.

As shown in Figure 8, the cofactor $NADP^+$ involved in the first step of the xylose-to-xylulose conversion can be regenerated via the pentose phosphate pathway. Next, the cofactor NADH can be oxidized via aerobic respiration. Hence, oxygen availability plays an important role in regeneration of the cofactor NAD necessary for the conversion of xylitol to xylulose (Fig. 8) (Hahn-Hägerdal et al., 1994). Xylulose is subsequently phosphorylated and then metabolized to ethanol via the pentose phosphate pathway and glycolysis (Jeffries, 1983). Yet, the cofactor dependent enzymes in the xylose-to-xylulose pathway limit the efficiency by which these yeasts convert xylose to ethanol. Some exceptions have been found where eukaryotic micro-organisms use the xylose isomerase route to convert xylose into xylulose and subsequently to ethanol (Schneider, 1989; Banerjee et al., 1994; Harhangi et al., 2003).

Another approach to obtain an ideal ethanol producing micro-organism is via metabolic engineering (Hahn-Hägerdal et al., 2007). Genes encoding enzymes playing a role in the xylose-to-ethanol pathway have been transferred successfully into ethanol-producing micro-organisms such as *S. cerevisiae*, *Z. mobilis* and *Escherichia coli*. Recently, the gene encoding xylose isomerase has been transferred from the fungus *Piromyces* sp. in the wild-type *S. cerevisiae* resulting in a recombinant strain capable of converting xylose into ethanol (Kuyper et al., 2004).

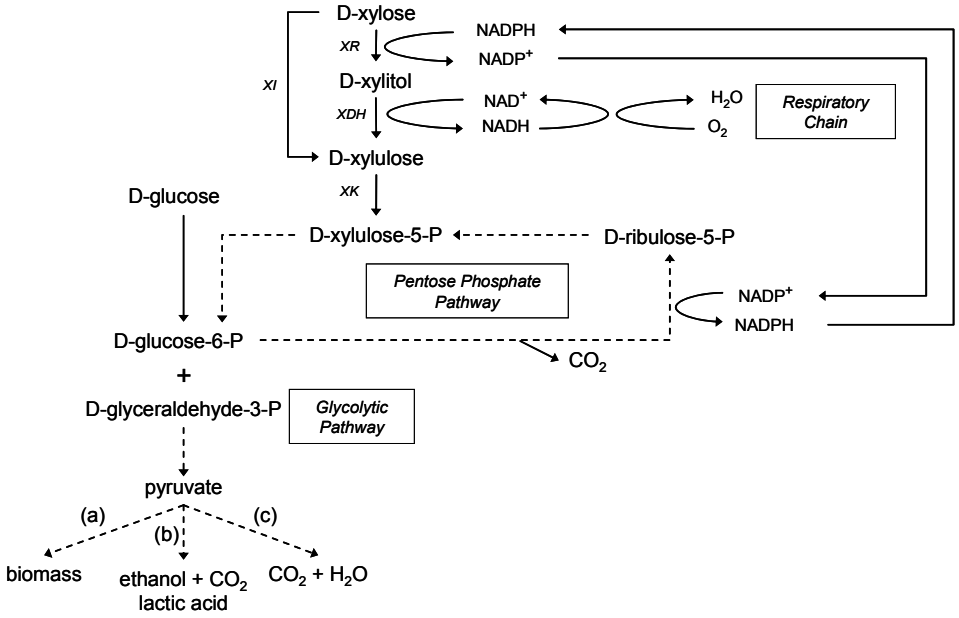

Figure 8. Pentose and hexose metabolism. The bacterial D-xylose conversion pathway via xylose isomerase (*XI*) and fungal route via xylose reductase (*XR*) and xylitol dehydrogenase (*XDH*). D-Xylulose is phosphorylated by xylulose kinase (*XK*) to xylulose-5-P and further metabolized via the pentose phosphate pathway followed by the glycolysis to pyruvate which can function as substrate for (a) growth, (b) fermentation or (c) respiration. The majority of the glucose is converted through the glycolytic pathway whereas a minor part enters the pentose phosphate pathway. Adapted partly from (Schneider, 1989).

Lactic acid production

Lactic acid is the most widely naturally occurring hydroxyl-carboxylic acid and can be produced by various bacterial and fungal species via homo-lactic or hetero-lactic routes. Homo-lactic (or homo-fermentative) can be defined as production of solely lactic acid from C6-sugar (*e.g.* glucose) or C5-sugar (*e.g.* xylose) with a theoretical maximal yield of 1 g/g (Fig. 9, Equation 1 and 2). Next, hetero-lactic or hetero-fermentative production results besides lactic acid in a two-carbon component such as acetic acid or ethanol (and CO_2) (Fig. 9, Equation 3 and 4). The molecule lactic acid can occur in two stereo-isomeric forms: D(+) and L(-). The stereospecifity of lactate dehydrogenase and the presence or absence of lactate racemase determines whether D-, L- or DL-lactate is formed.

Current commercially industrial processes for the biological conversion of glucose to lactic acid are carried out by homolactic lactobacilli. Despite of high lactic acid yields and high productivities with glucose as sole carbon source, most of these species are hetero-lactic when using pentose sugars as substrate. Lactic acid bacteria ferment pentoses at least partly via the phosphoketolase pathway splitting C5-sugars into a C2-compound (acetic

acid or ethanol) and a C3-compound (lactic acid) as shown by Equation 4. Yet, some exceptions have been reported who ferment xylose homolactically (Garde et al., 2002; Tanaka et al., 2002). Nevertheless, these bacteria require complex supplements in their growth medium which adds to the costs of lactic acid production and complicates purification of lactic acid.

Homo-fermentative:	$1\ C_6H_{12}O_6 \rightarrow 2\ C_3H_6O_3$ glucose lactic acid	Eq. 1
	$3\ C_5H_{10}O_5 \rightarrow 5\ C_3H_6O_3$ xylose lactic acid	Eq. 2
Hetero-fermentative:	$1\ C_6H_{12}O_6 \rightarrow 1\ C_3H_6O_3 + 1\ C_2H_5OH + 1\ CO_2$ (or $C_2H_4O_2$) glucose lactic acid ethanol acetic acid	Eq. 3
	$1\ C_5H_{10}O_5 \rightarrow 1\ C_3H_6O_3 + 1\ C_2H_4O_2$ xylose lactic acid acetic acid	Eq. 4

Figure 9. Homo- and hetero-fermentative lactic acid production from glucose and xylose.

Another group of well-known organic acid producers are the filamentous fungal species belonging to the genera *Rhizopus* (Magnuson and Lasure, 2004). Especially, the fungus *Rhizopus oryzae* can produce organic acids such as lactic acid (Soccol et al., 1994; Wright et al., 1996; Longacre et al., 1997; Skory et al., 1998), fumaric acid (Zhou et al., 2002) and ethanol (Millati et al., 2005). In general, two groups of *R. oryzae* strains can be identified: the fumaric acid-producing strains and the lactic acid-producing strains (Skory and Ibrahim, 2007). The lactic acid-producing *R. oryzae* strains convert hexose sugars homo-fermentatively to optically pure lactic acid and require mineral media composition with some inorganic minerals and ammonium salt as nitrogen source. The filamentous fungus is also capable to produce and secrete hydrolytic enzymes involved in the breakdown of complex polymers in biomass (Bakir et al., 2001; Murashima et al., 2002; Saito et al., 2003) and is known as Direct Microbial Conversion (DMC). A disadvantage of fungi is that they require oxygen for growth resulting in a lower lactic acid yield and lower productivity which is limited by oxygen transfer. Furthermore, *R. oryzae* cultures are morphologically complex. They can grow in submerged cultures as extended filamentous form, mycelial mats, mycelial pellets or clumps which are affected by various chemical and physical conditions and can have significant effect on the product formation (Bai et al., 2003; Papagianni, 2004).

A third group, the bacteria belonging to the genera *Bacillus* sp. such as *B. coagulans* are facultative anaerobic micro-organisms and known to produce chirally pure lactic acid with high efficiencies. An advantage of this kind of organisms is that they can grow at thermophilic conditions, especially when combining the fermentation with enzymatic

hydrolysis at temperatures around 50°C. *Bacillus* sp. is capable to ferment saccharides homolactically where pentoses are presumable metabolized via the transaldolase/transketolase pathway (Patel et al., 2004). In this thesis, we studied the potential of converting lignocellulose-derived carbohydrates into lactic acid by the fungus *R. oryzae* (Chapter 3 and 4) and *B. coagulans* (Chapter 7).

The down stream processing (DSP) of organic acids such as lactic acid is complicated and involves several recovery and purification steps. Traditional methods are liquid/solid separation to remove solids (*e.g.* microbial biomass), acidification to liberate acid and removal of salts from the lactic acid solution followed by a concentration step. Alternatives are the more advanced methods such as adsorption, membrane separations and ion exchange (Vaidya et al., 2005).

PROCESS INTEGRATION OF LIGNOCELLULOSE-TO-PRODUCT CONVERSION

A conventional lignocellulose-to-product conversion process can be carried out by performing the enzymatic hydrolysis and microbial fermentation separately (SHF) at their respective optimal reaction conditions (*e.g.* pH and temperature) (Fig. 10). However, the intermediate cellulose hydrolysis product, cellobiose inhibits cellulase activity at relatively low concentrations (K_i 5.85 g/l) (Philippidis et al., 1993). An even stronger inhibition is caused by the end-product glucose which inhibits β-glucosidase at very low concentration (K_i 0.62 g/l). Attempts have been made minimizing inhibition by the addition of high enzyme concentrations, supplementation of β-glucosidase and sugar removal during hydrolysis by ultrafiltration (Sun and Cheng, 2002). A successful approach to avoid inhibition on the hydrolytic system is by fermenting glucose, as soon as it appears in the medium, into product (*e.g.* ethanol K_i 50.35 g/l) and is known as Simultaneous Saccharification and Fermentation (SSF) (Fig. 10). Often, prior to the SSF processes, a short pre-hydrolysis phase or liquefaction phase (few hours) is introduced where the high-viscosity polysaccharide material is hydrolyzed to low-viscosity material resulting in easier pumpable slurry.

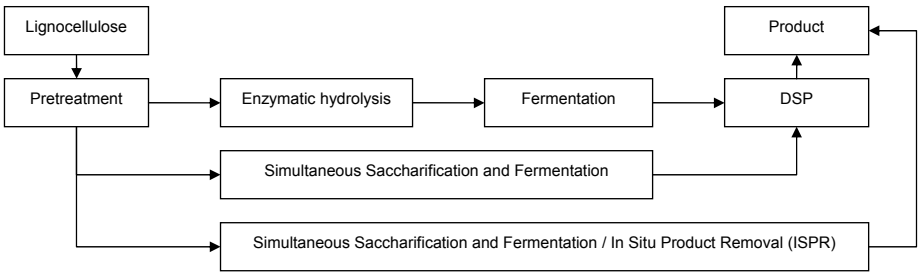

Figure 10. Schematic overview of lignocellulose-to-product process including the SHF and SSF approach.

In comparison to the two-stage SHF process, the SSF has some advantages: (1) continuous removal of sugars that potentially inhibit hydrolytic activity; (2) lower enzyme concentration; (3) higher product yields; (4) since substrate (*e.g.* glucose) is removed as soon as it appears, there is minor risk for microbial contamination; and (5) because both reactions occur in the same reactor less reactor volume is required. Yet, some disadvantages of SSF can be listed: (1) hydrolysis and fermentation occur at compromising sub-optimal conditions (*e.g.* temperature and pH); (2) introduction of inhibition of cellulases caused by fermentation products such as ethanol and lactic acid, however, these are lower than for glucose (Takagi, 1984; Philippidis et al., 1993); and (3) it is difficult to recycle the microbial biomass since the microbes are mixed together with the insoluble non-hydrolysable/non-fermentable fraction (*e.g.* lignin) (Galbe and Zacchi, 2002). Both the SSF method for the conversion of lignocellulose to ethanol (Spindler et al., 1991; Wyman et al., 1992; Bollók et al., 2000; Kádár et al., 2004) and the conversion of starch and cellulose to lactic acid have been described previously (Abe and Takagi, 1991; Anuradha et al., 1999; Sreenath et al., 2001).

The recovery and purification of ethanol from the lignocellulose-to-ethanol process often occurs via distillation, rectification and dehydration methods (Reith et al., 2002). In situ product removal (ISPR) such as gas stripping or cell retention (filtration) can be a suitable technique to minimize the product inhibition on hydrolytic and microbial activity (Fig. 10). The non-fermentable residues (mainly lignin) can be used as fuel for thermal conversion in a Combined Heat and Power (CHP) system providing the total steam and electricity requirement for the production process and an electricity surplus for export to the grid (Reith et al., 2002) and is further described in Chapter 6.

OUTLINE OF THIS THESIS

The research presented in this thesis was performed in a four-year project financed by the Economy, Ecology and Technology (EET) Program of the Dutch Ministries of Economic Affairs, Education, Culture and Science and of Housing, Spatial Planning and the Environment. Within this project, the Wageningen University Department of Agrotechnology and Food Sciences Group (AFSG) collaborated extensively with the industries Purac biochem, Royal Nedalco and Shell Global Solutions International and the R&D institutes ECN, TNO Environment, Energy and Process Innovation and TNO Nutrition and Food Research. The research described in this thesis is aimed to convert lignocellulose via physical pretreatment, chemical pretreatment and enzymatic hydrolysis into fermentable sugars. The fermentable sugars function as carbon source for microbial fermentation to ethanol by the yeast *Saccharomyces cerevisiae* and lactic acid by the fungus *Rhizopus oryzae* and the bacterium *Bacillus coagulans*. The outline of this thesis and the coherence between the chapters is schematically depicted in Figure 11.

In *Chapter 2*, the potential of mild-temperature alkaline (lime) treatment on wheat straw is described. The effects of the different pretreatment conditions are validated by enzymatic hydrolysis experiments and ethanol fermentation experiments by *S. cerevisiae* with regard to presence of inhibitors. The determined optimal reaction conditions of the alkaline treatment were standardized and used for the production of lignocellulosic hydrolysates in further studies.

Chapters 3 and 4 describe the conversion of the pentose sugar xylose, abundantly present in lignocellulose hydrolysates, to lactic acid by the filamentous fungus *R. oryzae*. In *Chapter 3*, ten *R. oryzae* strains are screened on their ability to convert xylose to lactic acid. Fermentation characteristics such as lactic acid productivity and yield with glucose and xylose as sole carbon source were determined and compared. Furthermore, artificial media with mixed sugars (glucose and xylose), variable sugar concentrations and sugars from lime-treated lignocellulosic hydrolysates were examined for lactic acid production. In *Chapter 4*, the differences in lactic acid production between glucose and xylose are discussed. In addition, the effect of presence of oxygen and of nutrients, essential for respiration and growth, respectively, on the conversion of glucose and xylose and product formation by *R. oryzae* are studied in more detail.

In *Chapter 5*, the conversion of lime-treated wheat straw to ethanol by *S. cerevisiae* is reported via two optional routes; in Separate Hydrolysis and Fermentation (SHF) and in Simultaneous Saccharification and Fermentation (SSF). Process characteristics of both routes like hydrolysis rate, ethanol production rate, and yield of sugar and ethanol were compared at lab-scale experiments. With the optimized process conditions, the SSF lignocellulose-to-ethanol process was further up-scaled to pilot-scale (100 l) which is

described in *Chapter 6*. In this chapter several aspects of the lignocellulose-to-ethanol process are studied: (1) the SSF performance at pilot-scale; (2) the quality of the produced ethanol for transportation fuel purposes; (3) the conversion of the residual soluble organic fraction for anaerobic digestion to biogas; (4) the thermal conversion of the solid distillate fraction (*e.g.* lignin) by combustion yielding energy; and (5) the composition of the inorganic ash after thermal conversion.

In *Chapter 7* the development of an integrated process at pilot-scale (20 l) is described where alkaline pretreated wheat straw is used to: (1) provide a carbon substrate for lactic acid production; and (2) to neutralise the lactic acid produced by *B. coagulans* in a simultaneous saccharification and fermentation process.

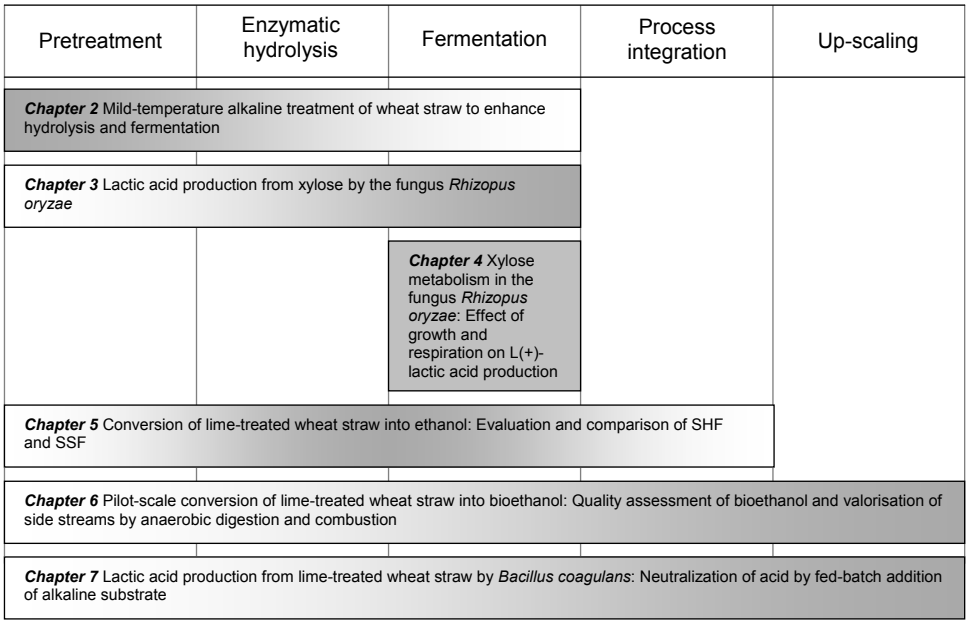

Figure 11. Overview of the thesis outline and the coherence between the chapters. The central issue of the respective chapter is colored dark.

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CHAPTER 2

Mild-Temperature Alkaline Pretreatment of Wheat Straw to Enhance Hydrolysis and Fermentation

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ABSTRACT

In this paper, mild-temperature lime ($Ca(OH)_2$) pretreatment of lignocellulosic biomass for enzymatic hydrolysis and fermentation purposes was investigated. Lime pretreatment has gained interest because of perceived advantages compared to other pretreatment techniques, including minor formation of fermentation inhibitors. Wheat straw was pretreated with $Ca(OH)_2$ at variable water-biomass ratios and alkali loading rates, followed by enzymatic hydrolysis. Solid and liquid analysis indicates that lime pretreatment at mild temperatures does not lead to significant solubilisation of lignin. Enzymatic hydrolysis under optimal conditions shows that lime pretreatment results in hydrolysis of 93.0% of glucan and 81.4% of xylan to monomeric sugars, a three to four fold improvement when compared to water treatment alone. At enzymatic hydrolysis conditions that would more resemble industrial applications, monomeric sugar yields were generally lower, providing evidence of enzyme-substrate inhibition. Fermentation tests of unwashed hydrolysates showed good fermentability to ethanol under standard pH conditions (5.0), however ethanol fermentation was significantly affected by acetic acid released from the substrate, in particular at lower pH or at higher biomass loading rates. Removal of acetic acid from the substrate led to improvements in fermentability.

INTRODUCTION

Due to limited oil reserves, rising oil prices and concerns for global climate change the interest in conversion of lignocellulosic biomass into renewable fuels and bulk chemicals by fermentation is increasing (Claassen et al., 1999). Lignocellulose consists primarily of cellulose, a polymer build up of solely hexose sugars and hemicellulose, a heterogenous polymer build up of pentose or hexose sugars, which are embedded in a matrix of the phenolic polymer lignin. The main pathway to derive fermentable sugars from lignocellulose is through hydrolysis by cellulolytic and hemicellulolytic enzymes. As most cellulose and hemicellulose present in lignocellulosic biomass is not readily accessible for these enzymes, a pretreatment is required to alter the structure of lignocellulosic substrates in order to enable efficient and cost-effective enzymatic hydrolysis. Pretreatment is considered by many as the most costly step in lignocellulosic biomass conversion (Mosier et al., 2005), which can significantly affect costs of subsequent processes (Wyman et al., 2005), including enzymatic hydrolysis (e.g. enzyme selection, enzyme loading), fermentation (e.g. toxicity of pretreated substrates) as well as down-stream processes (e.g. product purification, residue generation and disposal). Therefore, research towards the development of pretreatment technologies should not only be focused on reducing costs of the pretreatment process itself, but also on how pretreatment affects other process steps, in order to facilitate the design of full-scale biological conversion systems for lignocellulose.

While so far no pretreatment technology has been implemented at the industrial scale, a variety of pretreatment methods have been studied and some have been developed at the pilot scale. Mosier et al. (2005) characterized a number of methods that have potential for cost-effective pretreatment of lignocellulose, including steam explosion, liquid hot water, dilute acid, lime, and ammonia pretreatments. Pretreatment methods using organic solvents have been evaluated as well (Lynd et al., 2002). These pretreatment methods can be characterized by their physical and/or chemical nature used to break down the lignocellulosic biomass structure, and the use of chemical additives, generally acids or bases, to promote the pretreatment process. In addition, pretreatment methods are carried out under quite diverse process conditions including pressure, temperature, reaction time, as well as solids loading rate (Sun and Cheng, 2002; Wyman et al., 2005). Given this diverse nature, pretreatment may have distinctly different effects on the chemical and structural composition of lignocellulosic biomass, including cellulose depolymerization, hemicellulose solubilisation, lignin solubilisation, or lignin redistribution (i.e. alteration of lignin properties without significant solubilisation and removal; Lynd et al., 2002). All these effects are thought to be responsible for increasing accessibility to enzymes, and the various pretreatment processes have been shown to produce pretreated lignocellulose from which, under optimal reaction conditions, nearly all cellulose and hemicellulose can be

hydrolysed by cellulolytic and hemicellulolytic enzymes. Depending on the feedstock composition and the pretreatment process conditions however, pretreatment may also lead to the formation of sugar degradation products such as furfural and 5-hydroxymethylfurfural (5-HMF), lignin degradation products like phenolic compounds, and organic acids, which all are known inhibitors of microbial activity throughout fermentation processes (Olsson and Hahn-Hägerdahl, 1996). Furthermore, the presence of these compounds will likely incur detoxification steps accompanied with additional costs for further up- or downstream processes.

As part of a larger, integrated research programme investigating lignocellulosic biomass conversion, we selected lime ($Ca(OH)_2$) pretreatment as a model for further study. Lime pretreatment has gained industrial interest because of its perceived advantages over other pretreatment methods, including use of a low-cost chemical (lime) that is already in use in many agriculture-based crop processing schemes (e.g. sugar refining), lower reactor investment costs, as well as limited potential for degradation products formation. The aim of our study was to gain more insights in the effects of lime pretreatment on subsequent processes, including enzymatic hydrolysis, and fermentation into ethanol and lactic acid, resulting in facilitating further process development and economic process analysis. While several researchers have demonstrated the potential of lime pretreatment to enhance enzymatic hydrolysis (Chang et al., 1998; Kaar and Holtzapple, 2000; Kim and Holtzapple, 2005) as well as ethanol fermentation (Chang et al., 2001), so far only a limited number of results have been presented on the effects of lime pretreatment on biomass composition, and ethanol fermentability. In addition, most studies reported on employed enzymatic hydrolysis and fermentation conditions that are not likely to be relevant at the industrial scale, including high enzyme loading rates, low dry matter loading rates and high fermentation pH. Therefore, the specific goals of the present study were to determine effects of lime pretreatment on biomass composition, and to evaluate enzymatic hydrolysis and ethanol fermentability of lime-pretreated lignocellulose under a wider range of process conditions. Results of lactic acid fermentation of lime-pretreated substrates have been presented in a separate paper (Maas et al., 2006).

MATERIALS AND METHODS

Feedstock selection and mechanical pretreatment

Wheat straw was used as a feedstock for all the experimental work described in this study. The straw was collected by conventional harvest equipment from a commercial farmer's field in northeast Groningen province (The Netherlands) and packed by conventional baling equipment in 25 kg bales, with straw having an average dry matter

content of 91% (wet weight basis). Baled straw was then stored in a storage facility at room temperature and ambient relative humidity. Prior to alkaline pretreatment, baled wheat straw was milled with a bench-type knife mill (Pallmann type PS 3-5), equipped with a screen of 15mm×15mm square opening. Throughput of the mill was 169 kg/h on dry matter basis. For some of the pretreatment methods described in this report, batches of milled straw were further comminuted by grinding the straw in a laboratory wileymill (Retsch type SM 200) that was equipped with a 2mm screen.

Alkaline pretreatment

Alkaline pretreatment of the feedstock was done at bench scale and consisted of treating batches of 200-300 g milled wheat straw (2mm) in a 2 l laboratory pulp mixer (Quantum Mark V reactor) which was equipped with a variable-speed stirrer and automatic heating control. Straw was treated batch-wise at variable alkali:biomass ratios in demineralized water, and at dry matter concentrations of 10% up to 25% (*w/w*). For all batches, dry $Ca(OH)_2$ was used at loading rates varying from 0 to 0.15 g/g dry biomass. For comparison purposes, one batch was treated with NaOH (0.12 g/g). To allow for rapid mixing of all components, initial mixing was carried out at 2400 rpm for 6 intervals of 10 seconds. Pretreatment was carried out at 85°C, unless noted otherwise. During the pretreatment time and at intervals of 3 minutes, the reactor was stirred for 3 s at 600 rpm to allow for good heat transfer in the substrate. Pretreatment time varied from 16 - 20 h (overnight). The pretreatment time was selected to facilitate timely execution of further steps, such as neutralization and enzymatic hydrolysis. After completion of the alkaline pretreatment, batches were cooled to 50°C and neutralized to pH 5.0 with 20% (*v/v*) sulphuric acid. For one series of experiments, batches were cooled to 50°C and divided into three parts. Each part was adjusted to a pH of 4, 4.5 or 5 by using 20% (*v/v*) H_2SO_4. The final dry weight on straw biomass basis after addition of H_2SO_4 for this experiment series was 10.9 %, equivalent to 122 g/l biomass loading rate. Samples were stored at 4°C prior to enzymatic hydrolysis and fermentation. Samples used for lignin determination were suspended in demineralised water that was preheated to 90°C, and acetic acid was added to the suspension to achieve a neutral pH (7.0). Following neutralization, the suspension was filtered over a Buchner funnel (Whatman filter nr 595), the solids were dried at 50°C and submitted for lignin analysis.

Enzymatic hydrolysis of pretreated feedstock

Enzymatic hydrolysis was carried out both at laboratory scale and at bench scale. At laboratory scale, material from pretreated batches was collected in 50 ml PP-tubes and centrifuged. The material was washed twice with distilled water. The supernatants and wash-water were collected. Both the washed residue and the supernatant were freeze-dried

and weighed. The washed residues were submitted to enzymatic hydrolysis. Enzymatic hydrolysis took place in test tubes at 50°C in a rotary shaking device at 200 rpm. The first 5 h, the tubes were shaken head over tail manually every hour. At 0, 5, 24, 48 and 72 h samples of about 0.5 g were taken. Before enzymes were added the pH of the residues was adjusted to a pH of 4.0-5.0 and per enzyme incubation 6 ml of distilled water, 4 ml of 50 mM NaOAc (pH 5) was added to 100 mg of residue. Enzyme incubations were started by adding 100 µl of the commercial enzyme preparations Cellubrix (Novozymes) or GC 220 (Genencor) and lasted for 24h at 40°C. Cellubrix contains a cellulase activity of 56 IFPU/ml whereas GC 220 contains a cellulase activity of 116 IFPU (Kabel et al., 2006). The enzymes were inactivated by incubation for 5 min at 100°C before analysis of the formed monomers glucose and xylose. In another series, parts of 25 g of pretreated material were put in tubes without further washing and GC 220 was added at loading rates of 21.1, 42.3, 84.6 and 169.1 mg GC 220/g wheat straw (equivalent to 2.1, 4.1, 8.3 and 16.3 IFPU/g of biomass, respectively). Per enzyme dosage duplicates were made. Enzymatic hydrolysis took place at 50°C in a rotary shaking device at 200 rpm. The first 5 h, the tubes were shaken head over tail manually every hour. At 0, 5, 24, 48 and 72 h samples of about 0.5 g were taken. The enzyme activity was stopped by boiling the samples for 5 min. Samples were centrifuged and supernatants were analyzed for monomers present (glucose and xylose), and used for sugar composition analysis. To evaluate whether the residue could be further hydrolysed by adding new enzyme after the initial hydrolysis, selected samples were subjected to secondary hydrolysis or "restart hydrolysis" (Lynd et al., 2002). For this secondary incubation, pellets of samples incubated for 72 h were collected after centrifugation and washed until no monomeric glucose or xylose was detectable by HPLC. To the pellets 50 mM NaOAc buffer pH 5 was added in same amounts as liquid was removed after the first centrifugation-step. Again GC 220 was added (169.1 mg GC 220 per g dm pellet), and samples were incubated head over tail for 24 h at 50°C. The enzyme activity was stopped by boiling the samples for 5 min. Samples were then centrifuged and supernatants were analysed for monomers present (glucose and xylose), and used for sugar composition analysis.

At bench scale, enzymatic hydrolysis was carried out in the same 2l pulp reactor that was used for alkaline pretreatment, but with setting automatic preheat at 50°C and at lower rotational speed (300 rpm). Enzymatic hydrolysis was done by using GC 220 at enzyme loading rate of 152 mg/g for 24 h and at 50°C.

Fermentation into ethanol

Selected hydrolysate samples from both lab-scale and bench-scale pretreatment were used for ethanol fermentation tests with bakers' yeast *Saccharomyces cerevisiae*. Prior to fermentation, the pH of the hydrolysates were, if necessary, adjusted and conditioned by

using the method of Verduyn et al., (1990). Per 100 ml hydrolysate the following components were added: 0.5 g $(NH_4)_2SO_4$, 0.3 g KH_2PO_4, 0,05 g $MgSO_4.7H_2O$, vitamins, trace elements, fatty acids (tween 80 and ergosterol) and 0.05 ml antifoam Acepol 77 (Emerald Foam Control). Following the conditioning 0.63 g wet bakers' yeast/100 ml (approx. 0.17 g cell dry weight/100 ml) was added. All fermentations were carried out at 32°C and 300 rpm stirring speed by employing a 'Biological Activity Monitor' fermentation system (Halotec, Delft, the Netherlands). *S. cerevisiae* converts glucose in equimolar amount of ethanol and carbon dioxide. During fermentation, CO_2 production rate was monitored (and cumulative production volume), which was used as a measure of conversion rate of glucose into ethanol.

Analytical

Determination of Acid Soluble Lignin (ASL) and Acid Insoluble Lignin (AIL) was performed according to the Tappi method, where the insoluble lignin is measured by weight, and the soluble lignin was determined by spectrophotometrical determination at 205 nm (Tappi, 1999). Analysis of monomeric sugars was performed by using High-Performance Anion-ExChange (HPAEC) at pH 12. HPAEC was performed on a Thermo Separation Products system equipped with a Borate trap (Dionex), a Dionex CarboPac PA-20 column (3 mm ID×150 mm) in combination with a Dionex CarboPac PA-20 guard column (3 mm×25 mm) and PAD-detection (Dionex, Sunnyvale, CA). For the analysis of xylose and glucose in the hydrolysates, an isocratic elution (0.5 ml/min) of 20 min was carried out with a solution of 18 mM NaOH. Each elution was followed by a washing and equilibration step.

The neutral sugar composition of insoluble and soluble biomass fractions was determined by Gas Chromatography according to Englyst and Cummings (1984), using inositol as an internal standard. The samples were treated with 72% (*w/w*) H_2SO_4 (1 h, 30°C) followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100°C and the constituent sugars released were analysed as their alditol acetates. Selected supernatants from enzymatic hydrolysis at pH 4.5 were analysed by using the "neutral sugar composition"-analysis as well. With this method the total amount of (soluble) glucan and xylan was determined, giving the degradation of glucan to glucose plus glucose-oligomers and of xylan to xylose plus xylo-oligomers. The uronic acid content was determined as anhydro-uronic acid by an automated *m*-hydroxydiphenyl assay using an auto analyser (Skalar Analytical BV).

Xylan-to-xylose conversion and glucan-to-glucose conversion was calculated based on sugar concentrations in the supernatant following enzymatic hydrolysis, and neutral sugar composition of the pretreated biomass prior to enzymatic hydrolysis.

The samples withdrawn throughout fermentation were treated and analyzed on monosaccharides, organic acids, alcohols and polyols by High Pressure Liquid Chromatography as described earlier (Maas et al., 2006).

RESULTS AND DISCUSSION

Effect of $Ca(OH)_2$ pretreatment on wheat straw composition

Table 1 presents lignin and carbohydrate concentrations in the alkaline pretreated biomass, as well as the untreated and control samples (0 g/g $Ca(OH)_2$). Lime pretreatment yields an insoluble and a soluble (supernatant) fraction, with the insoluble fraction representing 85.1 to 87.4% of total dry matter for lime pretreatment, compared to 90.1% for water treatment and 63.5% for sodium hydroxide pretreatment. The lignin concentrations do not indicate significant delignification in lime-pretreated samples, even at the highest calcium hydroxide loading rate (0.15 g/g). By comparison, sodium hydroxide pretreatment leads to extensive delignification, with approximately half of the lignin removed.

TABLE 1. Effect of alkaline pretreatment on structural components: total biomass recovered, lignin and carbohydrate compositions of soluble and insoluble fractions.

			$Ca(OH)_2$				NaOH
		No treatment	0 g/g	0.075 g/g	0.10 g/g	0.15 g/g	0.12 g/g
Fraction recovered as insoluble fraction (% total d.w. biomass)		n.d.	90.8	85.1	87.4	85.6	63.5
Lignin (% total d.w. biomass)	Acid Insoluble lignin	23.5	24.0	24.4	23.5	24.2	12.0
	Acid Soluble lignin	0.5	0.5	0.3	0.2	0.3	0.7
	Total lignin[a]	24.0	24.5	24.7	23.7	24.4	12.7
Soluble fraction (% d.w. solubles)	Rhamnan	n.d.	0	0	0	0	0
	Arabinan	n.d.	3	5	5	5	3
	Xylan	n.d.	3	9	11	9	5
	Mannan	n.d.	2	1	1	2	0
	Galactan	n.d.	2	2	2	2	1
	Glucan	n.d.	9	5	3	3	3
	Uronic acids	n.d.	3	3	3	3	2
	Total carbohydrate		22	25	25	24	14
Insoluble fraction (% d.w. insolubles)	Rhamnan	0.0	0	0	0	0	0
	Arabinan	2.1	3	3	3	3	3
	Xylan	20.3	21	19	19	18	23
	Mannan	0.5	0	0	0	0	0
	Galactan	0.7	1	1	1	1	0
	Glucan	32.0	30	29	28	28	38
	Uronic acids	n.d.	4	3	3	3	3
	Total carbohydrate		59	55	54	53	67

n.d. not determined; [a] sum of acid insoluble lignin and acid soluble lignin.

The results of the lime pretreatment are in contrast with the earlier findings of Chang et al. (1997, 1998) who reported lignin solubilisation of 29% and 14% for lime-pretreated switchgrass and bagasse, respectively, while lime pretreatment was carried out at 100 to 120°C. Apparently, lime pretreatment at 85°C is mild with respect to lignin in wheat straw, even though pretreatment was carried out for 20 h. Results also show that following lime pretreatment, the soluble fraction, which represents 12.6 to 14.9% of total biomass dry matter, contains 24 to 25% carbohydrates (equivalent to 3.0 to 3.7% of total biomass) with xylose as primary component. When compared to water treatment therefore, lime pretreatment leads to an increase in xylan content in the soluble fraction, with on average 7.7% of total xylan solubilised for lime-pretreated straw (1.4% for water treatment). This increase is however moderate when compared to NaOH pretreatment, which results in 11.1% of total xylan solubilised. The carbohydrate composition of the insoluble fractions of lime pretreated straw closely resembles the carbohydrate composition of the untreated wheat straw, albeit with a slight decline in glucan and xylan content. The compositions of both soluble and insoluble fractions do not seem to be affected much by lime loading rate. Overall, lime pretreatment of straw yields 53 to 55% of carbohydrates on total dry matter basis in the insoluble fractions, with the major part being glucan (52% glucan on total carbohydrate basis) and xylan (33% on total carbohydrate basis).

In summary, lime pretreatment is shown to not significantly alter the structural composition of the solid residues compared to untreated wheat straw. Lignin concentration in particular is not affected by lime pretreatment, even at higher lime concentrations (0.15 g/g). In addition, only 7.1% on average of carbohydrates are solubilised, while the vast majority of carbohydrates are contained in the solid fraction. These results indicate potential advantages of lime pretreatment over other types of alkaline pretreatment: lignin is not solubilised and may be extracted later on in the process, after hydrolysis and fermentation. In addition, intermittent processing to recover alkali prior to enzymatic hydrolysis as proposed by Chang et al. (1998), e.g. by washing the pretreated lignocellulose prior to enzymatic hydrolysis, would not lead to extensive loss of soluble carbohydrates.

Effect of lime pretreatment on enzymatic hydrolysis of wheat straw

Figure 1 shows results of enzymatic hydrolysis tests of pretreated wheat straw (washed insoluble fraction from alkaline pretreatment) under laboratory conditions and high enzyme dosage, using either GC 220 or Cellubrix as enzyme preparation. Glucan-to-glucose conversion during incubation with GC 220 is shown to increase from 32.8% (water treatment at 85°C with no lime added) to 93.0% (lime loading rate 0.15 g/g), whereas xylan-to-xylose conversion increases from 20.9% to 81.4%. Hydrolysis rates for glucan and xylan are largely similar for the Cellubrix enzyme incubation.

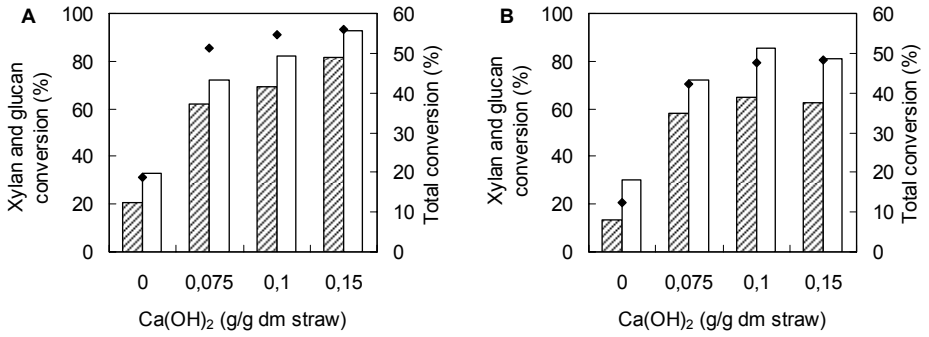

Figure 1. Effect of $Ca(OH)_2$ loading rate (g/g dm straw) on enzymatic hydrolysis of xylan to xylose (▨) and glucan to glucose (□) in lime pretreated wheat straw (washed insoluble fraction). Enzyme loading rate was 116 IFPU/g dm for GC 220 (*A*) and 56 IFPU/g dm for Cellubrix (*B*). Incubation was at 40°C, for 24h, and pH was buffered at 5.0. The total conversion (◆) is expressed as total dry matter solubilized after pretreatment and enzymatic hydrolysis.

These results suggest that lime pretreatment leads to a three to four-fold improvement in enzymatic degradability over wheat straw that is treated with water under similar conditions, even though pretreatment did not indicate any significant lignin solubilisation in the lime-pretreated solid biomass. Apparently, lignin structure is sufficiently modified to enhance enzymatic hydrolysis without severe lignin depolymerization that would result in lignin solubilisation as in the case of other, more aggressive alkaline pretreatments (de Vrije et al., 2002), or lime pretreatment at higher temperatures or longer pretreatment times (Chang et al., 1998). By comparison, the enzymatic degradation of glucan and xylan of NaOH-pretreated wheat straw (results not shown), were largely similar as the results for lime pretreatment at 0.15 g/g, with close to 90% of glucan converted to glucose, and 94% xylan-to-xylose conversion. On the basis of these results as well as earlier reports by Chang et al. (1997) who recommended a lime loading rate of 0.1 g/g for optimal lime pretreatment of switchgrass, for all further experimental work in this study the following pretreatment conditions were selected: 0.1 g lime/g dm substrate, 85°C, and reaction time of 16 h.

Figure 2 presents results of enzymatic hydrolysis experiments of lime pretreated straw (entire, unwashed fraction from pretreatment) under non-buffered conditions, varying enzyme dosage and at a lower hydrolysis pH (4.5). The main purpose of the experiment was to study the enzymatic degradability at more "practical" enzymatic hydrolysis conditions that would be more realistic for industrial scale applications, including lower enzyme dosage (2.1 to 16.3 IFPU/g dm biomass) and higher dry matter loading rate (122 g/l).

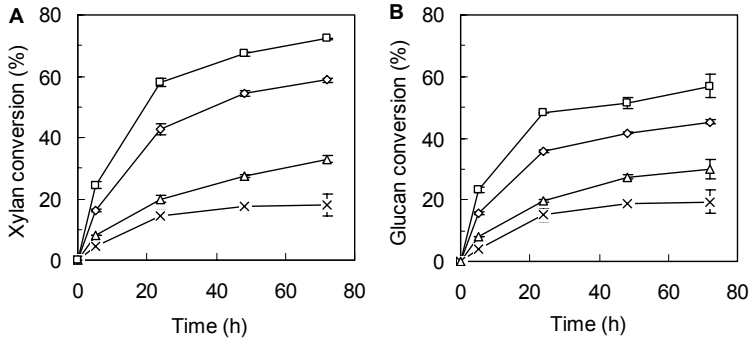

Figure 2. Conversion of xylan to xylose (*A*) and glucan to glucose (*B*) in lime-treated (0.1 g/g) wheat straw with GC 220 and dosage of 2.1 (×), 4.1 (△), 8.2 (◇) and 16.3 (□) IFPU/g dm straw at pH 4.5 and 50°C.

Both glucan and xylan hydrolysis profiles show a declining rate of hydrolysis in time (i.e. the rate of sugar release declines sharply as the reaction proceeds), which corresponds with well-known phenomena described by other authors. Factors that contribute to this rate decline include enzyme inactivation due to thermal effects, the formation of in-active enzyme-lignin complex, and inhibition by hydrolysis products (Lynd et al., 2002). Results from the restart or secondary hydrolysis experiments (results not shown) indicated that both glucan and xylan could be further hydrolysed to 78% and 100% of total glucan and xylan, respectively. These observations suggest that a declining substrate reactivity does not likely play a role in the hydrolysis rates shown in Figure 2, even though a fraction of cellulose (approx 22% glucan) apparently remains resistant to enzymatic hydrolysis even after secondary enzymatic hydrolysis.

In addition to the declining hydrolysis rate, results in Figure 2 show that enzyme dosage has a large effect on the yield of enzymatic hydrolysis. For the highest enzyme dosage (16.3 IFPU/g dm), glucan-to-glucose and xylan-to-xylose conversions amount to 57% and 72% respectively and are comparable to the results obtained earlier with Cellubrix (Fig. 1B). At the lowest enzyme dosage (2.1 IFPU/g dm) however, glucan and xylan conversions are significantly reduced (18 and 19%, respectively). The enzymatic hydrolysis rate in particular in the first 20 h of enzymatic hydrolysis seems directly proportional to the enzyme concentration, which suggests that a certain amount of enzyme is rapidly inactivated, most probably by non-productive binding of the enzyme to the lignin contained in the pretreated biomass. A variety of techniques to counter-effect enzyme-lignin inactivation have been researched such as adding proteins (Yang and Wyman, 2005) or surfactants (Eriksson et al., 2002), prior to enzymatic hydrolysis in order to reduce non-specific binding sites. The relevance of these methods at the industrial scale is not evident. Therefore, optimization of the enzyme cocktail with regards to binding capability to lignin, e.g. by selecting “weak lignin-binding” enzymes (Berlin et al., 2005) could likely lead to

better enzymatic hydrolysis at lower enzyme dosage, without the need for other chemical or biological additives. Enzyme modification was however beyond the scope of this study.
One potential additional factor leading to enzyme inactivation during hydrolysis is physical destruction of the proteins by shear stress, brought about by the high initial viscosity of the pretreated solids at the start of the enzymatic hydrolysis. Observations made during bench-scale hydrolysis (2l; Fig. 3) indicate that, soon after introducing the enzymes, the viscosity of lime-pretreated substrate is significantly reduced, therefore shear stress is not believed to be a large factor in enzyme inactivation. Regardless, the apparent changes in viscosity of the substrate after incubation with enzyme are notable.

Figure 3. View of bench-scale hydrolysis of lime pretreated wheat straw during enzymatic hydrolysis at t = 0 (top left), 0.5 (top right), 2 (bottom left) and 24 h (bottom right) after adding enzymes

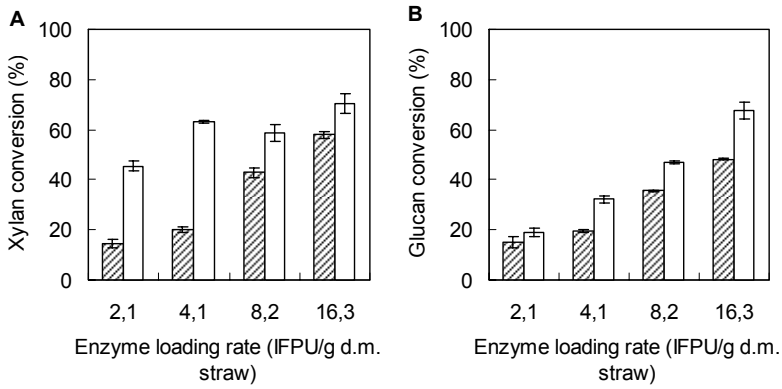

Figure 4. Effect of enzyme loading rate on xylan (*A*) and glucan (*B*) conversion from lime-treated wheat straw. Bars indicate conversion based on monomeric sugars in supernatants after enzymatic hydrolysis (▨), and monomeric sugars after supernatants were treated with 1 M sulphuric acid (□). Enzymatic incubation for 24 h with GC 220 at pH 4.5 and 50°C. Error bars represent minimum and maximum values

Figure 4 shows the neutral sugar yields after 24 h hydrolysis from the same experiments as shown in Figure 2, but includes monomeric sugar yields after supernatants were treated with 1M sulphuric acid, thereby indicating the total soluble (monomeric plus oligomeric) sugar yield from enzymatic hydrolysis. The results show that for enzymatic hydrolysis of xylan, a majority of xylan is converted into soluble sugars, irrespective of enzyme loading rate. However, at lower enzyme loading rates (2.1 and 4.1 IFPU/g dm) more than half of these soluble sugars are present in oligomeric form, while at the higher enzyme loading rates (8.2 and 16.3 IFPU/g dm) most of the soluble sugars appear in monomeric form. Results for enzymatic hydrolysis of glucan show a different trend: at low enzymatic loading rates total soluble sugar yield from glucan is low, but glucan conversion to soluble sugars increases significantly with enzyme loading rate. For all enzyme loading rates however, the majority of sugars derived from enzymatic conversion of glucan appear in monomeric form. These experiments confirm earlier reports (Eggeman and Elander, 2005) with regard to xylan for enzymatic hydrolysis of lime-pretreated corn stover, although reported results for glucan are different. The results in Figure 4 suggest that the limited enzymatic degradation of cellulase at low enzyme dosage is not likely a result of incomplete enzymatic degradation of hemicellulose, as the majority of xylan is converted in soluble sugars. In any case, there appears to be a need to further optimize the lime pretreatment with regard to increasing cellulose reactivity, as well as the enzyme selection with regard to reducing inactivation by lignin. Given the recent interest in fermentation of pentose sugars into ethanol (Kuyper et al., 2004), enzyme selection should also be geared towards optimizing enzymatic hydrolysis of xylan to xylose.

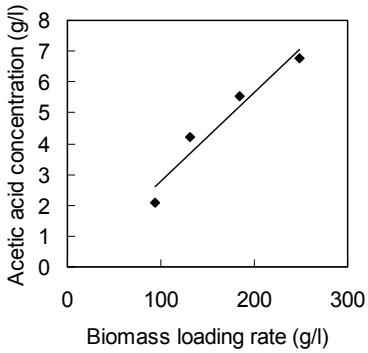

Figure 5. Acetic acid concentration in lime-pretreated wheat straw, as a function of dry matter loading rate (lime pretreatment conducted at 0.1 g/g on dry biomass basis).

Fermentability of lime-pretreated substrates to ethanol

To evaluate the formation of common fermentation inhibitors during lime pretreatment and their effect on fermentation into ethanol, four batches of lime pretreated wheat straw were prepared at bench scale (2 l) using standard pretreatment conditions (85°C, 0.1 g/g $Ca(OH)_2$, and in upgoing biomass loading rate (93, 132, 185 and 249 g dry biomass/l corresponding to 8.5, 11.7, 15.6 and 19.9% dry matter biomass content). HPLC-analysis of the soluble fraction of biomass after pretreatment indicated that both furfural and 5-hydroxymethylfurfural (5-HMF), two common fermentation inhibitors in lignocellulosic biomass conversion were below 0.01 g/l (highest concentrations were 0.008 g/l and 0.006 g/l for furfural and 5-HMF, respectively). These results are consistent with the mild temperature used during lime pretreatment, and provide evidence of the advantage of alkaline pretreatment over other techniques that require elevated temperatures, such as dilute acid pretreatment. However, a significant amount of acetic acid is produced during lime pretreatment. Figure 5 shows that the acetic acid concentration increases from 2.1 g/l at lowest biomass loading rate to 6.7 g/l at highest biomass loading rate, and acetic acid is shown to be largely proportional to biomass loading rate. The acetic acid formed during pretreatment originates from the acetyl-linkages in the hemicellulose fraction, which are broken at elevated pH that are typical for alkaline pretreatment.

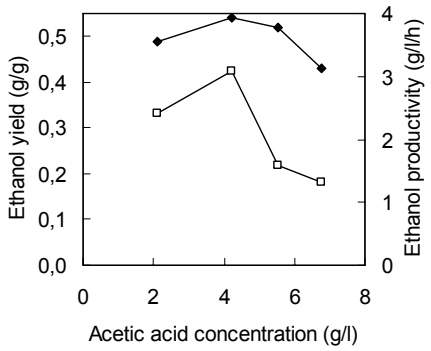

Figure 6. Effect of acetic acid concentration in lime-pretreated straw on ethanol yield (◆) and productivity (□) by *S. cerevisiae* fermented at pH 5 and 32°C.

The amount of acetic acid released is therefore associated to the degree of acetylation of hemicellulose, which is feedstock dependent. In the case of the wheat straw used in this study, on average 2.8% of dry matter biomass was converted into acetic acid. Figure 6 shows results of ethanol fermentation tests at pH 5.0 of the four pretreated substrates after 24 h of enzymatic hydrolysis and subsequent ethanol fermentation. While the ethanol yield for all samples remains close to the theoretical yield of 0.51 g ethanol/g C6-sugar, the increasing inhibiting effect of acetic acid on ethanol fermentation is clearly shown as ethanol productivity drops from 3.1 g/l/h to 1.6 g/l/h as acetic acid concentration increases from 4.2 to 5.5 g/l, with a further decline to 1.32 g/l/h at 6.7 g/l acetic acid, a decline of 53%. In other words, a higher acetic acid concentration leads to longer fermentation times, thereby reducing the feasibility of fermentation into ethanol of lime pretreated lignocellulose at the industrial scale. In addition, the inhibiting effect of acetic acid might be aggravated when fermentation is carried out at pH levels typically found at ethanol production facilities (pH 4.0).

In order to study the effect of acetic acid concentration on ethanol fermentation, a relatively simple washing technique subsequent to lime pretreatment was conducted by placing the pretreated wheat straw in a cotton bag, submerging the bag in 2 l of fresh demineralised water at room temperature, and dewatering the cotton bag by using a manual piston press containing a screen at the bottom. Results of HPLC analysis of the supernatants showed that acetic acid in the pretreated substrate could be reduced by a factor five by conducting three consecutive washing steps. Table 2 shows results of ethanol fermentability tests of three original and washed lime-pretreated wheat straw after 4.5 h of fermentation.

TABLE 2. Fermentation of non-washed and washed lime-treated wheat straw into fermentation products at pH 4, 4.5 and 5 at beginning and end[a] of fermentation.

	Acetic acid		Sugars (g/l)[b]			Fermentation products (g/l)	
	pH[c]	(g/l)	Glucose	Xylose	Arabinose	Ethanol	Glycerol
Non-washed substrate							
pH 5 t_0	5.0	3.1	23.6	10.8	2.1	0	0
pH 5 t_e	4.8	2.9	1.1	10.9	2.0	11.7	1.6
pH 4,5 t_0	4.5	3.0	23.3	10.5	2.1	0	0
pH 4,5 t_e	4.4	2.9	1.4	10.9	2.1	11.9	1.3
pH 4 t_0	4.0	3.0	23.0	10.4	2.0	0	0
pH 4 t_e	4.1	3.0	16.7	10.5	2.1	3.7	0.7
Washed substrate[d]							
pH 5 t_0	4.9	0.6	26.7	10.7	2.0	0	0
pH 5 t_e	4.3	0.5	0.2	11.8	2.0	11.3	1.4
pH 4,5 t_0	4.5	0.6	25.7	10.3	1.8	0	0
pH 4,5 t_e	3.9	0.5	0.2	11.6	1.9	11.7	1.2
pH 4 t_0	4.0	0.6	26.2	10.5	1.9	0	0
pH 4 t_e	3.6	0.5	0	11.6	2.0	11.5	1.1

[a]Time end (t_e) indicate the moment that yeast stopped producing carbon dioxide; [b]Mannose and rhamnose were not detected and galactose was below 0.2 g/l; [c]The hydrolysate was adjusted with 20% (*v/v*) sulphuric acid to desired pH prior to fermentation; [d]Prior to enzymatic hydrolysis, the lime-treated wheat straw was washed.

Fermentation of both non-washed (containing approx. 3 g/l acetic acid) and washed substrates (0.6 g/l acetic acid) are shown to lead to almost complete conversion of glucose to ethanol, with the exception of the non-washed substrate fermented at pH 4 where only 27% of the glucose was fermented to ethanol and other by-products. Apparently, the combination of lower pH and moderate acetic acid concentration in lime-pretreated wheat straw leads to severe inhibition. The increasing inhibition effect at lower pH is also indicated by the CO_2 production in time, which is shown in Figure 7.

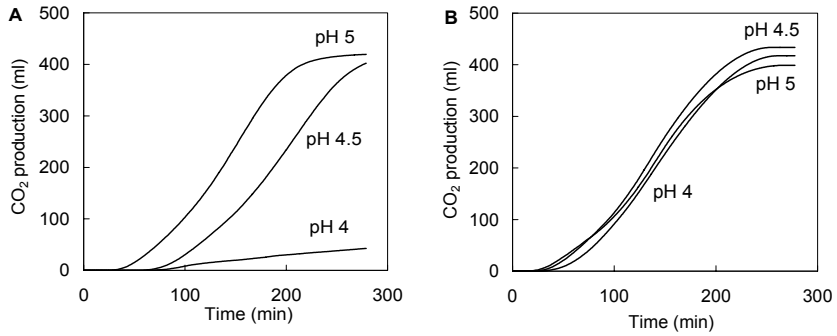

Figure 7. Production of CO_2 (as equimolar of ethanol) during fermentation of unwashed (*A*) and washed (*B*) lime-pretreated wheat straw by *S. cerevisiae* at 32°C and pH 4, 4.5 and 5.

The cumulative production of CO_2 in time for non-washed substrates clearly shows the inhibition of fermentation when pH is below 5.0, whereas fermentation of washed substrates under similar conditions does not exhibit such inhibition. These results confirm well-known effects of lowering pH on antimicrobial behaviour of organic acids (lowering the pH below the dissociation constant increases the amount of undissociated acid which can diffuse across the cell membrane; Narendranath et al., 2001). For industrial purposes therefore, acetic acid removal prior to hydrolysis and fermentation should be considered when employing lime pretreatment of lignocellulosic feedstocks, depending on the degree of acetylation of the hemicellulose fraction of the feedstock. A potential drawback of washing prior to enzymatic hydrolysis could be a loss of soluble sugars derived from the xylan fraction, however, the results presented in this paper (less than 10% of xylan-derived sugars are solubilised during lime pretreatment; Table 1) suggest that this loss would be rather limited.

CONCLUSIONS AND RECOMMENDATIONS

Results presented in this paper indicate that lime pretreatment of wheat straw, when carried out at mild temperatures (85°C) and intermediate pretreatment times (16-20 h), does not lead to any significant solubilisation of lignin, and only limited solubilisation of hemicellulose and cellulose. Therefore, lime pretreatment of wheat straw yields a large insoluble fraction with a composition that closely resembles the crude, untreated biomass.

Under high enzyme loading and controlled hydrolysis conditions, up to 93% of glucan and 81% of xylan contained in lime-pretreated wheat straw can be converted by 24 h enzymatic hydrolysis into monomeric sugars. This represents a three to four fold increase in enzymatic digestibility compared to control treatments. At lower enzyme loading rates (2.1-16.3 IFPU/g dm) and enzymatic hydrolysis conditions that would more resemble industrial applications (unbuffered pH, 122 g/l solids loading), the enzyme dosage had a significant effect on enzymatic hydrolysis yields, and monomeric sugar yields were generally lower. Results further indicated that a significant fraction (20% or more) of sugars was released in soluble, oligomeric form. The decline in monomeric sugar yields at lower enzyme dosage may be attributed to a number of factors, including end-product inhibition of the enzyme, non-productive binding of the enzyme to lignin, and incomplete conversion of soluble oligomeric sugars to monomeric sugars. More fundamental knowledge is needed on enzyme-substrate inhibition, in particular where lignocellulosic biomass is pretreated under mild conditions.

Almost no furfural or 5-HMF, two common degradation products that inhibit microbial activity, is formed during mild temperature lime pretreatment of wheat straw. Lime

pretreatment at mild temperatures however leads to a rapid release of acetic acid from the lignocellulosic biomass as a result of deacetylation of hemicellulose, which may benefit the enzymatic hydrolysis of lignocellulose. Hydrolysates of lime-pretreated wheat straw were suitable for ethanol fermentation, especially at higher pH (5.0) and intermediate solids loading rate (122 g/l). At lower fermentation pH however, acetic acid originating from the lignocellulosic biomass affected the ethanol productivity moderately (pH 4.5) to severely (pH 4.0). Ethanol fermentation was also affected when fermentation was carried out at higher solids loading rates. Inhibitory effects of acetic acid at lower fermentation pH could be overcome by washing the lime-pretreated wheat straw prior to enzymatic hydrolysis.

In summary, lime pretreatment at mild temperatures is a promising pretreatment route to enhance enzymatic hydrolysis of lignocellulose, and can be characterized by high enzymatic degradability, and no significant delignification or xylan degradation. During ethanol fermentation of sugars derived from lime-pretreated biomass, the primary fermentation inhibitor of concern is acetic acid, especially at low pH. Depending on feedstock type and composition, either acetic acid removal prior to enzymatic hydrolysis or selection of more acid-tolerant yeasts should be considered when employing lime pretreatment at the industrial scale.

ACKNOWLEDGEMENTS

This research is supported with a grant of the Dutch Programme EET (Economy, Ecology, Technology) a joint initiative of the Ministries of Economic Affairs, Education, Culture and Sciences and of Housing, Spatial Planning and the Environment. Co-financing was provided by Program 412 Green Resources of the Dutch Ministry of Agriculture, Nature and Food Quality. Jacinta vd Putten and Richard Gosselink are acknowledged for providing technical assistance.

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CHAPTER 3

Lactic Acid Production from Xylose by the Fungus *Rhizopus oryzae*

Ronald H.W. Maas, Robert R. Bakker, Gerrit Eggink and Ruud A. Weusthuis

ABSTRACT

Lignocellulosic biomass is considered nowadays to be an economically attractive carbohydrate feedstock for large-scale fermentation of bulk chemicals such as lactic acid. The filamentous fungus *Rhizopus oryzae* is able to grow in mineral medium with glucose as sole carbon source and to produce optically pure L(+)-lactic acid. Less is known about the conversion by *R. oryzae* of pentose sugars such as xylose, which is abundantly present in lignocellulosic hydrolysates. This paper describes the conversion of xylose in synthetic media into lactic acid by ten *R. oryzae* strains resulting in yields between 0.41 and 0.71 g/g. By-products were fungal biomass, xylitol, glycerol, ethanol and carbon dioxide. The growth of *R. oryzae* CBS 112.07 in media with initial xylose concentrations above 40 g/l showed inhibition of substrate consumption and lactic acid production rates. In case of mixed substrates, diauxic growth was observed where consumption of glucose and xylose occurred subsequently. Sugar consumption rate and lactic acid production rate were significantly higher during glucose consumption phase compared to xylose consumption phase. Available glucose (19.2 g/l) and xylose (10.3 g/l) present in a mild-temperature alkaline treated wheat straw hydrolysate was converted subsequently by *R. oryzae* with rates of 2.2 g glucose/l·h^{-1} and 0.5 g xylose/l·h^{-1}. This resulted mainly into the product lactic acid (6.8 g/l) and ethanol (5.7 g/l).

INTRODUCTION

The use of inexpensive and widely available lignocellulosic biomass materials such as wheat straw becomes more and more interesting as potential feedstock for the production of bulk chemicals. Deriving fermentable sugars from lignocellulosic biomass is a major R&D issue in the development of the 'lignocellulose-to-ethanol' production technology. Future prospects show an increasing demand of renewable sources for the production of high volumes of bulk chemicals. The pentose monosaccharide xylose, besides the hexose monosaccharide glucose, is one of the most abundant sugars found in nature. It is the predominant hemicellulosic sugar of hardwoods and agricultural residues, accounting for up to 25% of the dry weight biomass of some plant species (Ladisch et al., 1983). The abundance and ease of isolation of xylose makes it an important potential feedstock for the production of bulk chemicals such as lactic acid.

With a worldwide annually increasing production, lactic acid and its potential derivatives represent an important category of chemicals for industries producing food, chemicals and pharmaceutical products. Lactic acid can be manufactured in a racemic mixture by chemical synthesis and both in racemic mixtures and optically pure forms by microbial carbohydrate fermentation processes (Datta et al., 1995; Zhou et al., 1999). Highly purified, preferably L(+)-lactic acid anhydrous monomer is required for the production of the biodegradable polymer polylactic acid (PLA), which is an environmentally friendly replacement of plastics derived from petrochemical materials. Current commercial industrial processes for the biological conversion of glucose into almost optically pure lactic acid are carried out by homolactic lactobacilli with yields up to 80-90% (Longacre et al., 1997). Homolactic acid bacteria cannot efficiently ferment pentoses and require growth medium with complex supplements, which adds to the costs of lactic acid production and complicates purification of lactic acid (Skory, 2000; Tay and Yang, 2002). The separation of the D(-) from the L(+) optical form is also difficult and contributes to the production costs of PLA (Longacre et al., 1997).

The filamentous fungus *Rhizopus oryzae* produces optically pure L(+)-lactic acid and requires a mineral medium composition with some inorganic minerals and ammonium salt as sole nitrogen source (Lockwood et al., 1936; Bai et al., 2003). *R. oryzae* produces mainly lactic acid from glucose with yields of 60-80% and also ethanol, carbon dioxide and minor amounts of malic acid, fumaric acid and citric acid (Longacre et al., 1997; Skory, 2004). Product formation depends on cultivation conditions, it has been shown (Skory et al., 1998) that, under oxygen-limiting conditions, product formation shifts from lactic acid to ethanol.

A number of research groups reported the consumption of xylose by *R. oryzae* NRRL 395 for the production of lactic acid. Park et al. (2004) described the conversion of an enzymatic hydrolysate of waste office paper and artificial media with mixtures of glucose,

xylose and cellobiose. The conversion rate of xylose as sole carbon source into lactic acid was 7.3 g/l·d^{-1} and accounted for 28% of the conversion rate of glucose or cellobiose (Park et al., 2004). The conversion of xylose by *R. oryzae* NRRL 395 was also described by Yang et al. (1995) resulting in a lactic acid yield of 0.7 g/g and with glycerol and ethanol as by-products (Yang et al., 1995). Taherzadeh et al. (2003) reported the cultivation of *R. oryzae* in paper pulp spent liquor to achieve high biomass and ethanol yields. The consumption rate of hexoses was faster than that of pentoses in synthetic media (Taherzadeh et al., 2003).

In the present paper, we report on the conversion of xylose into lactic acid by ten different fungal strains of *Rhizopus oryzae*. A selected *R. oryzae* strain was used to convert sugars such as glucose and xylose present in a mild-temperature alkaline wheat straw hydrolysate. As lignocellulosic hydrolysates are complex mixtures with variable sugar concentration and composition, we first studied the effects of different initial xylose and glucose concentrations and mixtures on lactic acid production by *R. oryzae* in synthetic media.

MATERIALS AND METHODS

Fungal strains

Ten *Rhizopus oryzae* strains were used: *R. oryzae* CBS 147.22, CBS 128.08, NRRL 395, CBS 539.80, CBS 328.47, CBS 127.08, CBS 321.35, CBS 396.95, CBS 112.07 and CBS 264.28 (CBS-Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands and NRRL-Northern Regional Research Laboratory, Peoria, Illinois, USA). Strains were grown on Potato Dextrose Agar (Oxoid) at 30°C. Spores were collected after approximately 7 days by washing the mycelium with a sterile solution of 10% (*v/v*) glycerol and 0.05% (*v/v*) Tween 80. The number of spores was determined microscopically using a cell counter (Neubauer, Germany). The stock spore suspension was stored at –80°C.

Inoculum preparation

Spores of ten different *R. oryzae* strains were initially used to inoculate fermentation medium with different carbon sources. We observed germination and growth (~3 to 5 g dry biomass/l) of the ten tested strains with glucose (120 g/l) and with a mixture of glucose and xylose (both 10 g/l). However, attempts to germinate these spores in order to obtain considerable amounts of mycelial biomass in fermentation medium with xylose (30 g/l) as sole carbon source failed with six strains. The spores of *R. oryzae* apparently germinate and form mycelial biomass more efficiently in presence of glucose. It has also been observed by others that the percentage of spore germination of the related fungus *Rhizopus oligosporus*

is often determined by the presence of suitable carbon sources (Medwid and Grant, 1984). Because spores of some *R. oryzae* strains were not able to germinate and grow on xylose, another approach was developed. Mycelial biomass was produced in growth medium with 30 g/l glucose in order to inoculate fermentation medium with glucose or xylose as sole carbon source. The composition of growth medium per litre was: glucose, 30 g; $(NH_4)_2SO_4$, 1.25 g; KH_2PO_4, 0.6 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $ZnSO_4 \cdot 7H_2O$, 0.04 g; $Na_2HPO_4 \cdot 2H_2O$, 5.37 g and $NaH_2PO_4 \cdot H_2O$, 9.64 g. The chemicals, unless indicated otherwise, were purchased from Merck (Darmstadt, Germany). Growth medium was prepared by adding 25 ml of nutrient (4×) stock solution [$(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4 \cdot 7H_2O$ and $ZnSO_4 \cdot 7H_2O$], 50 ml of sodium phosphate (2×) stock solution ($Na_2HPO_4 \cdot 2H_2O$ and $NaH_2PO_4 \cdot H_2O$) and 25 ml of glucose (4×) stock solution to 250-ml baffled flasks with metal caps which allow gas transfer. Glucose solution served as carbon source and was filter sterilised (cellulose acetate filter with pore size 0.2 μm, Minisart, Sartorius). Nutrient solution and sodium phosphate buffer were sterilised (20 min at 125°C) separately. Growth medium was inoculated with $2 \cdot 10^5$ spores per millilitre and incubated in a rotary shaker with an agitation rate of 150 rpm at 37°C (Innova 4230, Edison, NJ, USA).

The separation of the mycelial biomass from the growth medium (100 ml) was performed by a wash treatment procedure. The medium was removed by 3 min of centrifugation at 2,000 rpm (Centaur 2, Beun de Ronde, Abcoude, The Netherlands) and the fungal biomass pellet was washed with 40 ml of sterile solution of 25 mM sodium phosphate buffer (pH 6.5). The pellet was re-suspended carefully and the centrifugation step was repeated. This washing procedure was carried out twice.

Shake flask experiments

In this study, we used baffled shake flasks instead of controlled stirred fermentors because of the following two reasons. First, our experiments with *R. oryzae* in a submerged controlled stirred fermentor often resulted in heterogeneous growth with mycelium attached around elements in the reactor such as baffles, electrodes and impellers (data not shown). Others also observed that fungal strain, nutrients, substrate, pH, aeration, agitation and concentration of inoculum can influence the growth form of *R. oryzae*. Pellet or clump growth caused sub-optimal growth conditions by a decrease in gas, and mass transfer resulted in long fermentation periods, cell death and low lactic acid yields due to by-product formation such as that of ethanol (Bai et al., 2003). We used baffled shake flasks in order to achieve reproducible and comparable growth forms of *R. oryzae*. Secondly, an advantage of using a controlled stirred fermentor is that percentage of oxygen and carbon dioxide of in- and outlet gas can be analysed to determine the respiration rate by *R. oryzae*. Yet, our results showed that due low gas transfer, probably caused by high viscosity of the fermentation broth, the differences in composition between gasses entering and leaving the

fermentor were so small that oxygen consumption and carbon dioxide production could not be determined accurately (data not shown). In shake flask cultures, we used the carbon recovery calculations to estimate the carbon dioxide produced by respiration.

The composition of the fermentation medium used in the shake flaks cultures was similar to the growth medium except that sodium phosphate salts were replaced by 52 g/l $CaCO_3$ (SA Omya, Benelux NV). Calcium carbonate neutralises the lactic acid and regulates the pH between 6.0 and 6.5 but is impure and contains several trace elements. Sugar solution with xylose (Sigma-Aldrich, Germany) or glucose served as carbon source and was filter sterilised (cellulose acetate filter with pore size 0.2 µm, Minisart, Sartorius). The mycelial biomass obtained after the wash treatment procedure of growth medium served as inoculum for fermentation medium [0.1 gram mycelial biomass (dry weight) per litre]. The cultures were incubated in a rotary shaker with an agitation rate of 150 rpm at 37°C (Innova 4230, Edison, NJ, USA).

Lignocellulosic hydrolysate

Wheat straw was purchased from a commercial farm located in the northeast of the Netherlands and was used for the manufacturing of a hydrolysate. The wheat straw was mechanically treated by extrusion (rotation of 100 rpm and throughput of 50 kg/h) and chemically treated by soaking with 5% calcium hydroxide (pH 9) for 16 h at 75°C. The liquid in pre-treated wheat straw was substituted by fresh water to regain alkali and to reduce concentration of fermentation inhibitors such as acetic acid, which was reduced to 1 g/l. This pre-treated wheat straw (dry matter 110 g/l) was enzymatically hydrolysed with the enzyme cocktails Cellubrix and Novozymes 188 (Novozymes, Denmark) for 24 h at 50°C and pH 4.8. The non-hydrolysed solid particles were removed from the hydrolysate by 3 min of centrifugation at 3,000 rpm (Centaur 2, Beun de Ronde, Abcoude, The Netherlands). The composition of the fermentation medium was 100 ml supernatant of hydrolysate with (per litre): $CaCO_3$, 52 g; $(NH_4)_2SO_4$, 1.25 g; KH_2PO_4, 0.6 g; $MgSO_4 \cdot 7H_2O$, 0.25 g and $ZnSO_4 \cdot 7H_2O$, 0.04 g. Cultivation was performed in 250-ml baffled flasks and the medium was inoculated with $2 \cdot 10^5$ spores per millilitre medium.

Analytical methods

Samples of fermentation medium were heated for 10 min at 70°C to dissolve precipitated calcium lactate. Immediately after heating, the samples were centrifuged for 3 min at 17,400×g (Eppendorf 5417C). The supernatant was diluted (1:1, *v/v*) with 1 M sulphuric acid. The samples were filtered to remove solids and precipitated proteins (Spartan filter 13, pore size of 0.2 µm, Schleicher & Schuell). Substrates and products were analysed by high pressure liquid chromatography using an Altech IOA-1000 Organic Acids column with 3 mM sulphuric acid as the mobile phase at 90°C and a flow rate of 0.4

ml/min. Monosaccharides, polyols and organic acids were detected by a refractive index detector (Waters 2414).

To measure cell dry weight (CDW), residual $CaCO_3$ was removed from biomass by washing approximately 90 ml of fermentation medium with 2 M hydrochloric acid and, subsequently, with distilled water. Broth was filtered on a pre-weighed Whatman filter paper and washed biomass was dried overnight at 50°C before weight analysis. The $CaCO_3$ contained 1.5% (*w/w*) insoluble impurities which remained on the filter. The dry weight of the biomass was corrected for this insoluble matter. Because of heterogeneous growth of mycelium and low concentration of biomass, cell dry weight analyses were only performed at the end of the fermentation.

Calculations

The yield of lactic acid ($Y_{p/s}$) is expressed as amount of product (g) synthesised divided by the amount of substrate (g) consumed. Biomass yield ($Y_{x/s}$) is calculated by the amount of biomass (g dry weight) divided by the amount of substrate (g) consumed. Carbon recovery is calculated with respect to moles of carbon produced divided by moles of carbon consumed. Carbon dioxide production could not be measured accurately (see above) and could, therefore, not be included in the carbon recovery calculations. To estimate the amount of carbon dioxide formed, we assumed that all missing carbon in the carbon recovery calculations is in the form of carbon dioxide. The amount of carbon dioxide synthesised in ethanol production can be estimated easily as it is produced in equimolar amounts with ethanol. The amount of carbon dioxide produced in respiration can then be calculated as the total amount of carbon dioxide formed minus the amount of ethanol produced. For the calculation of the carbon recovery, fungal biomass composition of $CH_{1.72}O_{0.55}N_{0.17}$ with an ash content of 7.5% (*w/w*) was used (Carlsen and Nielsen, 2001).

RESULTS

Conversion of xylose and glucose by R. oryzae

Xylose conversion by ten *R. oryzae* strains was studied in synthetic media in shake flask cultures. All tested strains showed similar fermentation profiles but with different lactic acid concentrations and yields. Figure 1 demonstrates a typical conversion of glucose (A) and xylose (B) by *R. oryzae* CBS 127.08. Table 1 represents an overview of fermentation characteristics of the conversion of glucose or xylose by ten different *R. oryzae* strains. The consumption of xylose started after approximately 30 h of incubation and it was nearly depleted after 120 h. The tested *R. oryzae* strains convert 30 g/l xylose into mainly lactic acid (10.4 to 21.1 g/l). By-products such as xylitol (0.3 to 3.8 g/l) and glycerol (0.2 to 1.2

g/l) were produced in all cultures and, in some cases, minor amounts of ethanol were formed (0 to 0.6 g/l). After the depletion of xylose, *R. oryzae* started to utilise xylitol.

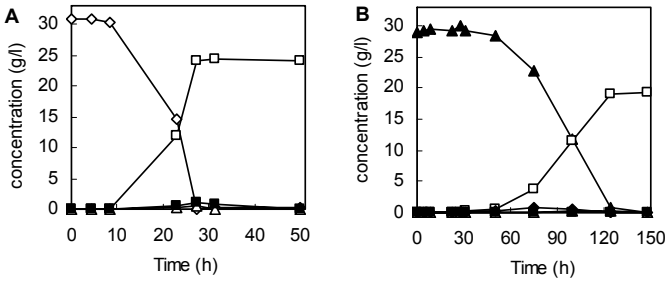

Figure 1. Typical conversion of (*A*) glucose (◇) and (*B*) xylose (▲) into L(+)-lactic acid (□) by *R. oryzae* CBS 127.08. The by-products were xylitol (◆), glycerol (△) and ethanol (■).

With 30 g/l glucose as the sole carbon source, consumption by *R. oryzae* started after approximately 8 h. Glucose was depleted after 27 h of cultivation and lactic acid was the main product (13.3 to 24.1 g/l). The by-products were glycerol (0.3 to 0.8 g/l) and ethanol (0.7 to 2.5 g/l).

The tested *R. oryzae* strains showed lactic acid yields ($Y_{p/s}$) on glucose from 0.42 to 0.79 g/g which corresponds to 42 and 79%, respectively, of the theoretical yield. The conversion of xylose resulted in lactic acid yields ranging from 0.41 to 0.71 g/g and accounted for 41 to 71% of the theoretical yield obtained with glucose (Table 1). In most cases, the biomass yield ($Y_{x/s}$) of *R. oryzae* is higher when cultivated on xylose compared with glucose.

TABLE 1. Fermentation characteristics of ten *R. oryzae* strains with an initial xylose concentration of 30 g/l or glucose concentration of 30 g/l as the sole carbon source.

R. oryzae strain	Fermentation time (h)		$Y_{p/s}$ (g lactic acid/g)		$Y_{x/s}$ (g CDW/g)		Carbon recovery (% C-mol)	
	Xyl	Gluc	Xyl	Gluc	Xyl	Gluc	Xyl	Gluc
CBS 147.22	124	27	0.71	0.74	0.05	0.06	79	91
CBS 128.08	124	27	0.42	0.69	0.10	0.05	56	89
NRRL 395	124	23	0.61	0.42	0.18	0.13	82	74
CBS 539.80	124	27	0.51	0.74	0.09	0.03	61	90
CBS 328.47	124	27	0.44	0.71	0.09	0.12	79	96
CBS 127.08	124	27	0.67	0.79	0.11	0.03	81	91
CBS 321.35	100	27	0.60	0.73	0.12	0.10	75	90
CBS 396.95	100	27	0.57	0.73	0.12	0.18	71	99
CBS 112.07	124	23	0.41	0.61	0.14	0.15	65	86
CBS 264.28	75	27	0.65	0.73	0.12	0.08	80	86

After the indicated fermentation time, a percentage of 95-100% of xylose and 99-100% of glucose was consumed. The initial biomass concentration was 0.1 g/l.

The calculations of carbon recovery from cultures grown on xylose were between 56 and 82% in contrast to calculations of cultures with glucose which recover between 74 and 99% of the total carbon used. This result indicates that carbon dioxide produced by respiration, which accounts for the missing carbon, was higher when *R. oryzae* was cultivated with xylose compared to with glucose.

R. oryzae strain CBS 112.07 was selected for the next experiments based on high production of biomass with glucose and xylose as carbon source and high lactic acid yield of 0.85 g/g with 120 g/l glucose (data not shown).

Effect of xylose concentration on lactic acid production by R. oryzae

The xylose concentrations in lignocellulosic hydrolysates can vary depending on the origin of the raw material, the efficiency of mechanical and chemical treatment and enzymatic hydrolysis. To study the effect of xylose concentration on lactic acid production, *R. oryzae* CBS 112.07 was cultivated in fermentation medium with different initial concentrations of xylose in a range of 20 to 111 g/l (Fig. 2, I). The consumption of xylose started after approximately 25 to 40 h. Increasing initial xylose concentrations (20 to 111 g/l) resulted in longer lag phases and lower consumption rates. After 90 h of incubation, xylose in the culture with an initial concentration of 20 g/l was completely depleted. The other *R. oryzae* cultures with initial xylose concentrations above 40 g/l showed consumption of approximately 40 g/l xylose. After this amount of xylose was consumed, the substrate consumption rate decreased approximately ten-fold. Although sufficient substrate was available and slowly consumed, neither lactic acid nor any other fermentation product was produced (Fig. 2, I). This suggests that *R. oryzae* switched to respiratory metabolism and converted the available xylose slowly to carbon dioxide. To determine if this phenomenon is specific for xylose, similar experiments were performed with glucose as substrate. To exclude an osmolarity effect in the comparison between xylose and glucose, the same molar concentrations were used for both sugars, resulting in somewhat higher amounts of glucose (g/l) compared with xylose. The profile of xylose uptake and product formation was not observed when *R. oryzae* CBS 112.07 was cultivated on different initial concentrations of glucose. Available glucose (20 to 121 g/l) was consumed completely starting after 16 h of incubation and lactic acid production rates were significantly higher compared with fermentation on xylose (Fig. 2, II).

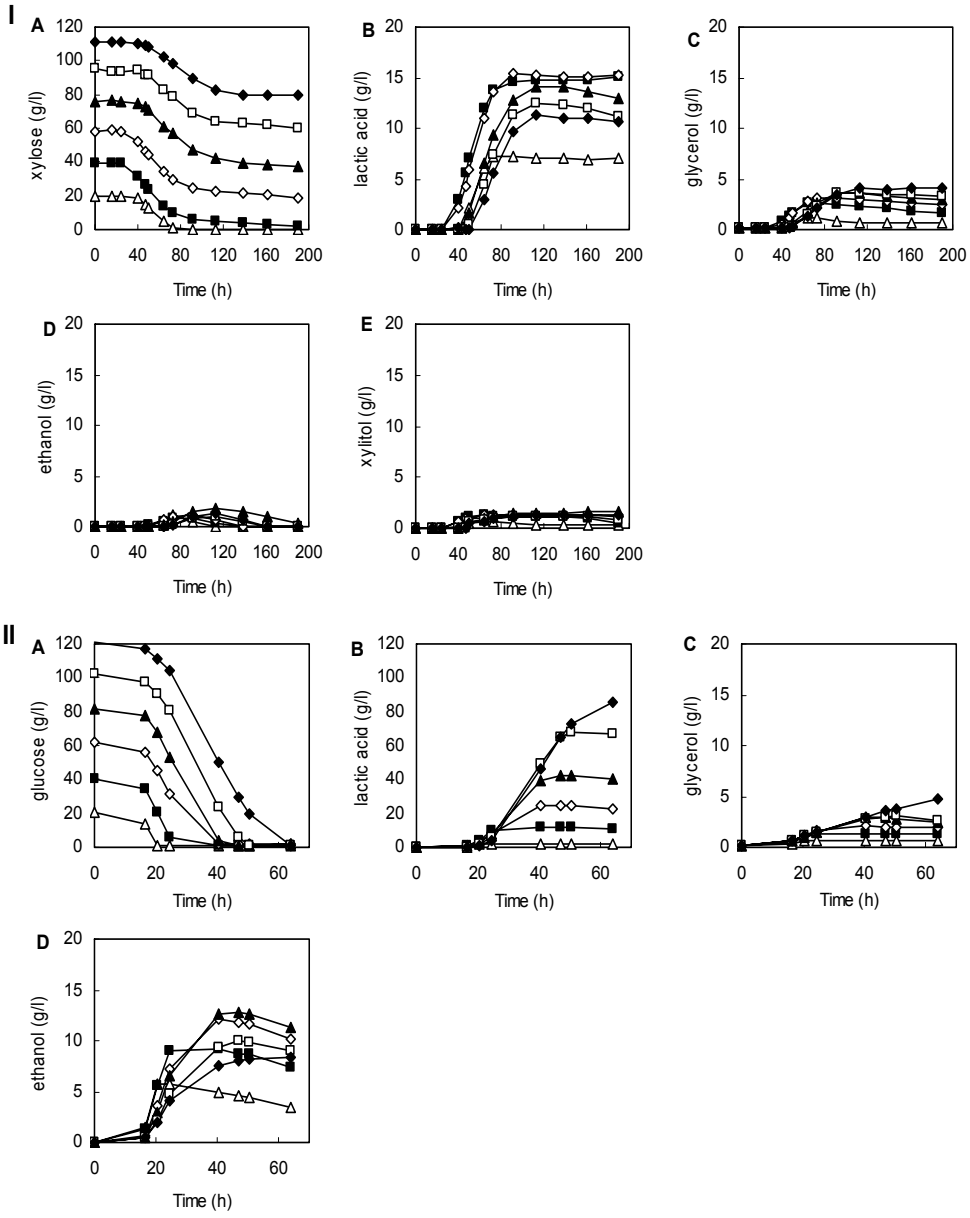

Figure 2. **I** Consumption of (*A*) xylose and formation of (*B*) lactic acid, (*C*) glycerol, (*D*) ethanol and (*E*) xylitol by *R. oryzae* CBS 112.07. Initial concentrations of xylose were 20 (△), 39 (■), 58 (◇), 76 (▲), 95 (□) and 111 g/l (◆). **II** Consumption of (*A*) glucose and formation of (*B*) lactic acid, (*C*) glycerol and (*D*) ethanol by *R. oryzae* CBS 112.07. The initial concentrations of glucose were 20 (△), 41 (■), 62 (◇), 82 (▲), 102 (□) and 121 g/l (◆).

TABLE 2. Fermentation characteristics of different initial concentrations of xylose and glucose by *R. oryzae* CBS 112.07.

Initial concentration (g/l)	Produced lactic acid (g/l)	$Y_{p/s}$ (g lactic acid/g)	Produced cell dry weight (g/l)	$Y_{x/s}$ (g CDW/g)	Carbon recovery (% C-mol)
Xylose					
19.8	7.2	0.36	4.4	0.23	78
39.3	15.2	0.40	4.4	0.11	63
58.5	15.2	0.38	6.9	0.18	73
75.6	14.1	0.39	4.3	0.11	75
95.1	12.4	0.40	6.4	0.18	85
111.3	11.3	0.39	3.9	0.12	81
Glucose					
20.4	1.7	0.09	2.3	0.12	82
40.8	11.7	0.30	2.7	0.07	83
61.9	25.0	0.41	2.5	0.04	88
81.9	42.5	0.53	2.9	0.04	91
102.3	68.2	0.67	3.9	0.04	94
121.0	85.9	0.72	4.4	0.04	93

Independent of the initial xylose concentration (20, 39, 59, 76, 95 or 111 g/l), *R. oryzae* CBS 112.07 consumed a maximum of approximately 40 g/l xylose and produced 11 to 15 g/l lactic acid which resulted in yields ($Y_{p/s}$) around 0.4 g/g (Table 2). On the other hand, *R. oryzae* consumed glucose concentrations of 20, 41, 62, 82, 102 and 121 g/l and produced 2, 12, 25, 43, 67 and 86 g/l lactic acid, respectively, which resulted in lactic acid yields of 0.09 to 0.72 g/g (Table 2). The by-products of fermentation with xylose were xylitol, glycerol and ethanol and with glucose as substrate, glycerol and ethanol (Fig. 2, I, II). At the end of the fermentation, the biomass concentrations of cultures with different initial xylose concentrations were between 3.9 and 6.9 g/l, whereas the cultures with different glucose concentrations ranged between 2.3 and 4.4 g/l. This resulted in biomass yields ($Y_{x/s}$) of 0.12 to 0.23 g/g xylose and of 0.04 to 0.12 g/g glucose. Again the carbon recovery of cultures with xylose was between 63 and 85% and with glucose between 82 and 94% (Table 2), indicating that respiration was higher when *R. oryzae* was cultivated with xylose compared to with glucose.

Conversion of glucose/xylose mixtures in synthetic media by R. oryzae

Lignocellulosic hydrolysates often contain mixtures of hexose and pentose sugars. To study the effect of sugar mixtures on lactic acid production by the ten different strains of *R. oryzae*, a synthetic medium with glucose (10 g/l) and xylose (10 g/l) was used. All tested cultures showed consumption profiles where glucose was first depleted followed by xylose (data not shown). *R. oryzae* CBS 147.22, CBS 127.08 and CBS 264.28 produced mainly

ethanol whereas the other strains produced mainly lactic acid. Strains NRRL 395 and CBS 396.95 produced ethanol during glucose consumption and lactic acid during xylose consumption.

Another experiment where *R. oryzae* CBS 112.07, pre-grown on xylose, was cultivated in fermentation medium with a mixture of glucose and xylose (both 30 g/l) showed that glucose was first completely utilised followed by consumption of xylose (Fig. 3A). The consumption of glucose began after 11 h of incubation and after 23 h the glucose was depleted and consumption of xylose started. The rate of substrate consumption was significantly higher during glucose consumption (1.8 g/l·h^{-1}) compared with xylose consumption (0.6 g/l·h^{-1}) which is similar to results obtained by cultivations on individual carbon sources. The main fermentation products were lactic acid (33.4 g/l), glycerol (1.9 g/l) and ethanol (2.1 g/l) and, during xylose conversion, xylitol (0.5 g/l) was produced. Lactic acid production rate during glucose consumption was 0.7 g/l·h^{-1} and during xylose consumption 0.4 g/l·h^{-1}. In case of cultivation in fermentation medium with 100 g/l glucose and 30 g/l xylose (Fig. 3B), a similar profile was observed where glucose was first consumed (3.2 g/l·h^{-1}) followed by xylose (0.3 g/l·h^{-1}). Due to the higher concentration of glucose, start of xylose consumption shifted to 35 h of incubation. This indicates that the consumption of xylose by R. oryzae is repressed by glucose and is called bi-phasic or diauxic growth.

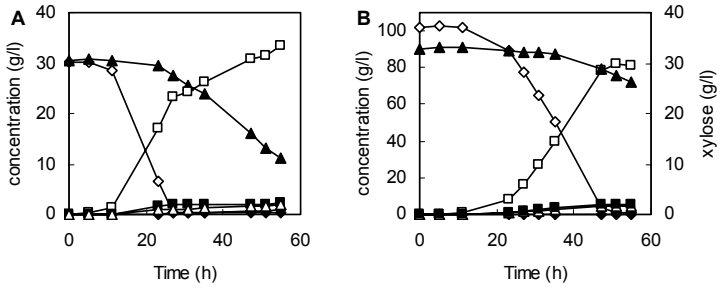

Figure 3. Conversion of sugar mixtures with (*A*) 30 g/l glucose and 30 g/l xylose and (*B*) with 100 g/l glucose and 30 g/l xylose by *R. oryzae* CBS 112.07. Substrate consumption of glucose (◇) and xylose (▲) and product formation of lactic acid (□), xylitol (◆), glycerol (△) and ethanol (■).

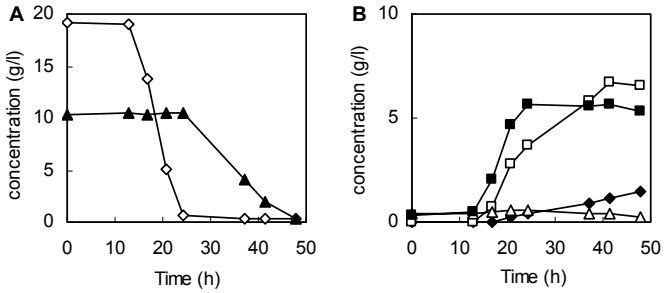

Figure 4. Fermentation of mild-temperature alkaline-treated wheat straw hydrolysate by *R. oryzae* CBS 112.07 (*A*) Substrate consumption of glucose (◇) and xylose (▲). (*B*) Product formation of lactic acid (□), xylitol (◆), glycerol (△) and ethanol (■).

Conversion of a lignocellulosic hydrolysate

Wheat straw was pre-treated by mild-temperature alkaline treatment followed by an enzymatic hydrolysis which resulted in a hydrolysate that contained a mixture of different monosaccharides released from (hemi)-cellulose. Glucose (19.2 g/l) and xylose (10.3 g/l) were the main monosaccharides present in the hydrolysate. *R. oryzae* CBS 112.07 was cultivated in the hydrolysate and substrate consumption and product formation were followed. Glucose consumption started after 13 h of incubation and xylose after 24 h. As with the glucose/xylose mixtures in synthetic media, glucose was first completely utilised followed by depletion of xylose (Fig. 4A). In the lignocellulosic hydrolysate, glucose (2.2 $g/l \cdot h^{-1}$) was also utilised faster than xylose (0.5 $g/l \cdot h^{-1}$). Lactic acid (3.7 g/l) and ethanol (5.7 g/l) were the main fermentation products during glucose consumption and lactic acid (3.1 g/l) and xylitol (1.5 g/l) during xylose consumption (Fig. 4B). Lactic acid yield was 0.23 g/g total sugar, whereas the ethanol yield was 0.19 g/g total sugar.

DISCUSSION

Lignocellulosic materials are cheap and widely available and, therefore, potentially interesting feedstocks for the production of organic acids such as lactic acid (Tsao et al., 1999). Both hemicellulose and cellulose can be hydrolysed into fermentable monosaccharides such as glucose and xylose. The biodegradable polymer PLA can be commercially competitive to petrochemical-derived plastics if both hexose and pentose sugars will be converted into lactic acid. Others described that commercial bioconversion of lignocellulose to ethanol also requires efficient fermentation of sugar mixtures including

xylose (Hinman et al., 1989). Without the conversion of xylose, the product yields are low. Because lignocellulosic hydrolysates are complex media with sugar mixtures, it is necessary to study first the conversion of sole carbon sources in synthetic media by *R. oryzae*.

The results obtained from this study showed that ten tested *R. oryzae* strains converted xylose and glucose mainly into lactic acid with yields which agrees very well with data reported by other researchers (Yang et al., 1995; Kosakai et al., 1997; Zhou et al., 1999; Park et al., 2004). Carbon recovery calculations showed differences between *R. oryzae* cultures with glucose (74 to 99%) and xylose (56 to 82%). These results suggest that, during xylose metabolism, relatively more substrate is respired into carbon dioxide, which has two possible explanations. First, a longer fermentation time enabled *R. oryzae* to take oxygen over a longer period resulting in a relatively higher contribution of respiration to cellular metabolism. Secondly, others described xylose-fermenting yeasts such as *Pichia stipitis*, *Candida shehatae* and *Pachysolen tannophilus* which follow the oxido-reductive route via xylitol in the conversion of xylose to xylulose (Hahn-Hägerdal et al., 1994). In these xylose converting cultures, respiration is required to balance the cofactors NADH and NAD^+ playing a role in the xylitol into xylulose conversion. More respiration results in higher energy generation accompanied by higher amount of biomass. More respiration and production of the intermediate xylitol also indicates that *R. oryzae* converts xylose through the oxido-reductive route but requires further research.

To achieve an efficient process with lignocellulosic hydrolysate as feedstock, variable sugar concentrations should be completely converted into products such as lactic acid. Therefore, the capability of *R. oryzae* CBS 112.07 to convert different initial xylose and glucose concentrations in synthetic media was tested. *R. oryzae* stopped converting xylose into lactic acid after approximately 40 g/l xylose was converted while carbon source was still in excess. This was not the case with different glucose concentrations where all glucose was converted into lactic acid. Moreover, at the end of the fermentation, biomass concentrations obtained with xylose were higher than biomass concentrations with glucose as substrate. This suggests that a nutrient, other than carbon source, was limiting the conversion of xylose. To convert higher concentrations of xylose, medium optimisation is required and may contribute to higher productivity and lactic acid yield.

Lignocellulosic hydrolysates were also simulated by mixing xylose and glucose in synthetic media. Subsequent or simultaneous utilization will influence the lactic acid productivity and, consequently, the total fermentation time required in converting sugars. When the ten *R. oryzae* strains were exposed to a mixture of glucose and xylose, diauxic

growth was observed with consumption of glucose first followed by xylose consumption. As shown in Fig. 3A, B, the start of xylose consumption is determined by the presence of glucose. Therefore, we can conclude that biphasic growth occurs due to repression of xylose metabolism by glucose.

In mild-temperature alkaline pre-treated wheat straw hydrolysate, glucose and xylose were converted subsequently with rates of 2.2 $g/l \cdot h^{-1}$ and 0.5 $g/l \cdot h^{-1}$, respectively, resulting into the products lactic acid and ethanol. These consumption rates agreed very well with results achieved in synthetic media, indicating that the level of inhibitors such as acetic acid (1 g/l) present in the wheat straw hydrolysate was not inhibiting the sugar consumption rates by *R. oryzae*. Due to the formation of high-concentration by-products, a relatively low lactic acid yield (0.23 g/g) was obtained. The production of the by-product ethanol by *R. oryzae* is probably due to limited oxygen transfer in the viscous hydrolysate (Skory et al., 1998). Usage of a controlled tank reactor such as an airlift fermentor can enhance gas and nutrient transfer and improve the lactic acid yield.

From the results obtained in this study, we can conclude that the filamentous fungus *R. oryzae* is an excellent microbial producer of L(+)-lactic acid from glucose. Furthermore, *R. oryzae* also converts xylose into mainly lactic acid but with a lower rate and lower yield. To produce lactic acid by *R. oryzae* from wheat straw with conversion of glucose and xylose in an economically feasible process, the consumption rate and yield of xylose must improve. The effect of higher xylose concentrations on lactic acid production and the influence of oxygen on the xylose conversion by *R. oryzae* will be further studied.

ACKNOWLEDGEMENTS

This work was supported with a grant of the Dutch Programme EET (Economy, Ecology, Technology), a joint initiative of the Ministries of Economic Affairs, Education, Culture and Sciences and of Housing, Spatial Planning and the Environment. Co-financing was provided by Program 412 Renewable Resources of the Dutch Ministry of Agriculture, Nature and Food Quality.

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CHAPTER 4

Xylose Metabolism in the Fungus *Rhizopus oryzae*: Effect of Growth and Respiration on L(+)-Lactic Acid Production

Ronald H.W. Maas, Jan Springer, Gerrit Eggink and Ruud A. Weusthuis

ABSTRACT

The fungus *Rhizopus oryzae* converts both glucose and xylose under aerobic conditions into chirally pure L(+)-lactic acid with by-products such as xylitol, glycerol, ethanol, carbon dioxide and fungal biomass. In this paper, we demonstrate that the production of lactic acid by *R. oryzae* CBS 112.07 only occurs under growing conditions. Deprivation of nutrients such as nitrogen, essential for fungal biomass formation, resulted in a cessation of lactic acid production. Complete xylose utilization required a significantly lower C/N ratio (61/1) compared to glucose (201/1), caused by higher fungal biomass yields that were obtained with xylose as substrate. Decreasing the oxygen transfer rate resulted in decline of xylose consumption rates whereas the conversion of glucose by *R. oryzae* was less affected. Both results were linked to the fact that *R. oryzae* CBS 112.07 utilises xylose via the two-step reduction/oxidation route. The consequences of these effects for *R. oryzae* as a potential lactic acid producer are discussed.

INTRODUCTION

Lactic acid and its derivatives represent an important category of compounds for industries producing food, chemical and pharmaceutical products. Lactic acid can be synthesized chemically, but this results in racemic mixtures of D and L-isomers. Chirally pure lactic acid can be produced by microbial fermentation and is required for the manufacturing of the biodegradable polymer polylactic acid, which is an alternative to petrochemically derived plastics (Drumright et al., 2000). Furthermore, the microbial production of lactic acid is of economic importance due to the prospect of using cheap and widely available feedstock materials such as lignocellulose (Åkerberg and Zacchi, 2000).

Lignocellulosic biomass and agricultural residues are rich in polysaccharides such as cellulose (~35-50%) and hemi-cellulose (~5-20%) which are embedded in a matrix of lignin (Jeffries, 1983). A broad range of physical, chemical and enzymatic treatments can be applied in order to remove/modify the lignin and to hydrolyse the polysaccharides to fermentable monosaccharides (Mosier et al., 2005). The resulting hydrolysates contain a mixture of hexoses such as glucose and pentoses such as xylose which all form potential substrates for the microbial production of lactic acid.

The Zygomycete fungus *Rhizopus oryzae* is a well known producer of chirally pure L(+)-lactic acid from glucose and starch (Yin et al., 1997; Zhou et al., 1999; Tay and Yang, 2002; Ping Huang et al., 2005) and capable to form cellulolytic and xylanolytic enzymes (Bakir et al., 2001; Murashima et al., 2002). In earlier work, we studied the conversion of xylose to lactic acid by ten different wild-type strains of *R. oryzae* (Maas et al., 2006). When cultures of *R. oryzae* CBS 112.07 were exposed to relatively high xylose concentrations (60-120 g/l), only ±40 g/l xylose was converted to mainly lactic acid. Furthermore, most of the tested *R. oryzae* xylose-converting cultures showed a lower lactic acid yield in comparison to the cultures grown on glucose as sole carbon source. Carbon dioxide as a product of aerobic respiration was found in higher amounts with the cultures grown on xylose in comparison to the ones on glucose. This indicated that throughout the conversion of xylose by *R. oryzae*, the respiratory metabolism and xylose-to-lactic acid fermentation are related.

Incomplete substrate utilization, lactate yields and the requirement of oxygen for product formation are important aspects for the suitability of *R. oryzae* as lactic acid producer. Therefore, in the present study, the influence of growth and respiration on the conversion of xylose into lactic acid was studied.

MATERIALS AND METHODS

Fungal strain and inoculum preparation

The strains *Rhizopus oryzae* CBS 112.07 (CBS-Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) and *R. oryzae* NRRL 395 (NRRL-Northern Regional Research Laboratory, Peoria, IL, USA) were grown on Potato Dextrose Agar at 30°C. Spores were harvested as described previously (Maas et al., 2006) and stored at -80°C. Mycelial biomass was produced in growth medium containing per litre: glucose, 30 g; $(NH_4)_2SO_4$, 1.25 g; KH_2PO_4, 0.6 g; $MgSO_4.7H_2O$, 0.25 g; $ZnSO_4.7H_2O$, 0.04 g; $Na_2HPO_4.2H_2O$, 5.37 g and $NaH_2PO_4.H_2O$, 9.64 g. All chemicals, unless indicated otherwise, were purchased from Merck (Darmstadt, Germany). The growth medium was prepared as described previously (Maas et al., 2006), inoculated with $2 \cdot 10^5$ spores per ml and incubated in a rotary shaker with an agitation rate of 150 rpm at 37°C. Mycelial biomass was harvested by a wash treatment and served as inoculum for shake flask experiments (Maas et al., 2006).

Shake flask experiments

Composition of the fermentation medium used in the baffled Erlenmeyer shake flask cultures was similar to the growth medium except sodium phosphate salts were replaced by 52 g/l $CaCO_3$ (SA Omya, Benelux NV). A filter sterilised xylose (Sigma-Aldrich, Germany) or glucose solution served as carbon source (cellulose acetate filter with pore size 0.2 µm, Minisart, Sartorius). Fermentation medium was inoculated with washed mycelial biomass (0.1 gram mycelial biomass (dry weight) per litre). Cultures were incubated in a rotary shaker with an agitation rate of 150 rpm and 37°C. Homogenous samples of 1 ml were withdrawn and analyzed for substrate and product concentration by High Pressure Liquid Chromatography.

Preparation of cell-free extract

Fungal cells cultivated in growth medium were centrifuged (5 min at 3000 rpm) and the wet pellet was stored at -80°C. Prior to use, the pellet was washed with 0.85% NaCl solution to remove residual medium components. The cell-free extract was prepared by freezing the washed cells in liquid nitrogen and grinding in a mortar. An extraction solution containing Bis-Tris buffer pH 6.5, 20 mM; $MgCl_2$, 5 mM and ß-mercaptoethanol, 1 mM, was added to the powder (1:1 *w/v*). After extraction for one hour, the protein suspension was centrifuged for 15 minutes at 14000×g and 4°C to obtain a clear supernatant which serves as a source of intracellular enzymes.

Enzyme assays

Xylose reductase (XR) and xylitol dehydrogenase (XDH) activity assays were performed in 200-µl volumes. Oxidation of cofactors was monitored in a temperature controlled spectrophotometer by measuring absorbance at 340 nm. XR activity in cell-free extract (20 µl) was assayed in a sodium phosphate (pH 6.5, 50 mM) buffered mixture containing D-xylose (100 mM) as substrate and NADPH (0.2 mM) as cofactor (Witteveen et al., 1989). The XDH activity in cell-free extract (20 µl) was assayed in a PIPES KOH (pH 7.0, 50 mM) buffered mixture containing D-xylulose (10 mM) as substrate and NADH (0.2 mM) as cofactor (Richard et al., 1999). Background activities (assays without xylose or xylulose) were also measured and included into the calculations. One unit of enzyme activity corresponds to 1 nmol of cofactor converted per mg cell free extract protein per minute.

Xylose isomerase (XI) activity was tested in 1-ml volumes by incubating 20 µl cell-free extract in a 50 mM sodium phosphate buffered (pH 7.0) mixture containing D-xylose (10 mM) and $MgCl_2$ (10 mM) for 17 h at 37°C (Harhangi et al., 2003). The enzymatic activity was stopped by adding 0.05 ml 50% trichloroacetic acid and xylulose was assayed by the cysteine-carbazole-sulphuric acid method (Dische and Borenfreund, 1951). In order to determine the protein content, the proteins in the cell-free extract were separated from the extraction buffer by centrifugation over a membrane (Microcon YM-3, Millipore, USA) and resolved in de-ionized water. The protein content was analysed by the BCA Protein Assay Kit (Pierce, USA) using diluted bovine serum albumin as standard.

Evaluation of gene sequences encoding enzymes involved in xylose metabolism

As part of the Fungal Genome Initiative at the Broad Institute of Harvard and MIT the genome sequence plus automated annotation from the *Rhizopus oryzae* strain RA 99-880 has been released on http://www.broad.mit.edu/annotation/fungi/rhizopus_oryzae/ . This database enabled us to search for putative genes encoding the enzymes involved in xylose metabolism in *R. oryzae*. Enzymes of interest are XR (EC 1.1.1.21), XDH (EC 1.1.1.9) and xylulose kinase (XK) (EC 2.7.1.17) catalyzing the conversion from xylose to xylulose-5-P via xylitol and xylulose, respectively, and XI (EC 5.3.1.5) which converts xylose directly in xylulose. A selection of sequences obtained from NCBI and EMBL databases was used to search the *R. oryzae* genome using the 'tblastn' algorithm. For XR, XDH and XK, genes from fungi were selected to create a high probability of finding the corresponding gene. Accession numbers of XR were: *Pichia stipitis* (X59465), *Neurospora crassa* (AY876382), *Aspergillus niger* (AF219625), *Candida sp.* (AY854501). Accession numbers of XDH were: *Pichia stipitis* (A16166), *Neurospora crassa* (XP_325071), *Aspergillus oryzae* (AB109101), *Candida albicans* (XP_719434). Accession numbers of XK were: *Piromyces sp.* (AJ249910), *Pichia stipitis* (AAF72328), *Aspergillus niger* (AJ305311), *Neurospora*

crassa (EAA33587), *Candida sp.* (DQ087275). Since no fungal genes other than the *Piromyces* sp. gene (Harhangi et al., 2003) are known, the search for XI was also performed using genes from different types of organisms having accession numbers: *Piromyces sp.* (AJ249909), *Arabidopsis thaliana* (Q9FKK7), *Xanthomas campestris* (Q8P9T9), *Streptomyces albus* (P24299), *Escherichia coli* (P00944), *Bacillus subtilis* (P04788), *Lactobacillus brevis* (P29443).

Analytical methods

The treatment of samples and analysis of monosaccharides, organic acids, alcohols and polyols were performed by High Pressure Liquid Chromatography as described earlier (Maas et al., 2006). The cell dry weight determination was performed by filtration of fermentation broth over a pre-weighed filter and drying overnight (Maas et al., 2006). The ammonium concentration in fermentation samples was analysed with an Ammonium Test (Spectroquant, Merck, Germany). The chiral purity (%) of lactic acid was determined by derivatization of all lactates using methanol, after which both enantiomers of methyl lactate were separated on a chiral Gas Chromatography column and detected using a Flame Ionization Detector. The chiral purity was expressed as the area of the main enantiomer divided by the sum of areas of both enantiomers.

Calculations

The maximal volumetric consumption rate ($q_s max$) is defined as the amount of sugar consumed (g) per litre per hour whereas the maximal volumetric production rate ($q_p max$) can be defined as amount of product formed (g) per litre per hour. The yield of lactic acid ($Y_{p/s}$) is based on the amount of product synthesised (g) divided by the amount of substrate consumed (g). Biomass yield ($Y_{x/s}$) is calculated by the amount of biomass (g dry weight) divided by the amount of substrate consumed (g). The carbon recovery is calculated with respect to moles of carbon produced divided by moles of carbon consumed. Carbon recovery calculations were made to estimate carbon dioxide production by respiration. In these calculations, ethanol was considered to be a 3-carbon compound to compensate for equimolar carbon dioxide formation in ethanol synthesis.

RESULTS

Effect of nutrient limitation on sugar utilization and product formation by R. oryzae

As described in previous work, cultures of *R. oryzae* CBS 112.07 grown in fermentation media with relatively high xylose concentrations (60-120 g/l) decreased the consumption rate when approximately 40 g/l was converted, although sufficient carbon source was

available (Maas et al., 2006). This result suggests that a nutrient, other than xylose, was limiting. According to the composition of the fermentation medium and an assumed fungal biomass composition of $CH_{1.8}O_{0.5}N_{0.16}S_{0.0045}P_{0.0055}$, it was calculated that nitrogen was the limiting compound.

To study the effect of N-limitation on the lactic acid production from xylose by *R. oryzae*, the fungus was cultivated in fermentation medium containing 80 g/l xylose and 1.25 g/l $(NH_4)_2SO_4$ corresponding to a C/N ratio of 121/1 (g/g) (Fig. 1A). After 115 hours of incubation, approximately 40 to 45 g/l xylose was converted into chirally pure L(+)-lactic acid (21.1-24.4 g/l, Fig. 1B). So, for complete xylose conversion, a C/N ratio of approximately 61/1 is required. The by-products glycerol (3.5-4.0 g/l, Fig. 1C) and xylitol (1.0-1.2 g/l, Fig. 1E) were produced at lower levels. Between 115 and 160 hours of incubation, the consumption of xylose by *R. oryzae* was continued at a low linear rate. To test if this was caused by N-limitation, cultures were pulsed with 2.5 ml $(NH_4)_2SO_4$ solution, $(NH_4)_2PO_4$ solution (both 50 g/l) or demi water (blank) after 160 h incubation.

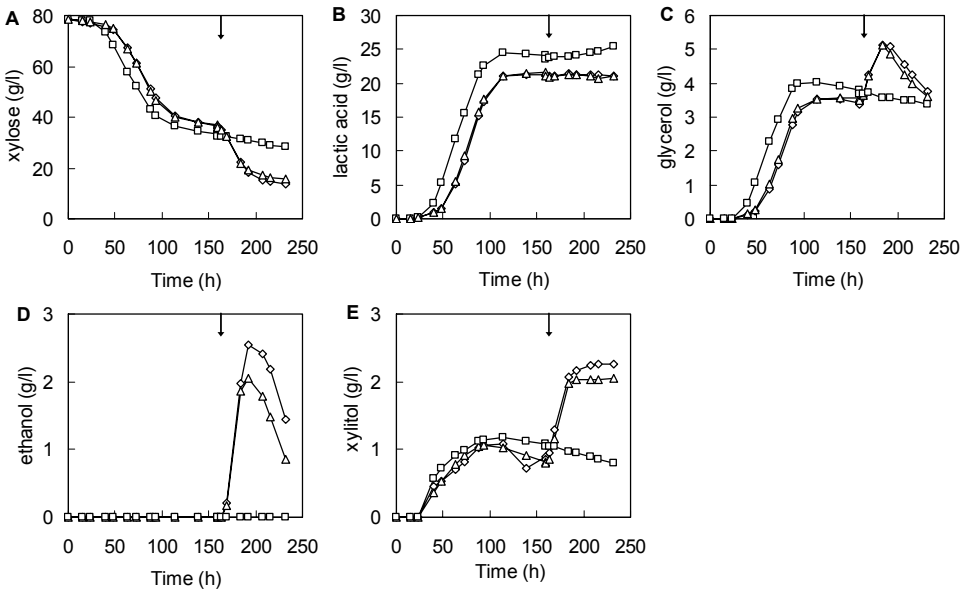

Figure 1. Conversion of xylose (*A*) by *R. oryzae* CBS 112.07 to lactic acid (*B*), glycerol (*C*), ethanol (*D*) and xylitol (*E*). The arrow represent the moment of an extra addition of $(NH_4)_2SO_4$ (◇), $(NH_4)_2PO_4$ (△) and blank with demi water (□).

In comparison to the blank, the ammonium pulsed cultures both showed an accelerated xylose consumption rate. Production rates of glycerol (Fig. 1C), ethanol (Fig. 1D) and xylitol (Fig. 1E) increased whereas no significant lactic acid production (Fig. 1B) was determined after ammonium addition. After 190 hours of incubation, the glycerol and ethanol concentrations decreased, suggesting these compounds can serve as carbon source for *R. oryzae* and can be used simultaneously with xylose. At the end of the fermentation (240 h), the biomass concentration of the $(NH_4)_2SO_4$ and $(NH_4)_2PO_4$ pulsed cultures was 10.2 and 7.6 g/l, respectively, corresponding to a biomass yield ($Y_{x/s}$) of 0.16 and 0.12 g/g. These biomass concentrations - in the cultures with double ammonium concentration - were approximately twice as high in comparison to the biomass concentration obtained in the culture without the extra ammonium addition.

Former results showed that cessation of product formation, which was observed during growth on xylose and can now be attributed to nitrogen limitation, did not occur under further identical conditions with glucose as substrate (Maas et al., 2006). However, utilization of glucose as carbon source resulted in lower microbial biomass formation compared to xylose, indicating that N-limited conditions might not yet have been established. To investigate this, *R. oryzae* was cultivated in glucose containing media with higher C/N ratios. The cultures with C/N ratio of 201/1 and 151/1 converted glucose completely within 70 hours of incubation into 74 and 75 g/l chirally pure L(+)-lactic acid (Fig. 2A, 2B), respectively, indicating no N-limitation. The glucose consumption rate of *R. oryzae* cultivated at higher C/N ratios of 603/1 and 302/1, declined after consumption of 15 and 60 g/l glucose, respectively. By-products such as ethanol (Fig. 2C) and glycerol (Fig. 2D) were formed at different levels depending on the C/N ratio with a maximum of 4.5 and 5.3 g/l, respectively. The addition of extra $(NH_4)_2SO_4$ restored glucose consumption indicating N-limitation. The concentration of glycerol and ethanol decreased after glucose was completely consumed, suggesting catabolite repression by glucose. Figure 2F shows that whereas growth and lactic acid production continued up to 65 h, in all tested *R. oryzae* cultures the concentration of $(NH_4)_2SO_4$ was already nearly depleted from the medium after 44 hours of incubation.

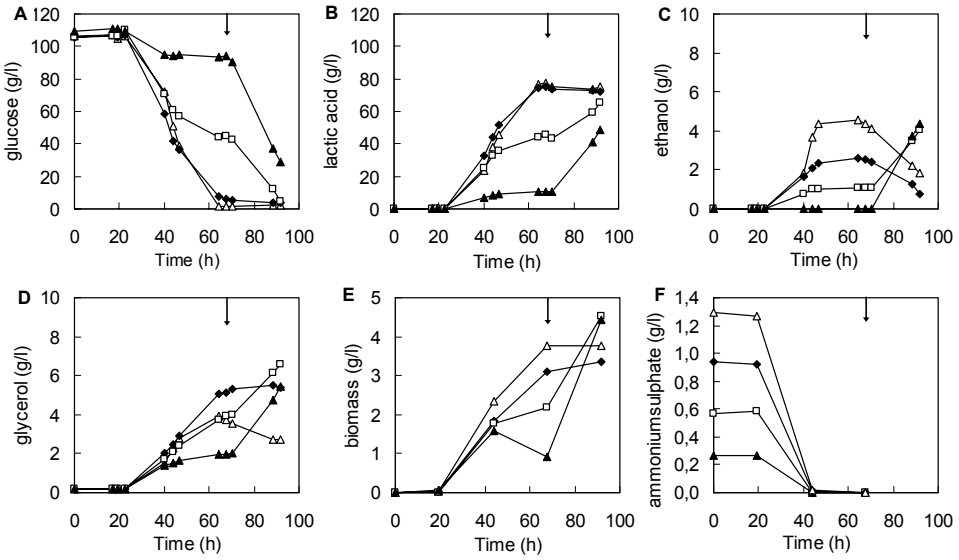

Figure 2. Conversion of glucose (*A*) by *R. oryzae* CBS 112.07 to lactic acid (*B*), ethanol (*C*), glycerol (*D*) and fungal biomass (*E*) with initial C/N ratio of 603/1 (0.31 g/l $(NH_4)_2SO_4$) (▲), 302/1 (0.63 g/l $(NH_4)_2SO_4$) (□), 201/1 (0.94 g/l $(NH_4)_2SO_4$) (◆) and 151/1 (1.25 g/l $(NH_4)_2SO_4$) (△). Figure *F* represent the $(NH_4)_2SO_4$ concentration in the fermentation medium. The arrow represent the moment of addition of ammonium sulphate.

A possible explanation is intracellular accumulation of nitrogen or the formation of reserve material such as glycogen. Furthermore, after 70 h of incubation and prior to the ammonium pulse, the cultures with C/N ratios of 608/1, 302/1, 201/1 and 151/1 showed biomass concentrations of 0.9, 2.2, 3.1 and 3.8 g/l (Fig. 2E), respectively, corresponding to a biomass yield ($Y_{x/s}$) between 0.03 and 0.06 g/g. To study if growth-associated product formation is a more general phenomenon for *R. oryzae*, it was also tested for *R. oryzae* CBS 112.07 with glucose under P-limitation and for *R. oryzae* NRRL 395 with xylose as substrate under N-limitation. Also, in these cases the conversion of substrate to lactic acid stopped when growth limiting conditions were reached (results not shown).

Thus, lactic acid production from both glucose and xylose by *R. oryzae* CBS 112.07 only occurs under growing conditions. Furthermore, in comparison to glucose, conversion of xylose by *R. oryzae* required a lower C/N ratio due to a higher biomass yield. This indicates that with xylose as substrate the energetically more efficient respiration plays a more important role compared to the situation with glucose. The higher respiratory flux could be related to the occurrence of the reduction/oxidation pathway of xylose utilization in *R. oryzae* as suggested by subsequent production and consumption of xylitol. In order to better understand the relation between growth, aerobic respiration and lactic acid formation via fermentation, the initial steps in the xylose metabolism of *R. oryzae* were identified.

Identification of enzymes involved in xylose metabolism by R. oryzae

Most eukaryotic organisms use a two-step reduction/oxidation route to convert xylose via xylitol to xylulose. Some exceptions have been reported where yeast and fungal species employ the isomerase route (XI) (Schneider, 1989; Banerjee et al., 1994; Harhangi et al., 2003). As proposed in Figure 3, the first enzyme involved in the two-step reduction/oxidation route is the NADPH-dependent xylose reductase (XR) catalyzing the conversion of xylose to xylitol. The polyol xylitol can be excreted or oxidised to xylulose by a NAD-dependent xylitol dehydrogenase (XDH). Further metabolism is from that point on identical, starting with the phosphorylation of xylulose to xylulose-5-phosphate by xylulose kinase (XK) and further converted to glyceraldehyde-3-phosphate via the pentose phosphate pathway. The resulting intermediate pyruvate can function as substrate for fermentation via the glycolytic pathway to lactic acid or ethanol, for biomass formation or for oxidation to carbon dioxide and water via respiratory processes (Jeffries, 1983; Schneider, 1989).

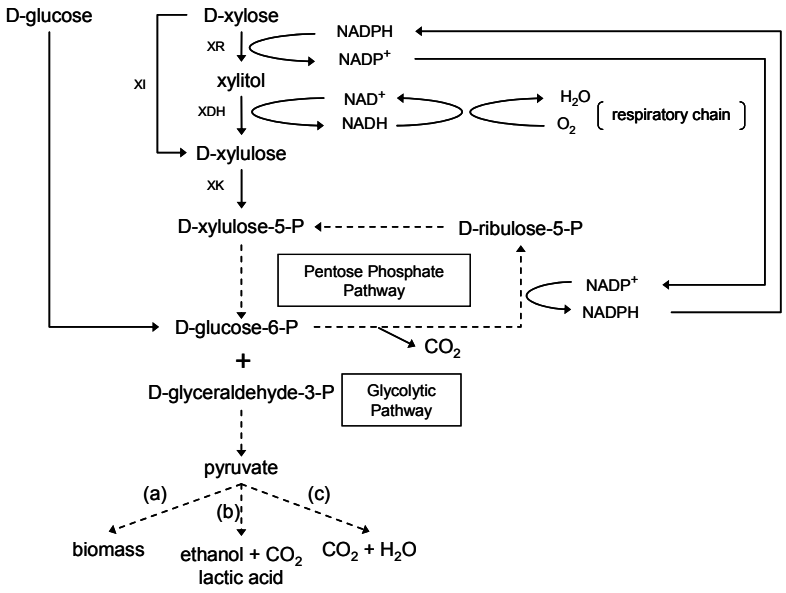

Figure 3. Proposed pathways for the catabolism of xylose, adapted from Schneider (1989). XI, xylose isomerase; XR, xylose reductase; XDH, xylitol dehydrogenase; XK, xylulose kinase. Pyruvate functions as substrate for growth (a), fermentation (b) and/or respiration (c).

TABLE 1. In vitro analysis of xylose reductase (XR), xylitol dehydrogenase (XDH) and xylose isomerase (XI) activity in cell-free extracts of glucose or xylose-converting *R. oryzae* CBS 112.07.

C-source	Enzyme activity		
	XR [a]	XDH [a]	XI
Xylose	75.1	0.53	ND
Glucose	ND	ND	ND

[a]One unit of activity is defined as nmol of NADPH or NADH converted per mg total cell-free extract protein per minute; ND Not Detected.

To identify the presence of the isomeration route and/or the two-step reduction/oxidation route in *R. oryzae* CBS 112.07, activities of XI, XR and XDH were assayed by *in vitro* analysis. Cell-free extracts of *R. oryzae* CBS 112.07 cultivated in growth medium containing 30 g/l xylose or glucose were used for the enzyme assays. In the glucose culture, *R. oryzae* converted approximately 30 g/l glucose to 13.5 g/l lactic acid within 48 h of incubation whereas the fungus grown in xylose-containing medium utilises approximately 10 g/l xylose and produces 4.5 g/l lactic acid. The cell-free extract made of fungal biomass cultivated with glucose contained soluble proteins without detectable XI, XR or XDH activity. On the other hand, as shown in Table 1, the cell-free extract of the xylose-converting culture contained detectable XR and XDH activity whereas no XI activity was analysed, indicating the presence of the two-step reduction/oxidation route in *R. oryzae*.

The rDNA sequence of *R. oryzae* strain RA 99-880 showed 100% homology with strain CBS 112.07 (Abe et al., 2006) whereas a lower homology (~80%) was obtained when comparing with other eukaryotes such as *S. cerevisiae* and *Trichoderma*. This indicates that the sequence of strain RA 99-880 is representative for strain CBS 112.07. A selection of sequences obtained from NCBI and EMBL databases was used to search the *R. oryzae* genome using the 'tblastn' algorithm. The Blast search on XR (Locus RO3G_16589.1; E-value 3e-100) revealed 6 possible transcripts (>38% amino acid identity) in the *R. oryzae* genome all belonging to the aldo/keto reductase family (Pfam domain search). The *R. oryzae* transcript with the highest homology with the target sequences (47-53% amino acid identity) is likely to be a XDH (Locus RO3G_02257.1; E-value 9e-78). The Blast search on XDH revealed 4 possible transcripts (>30% amino acid identity) in the *R. oryzae* genome all harboring an alcohol dehydrogenase GroES-like domain and zinc-binding dehydrogenase domain (Pfam domain search). The transcript with the highest homology with the target sequences (44-46% amino acid identity) is likely to be a XDH. The Blast search on XK revealed 1 possible transcript (>36% amino acid identity) in the *R. oryzae* genome harboring the FGGY family of carbohydrate kinases, N-terminal domain and the FGGY family of carbohydrate kinases, C-terminal domain (Pfam domain search). This transcript is likely to be a XK (Locus RO3G_11716.1; E-value 2e-130). The Blast search on XI revealed no possible transcripts in the *R. oryzae* genome. It seems likely that no gene

encoding this enzyme is present in *R. oryzae*. The findings of this molecular analysis confirm the results obtained by the *in vitro* enzyme analysis indicating the presence of the two-step reduction/oxidation route in *R. oryzae* CBS 112.07.

Effect of oxygen transfer rate on metabolism of R. oryzae

Conversion of xylose via the two-step reduction/oxidation route suggests an enhanced negative effect of oxygen limitation on sugar consumption and product formation compared with glucose (Schneider, 1989; Hahn-Hägerdal et al., 1994). In order to study the influence of oxygen transfer rate (OTR) on the conversion of glucose and xylose by *R. oryzae*, reactors with monitoring and control of the oxygen concentration are preferably used. However, as described in previous work (Maas et al., 2006), growth of the filamentous fungus *R. oryzae* in submerged controlled stirred fermentor often resulted in heterogeneous growth with mycelium attached around elements in the reactor such as baffles, electrodes and impellers. Therefore, to examine the effect of OTR on the conversion of glucose and xylose by *R. oryzae* CBS 112.07, another experimental set-up was used where the fungus was cultivated in different medium volumes of 75, 100, 125 and 150 ml.

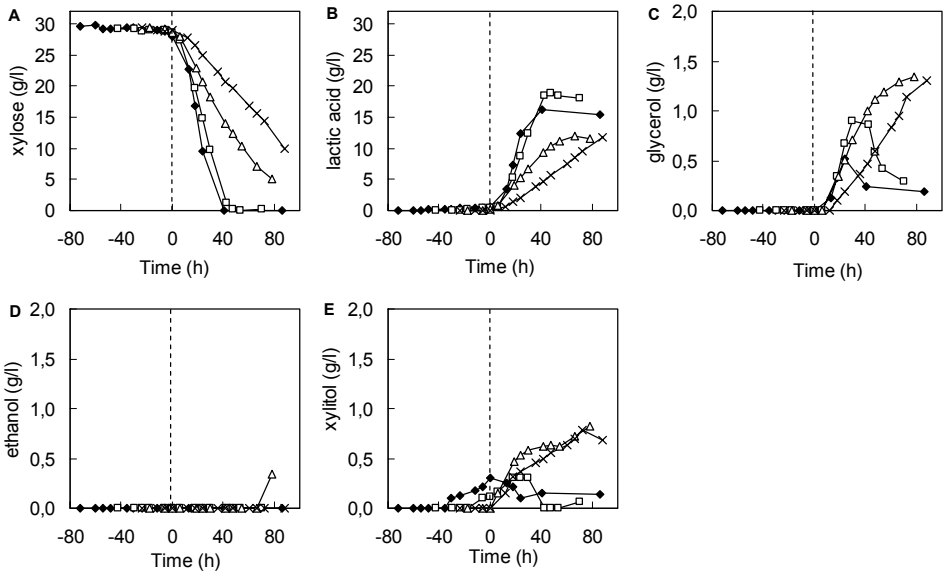

Figure 4. Effect of different medium volumes (aeration rates) on the conversion of xylose (*A*) by *R. oryzae* CBS 112.07 to lactic acid (*B*), glycerol (*C*), ethanol (*D*) and xylitol (*E*) in 250-ml baffled shake flasks with medium volumes of 75 (◆), 100 (□), 125 (△) and 150 ml (x). Dotted lines represent the start of detectable consumption of xylose.

TABLE 2. Fermentation data of *R. oryzae* CBS 112.07 cultures grown in 250-ml baffled shake flasks with variable culture volumes containing 30 g/l xylose or glucose.

C-source	Medium volume (ml)	Lactic acid (g/l)[a]	$q_s max$ (g/l/h)[b]	$q_p max$ (g/l/h)[b]	$Y_{p/s}$ (g lactic acid/g)[a]	Carbon recovery (% C-mol)[a]
Xylose	75	16.1	1.16	0.78	0.55	66
	100	18.8	0.73	0.60	0.65	83
	125	12.0	0.35	0.22	0.54	70
	150	16.2	0.22	0.16	0.59	81
Glucose	75	21.6	1.77	1.53	0.71	96
	100	22.3	2.50	1.97	0.77	98
	125	23.5	2.50	1.97	0.78	90
	150	23.2	1.96	1.57	0.79	94

[a]Calculations with data obtained when at least 83% of xylose and 98% of glucose was consumed; [b]Rates calculated over at least three data points.

By altering the volume of the medium in baffled Erlenmeyer flasks, the oxygen transferring liquid/air surface can be varied (Koning de et al., 1990). In shake flask cultures, we used the carbon recovery calculations in order to estimate the carbon dioxide produced by respiration. Decreasing culture volumes resulted in significantly increased xylose consumption and lactic acid production rates by *R. oryzae* (Fig. 4A, 4B). Table 2 shows the maximal xylose consumption rate, $q_s$max of 0.22 to 1.16 g/l/h and the maximal lactic acid production rate, $q_p$max of 0.16 to 0.78 g/l/h by *R. oryzae* in different culture volumes. The cultures with volume of 125 and 150 ml resulted, in comparison to the cultures in 75 and 100 ml, in higher concentrations of glycerol and xylitol (Figures 4C, 4E). The concentration of these fermentation products decreased at the moment that xylose nearly depleted, indicating that the fungus utilises glycerol and xylitol as carbon source. The lactic acid yields ($Y_{p/s}$) varied between 0.54 and 0.65 g/g where the culture of 100 ml yielded the highest amount of lactic acid. The relatively low carbon recoveries show that a significant amount of carbon substrate utilization can be ascribed to respiratory CO_2 production (missing carbon in this calculation).

With glucose as carbon source the situation is different. Figures 5A and 5B show that the conversion rate of glucose into lactic acid by *R. oryzae* is less affected by the tested culture volume and therefore by the OTR. Lactic acid yields of 0.71 to 0.79 g/g were calculated and are significantly higher than the yields obtained with xylose (Table 2). By-products such as glycerol (0.3-0.5 g/l) and ethanol (1.2-1.5 g/l) were formed in all cultures up to comparable levels (Fig. 5C, 5D). When glucose was depleted, the formerly produced glycerol and ethanol concentrations decreased again. Table 2 shows that in comparison to the data obtained with xylose, significantly higher maximal consumption rates (1.77-2.50 g/l/h), lactic acid production rates (1.53-1.97 g/l/h) and lactic acid yields (0.71-0.79 g/g) were obtained with glucose as carbon source. Furthermore, the carbon recovery data were higher in case of glucose cultures (~95%) compared to xylose cultures (~75%) suggesting less conversion of substrate to carbon dioxide.

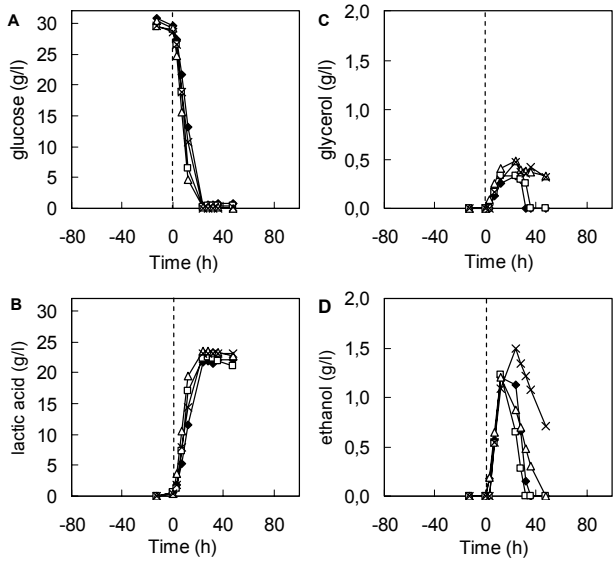

Figure 5. Effect of different medium volumes (aeration rates) on the conversion of glucose (*A*) by *R. oryzae* CBS 112.07 to lactic acid (*B*), glycerol (*C*) and ethanol (*D*) in 250-ml baffled shake flasks with medium volumes of 75 (◆), 100 (□), 125 (△) and 150 ml (x). Dotted lines represent the start of detectable consumption of glucose.

DISCUSSION

In a previous article we reported that *R. oryzae* is able to convert xylose into lactic acid and ethanol. Compared to glucose utilization, the conversion of xylose requires additional enzymatic steps. In this study, we showed several results indicating that *R. oryzae* utilizes xylose via the two-step reduction/oxidation route.

First, *in vitro* enzyme analysis of cell-free extracts, made from xylose-converting *R. oryzae* biomass, showed activity of XR and XDH whereas XI activity was lacking. The activity of XR in the cell-free extract was approximately 150-fold higher in comparison to the XDH activity. A possible explanation for this discrepancy in activity can be found in the different hydrophilic characters, determined by the approach described by Kyte and Doolittle (1982) (Kyte and Doolittle, 1982), which seems significantly higher for XR than for XDH. As a result, the used extraction procedure may have caused some loss of XDH activity. Furthermore, the stability of the enzymes throughout the extraction procedure was not tested and therefore unknown. Anyhow, detection of XR and XDH indicates the presence of the two-step reduction/oxidation route in *R. oryzae*.

Secondly, results from previous study showed that *R. oryzae* CBS 112.07 secreted xylitol throughout xylose conversion (Maas et al., 2006). As soon as the xylose nearly

depleted from the medium, the fungus started to utilise the formerly produced xylitol from the medium. Furthermore, *R. oryzae* cultures converted xylitol, as sole carbon source, to mainly lactic acid (data not shown). As shown in Figure 3, xylose-fermenting micro-organisms can produce xylitol from xylose either by XR activity or, more unlikely, via activities of XI and XDH. Therefore, the formation and utilisation of xylitol by *R. oryzae* strongly implies the presence of the two-step reduction/oxidation route to convert xylose.

Finally, comparison of the genome sequence of *R. oryzae* strain RA 99-880 – representative for the used strain CBS 112.07 based on rDNA sequence homology – and sequences of genes representing XR and XDH from the NCBI and EMBL databases showed the presence of genes encoding these enzymes. No homology was found between the genes representing XI and the genome sequence of strain RA 99-880 suggesting once more that the two-step reduction/oxidation route is present in *R. oryzae*.

The two-step reduction/oxidation route bas been extensively investigated for the production of ethanol by various xylose utilizing yeast species, e.g. *Pachysolen tannophilus* and *Candida shehatae* (Jeffries, 1983; Schneider, 1989; Hahn-Hägerdal et al., 1994). A consequence of the difference in co-factor dependency of the two key enzymes is that both reactions do not balance out each others co-factor requirement. Other metabolic routes have to be applied for this purpose: the pentose phosphate cycle to regenerate NADPH used by XR, and oxygen-requiring respiration to regenerate the NAD used by XDH (Fig. 3). Whereas xylose conversion requires the input of oxygen, ethanol production in these yeasts only occurs under oxygen limitation, with higher ethanol yields at stronger oxygen limitation. Consequently, ethanol production by these yeasts has to occur under suboptimal conditions for both xylose conversion and ethanol production. This rendered the conversion of xylose into ethanol by yeasts harbouring the two-step reduction/oxidation route - both wild type and genetically engineered strains - uneconomical, at least until xylose utilization by a genetically engineered *S. cerevisiae* was realised, transformed with a gene encoding XI (Kuyper et al., 2004).

In our study, a comparable effect of oxygen transfer rate on xylose utilisation and product formation was found for *R. oryzae*. Decreasing oxygen transfer rate had no significant effect on glucose consumption, but strongly affected the xylose uptake rate. Furthermore, with glucose as substrate ethanol was formed under all experimental conditions whereas with xylose as substrate ethanol production was absent in most cases. So, the disadvantage of the two-step reduction/oxidation pathway for ethanol production from xylose also seems to hold for *R. oryzae*. This is however not the case for lactic acid since it is produced both under strict aerobic and oxygen limiting conditions.

Our results showed that the conversion of xylose by *R. oryzae* strain CBS 112.07 and NRRL 395 not only depends on the availability of a carbon source in the medium but also on the presence of other nutrients, such as nitrogen and phosphorous. This occurred for both xylose and glucose, but at different C/N ratios. These different C/N ratios can be explained by the higher biomass yield on xylose compared to glucose. This, in turn, is caused by the fact that with xylose as substrate respiration is necessary for the re-oxidation of NADH generated by xylitol dehydrogenase. Due to the higher energetic efficiency of respiration compared to lactic acid production more fungal biomass can be produced from the same amount of sugar. Or, in other words, less sugar (C) is necessary to convert the same amount of ammonium (N) into biomass, resulting in lower C/N ratios for complete sugar utilization.

Product formation, limited to conditions at which growth occurs, has - as far as we are aware of - not been reported for a fermentation product as lactic acid. In its natural habitat (mostly soil*)*, *R. oryzae* will not encounter the high sugar concentrations used in our experiments. Its regulation of sugar metabolism might therefore not be adapted to these situations resulting in lactic acid production as a consequence of overflow metabolism (Magnuson and Lasure, 2004). One could hypothesize that during growth under carbon and energy limitation tight regulation of sugar uptake and/or glycolysis is not necessary because they are rate-limiting anyhow, whereas under non-growing conditions a tight regulation is more important to prevent spillage of resources. This could be an explanation of the growth associated lactic acid production by *R. oryzae*. Growth associated product formation could prove to be a disadvantage for using *R. oryzae* for lactic acid production since compulsory fungal biomass synthesis has a negative impact on lactic acid yield. The extent of this negative influence depends on the biomass yield on glucose and xylose. In general, the biomass yield for glucose is rather low, 0.03-0.06 g/g, resulting in lactic acid yields of 0.7-0.8 g/g. This corresponds well with lactate yields for homolactic lactobacilli (Longacre et al., 1997). The biomass yield on xylose is higher due to the relatively higher contribution of respiration to cellular energy generation, resulting in considerably lower lactate yields of 0.55-0.65 g/g. However, considering the fact that lignocellulosic hydrolysates generally contains about two times higher glucose than xylose concentrations, the negative effect of growth on lactate production from xylose will have a minor impact on overall lactic acid yield.

Current industrial lactic acid production occurs at near neutral pH. At this pH not lactic acid but Ca-lactate is formed. By treatment with sulphuric acid this is converted into lactic acid with $CaSO_4$ (gypsum) as by-product (Vaidya et al., 2005). At the scale necessary for lactic acid as food product it is still possible to deal with gypsum as by-product. However, if lactic acid is to be used at larger scale as building block for the chemical industry it

would be advantageous to perform the fermentation process at lower pH, producing lactic acid itself directly. The strains used in contemporary processes are not able to produce lactic acid at these low pH values. Alternatives are sought among yeasts and fungi able to grow at lower pH, e.g. *R. oryzae* (Tay and Yang, 2002). It however appears that, as reviewed by van Maris et al. (2004), cellular export of weak acids such as lactic acid at low pH proceed via active transport and costs metabolic energy (1 ATP/acid). This would imply that at low pH lactic acid production does not result in net energy gain and that lactic acid production should always be accompanied by energy generating processes like alcoholic fermentation or aerobic respiration in order to meet maintenance requirements. So, whereas the isomerase route for xylose conversion is the pathway of choice for synthesis of ethanol because it can function without oxygen input, the situation is not as clear cut in case of lactic acid production at low pH since this requires oxygen for energy generation anyway.

R. oryzae is considered to be a potential production organism for lactic acid with advantages compared to lactic acid bacteria, its ability to convert xylose homolactically, being able to grow without complex nutrients and with a high chiral purity of the produced lactic acid (Maas et al., 2006). Because *R. oryzae* is able to grow at low pH, the possibility to use this micro-organism for the synthesis of lactic acid at low pH has been discussed.

The experiments described in this paper show that lactic acid formation is growth associated. This can be considered as a disadvantage for industrial production; however, it was shown that due to the relatively low biomass yields this will have only minor consequences for the conversion of lignocellulosic hydrolysates into lactic acid.

Lactic acid production by *R. oryzae* occurs under aerobic conditions. This can be considered to be a disadvantage, since the productivity of the industrial process may become limited by oxygen transfer. However, if lactic acid production at low pH does not generate net ATP as described by van Maris et al. (2004), lactic acid production has to occur under aerobic conditions anyway.

ACKNOWLEDGEMENTS

This project is supported with a grant of the Dutch Programme EET (Economy, Ecology, Technology) a joint initiative of the Ministries of Economic Affairs, Education, Culture and Sciences and of Housing, Spatial Planning and the Environment. The programme is run by the EET Programme Office, SenterNovem. The authors thank J. Voogt (AFSG, Wageningen, The Netherlands) for his contribution to this work and D. Visser (PURAC, Gorinchem, The Netherlands) for chiral lactic acid purity determination.

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CHAPTER 5

Conversion of Lime-Treated Wheat Straw into Ethanol: Evaluation and Comparison of SHF and SSF

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ABSTRACT

The conversion of lime-treated wheat straw to ethanol was studied by separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) routes with a commercial cellulase preparation and the yeast *Saccharomyces cerevisiae*. The objective was to evaluate reaction conditions and to compare both routes with regard to hydrolysis rates and yields. At optimal hydrolysis reaction conditions (50°C, pH 5.0 and 4.5) the highest tested enzyme concentration (169.1 mg/g dry matter substrate) resulted after 72 h of incubation in glucan-to-glucose and xylan-to-xylose yields of 56 and 67%, respectively, of the theoretical maximum. Acetic acid (2.7 g/l) present in lime-treated wheat straw seemed to inhibit the yeast fermentation at 37°C drastically and was reduced to 0.4 g/l by a simple washing procedure. This resulted in an improved glucan-to-ethanol yield after 72 h of fermentation of 47% of the theoretical maximum. Under optimized reaction conditions and at a relatively high enzyme concentration (141 mg/g dry matter substrate), comparable volumetric ethanol productivities of 0.25 g/l/h and glucan-to-ethanol conversions of 55% of theoretical maximum yield were achieved after 56 h of incubation by the SHF and SSF processes whereas xylan was converted slightly better to xylose by SHF (42%) in comparison to SSF (28%).

INTRODUCTION

Because the world-wide demand and costs of petrochemically derived energy are increasing, alternative resources for production of energy become more attractive. Ethanol as transport fuel made from renewable biomass has been considered as a clean alternative to fossil fuels (Lin and Tanaka, 2006). The carbon dioxide produced from ethanol combustion is taken up by growing plants through a short-cycle. Hence, the substitution of fossil fuels by ethanol will reduce net carbon dioxide emissions and will therefore contribute less to global warming (Claassen et al., 1999; Ogbonna, 2004).
Lignocellulosic biomass obtained from agro-industrial processes can be used as potential feedstock for ethanol production by using microbial fermentation (Galbe and Zacchi, 2002; Ogbonna, 2004). Lignocellulose is a renewable resource, relatively inexpensive and available throughout the world. The composition of lignocellulose materials such as wheat straw is complex and consists of the carbohydrate polymers cellulose and hemicellulose which are embedded in a matrix of lignin (Claassen et al., 1999).

The conventional process for converting lignocellulose into ethanol requires a pretreatment step, enzymatic hydrolysis and microbial fermentation. Ethanol can be recovered from the fermentation broth by distillation whereas the non-fermentable fraction (mainly lignin, some residual carbohydrates and microbial biomass) can be used as feed for combustion yielding heat and power (necessary in other parts of the process and making the whole process net energy sufficient) (Galbe and Zacchi, 2002). The pretreatment process generally consists of a mechanical and chemical treatment to reduce particle size and to increase surface area, to modify and/or remove lignin, and to make the crystalline cellulose structure more accessible for enzymatic hydrolysis (Mosier et al., 2005). Chang et al. (1998) listed several mechanical and chemical treatments, with acids, alkali, gasses, cellulose solvents, alcohols, oxidizing agents, and reducing agents, of wheat straw which have been studied in the past. Among these chemical agents, lime (calcium hydroxide) as pretreatment agent has potential because of its low costs, safety and its use hardly results in formation of inhibitors.

After a chemical pretreatment, the polysaccharides present in lignocellulose can be hydrolyzed by enzymes. The hydrolysis of cellulose and hemicellulose (i.e. xylan) is a complex process involving the combined action of exo- and endocellulases and cellobiases yielding glucose, and xylanases yielding xylooligomers and xylose (Kabel et al., 2006). Finally, these hydrolysis products can be fermented to ethanol by a variety of naturally occurring micro-organisms including bacteria, yeast and fungi (Olsson and Hahn-Hägerdal, 1996).

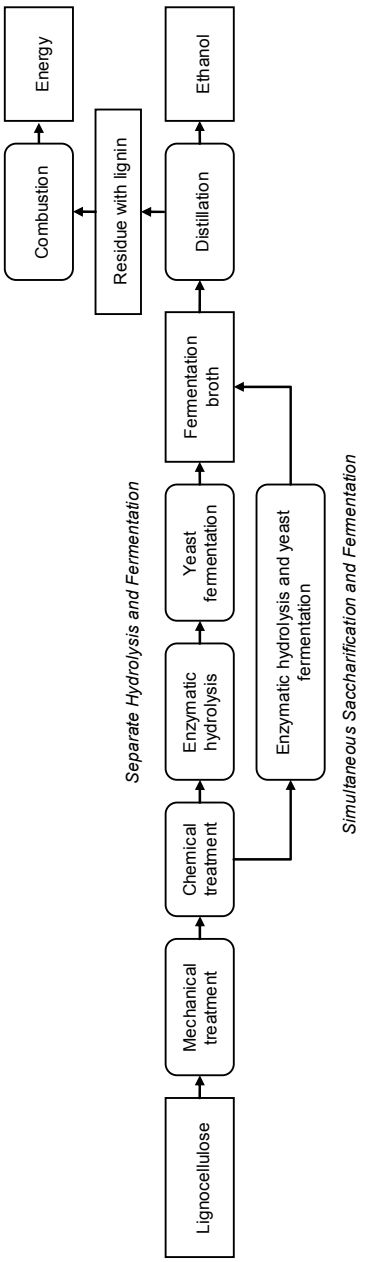

Figure 1. Schematic overview of the lignocellulose-to-ethanol process by separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) routes. In the scope of this study, the focus was on the enzymatic hydrolysis and yeast fermentation steps.

The most commonly used micro-organism is bakers' yeast, *Saccharomyces cerevisiae,* which is tolerant to high concentrations of sugars, produces high yields of ethanol and is robust and well suited for fermentation of lignocellulosic hydrolysates (Galbe and Zacchi, 2002; Lin and Tanaka, 2006; Olsson and Hahn-Hägerdal, 1993).

The enzymatic hydrolysis and fermentation of lignocellulose can be designed in different configurations (Fig. 1). In a separate hydrolysis and fermentation (SHF) route, the enzymatic hydrolysis occurs first and is followed by a fermentation of monomeric sugars into ethanol, both at their respective optimal reaction conditions (Lin and Tanaka, 2006). Yet, cellulases and cellobiase are strongly repressed by the presence of the hydrolysis products cellobiose and glucose, respectively (Philippidis et al., 1993). Also, enzymes involved in the degradation of xylan, such as ß-xylosidase, are inhibited by the presence of their hydrolysis products such as xylose (Poutanen and Puls, 1988). The costs of enzymes play a major role in the overall costs in the ethanol production process (Reith et al., 2002). Therefore, enzymes should be used as efficiently as possible by using favourable conditions for the hydrolysis step (Tengborg et al., 2001). One way to overcome the inhibition of hydrolyzing enzymes by hydrolysis products is to convert the monomeric sugars as soon as they appear in the liquid into ethanol, by performing enzymatic saccharification and fermentation simultaneously (SSF) in the same reactor. Ethanol produced during SSF inhibits cellulases less than glucose and cellobiose (Philippidis et al., 1993; Wu and Lee, 1997).

In the present report, we examined two routes to convert lignocellulose to ethanol; separate hydrolysis and fermentation and simultaneous saccharification and fermentation. For pretreatment, we selected lime pretreatment which was considered as a representative method for mild temperature treatments of lignocellulosic materials. The objective was to evaluate the influence of pH and enzyme concentration on the hydrolysis and fermentation of lime-treated wheat straw by a commercial cellulase preparation and the yeast *S. cerevisiae*. This is the first study describing an evaluation and comparison of SHF and SSF of lime-treated wheat straw with regard to hydrolysis rates and yields of xylan to xylose and glucan via glucose to ethanol.

MATERIALS AND METHODS

Feedstock and enzymes

Wheat straw was purchased from a commercial farm located in the Northeast of the Netherlands. The wheat straw was air dry (89.5% (*w/w*) dry matter) and was ground through a 2-mm screen. The chemical composition was analysed as described by van den Oever et al. (2003).

For all enzymatic hydrolysis work, a commercial enzyme preparation GC 220 (Genencor-Danisco, Rochester, USA) was used. This enzyme preparation contained cellulase (Filter Paper Units), cellobiase and xylanase activity of 116, 215 and 677 units/ml, respectively (Kabel et al., 2006). The specific gravity of GC 220 was 1.2 g/ml. The enzyme preparation contained 4.5 mg/ml glucose, 2.9 mg/ml mannose and 0.8 mg/ml galactose.

Alkaline pretreatment

A Quantum Mark V laboratory pulp mixer (Quantum Technologies, Inc., USA) with working volume of two litre was filled with 228 g of grounded wheat straw (89.5% (*w/w*) dry matter), 1600 ml demineralised water and 20.48 g lime ($Ca(OH)_2$). The pretreatment was started by mixing at 2400 rpm for one minute at intervals of 10 seconds and electric heating at 85°C for 20 h. At intervals of three minutes, the wheat straw was mixed for 3 seconds at 1200 rpm to allow for good heat transfer. After the pretreatment, the lime-treated wheat straw (LTWS) was cooled down to 50°C and was divided into three equal parts. The pH of each part was adjusted to 4.0, 4.5 or 5.0 by using a 20% (*v/v*) solution of sulphuric acid. The final concentration of LTWS was 122.3 g pretreated biomass per litre. Batches were stored overnight at room temperature; pH was measured again and if necessary adjusted to 4.0, 4.5 or 5.0 with a 20% (*v/v*) solution of sulphuric acid.

Washing of LTWS

A washing procedure was performed after alkaline pretreatment by placing the LTWS in a cotton bag, submerging the cotton bag in two litre of fresh demineralised water for five minutes, and dewatering the LTWS in the cotton bag by using a manual piston press at room temperature and at maximum pressure of 9.7 kg/m^2. The sequence of submersion and dewatering was repeated two times. After the third and final dewatering step, the LTWS was removed from the cotton bag and placed in the pretreatment reactor for neutralization purposes. Fresh demineralised water was then added to the dewatered LTWS to achieve the same biomass concentration (122.3 g/l) as during the pretreatment. The pH of the washed lime-treated wheat straw was then adjusted to 4.0, 4.5 or 5.0 by using a 20% (*v/v*) solution of sulphuric acid. Batches were stored overnight at room temperature; pH was measured again and if necessary adjusted to 4.0, 4.5 or 5.0 with a 20% (*v/v*) solution of sulphuric acid.

Enzymatic hydrolysis

Non-washed LTWS (25.0 g) with initial pH of 4.0, 4.5 or 5.0 were placed in a pre-heated incubator of 50°C. The enzymatic hydrolysis was initiated by the addition of 21.1, 42.3, 84.6 and 169.1 mg of the cellulase preparation GC 220 per g DM non-washed LTWS and incubated in a shaking incubator (Innova 4000, New Brunswick Scientific, USA) with

an agitation rate of 200 rpm at 50°C, which is reported by Genencor-Danisco as optimal temperature for activity of GC 220. All enzymatic hydrolysis experiments were conducted in duplicate and homogeneous samples of 0.5 g were withdrawn after 0, 5, 24, 48 and 72 h of incubation. Samples were boiled for five minutes in order to inactivate the enzymes. Several enzymatic hydrolysis experiments were continued by a "restart" hydrolysis which principle has been described by Lynd et al. (2002). After 72 h of incubation, the residual LTWS was centrifuged and the pellet was washed with 50 mM Sodium Acetate buffer (pH 5.0) until no monomeric glucose or xylose was detected by high-performance anion-exchange chromatography. Again 169.1 mg enzyme preparation per g DM was added and incubated for 24 h at 50°C. The samples were centrifuged and supernatants were analysed for glucose and xylose which are the main hydrolysis products of glucan and xylan, respectively.

Simultaneous saccharification and fermentation

Equal amounts of 195.2 g of non-washed or washed LTWS were added to 500-ml Scott flasks. Wheat straw adsorbs water easily resulting in a relative high viscosity suspension. As the LTWS was difficult to mix by magnetic stirring, a pre-hydrolysis was introduced with 25% (*v/v*) of the total enzyme concentration corresponding to 8.8, 17.6 or 35.2 mg enzyme preparation per g DM LTWS during 6 h in a shaking water bath at 50°C in order to achieve better mixing properties.

After the pre-hydrolysis, the hydrolysate was cooled down to room temperature and nutrients were added to the medium. All chemicals, unless indicated otherwise, were purchased from Merck (Germany). The LTWS hydrolysates were enriched with a (50×) stock solution of salts with final concentration of (per litre) $(NH_4)_2SO_4$, 4.2 g; KH_2PO_4, 2.5 g; $MgSO_4.7H_2O$, 0.42 g; trace element solution with final concentrations of EDTA, 15.0 mg; $ZnSO_4.7H_2O$, 4.5 mg; $MnCl_2.2H_2O$, 0.84 mg; $CoCl_2.6H_2O$, 0.3 mg; $CuSO_4.5H_2O$, 0.3 mg; $Na_2MoO_4.2H_2O$, 0.4 mg; $CaCl_2.2H_2O$, 4.5 mg; $FeSO_4.7H_2O$, 3.0 mg; H_3BO_3, 1.0 mg; and KI, 0.1 mg; vitamins solution with final concentrations of biotin, 0.05 mg; calcium panthotenate (Fluka, The Netherlands), 1.0 mg; nicotinic acid (Calbiochem, Germany), 1.0 mg; inositol, 25.0 mg; thiamine HCl (Fluka, The Netherlands), 1.0 mg; pyridoxine HCl (Calbiochem, Germany), 1.0 mg; and para-aminobenzoic acid, 0.2 mg; fatty acids solution with final concentrations of ergosterol (Sigma, The Netherlands), 10.0 mg; and tween 80, 420.0 mg dissolved in 1.25 ml ethanol; and antifoam Acepol 77, 0.5 ml (Emerald Foam Control, Germany). The trace elements solution, vitamins solution and fatty acid solution were prepared separately in stocks of 1000×, 1000× and 800×, respectively, as described by Verduyn et al. (1992). The residual part (75% (*v/v*)) of the total enzyme concentration which is equivalent to 26.4, 52.9 or 105.8 mg enzyme preparation per g DM LTWS was added at a solid loading concentration of 119.2 g pretreated biomass per litre. An amount of

12.6 g fresh wet bakers' yeast (Koningsgist, DSM, The Netherlands) per kg LTWS was added to the hydrolysate. Immediately after yeast addition, flasks were connected to the Biological Activity Monitor (Halotec, The Netherlands) in order to start carbon dioxide measurement continuously by a mass flow analyser (Brooks Instruments BV, The Netherlands). As carbon dioxide is produced in equimolar amounts with ethanol from glucose by bakers' yeast, measurements of carbon dioxide production rates were used to estimate production rates of ethanol.

Incubations were carried out at 37°C and under continuously mixing of 300 rpm by a magnetic stirrer. All SSF experiments were conducted either in duplicate or triplicate and homogeneous samples of 1-ml were withdrawn after 6 (end of pre-hydrolysis), 8, 22, 32, 46, 56 and 78 h of incubation in order to analyse concentrations of monosaccharides, organic acids, glycerol and ethanol.

Analyses

The dry matter (DM) content of LTWS was determined by drying a known weight of a homogenized sample overnight at 105°C. After cooling to room temperature in a dessicator, the samples were weighed again and DM content was calculated by the weight difference.

Samples withdrawn during enzymatic hydrolysis experiments were centrifuged (3 min at 17400×*g*) and analysed by high-performance anion-exchange on a Thermo Separation Products HPLC-system equipped with a Carbopack PA-20 column and pulsed amperometric detection (PAD) (Dionex, Sunnyvale, CA) (Lee, 1996). The mobile phase was an isocratic elution of 18.0 mM sodium hydroxide at a flow rate of 0.5 ml/min.

The samples of simultaneous saccharification and fermentation experiments were centrifuged (3 min at 17400×*g*), the pH of the supernatant was adjusted to 5.0 with barium carbonate (Fluka, Germany) using a pH-indicator (Bromophenolblue) and the liquid was filtered prior to analysis of monomeric sugar composition. The analysis was performed by high-performance anion-exchange chromatography using a Carbopack PA-1 column at 30°C and a pulsed amperometric detector (ED50) (Dionex, Sunnyvale, CA). Prior to injection, the system was equilibrated with 25.5 mM NaOH for 10 min at a flow rate of 1.0 ml/min. For the separation of monomeric sugars, at injection the mobile phase was shifted to de-ionized water for 30 min. Post-column addition of sodium hydroxide was used for detection of the neutral monomeric sugar composition.

For the analysis of ethanol, glycerol and organic acids, supernatant of centrifuged samples (3 min at 17400×*g*) was diluted (1:1, *v/v*) with 1.0 M sulphuric acid and filtered in order to remove solids and precipitated proteins. Samples were analysed by high pressure liquid chromatography system (Waters, USA) using an Organic Acids column IOA-1000 (Alltech, The Netherlands) with 3.0 mM sulphuric acid as the mobile phase at 90°C and a flow rate of 0.4 ml/min. Detection occurred by a refractive index detector (Waters 2414).

Determination of conversion yields

Conversion or yield (% *w/w*) of polymeric sugar to monomeric sugar ($Y_{monomer/polymer}$) such as glucan to glucose ($Y_{glucose/glucan}$) and xylan to xylose ($Y_{xylose/xylan}$) was calculated by the analysed monomeric sugar concentration divided by the theoretical maximum concentration of monomeric sugar [Eq. 1].

$$Y_{monomer\,/\,polymer}(\%) = \frac{[monomer]}{[biomass] * F_{polymer} * HF_{polymer}} * 100\% \qquad \text{[Eq. 1]}$$

The conversion or yield (% *w/w*) of polymeric sugar to fermentation product ($Y_{product/polymer}$) such as glucan to ethanol ($Y_{ethanol/glucan}$) was calculated by the analysed ethanol concentration divided by the theoretical maximum concentration of ethanol [Eq. 2].

$$Y_{product\,/\,polymer}(\%) = \frac{[product]}{[biomass] * F_{polymer} * HF_{polymer} * FY_{yeast}} * 100\% \quad \text{[Eq. 2]}$$

Where [*monomer*] = concentration of monomeric sugar at a time point (*g/l*); [*product*] = concentration of fermentation product at a time point (*g/l*); [*biomass*] = dry biomass concentration at the beginning of the fermentation (*g/l*); $F_{polymer}$ = fraction polymer per dry matter biomass (*g/g*); $HF_{polymer}$ = hydrolysis factor, introduction of water results in 1.11 g glucose from 1.00 g glucan and 1.14 g xylose from 1.00 g xylan (*g/g*); FY_{yeast} = maximum theoretical fermentation yield from glucose to ethanol by *S. cerevisiae* is 0.51 (*g/g*) (Ogbonna, 2004). The ethanol measurements were corrected by the amount of ethanol introduced with the fatty acid solution (0.6 g/l).

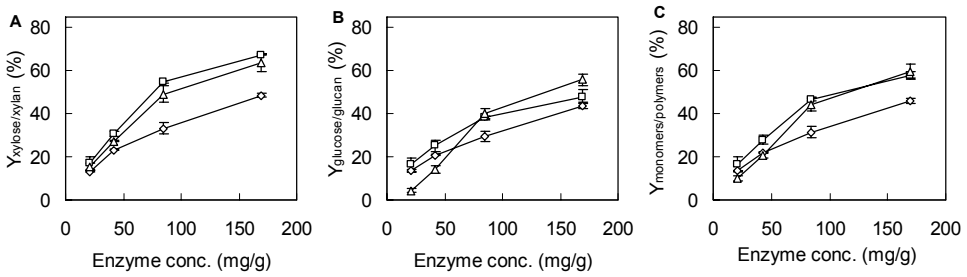

Figure 2. Effect of enzyme concentration at pH 4.0 (◇), 4.5 (□) and 5.0 (△) on the hydrolysis yield of xylan to xylose (*A*) and yield of glucan to glucose (*B*) in non-washed LTWS (72 h, 50°C). Figure *C* represents the overall yield of xylose and glucose (%) formed from xylan and glucan. The error bars in symbols denote deviation of duplicate experiments.

RESULTS

Enzymatic hydrolysis of non-washed lime-treated wheat straw

To study the effect of reaction conditions on the hydrolysis of non-washed lime-treated wheat straw (LTWS), the substrate was enzymatically hydrolyzed at three different initial pH values and four enzyme concentrations. The non-washed LTWS consisted of glucan and xylan levels of 33.0 and 19.0% (*w/w*), respectively. These values were used in Equation 1 and resulted in a theoretical maximum concentration of 44.8 g/l glucose and 26.5 g/l xylose at a solids loading concentration of 122.3 g non-washed pretreated biomass per litre. An overview of the hydrolysis yields of non-washed LTWS after 72 h of incubation is presented in Figure 2. The highest glucan conversion of 56% (Fig. 2B) at pH 5.0 and xylan conversion of 67% at pH 4.5 (Fig. 2A) was obtained with 169.1 mg enzyme preparation per g non-washed LTWS. Figure 2C describes the yield of the total monomers glucose and xylose hydrolysed from glucan and xylan, respectively, and showed a maximum of 60% of the theoretical yield.

The formation of glucose and xylose is represented in more detail i.e. in time at initial pH of 4.0, 4.5 and 5.0 and with 169.1 mg enzyme preparation per g DM non-washed LTWS (Fig. 3). As the figure shows, the highest glucose and xylose formation rates are obtained during the initial hours of incubation. The maximum amount of glucose (25.1 g/l) was formed after 72 h of hydrolysis at pH 5.0 and the highest amount of xylose (17.9 g/l) was observed at pH 4.5 and 5.0. Yet, in contrast to glucan conversion, the conversion of xylan to xylose is clearly influenced by lower pH of 4.0. At the end of the enzymatic hydrolysis of non-washed LTWS, a slight pH increase was observed with initial pH of 4.5

and 5.0 to pH 4.7 and 5.5, respectively, which is probably caused by slow release of calcium hydroxide from the wheat straw.

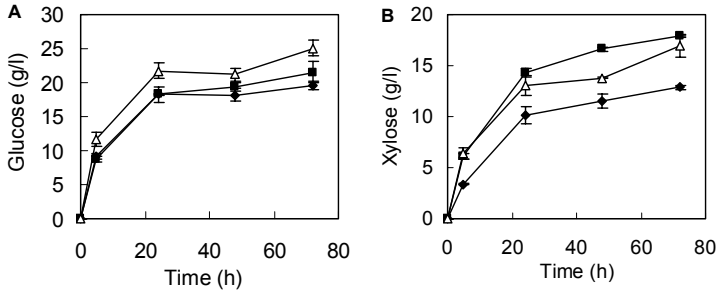

Figure 3. Influence of pH 4.0 (◆), 4.5 (■) and 5.0 (△) on the formation of glucose (*A*) and xylose (*B*) as a function of time during enzymatic hydrolysis of non-washed LTWS (169.1 mg enzyme preparation per g DM LTWS, 50°C). The error bars in symbols denote deviation of duplicate experiments.

The effect of different enzyme concentrations on the glucose and xylose formation rates and yields is presented in Figure 4. After 5 h of hydrolysis at pH 4.5, enzyme concentrations of 21.1, 42.3, 84.6 and 169.1 mg enzyme preparation per g DM non-washed LTWS resulted in glucose formation of 1.5, 3.0, 6.0 and 9.0 g/l, respectively (Fig. 4A), and xylose formation of 1.0, 2.0, 4.0 and 6.0 g/l, respectively (Fig. 4B). The largest part of the total formed sugars (after 72 h) was produced within 24 h of hydrolysis after which glucose and xylose appear to be formed by a low linear production rate. After 72 h of hydrolysis, 169.1 mg enzyme preparation per g DM non-washed LTWS yielded the highest amount of glucose and xylose.

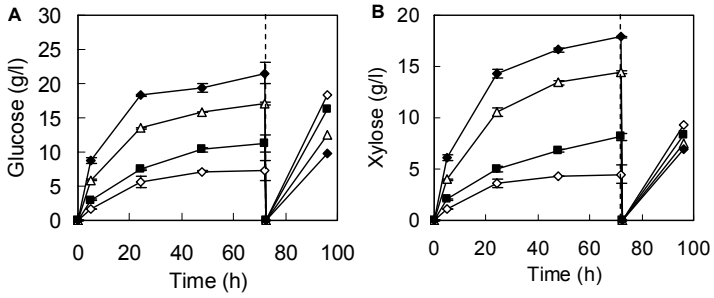

Figure 4. Influence of enzyme concentration of 21.1 (◇) 42.3 (■) 84.6 (△) 169.1 (◆) mg per g DM on the formation of glucose (*A*) and xylose (*B*) as a function of time during enzymatic hydrolysis of non-washed LTWS (pH 4.5, 50°C). The dotted lines represent the "re-start" hydrolysis of washed residue with 169.1 mg enzyme preparation per g DM LTWS. The error bars in symbols denote deviation of duplicate experiments.

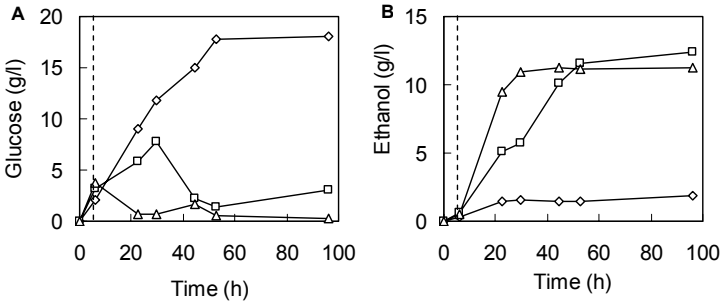

Figure 5. Formation of glucose (*A*) and ethanol (*B*) by *S. cerevisiae* during SSF of non-washed LTWS at pH 4.0 (◇), 4.5 (□) and 5.0 (△). Dotted lines represent the end of pre-hydrolysis and moment of yeast addition.

To study whether the remaining polymeric sugars could still be hydrolysed further, the LTWS suspension was washed in order to remove dissolved sugars and the pellet was re-incubated with fresh enzymes (169.1 mg enzyme preparation per g DM substrate). The "restart" hydrolysis resulted after 24 h of additional incubation in a further conversion of glucan to glucose and xylan to xylose (Fig. 4A, B). The highest initial tested enzyme concentration (169.1 mg) resulted in a total glucose and xylose concentration of 31.3 and 24.9 g/l, respectively, and accounted for total conversion of 70% of glucan to glucose and 94% of xylan to xylose, of the theoretical maximum yields.

SSF of non-washed lime-treated wheat straw

The effect of reaction conditions on simultaneous saccharification and fermentation (SSF) of non-washed LTWS was examined at three different pH values. Temperature was set at 37°C, which is a compromise between the optimal temperature for the activity of the enzyme preparation and the yeast *S. cerevisiae*. The production of glucose and ethanol during SSF of non-washed LTWS is shown in Figures 5A and B. After 96 h of SSF at pH 5.0, ethanol (11.2 g/l) was formed without accumulation of glucose corresponding to 49% of theoretical ethanol yield. During the first 30 h of SSF at pH 4.5, we observed a moderate accumulation of glucose (7.7 g/l) and a minor ethanol production (5.7 g/l) in comparison to SSF at initial pH of 5.0. However, SSF at initial pH of 4.0 clearly showed accumulation of glucose (18.1 g/l) indicating that ethanol formation (4.0 g/l) by this yeast was inhibited.

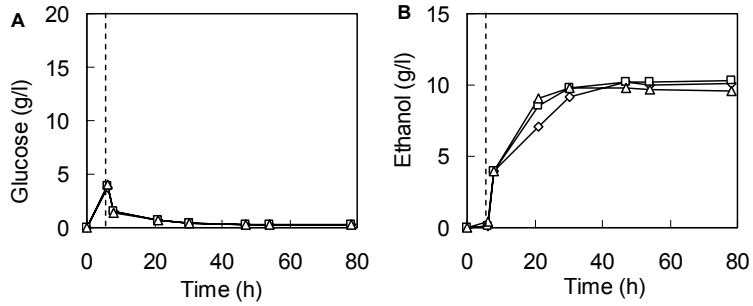

Figure 6. Formation of glucose (*A*) and ethanol (*B*) by *S. cerevisiae* during SSF of washed LTWS at pH 4.0 (◇), 4.5 (□) and 5.0 (△). Dotted lines represent the end of pre-hydrolysis and moment of yeast addition.

SSF of washed lime-treated wheat straw

It is described in literature that during chemical treatment of lignocellulosic materials a broad range of organic acids, sugar degradation products and aromatic degradation products can be formed that are able to inhibit fermentation processes (Klinke et al., 2004). A simple washing procedure was introduced aiming to detoxify the LTWS. By this treatment, the concentration of acetic acid was reduced from 2.7 to 0.4 g/l and no monomeric sugars were detected. Analysis of the chemical composition of washed LTWS showed that the glucan content per dry matter substrate was slightly higher (33.8%) in comparison to non-washed LTWS (33.0%) which is most likely due to removal of soluble components. The xylan content per dry matter washed LTWS (17.0%), however, was significantly lower in comparison to non-washed LTWS (19.0%), most probably as a result of removal of soluble xylooligomers. Other inhibitors such as furfural and hydroxymethyl furfural (HMF), degradation products of pentoses and hexoses, respectively and known to be formed during acid pretreatment processes, were not detected in the LTWS. Figures 6A and B show glucose and ethanol formation of washed LTWS during SSF at initial pH of 4.0, 4.5 and 5.0. In comparison to the results obtained by SSF with non-washed LTWS (Fig. 5A, B), only a minor effect of pH on glucose consumption and ethanol formation was observed. These results suggest that inhibitors such as acetic acid were below inhibiting concentrations in washed LTWS. Thus, we showed that due to the presence of acetic acid in non-washed LTWS there is effect of pH on the SSF process. In order to study whether pH has other effects on the SSF, we decided to use the washed LTWS in further experiments.

The effects of pH and enzyme concentration on the xylan-to-xylose and glucan-to-ethanol conversion of washed LTWS after 72 h of SSF were described by Figures 7A and B. The calculations of the yields were based on the glucan and xylan levels of 33.8 and 17.0% (*w/w*), respectively.

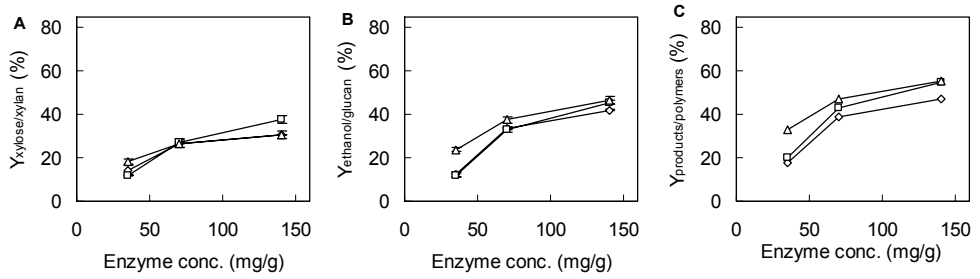

Figure 7. Effect of enzyme concentration at pH 4.0 (◇), 4.5 (□) and 5.0 (△) on the SSF of xylan-to-xylose (*A*) and glucan via glucose to ethanol (*B*) in washed LTWS (72 h). Figure *C* represents the overall conversion of xylan and glucan to remaining glucose and xylose and to ethanol, carbon dioxide, glycerol, acetic acid and lactic acid. The error bars in symbols denote deviation of duplicate experiments.

These values were used in Equation 1 and 2 and resulted in a theoretical maximum concentration of glucose, xylose and ethanol of 44.7, 23.1 and 22.8 g/l, respectively; at a solid loading concentration of 119.2 g washed pretreated biomass per litre. The highest xylan-to-xylose conversion of 38% was achieved after 72 h of incubation at pH 4.5 and 141.0 mg enzyme preparation per g washed LTWS and the highest glucan-to-ethanol conversion of 47% at pH 5.0. After 26 h of incubation, glucan conversion of 44% was already obtained indicating that during the last 46 h of incubation only 3% of the glucan was converted into ethanol.

However, during the SSF experiments, the washed LTWS was not converted into ethanol and carbon dioxide only but also in other products such as glycerol, acetic acid and lactic acid (Table 1). The SSF experiments with various enzyme concentrations at pH 5.0 resulted in different ethanol concentrations (6.0 – 10.7 g/l) accompanied with lactic acid production (6.2 – 6.7 g/l). Lowering the pH to 4.0 resulted in slightly lower ethanol concentrations (3.3 – 10.2 g/l) but clearly a much lower lactic acid formation (1.4 – 3.0 g/l).

TABLE 1. Production of ethanol, glycerol, acetic acid and lactic acid (*g/l*) after 72 h of SSF of washed LTWS (at a solids loading concentration of 119.2 g DM LTWS/l) and variable enzyme concentrations at different pH.

Enzyme conc. (mg/g)	pH 4.0				pH 4.5				pH 5.0			
	Eth	Gly	Aa	La	Eth	Gly	Aa	La	Eth	Gly	Aa	La
35.2	3.3	0.2	0.9	1.4	2.7	0.3	1.3	4.0	6.0	0.7	1.0	6.2
70.5	8.3	1.0	1.3	1.9	8.2	1.0	1.2	5.5	8.6	0.9	1.2	6.6
141.0	10.2	1.0	1.7	3.0	10.3	1.0	1.3	6.0	10.7	1.2	1.7	6.7

Eth, ethanol; Gly, glycerol; Aa, acetic acid; La, lactic acid. The presented data are averages of duplicate experiments.

The formation of glycerol and acetic acid was not clearly affected by the pH. In order to quantify the efficiency of the enzymatic saccharification during SSF of washed LTWS, the yield was determined by including all fermentation and hydrolysis products, except yeast biomass formation, formed per total amount of glucan and xylan present (assuming an ethanol yield of 0.51 g/g and other fermentation products 1.0 g/g monomeric sugar). This resulted in a maximal conversion of glucan and xylan of 56% of the theoretical maximum yield (Fig. 7C).

Effect of non-sterile conditions on SSF

The wheat straw used is not sterile and also the lime pretreatment of the wheat straw occurs in an open system under non-sterile conditions. It is therefore possible that microbial contamination occurs during SSF experiments which cause formation of unwanted by-products. In Figures 8A and B, results of the SSF experiment with 141.0 mg enzyme preparation per g DM washed LTWS at pH 5.0 are presented. After 24 h of incubation at 37°C, acetic acid and lactic acid emerged suddenly, likely as a result of undesirable microbial contamination. The conversion of glucan to ethanol (10.8 g/l) and xylan to xylose (7.8 g/l) accounted for 47 and 34%, respectively, of theoretical maximum yield. In order to inhibit unwanted microbial contamination and therefore formation of by-products completely, we added the antibiotics penicillin and streptomycin (2.5 ml PenStrep per kg LTWS suspension, Sigma-Aldrich). These antibiotics did not have a negative effect on the ethanol formation from glucose by yeast (results not shown).

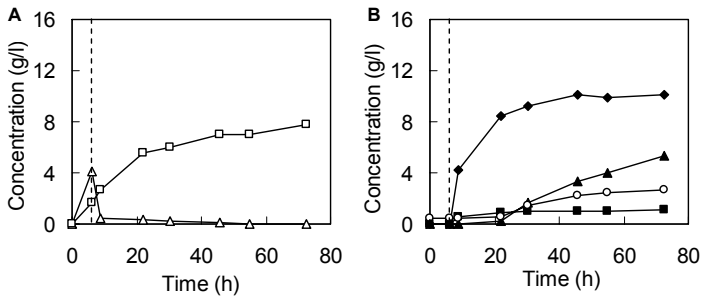

Figure 8. Typical SSF of washed LTWS at pH 5.0 with formation of glucose (△), xylose (□) (*A*) and ethanol (◆), glycerol (■), acetic acid (○) and lactic acid (▲) (*B*). Dotted lines represent the end of pre-hydrolysis and moment of yeast addition.

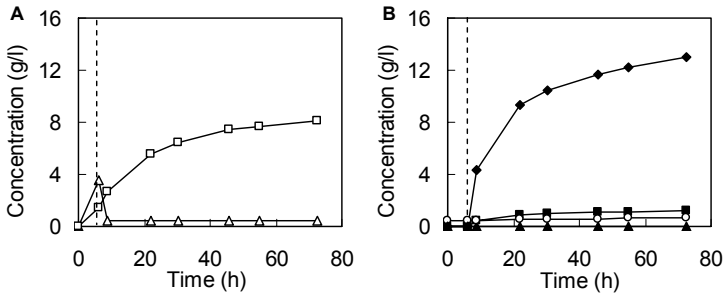

Figure 9. SSF of washed LTWS at pH 5.0 in the presence of PenStrep with formation of glucose (△), xylose (□) (*A*) and ethanol (◆), glycerol (■), acetic acid (○) and lactic acid (▲) (*B*). Dotted lines represent the end of pre-hydrolysis and moment of yeast addition.

As shown in Figures 9A and B, the addition of these antibiotics reduced acetic acid production (0.3 g/l) and prevented lactic acid formation and consequently resulted in a higher ethanol production (13.1 g/l). This ethanol concentration accounted for 58% of theoretical maximum yield whereas the conversion of xylan to xylose remained approximately equal at 35% of theoretical maximum yield.

Comparison of SHF and SSF

To compare the SHF and SSF routes properly, experiments were conducted in parallel at their optimal reaction conditions which were determined by the previous experiments. SHF and SSF were compared both at enzyme concentrations of 141.0 and 35.0 mg per g DM washed LTWS. After the lime pretreatment, the wheat straw was washed in order to reduce the acetic acid concentration. Furthermore, penicillin and streptomycin (2.5 ml per kg LTWS suspension) were added to the washed LTWS to prevent unwanted by-product formation by microbial contamination. The enzymatic hydrolysis of the SHF experiment was performed at pH 5.0 and 50°C. In the fermentation step the same pH was used and the temperature was decreased to 37°C.

The enzymatic hydrolysis of the SHF route with 141.0 mg enzyme/g LTWS resulted after 48 h of incubation in the formation of glucose (26.3 g/l) and xylose (9.8 g/l) (Fig. 10A). This corresponded to a glucan-to-glucose ($Y_{glucose/glucan}$) and xylan-to-xylose ($Y_{xylose/xylan}$) conversion of 58.8 ± 1.2% and 42.4 ± 1.4%, respectively, of theoretical maximum yield. Throughout the enzymatic hydrolysis we observed also the release of the pentose sugar arabinose (1.4 g/l) from the hemicellulose present.

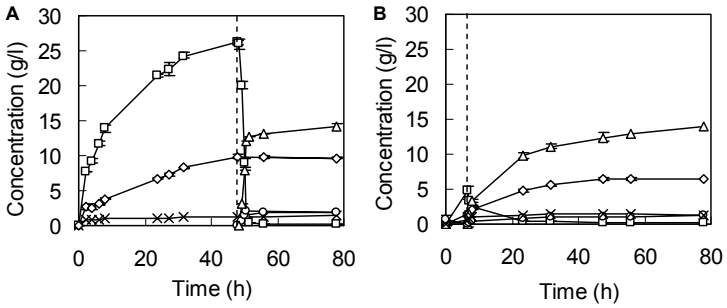

Figure 10. Comparison of SHF (hydrolysis at 50°C and fermentation at 37°C) (*A*) and SSF (pre-hydrolysis at 50°C and SSF at 37°C) (*B*) of washed LTWS in the presence of PenStrep with the formation of saccharification products glucose (□), xylose (◇) and arabinose (×) and fermentation products, ethanol (△) and glycerol (○) by *S. cerevisiae*. Dotted lines represent moment of yeast addition. The error bars in symbols denote deviation of triplicate experiments.

During the second phase of the SHF, the fermentation by *S. cerevisiae*, glucose was converted into ethanol (12.7 g/l), glycerol (1.9 g/l) and carbon dioxide within 3.2 h of incubation. The available glucose was converted into mainly ethanol and carbon dioxide resulted in an overall ethanol yield ($Y_{ethanol/glucan}$) of 55.7 ± 0.4% of the theoretical maximum. An average volumetric ethanol productivity of 0.25 g/l/h was determined over 51 h of incubation. Continuation of the process resulted in a SSF fashion with a linear ethanol production rate and low glucose concentrations. The SHF experiment was also performed at a fermentation temperature of 33°C (results not shown) which is described as the optimal temperature for ethanol production by *S. cerevisiae* (Aldiguier et al., 2004). However, at this temperature, the ethanol yield was actually 3% lower in comparison to the yield obtained at a fermentation temperature of 37°C.

The SSF experiment with 141.0 mg enzyme/g LTWS constituted of a pre-hydrolysis of the washed LTWS (6 h, pH 5.0, 50°C), resulting in a reduction of viscosity and the formation of the hydrolysis products glucose (4.7 g/l), xylose (1.4 g/l) and arabinose (0.7 g/l). After pre-hydrolysis, temperature was set at 37°C and yeast was added to the LTWS hydrolysate. Due to the presence of enzymes and yeast in the same reactor during SSF (Fig. 10B), the glucose concentration remained below 0.4 g/l. *S. cerevisiae* fermented the available hydrolysed glucose within 51 h to ethanol (12.5 g/l), glycerol (1.1 g/l) and carbon dioxide. This resulted in an ethanol yield ($Y_{ethanol/glucan}$) of 54.8 ± 2.3% of theoretical maximum yield, and an average volumetric ethanol productivity of 0.25 g/l/h, equal to the values obtained with SHF. Furthermore, the ethanol production profile of SSF (Fig. 10B) was used to recalculate the corresponding 'virtual' glucose production profile (fermentation yield of 0.49 g/g). This glucose curve fitted exactly over the glucose profile during the enzymatic hydrolysis of the SHF (Fig. 10A) at optimal temperature suggesting similar

volumetric productivities. The lower amount of glycerol production by SSF in comparison to SHF suggests lower yeast biomass formation. Conversion of LTWS by the SSF approach resulted in the formation of arabinose (1.4 g/l) and a yield of xylan to xylose (6.4 g/l) of 27.6 ± 1.1% of theoretical maximum yield which is significant lower in comparison to the xylan conversion achieved by the SHF route. These experiments were also performed with 35.0 mg enzyme/g LTWS; also under these conditions the ethanol yields of SSF and SHF differs after 56 h of incubation less than 5% (results not shown).

DISCUSSION

Lignocellulosic resources such as wheat straw have great potential as feedstock for the production of ethanol and can be a clean alternative to fossil fuels. In order to achieve an efficient lignocellulose-to-ethanol conversion, the pretreatment process, enzymatic hydrolysis and microbial fermentation should occur at optimal conditions. In this study, both SHF and SSF were examined and evaluated with regard to the process performance such as yield and productivity. The used wild type *S. cerevisiae* converts hexoses efficiently into ethanol, however, this yeast is lacking the ability to ferment pentose sugars present in lignocellulosic hydrolysates (Kuyper et al., 2003; Ogbonna, 2004). Anyhow, an efficient conversion of the pentose sugars in addition to hexoses is necessary for the overall economy of ethanol production from lignocellulosic resources (Olsson and Hahn-Hägerdal, 1996). Recently, *S. cerevisiae* has been genetically engineered to ferment xylose efficiently to ethanol (Kuyper et al., 2004). Therefore, in this research both glucan and xylan conversion is taken into account. An economical analysis was not part of this study.

Our results showed that the glucose consumption by yeast during SSF of LTWS was inhibited due to the presence of inhibitors. Analysis of the culture broth identified the presence of substantial amounts of acetic acid while quantities of furfural and hydroxymethyl furfural were nihil. In general, during the chemical treatment of lignocellulose, acetic acid is released upon solubilisation and hydrolysis of hemicellulose (Palmqvist et al., 1999). At pH below the dissociation constant (pK_a 4.75), acetic acid is mostly in the un-dissociated form and diffuses easily over the cell membrane. In the yeast cell, acetic acid dissociates and causes a decrease of intracellular pH (Taherzadeh et al., 1997). Consequently, inhibition of growth and ethanol production by yeast is dependent of the acetic acid concentration and the extracellular pH.

TABLE 2. Advantages and drawbacks of separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) routes in the lignocellulose-to-ethanol process.

Route	Advantage	Reference	Drawback	Reference
SHF	• Optimal reaction conditions • No ethanol inhibition • Simple recycling of microbial biomass since the soluble sugar fraction and microbial biomass are separated from the insoluble lignin fraction	(Lin and Tanaka, 2006) (Wyman et al., 1992) (Galbe and Zacchi, 2002)	• Sugar inhibition • Use of two reactors • Risk for microbial infection	(Philippidis et al., 1993) (Galbe and Zacchi, 2002) (Wyman et al., 1992)
SSF	• No sugar inhibition • Use of one reactor • Minor risk for microbial infection	(Philippidis et al., 1993) (Galbe and Zacchi, 2002) (Wyman et al., 1992)	• Suboptimal reaction conditions • Ethanol inhibition • Difficult recycling of microbial biomass since the microbes are mixed together with the insoluble lignin fraction	(Bollók et al., 2000) (Wyman et al., 1992) (Galbe and Zacchi, 2002)

Experiments indeed revealed more inhibition of acetic acid (2.7 g/l) on the glucose consumption and ethanol production at initial pH of 4.0 compared to pH 5.0. In order to reduce inhibiting components and improve fermentability of pretreated lignocellulosic materials, a detoxification step is required. Several detoxification techniques have been described, for an overview see Mussatto and Roberto (2004). The advantage of the mild-temperature lime pretreatment is that in comparison with other pretreatment techniques monomeric pentose sugars are hardly liberated from the lignocellulosic matrix. This enables the application of a solid/liquid separation to reduce the amount of acetic acid in the pretreated biomass without losing valuable pentose monomeric sugars. Nevertheless, our results showed that the washed LTWS resulted in an improved ethanol productivity and yield in comparison to non-washed LTWS while approximately 10% of the total xylan was lost, most probably as soluble xylooligomers throughout the washing procedure. The effect of applying a solid/liquid separation step on overall economics of the process compared to other detoxification steps and the potential to add value to the acetic acid rich stream remains to be studied.

It is often stated in literature that SSF is preferred to the SHF configuration to convert lignocellulose to ethanol. Benefits and drawbacks of both routes are summarized in Table 2. A lower sensitivity of SSF for microbial infection compared to SHF is mentioned. However, our SSF results showed after 24 h of incubation a sudden formation of atypical yeast fermentation by-products such as lactic acid and acetic acid by microbial contaminants. The formation of the unwanted by-product lactic acid was also observed by

other researchers during simultaneous saccharification and fermentation of steam-pretreated softwood (Stenberg et al., 2000). In our study, growth and by-product formation by these microbial contaminants were effectively inhibited by the addition of antibiotics and resulted in improved ethanol yields. Yet, addition of antibiotics at industrial scale of bio-ethanol production is not an option. Alternatively, as showed by previous results (Table 1) and reported by Olsson and Hahn-Hägerdal (1996), by-product formation by microbial contaminants can be inhibited by performing SSF at relatively low pH. However, low acetic acid concentration is than a necessity. Moreover, if the microbial contaminants originated from feedstock materials or used equipment, reduction of microbes can possibly be achieved by more intense and severe pretreatment of wheat straw e.g. higher temperature or higher lime concentration.

The primary advantage of SHF is that both enzymatic hydrolysis and fermentation occur separately at their respective optimal reaction conditions (Lin and Tanaka, 2006). The results obtained in this study illustrated that the enzymatic hydrolysis yield of glucan (56% of theoretical maximum) and xylan (67% of theoretical maximum), present in non-washed LTWS, is optimal at pH 5.0 and 4.5, respectively, and 50°C. The hydrolysis rate and yield of non-washed LTWS is also clearly influenced by the enzyme concentration. As illustrated in Figure 4, the sugar yield of LTWS increased proportional with increasing enzyme concentration. This effect of enzyme concentration is also observed by Tengborg et al. (2001) who studied the enzymatic hydrolysis of steam-pretreated softwood. Only, our highest tested enzyme concentration (169.1 mg) showed no proportional increase of hydrolysis products in comparison to half the dose. This indicates that at these enzyme concentrations, the saturation level towards the substrate was nearly reached. This enzyme saturation effect was also described previously by Spindler et al. (1989). In general, the highest hydrolysis rates of glucan and xylan were achieved during initial hours followed by a lower but linear production rate. This decline of hydrolysis rate can be explained by different substrate related causes such as the heterogeneous structure of the substrate or substrate recalcitrance, and by enzyme-related causes such as thermal instability of enzymes, enzyme inactivation or inhibition of enzymes by hydrolysis products (Yang et al., 2006). However, re-incubation of the remaining LTWS solids, after washing, with fresh enzymes resulted in a cumulative conversion of both glucan (70%) and xylan (94%) indicating that the decrease of hydrolysis rate and yield can be interpreted partially by enzyme-related causes such as inactivation due to pH or temperature or by inhibition due to the presence of hydrolysis products.

The main benefit of the SSF route is the reduction of inhibition of enzyme activity by hydrolysis products, as a result of the conversion of glucose, as soon as it appears in the medium, by yeast. Indeed, results showed no accumulation of glucose during SSF of washed LTWS. Moreover, the conversion of washed LTWS by our SSF technique (i.e. pre-

hydrolysis of 6 h and SSF of 72 h) resulted in the highest xylan-to-xylose conversion of 35% at pH 4.5. The highest glucan-to-ethanol conversion of 58% was obtained at pH 5.0 and agrees relatively well with the data reported by others. Chang et al. (2001) reported ethanol yields of lime pretreated switchgrass after 7 days of incubation, pretreated corn stover after 7 days of incubation and pretreated washed poplar wood after 5 days of incubation of 72%, 62% and 73% of theoretical, respectively. However, the variation in substrate composition, use of specific pretreatment conditions and the complexity of enzymatic hydrolysis makes it difficult to compare lignocellulose-to-ethanol results described in other studies.

This study enables a comparison of the conversion of lime-treated wheat straw by the SHF and SSF route due to comparable reaction conditions. At a relatively high enzyme concentration (141.0 mg per g DM washed LTWS), SHF and SSF resulted in similar yields of glucan to ethanol (55%) and comparable ethanol productivities of 0.25 g/l/h. This indicates that the positive effect of low sugar concentration on cellulolytic activity and the negative effect of suboptimal reaction conditions for growth and enzymic conversion in the SSF setup are equal and balance each other out. With the xylan-to-xylose conversion the situation is different (Fig. 10); the wild-type *S. cerevisiae* is not able to consume the xylose and therefore xylose accumulates. So, the negative effect of suboptimal temperature is not balanced out by the removal of inhibiting xylose. This results in lower yield and productivity for the xylan-to-xylose conversion with SSF. However, by using for example a genetically engineered *S. cerevisiae* strain, which is able to convert glucose and xylose efficiently to ethanol (Kuyper et al., 2004), it is possible that this repression by hydrolysis products of the xylan degrading enzymes can be minimized and may result in comparable conversion efficiencies of xylan for both SHF and SSF.

In this study, the comparison of SHF and SSF of LTWS to ethanol under optimized conditions for both processes – using the same type and concentration of LTWS, yeast and hydrolysing enzyme concentration – resulted in equal conversions and production rates. So a choice between SSF and SHF of LTWS for the production of ethanol cannot merely be based on yields and productivities of both processes, but should be based on an economic comparison of both processes. Wingren et al (2003) described an economic comparison of the conversion of softwood to ethanol by SHF and SSF. In that comparison the lower yield of SHF, the higher enzyme costs of SSF, costs of yeast production for SSF and higher capital costs for SHF were important factors. In the case of ethanol production from LTWS yield and enzyme costs are equal suggesting that production costs of yeast and capitol cost are decisive parameters. A thorough economic evaluation will be subject of further study.

CONCLUSION

This study showed that the glucan and xylan saccharification rates and yields of LTWS by SHF and SSF were improved by increasing the enzyme concentration per gram dry matter lime-treated wheat straw at optimal pH and temperature. The yields of glucan-to-ethanol by SSF were enhanced by reducing the inhibitor concentration, e.g. acetic acid, by a simple detoxification/washing procedure of the pretreated substrate. The main advantage of SSF in comparison to SHF is that due to continuous removal of glucose by the yeast, there is minor inhibition of cellulolytic activity offering the possibility of high hydrolysis rates. Indeed, our work showed that during the SSF of lime-treated wheat straw at suboptimal reaction conditions, a low glucose concentration in the medium was continuously maintained by the yeast. Despite of this advantage, SSF of lime-treated wheat straw did not result in significantly higher ethanol yields, ethanol productivities and xylan-to-xylose conversion in comparison to SHF. To distinct properly between the overall feasibility of the SHF and SSF route in order to convert lime-treated wheat straw into ethanol, process economics such as capital costs and operating costs, besides the product yield and productivity, should be taken into account.

ACKNOWLEDGEMENTS

This project is supported with a grant of the Dutch Programme EET (Economy, Ecology, Technology) a joint initiative of the Ministries of Economic Affairs, Education, Culture and Sciences and of Housing, Spatial Planning and the Environment. The programme is run by the EET Programme Office, SenterNovem. Jacinta C. van der Putten and Sela Combarros Castrillo (AFSG, Wageningen, The Netherlands) are gratefully acknowledged for technical assistance.

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CHAPTER 6

Pilot-Scale Conversion of Lime-Treated Wheat Straw into Bioethanol: Quality Assessment of Bioethanol and Valorisation of Side Streams by Anaerobic Digestion and Combustion

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ABSTRACT

Limited availability of fossil fuel sources, worldwide rising energy demands and anticipated climate changes attributed to an increase of greenhouse gasses are important driving forces for finding alternative energy sources. One approach to meet the increasing energy demands and reduction of greenhouse gas emissions is by large-scale substitution of petrochemically derived transport fuels by the use of CO_2-neutral biofuels such as ethanol. This paper describes an integrated pilot-scale process where lime-treated wheat straw with high dry matter content (~20% *w/w*) is converted to ethanol via simultaneous saccharification and fermentation by commercial hydrolytic enzymes and bakers' yeast *Saccharomyces cerevisiae*. After 53 hours of incubation, an ethanol concentration of 21.4 g/l was detected, corresponding to a 48% glucan-to-ethanol conversion of theoretical maximum. The xylan fraction remains - probably due to inability of this yeast to convert pentoses - mostly in the soluble oligomeric form (52%) in the fermentation broth. A preliminary assessment of the distilled ethanol quality shows that it meets the transportation ethanol fuel specifications. The distillation residue, containing non-hydrolysable and non-fermentable (in)organic compounds, was divided in a liquid fraction and a solid fraction. The liquid fraction served as substrate for the production of biogas (methane) whereas the solid fraction functioned as fuel for thermal conversion (combustion), yielding thermal energy which can be used for heat and power generation. Based on the achieved experimental values, in total 16.7 kg of pretreated wheat straw could be converted to 1.7 kg ethanol, 1.1 kg methane, 4.1 kg carbon dioxide, ~3.4 kg compost and 6.6 kg lignin-rich residue. The Higher Heating Value of the lignin-rich residue was 13.4 MJ thermal energy per kg (dry basis).

INTRODUCTION

The limited availability of oil reserves and growing worldwide energy demands result in increasing energy prices. Furthermore, the utilization of fossil fuels has some negative impacts such as air pollution and generation of the greenhouse gas CO_2 which is presumably one of the main anthropogenic contributors to the global warming effect. These factors stimulate the exploitation of alternative renewable energy sources such as biomass (Sanders et al., 2007). According to the Kyoto protocols, many of the industrialized nations need to reduce their CO_2-emisions by ~5% in 2010 as compared to the 1990 level while in the long-term a further decline is compulsory (Bolin, 1998). One strategy to meet the global increasing energy demand and the reduction of CO_2-levels is by substitution of petrochemically derived transport fuels by CO_2-neutral bio-fuels such as ethanol (Reith et al., 2002).

Bio-fuels are produced by microbial conversion of carbohydrates derived from biomass feedstocks such as agro-industrial residues. Lignocellulose containing feedstocks are widely available, relatively inexpensive, non-competitive with food applications, sustainable in terms of CO_2 emissions, and therefore of potential interest for the large-scale production of biofuels. Lignocellulose consist of a complex fibrous structure of polymeric sugars like (hemi-)cellulose embedded in a matrix of the aromatic polymer lignin (Claassen et al., 1999; Mosier et al., 2005). The conventional lignocellulose-to-ethanol conversion consists of (1) a pretreatment step; (2) a hydrolysis step; and (3) a fermentation step. Various physical and chemical pretreatments are developed to alter the structure of lignocellulosic substrates (Mosier et al., 2005). An example is with lime ($Ca(OH)_2$) at mild temperatures (<100°C) which enhances the accessibility of (hemi-)cellulose for the subsequent enzymatic hydrolysis (Chang et al., 1998; Kaar and Holtzapple, 2000; Bakker et al., 2007). The hydrolysis, often performed by a mixture of cellulolytic and hemi-cellulolytic activities, results in a hydrolysate containing mainly soluble monosaccharides (Maas et al., 2007b). Throughout the subsequent fermentation, these monomeric sugars can be converted to ethanol by yeasts or bacteria with high productivity and efficiency. Alternatively, enzymatic saccharification and fermentation are performed simultaneously (SSF) at compromising reaction conditions in one reactor.

In a previous study on the conversion of washed lime-treated wheat straw to ethanol, the optimal reaction conditions were determined at laboratory scale (Maas et al., 2007b). The objective of the present paper is to study the effect of up-scaling on the performance of the lignocellulose-to-ethanol SSF process, quality assessment of the bioethanol and valorization of by-product streams.

In the present study, ethanol was recovered from the fermentation broth via a distillation process and its composition was analyzed to assess its suitability as transport fuel. The

remaining distillation residue - consisting of water, lignin, non-hydrolyzed and non-fermented organic components, minerals from the feedstock, the added process chemicals and other non-ethanol fermentation products – was separated into a solid and liquid fraction. Combustion of the solid fraction, which represents a significant part of the energy input, was analysed and tested using a bench-scale experimental fluidized bed combustor to assess the suitability of the solid fraction as fuel for combined heat and power generation. The liquid fraction - containing soluble organic components – was tested for its suitability for anaerobic biodegradation yielding biogas (methane). Utilisation options of the ashes derived from combustion and sludge derived from anaerobic digestion are briefly discussed. This paper represents the first integral assessment of the conversion of lignocellulosic material into bioethanol including the valorisation of side-streams.

MATERIALS AND METHODS

Lignocellulosic feedstock, enzymes and yeast

Wheat straw was purchased from a farm located in the Northeast of the Netherlands. The wheat straw was air dry (89.5% (*w/w*) dry matter) and was ground to pass through a 4-mm screen. The chemical composition of the wheat straw was analyzed as described elsewhere (Maas et al., 2007b). The commercial enzyme cocktail GC 220 (Genencor-Danisco, Rochester, USA) contained cellulase, cellobiase and xylanase activities of 116, 215 and 677 U/ml, respectively (Kabel et al., 2006), with a specific gravity of 1.2 g/ml. The ethanol fermentation was performed with the wild-type bakers' yeast *Saccharomyces cerevisiae* (Koningsgist, DSM, The Netherlands). Chemicals, unless indicated otherwise, were purchased from Merck (Darmstadt, Germany).

Alkaline pretreatment and washing procedure

The alkaline pretreatment of air dried wheat straw was performed in a 215-l heated vessel (diameter of 67.5 cm, height of 60 cm). An amount of 22.5 kg ground wheat straw (approx. 20.1 kg dry matter) was added to the preheated vessel and moistened with 40 l water under manually mixing. Then, an amount of 2019 g lime ($Ca(OH)_2$) was dissolved in 145 l water and added to the moistened wheat straw. This suspension was heated by steam to 80-85°C under continuously mixing by a stirrer with an open framework. The vessel was closed in order to prevent evaporation of water. The temperature was measured every hour and, if necessary, adjusted to 85°C by adding steam. After 8 h of incubation (80-85°C), the vessel was thermally isolated in order to maintain the temperature. Throughout the next 16 h of incubation, the temperature of the wheat straw suspension decreased to 66°C. After a total of 24 h of incubation, the lime-treated wheat straw (LTWS) was transferred to a

drainage vessel and dewatered through two sieves by gravitation (sieve A contains pores of 3 mm with a total open surface of 10.3%; sieve B contains pores of 0.6 mm and a total open surface of 23%). The solid fraction was returned together with 215 l fresh hot water (55-60°C) in the drainage vessel under continuously mixing for 30 min. Then, the soaked pulp was dewatered again by the same approach. The washing/dewatering procedure was repeated five times. The pH of the pulp was adjusted to pH 6.5 with a 25% (*v/v*) H_2SO_4 solution. Finally, the pulp was dewatered once more to a dry matter content of approx. 35% and stored at 4°C.

The dry matter (dm) content was determined by drying a known weight of homogenized sample overnight at 105°C. After cooling to room temperature, the samples were weighed again and dm content was calculated by weight difference.

For the determination of insoluble solid content, the samples were centrifuged for 5 min at 3000 rpm, supernatant was removed and the remaining pellet was washed by re-suspension with water. The sequence of washing and centrifugation was repeated three times followed by drying the pellet overnight at 105°C.

Simultaneous saccharification and fermentation

The simultaneous saccharification and fermentation (SSF) of LTWS was carried out in a 100-l fermentor (Applikon, Schiedam, The Netherlands) with pH and temperature control (Biocontroller ADI 1020). The reactor was filled with 35 l tap water. The following salts were added to the water; $(NH_4)_2SO_4$, 425 g; KH_2PO_4, 255 g and $Mg_2SO_4.7H_2O$, 42.5 g. The pH was adjusted and maintained at 5.0 with a 2 M H_2SO_4 solution and a 20% (*w/v*) $Ca(OH)_2$ suspension. The process started with a pre-hydrolysis phase (I) of 8 h at 37°C and an agitation rate varying between 450 and 700 rpm. Throughout this phase, 48.42 kg of washed LTWS (with a dry matter content of approx. 35%) and 1105 ml GC 220 were added manually. The rate of substrate addition depended on the viscosity of the LTWS suspension in the reactor. After the pre-hydrolysis the following components were added to the LTWS; trace element solution, 85 ml; vitamin solution, 85 ml; fatty acid solution, 106.25 ml; anti-foam Acepol 77 (Emerald Foam Control), 42.5 ml; antibiotics Penicillin and Steptomycine (Sigma-Aldrich), 212.5 ml; second dosage of enzyme preparation GC 220, 787 ml and fresh wet bakers' yeast (Koningsgist, DSM, The Netherlands), 1071 g, all according to the methods as described previously (Maas et al., 2007b). The SSF phase (II) was initiated by the addition of the yeast cells and the reactor was closed in order to analyze CO_2 production (Uras 10E, Hartmann & Braunn). A nitrogen gas flow between 5 and 40 l/min was added to the fermenter functioning as carrier gas of the produced CO_2. As CO_2 is produced in equimolar amounts with ethanol from glucose by bakers' yeast, measurements of CO_2 production rates can be used to estimate the production rate of ethanol. Samples were taken throughout the experiment, treated and analyzed for composition of monomeric

sugars, organic acids, glycerol and ethanol by HPLC methods as described previously (Maas et al., 2007b). The soluble oligomeric sugars and insoluble polymeric sugars were determined as described before (Maas et al., 2007a).

Recovery of ethanol and quality assessment

After completion of the SSF process, the fermentation broth in the fermentor was heated up to 83°C. Nitrogen gas (15 l/min) functioned as carrier gas of evaporated ethanol. The ethanol was condensed by a cold fall spiral (length of 270 cm, diameter of 2 mm) which was cooled by water of 4°C. The distillate fraction of condensed ethanol/water mixture was collected in a bottle cooled with melting ice water.

Further upgrading of the ethanol content was performed in a second distillation step which was carried out in a batch distillation unit with 3 l boiler volume. An Oldershaw type distillation column containing 28 theoretical stages was used. The boiler was filled with 2 to 2.5 l of the ethanol/water mixture obtained by the first distillation step. Hence, several distillation runs were required to produce sufficient material. Distillate fractions of approximately 20 g were collected separately and analyzed on ethanol content. During the first phase of the distillation the reflux ration was kept at 2. After having received the first 100 g after the start, the reflux ration was increased to 3. This was done to ensure the required ethanol content of the distillate (during a batch distillation the most volatile component is continuously depleted from the liquid in the boiler). All distillate fractions were analyzed on their ethanol content by means of Gas Liquid Chromatography using a Flame Ionization Detector (GLC-FID). The final distillate sample (all combined distillate fractions with sufficient ethanol content) was analyzed on water content (Karl Fischer titration (ASTM D 1364)); acidity (acid-base titration (ASTM D 1613)); anion content (chloride, sulphate, nitrate and carbonate measured by Ion Exchange Chromatography (ICE); and organic components other than ethanol (Gas Liquid Chromatography-Mass Spectrometry (GLC-MS)).

Solid/liquid separation of distillation residue

After the distillation step, the distillation residue was separated by centrifugation (Heine Zentrifuge, type DM-K-Z 2237-2V2, 1977) resulting in a liquid fraction and a fraction containing mainly solid particles. The feeding rate of the distillation residue was around 6 l/min and an agitation rate of 3500 rpm.

Anaerobic biodegradation of liquid fraction and biogas production

The anaerobic biodegradability of the liquid fraction to methane was studied by adding 20 ml liquid fraction to 21.3 ml containing macro nutrients, trace elements and a 10 mM $K_2HPO_4.3H_2O/NaH_2PO_4.2H_2O$ phosphate buffer (pH 7) (Alphenaar, 1994), inoculated with

75 g wet sludge, adjusted the mixture with water to 200 ml total volume and incubated at 30°C. The used inoculum was anaerobic granular sludge from a reactor treating potato processing wastewater with an organic matter content of 0.08 g/g sludge. The sludge concentration was 30 g/l. A blank without sludge was used to correct for methane production from the inoculum itself. During the test the gas production and composition, the pH, dissolved chemical oxygen demand (COD) and volatile fatty acids (VFA) were measured. The dissolved COD concentration was determined by the micro method with dichromate (Dr. Lange, Germany). For determination of the VFA concentration, samples were withdrawn, centrifuged for 10 min at 10000 rpm, and analysed on a Gas Chromatograph (Hewlett Packard 5890serie II) with a 30 m Alltech column (AT-Aquawax-DA 0.32 × 0.25mm). The temperatures of the column, the injection port and the FID detector are 80°C (beginning) - 220°C (end), 275°C and 300°C respectively. Nitrogen saturated with formic acid was used as the carrier gas (20 ml/min). The volume of produced biogas was determined using the Oxitop pressure measurement system. The quantities of these components in the reactor gas were determined on a Gas Chromatograph (Hewlett Packard 5890) with two different columns: 30 m Molsieve 5A 0.53 mm × 15 µm and 25 m Poraplot Q 0.53 mm × 20 µm. The carrier gas was helium and the temperatures of the column, the injection port and the TCD detector were 45°C, 110°C and 99°C respectively.

The potential toxicity of the liquid fraction was studied in an accumulation test with undiluted liquid fraction. The liquid fraction was added to the reactors in small quantities; increasing the concentration with each addition until a final concentration of 80% liquid fraction was reached with respect to the substrate/sludge relation (due to time limitations, reaching 100% was not possible). With each liquid fraction addition also macro nutrients, trace elements and a phosphate buffer were added to the system. The test was carried out at 30°C. The initial sludge concentration was 63 g organic matter per l, after all additions this was reduced to 15 g/l. A blank without liquid fraction was used to correct for methane production from the inoculum itself. During the test the gas production and composition were measured, as well as the pH to check for acidification of the system.

Determination of quality of solid fraction for combustion

The solid fraction was tested as fuel for combustion experiments. The solid fraction was analyzed using standard methods for chemical analysis of biomass fuels according to the NEN NTA 8200 series. The results were used to calculate Fluidized Bed Combustion (FBC) agglomeration and fouling indicators. Suitable conversion technology was selected using in-house expertise and data from literature (Baxter et al., 1998; Jenkins et al., 1998; Kaltschmitt, 2001; Van Loo and Koppejan, 2003). Combustion experiments were performed in a bench-scale experimental fluidized bed combustor (feed rate: approx. 0.15 kg/h) (Drift van der and Meijden van der, 2004). Air was used to fluidize the sand bed. The

fluidized bed was operated at a temperature of approx. 850°C and 5-6% *v/v* oxygen in the flue gas. The solid fraction was dried before combustion (approx. 3 wt% moisture) and milled (sieve diameter: 2 mm). The fuel was transported into the reactor by a screw feeder. A deposition probe was placed in the flue gas to investigate fouling behaviour. After finishing the experiment, the probe was carefully removed and visually inspected. Fly ash was collected in quartz filters.

RESULTS

Conversion of LTWS into ethanol by SSF

After the chemical treatment, the lime-treated wheat straw (LTWS) suspension was washed and dewatered as described by Bakker et al. (2007). The resulting biomass had a dry matter content of approximately 35% (*w/w*) and contained 0.1 g acetic acid per liter. The polysaccharide composition of the washed/dewatered lime-treated wheat straw (LTWS) was: glucan, 37.2; xylan, 20.1; arabinan, 2.8; galactan, 0.4; rhamnan, 0.2; and mannan, 0.2% (*w/w*), whereas the remaining mass constituted of lignin, ash and uronic acids. The polysaccharide fraction glucan and xylan accounted for 94% of the total polymeric sugars present in the LTWS. Therefore, we focused mainly on the conversion of these polysaccharides into fermentable sugars and ethanol via simultaneous saccharification and fermentation (SSF) in the 100-l fermentor. The SSF process consisted of two phases: (I) a pre-hydrolysis phase and (II) the SSF phase (Fig. 1), both controlled at 37°C and pH 5.0.

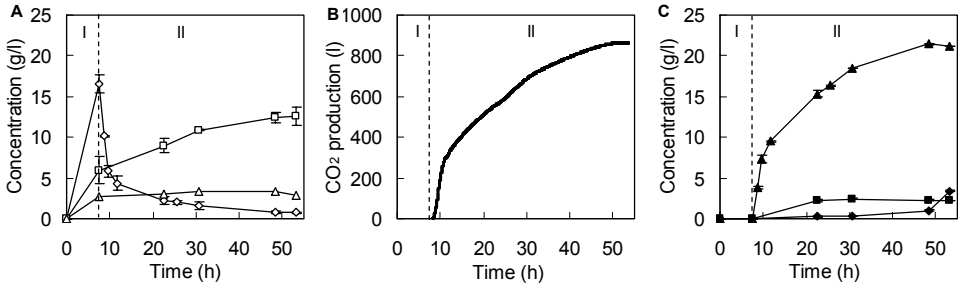

Figure 1. Profiles of the formation of glucose (◇), xylose (□) and arabinose (△) (A), the production of CO_2 (B), ethanol (▲), glycerol (■) and lactic acid (◆) (C) throughout the simultaneous saccharification and fermentation of lime-treated wheat straw by commercial enzyme preparation and *Saccharomyces cerevisiae* at pH 5.0 and 37°C. The dotted line represents the moment of yeast addition and discriminates between a pre-hydrolysis phase (I) and SSF phase (II). The error bars in symbols denote deviation of duplicate measurements.

Throughout the pre-hydrolysis phase, LTWS was added manually together with the commercial enzyme preparation GC 220, containing hydrolytic enzymes, in order to reduce the viscosity (liquefaction) and achieve a suitable amount of fermentable sugars. A total of 48.4 kg of LTWS (16.7 kg dry matter) was added during 6 h of the pre-hydrolysis phase to the reactor. Another 2 h of pre-hydrolysis (total of 8 h) resulted in a glucose, xylose and arabinose concentration of 15.4, 4.2 and 2.5 g/l, respectively (Fig. 1A). As soon as the bakers' yeast *S. cerevisiae* was added to the reactor, the accumulated glucose was rapidly fermented to ethanol and carbon dioxide (Fig. 1B, 1C). The wild-type bakers' yeast only converts glucose and the pentose sugars xylose and arabinose remain present in significant amounts. After 24 h of incubation, the glucose concentration was low (<3 g/l) suggesting that the enzymatic hydrolysis was the rate limiting step. After 48 h of incubation, the ethanol concentration increased to 21.4 g/l corresponding to 48% of theoretical maximum glucan-to-ethanol conversion. Based on this concentration, we conclude that 71% of the glucose monomers, liberated by hydrolysis, were converted into ethanol. At the end of the SSF, a sudden production of lactic acid was observed, most probably as a result of microbial contamination by e.g. lactic acid bacteria (Fig. 1C). During the SSF phase, the concentration of xylose increased to 11.8 g/l whereas the arabinose concentration remained constant at 3.3 g/l (Fig. 1A). The efficiency of the enzymatic saccharification and fermentation and the fate of polymeric glucan and xylan after 53 h of incubation are presented in Table 1. Part of the insoluble glucan initially present in the LTWS is still present as polysaccharide (25%), whereas minor parts are present as oligomeric sugar (6%) and monomeric sugar (1%). The largest part of the glucan is hydrolyzed and converted via glucose into fermentation products such as ethanol (and CO_2) (48%) and glycerol (5%) whereas the remaining part (15%) is most likely used mainly for yeast growth. With xylan, the situation is different. The insoluble xylan remains present as polysaccharide (26%), oligomeric sugars (52%) and xylose (24%).

TABLE 1. Fate of polysaccharide glucan and xylan initially present in washed lime-treated wheat straw after 53 hours of simultaneous saccharification and fermentation.

	Fraction	Glucan (% *w/w*)	Xylan (% *w/w*)
Hydrolysis products	Polysaccharides (insoluble)[a]	25	26
	Oligosaccharides (soluble)	6	52
	Monosaccharides (soluble)	1	24
Fermentation products	Ethanol and carbon dioxide (soluble)	48	[c]
	Glycerol (soluble)	5	[c]
Unaccounted[b]	(insoluble/soluble)	15	[c]

[a]Part of the initial polysaccharides remained present as insoluble polysaccharides; [b]Part of the initial polysaccharides was not recovered and therefore denoted as 'unaccounted'; [c]Xylan-derived sugars were not converted by yeast.

TABLE 2. Analysis of the ethanol distillate and comparison with ethanol fuel specifications (Bailey, 1996).

Component	Ethanol by fermentation		Specifications[a]	
	Ethanol distillate	Used test method	Values	Test method
Ethanol	90.7% wt (=72.6% *v/v*)	GLC-FID / GLC-MS	92% *v/v* (min)	ASTM D 3545-90
Methanol	0.01% wt	GLC-FID / GLC-MS	2% *w/w* (max)	ASTM D 4815-89[c]
Anion content (e.g. chloride)	<1 mg/l	ICE	Chloride 0.0004% *w/w* (max)	ASTM D 3120-87[d]
Water	9.6% wt	ASTM D1364	0.5% *w/w* (max)	ASTM E 203-75
Acidity	0.0006% wt HAc 0.01 mg KOH/g	ASTM D1613	HAc 0.007% *w/w* (max) KOH[b]	ASTM D 1613-85

[a]Specifications for E-95 (denatured) ethanol fuel according Detroit Diesel Corporation (DDC); [b]No specifications available; [c]Other alcohols and ethers; [d]ASTM D 3120-87 modified for determination of organic chlorides, and ASTM D 2988-86.

Quality of ethanol

To determine the quality of the produced ethanol for transportation fuel application, ethanol was distilled from the fermentation broth. The distillate contained an ethanol concentration of 11.5 % wt. The ethanol content was upgraded to approximately 90% wt by a second distillation step. The distillate was subsequently analyzed on potential contaminants (Table 2). These results show that the obtained product is fairly clean (on a water free water basis the contaminant levels will only slightly increase). With regard to the specifications of ethanol fuel described by Detroit Diesel Corporation (Bailey, 1996), the concentration of methanol, chloride ions and acetic acid are below the maximum limits. Furthermore, higher alcohols, esters or furfural type of components were not traceable in the ethanol distillate.

Anaerobic conversion of liquid fraction to biogas

Directly after SSF and subsequent distillation, the distillation residue was collected and a solid/liquid separation was performed by centrifugation. By this method, 71.6 kg distillation residue (17.3% dm and 9.7% insoluble solids) was divided in two fractions: (1) 52.7 kg (10.9% dm) liquid fraction serving as substrate for biogas production and (2) 18.6 kg (35.6% dm) of wet solid fraction functioning as fuel for thermal conversion tests. The centrifugation step however, was not optimized resulting in a liquid fraction containing a significant amount of solids, present in the form of small particles such as fines and salts.

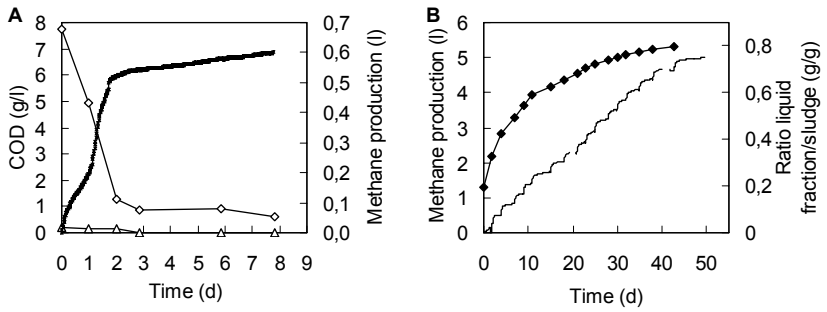

Figure 2. (A) Dissolved chemical oxygen demand (COD) (◇), dissolved volatile fatty acids (VFA-COD) (△) and methane production (line) during the anaerobic biodegradability test with ten times diluted liquid fraction (20 ml in total of 200 ml). Data were corrected for the blank. (B) Methane production (line) during the accumulation test where the ratio liquid fraction per sludge (◆) is increased. All data are represented as averages of duplicate experiments.

The liquid fraction, including dissolved organic compounds (e.g. xylose, arabinose, glycerol, acetic acid and lactic acid), was tested for potential anaerobic biodegradability to biogas (methane). Figure 2A shows the changes in dissolved chemical oxygen demand (COD), volatile fatty acids (VFA-COD) and methane production during the anaerobic fermentation. Throughout the fermentation, no VFA accumulation or acidification occurred (initial pH 6.7). The methane content of the biogas was 57% ± 5%. Furthermore, Table 3 shows an anaerobic biodegradability of 57% ± 1% on a COD basis. The remaining non-degraded fraction is most likely partly composed of non-biodegradable dissolved COD (Fig. 3A) and partly of non- or very slowly biodegradable solid matter, as visual inspection after 8 days showed that the solid material present in the effluent had not disappeared. Table 3 shows a COD balance of the experiment. From the values it can be concluded that the dissolved fraction was almost completely degraded whereas the non-dissolved organic matter was partly degraded.

TABLE 3. Chemical oxygen demand balance for the degradability of liquid fraction (in g O_2 based on a litre effluent)[a].

		COD (g O_2)	% of initial total COD value
Start situation	Total	134,9	100
	Dissolved	54,6	40
	Non-dissolved[a]	80,3	60
End situation	CH_4	77,2	57
	Dissolved	9,4	7
	Non-dissolved[a]	48,2	36

[a]Non-dissolved COD was calculated from the measured values for total and dissolved COD.

The effect of potential presence of toxic components in the liquid fraction was tested by performing an accumulation experiment. Figure 2B shows the methane production in the accumulation test where the liquid fraction increased. No acidification took place and the average methane content of the biogas was 53% ± 2%. Per feed roughly the same amount of methane was produced, with an average degradability of 60% ± 1%. In conclusion, no adaptation of the sludge or toxic effects of the liquid fraction was observed suggesting feasible biodegradability of the liquid distillate residue.

Thermal conversion of solid fraction

The wet solid fraction was after drying used as fuel for thermal conversion via combustion. The composition of the solid fraction is important for its thermal conversion behaviour. Data of the analysis of the solid fraction are presented in Table 4 and compared with the original wheat straw and typical values for clean, untreated wood. The ash content of the experimental solid fraction is high (27.0 wt%) compared to straw (11.5 wt%) and wood (1.5 wt%). The silica content of the solid fraction has increased significantly (>4 wt%) compared to the wheat straw (2.8 wt%), due to the removal and conversion of cellulose and hemi-cellulose. As a result of the addition of calcium hydroxide and sulphuric acid, the concentrations of calcium (5.2 wt%) and sulphur (3.6 wt%) are increased compared to wheat straw and high compared to other (biomass) fuels. The content of phosphorus has increased compared to wheat straw due to addition of phosphorus (0.51 wt%) containing additives during the fermentation process. Relatively small amounts of potassium (0.30 wt%) and chlorine (0.15 wt%) can be found; more than in clean wood, but less than in the original straw. The Higher Heating Value (HHV) of the $Ca(OH)_2$ wheat straw solid fraction (experimental) was 13.4 MJ/kg (dry basis) which is lower in comparison to the original wheat straw feedstock (16.6 MJ/kg) and wood (19.8 MJ/kg). The Lower Heating Values (assuming 0, 15 and 50% moisture content during combustion) are presented in Table 4.

TABLE 4. Fuel analysis results of the $Ca(OH)_2$ treated wheat straw solid fraction (experimental), original wheat straw feedstock and untreated wood.

Source Composition		$Ca(OH)_2$ Treated Wheat Straw Solid Fraction (Experimental)	Wheat Straw Original feedstock (Harvest 2003)	Wood (untreated, average) (Phyllis, 2006)
Parameter	Unit			
Ash (550°C)	$wt\%_{dry}$	27.2	12.6	-
Ash (815°C)	$wt\%_{dry}$	27.0	11.5	1.5
Volatiles	$wt\%_{dry}$	68.4	70.1	80.7
Moisture	$wt\%_{wet}$	3.4	7.7	49.2
HHV, measured	MJ/kg_{dry}	13.4	16.6	19.8
LHV	MJ/kg_{dry}	12.4	15.6	18.5
LHV (15% MC)	MJ/kg_{wet}	10.2	12.9	15.4
LHV (50% MC)	MJ/kg_{wet}	5.0	6.6	8.0
C	$wt\%_{daf}$	43.1	46.5	51
H	$wt\%_{daf}$	5.8	5.6	6.1
N	$wt\%_{daf}$	1.5	1.7	0.3
O	$wt\%_{daf}$	44.8	45.8	43
S	$wt\%_{daf}$	4.56	0.15	0.06
Cl	$wt\%_{daf}$	0.20	0.31	0.04
F	$wt\%_{daf}$	0.0031	-	0.0024
Al	mg/kg_{dry}	121	185	211
As	mg/kg_{dry}	5.4	< dl	<dl
B	mg/kg_{dry}	5.2	4.1	9
Ba	mg/kg_{dry}	3.2	3.6	143
Ca	mg/kg_{dry}	52000	4700	13000
Cd	mg/kg_{dry}	<dl	0.15	0.5
Co	mg/kg_{dry}	7.3	55	0.51
Cr	mg/kg_{dry}	10.2	0.2	5
Cu	mg/kg_{dry}	9.1	1.6	17
Fe	mg/kg_{dry}	160	220	120
K	mg/kg_{dry}	3000	15000	2000
Li	mg/kg_{dry}	<dl	0.2	<dl
Mg	mg/kg_{dry}	870	1105	769
Mn	mg/kg_{dry}	33	14	214
Mo	mg/kg_{dry}	<dl	1.9	0.55
Na	mg/kg_{dry}	1500	200	300
Ni	mg/kg_{dry}	5	0	8
P	mg/kg_{dry}	5100	485	486
Pb	mg/kg_{dry}	<dl	1.1	32
Sb	mg/kg_{dry}	1.4	1.8	1.8
Se	mg/kg_{dry}	<dl	1.9	1
Si	mg/kg_{dry}	40000	28000	900
Sn	mg/kg_{dry}	0	0.20	1.2
Sr	mg/kg_{dry}	23	18	14
V	mg/kg_{dry}	0.3	-	-
Zn	mg/kg_{dry}	25	0.6	0.4

The agglomeration indicators (not shown here) were calculated, but give no clear answer whether agglomeration behaviour will be better or worse compared to the straw feedstock. The risk on sinter induced agglomeration seems to be small. Fouling might be expected, but could also not be detected. Due to the higher content of sulphur, the emissions of SO_2 will be a point of attention and this is significantly different from regular combustion of straw or wood, but well known in coal combustion.

The combustion test showed good process stability and no signs of bed agglomeration were observed, but for being conclusive the duration of the test was too short due to the small amount of solid fraction sample. Also, in the SEM analyses no potassium silica coating (an indication for possible agglomeration) could be detected on the bed material. Compared to wood combustion the deposition rates of the ash particles on the probe were high due to the much higher ash content of the solid fraction. The ash depositions could however be easily removed from the probe surface. Fouling is likely to occur, but will not easily stick. Vibration or soot blowing can remove ash deposition.

Overall balance

An overview of the lignocellulose-to-ethanol process with the respective process steps of side-streams is presented in Figure 3. Furthermore, the input and output streams were quantified. With the results obtained by the SSF to ethanol, anaerobic digestion to methane and the thermal conversion, we calculated that a 1000 kg LTWS can be converted into 100 kg ethanol (equal to 3.0 GJ thermal energy calculated with Higher Heating Value (HHV) of 29.7 MJ/kg), 69 kg methane (equal to 3.8 GJ thermal energy calculated with HHV of 55.5 MJ/kg), approximately 204 kg sludge, 246 kg carbon dioxide and 398 kg lignin-rich residue. The solid lignin-rich fraction contains a Higher Heating Value of 13.4 MJ/kg (dry basis) equal to a total of 5.3 GJ thermal energy for heat and power generation. Furthermore, the combustion of the lignin-rich fraction results mainly in 458 kg carbon dioxide, 151 kg water and 107 kg inorganic ash.

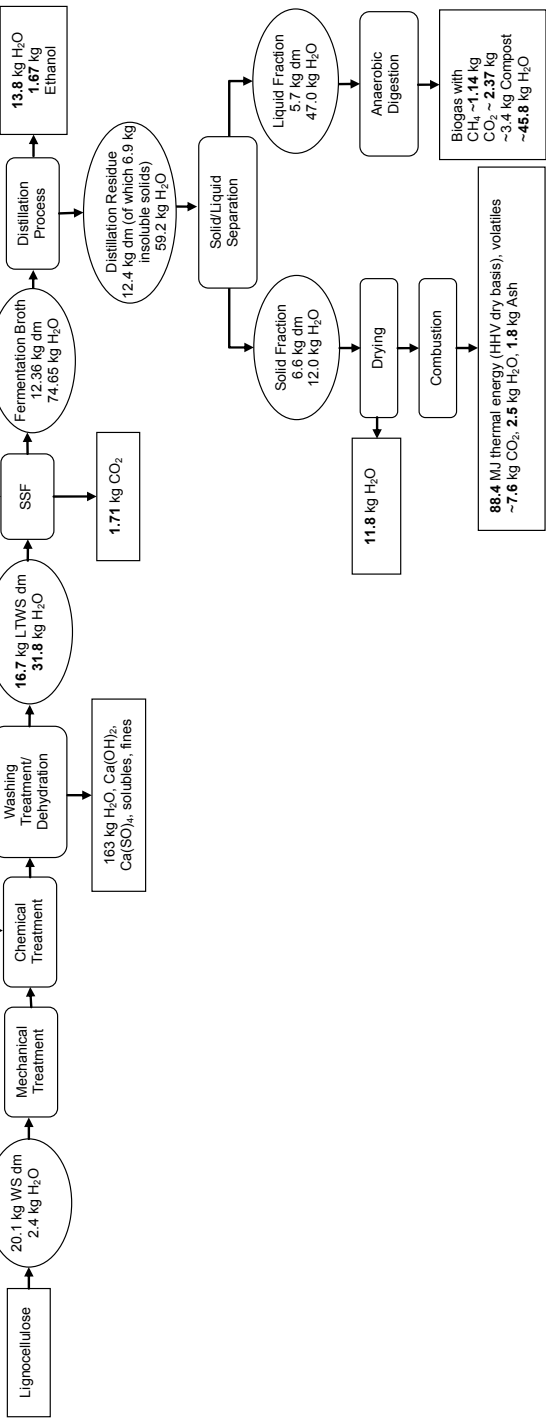

Figure 3. Schematic depiction of an integrated process converting lignocellulose into ethanol, biogas (methane), carbon dioxide, compost, energy (heat and power) and ash. The energy can be used for the process or can be delivered to the grid. The 'clean' water can be recycled into several steps of the process.

DISCUSSION

Lignocellulosic biomass has great potential with regard to its usage as substrate for large-scale production of bio-fuels. The lignocellulose-to-ethanol conversion via simultaneous saccharification and fermentation is a complex process involving various enzymatic and microbial activities. Results from previous studies showed that the mild-temperature lime treatment (0.1 g $Ca(OH)_2$ per g substrate, 16-20 h, 85°C) enhances the accessibility of polymeric carbohydrates towards hydrolytic enzymes resulting in the release of fermentable sugars and improved ethanol yield (Bakker et al., 2007; Maas et al., 2007b). Nevertheless, these studies were performed at laboratory- and bench scale. This paper describes the pretreatment, enzymatic hydrolysis and microbial conversion of lime-treated wheat straw via SSF to ethanol at pilot-scale. Prior to using the lime-treated substrate for hydrolysis and fermentation, the inhibitor acetic acid was successfully removed by a relatively simple washing procedure.

Reaction conditions and enzyme loadings determined in previous optimization studies were used for the hydrolysis and fermentation. This resulted in a glucan-to-ethanol conversion (48%) which is lower than the yields obtained by laboratory tests (56%) (Maas et al., 2007b). A possible explanation for this difference in ethanol yield can be found in the higher dry matter content, which was approximately twice as high in the pilot-scale ethanol fermentation (~20% *w/w*), influencing the enzymatic hydrolysis negatively. Approximately 25% of the glucan in LTWS remained present as insoluble polymeric sugar suggesting the presence of crystalline and hardly degradable cellulose and/or inactivation of the hydrolytic system.

In case of xylan, the situation is different. Due to inability of the wild type yeast *S. cerevisiae* to convert pentose sugars, possibly end product inhibition by xylose occurs resulting in the accumulation of mainly xylo-oligomeric sugars. Previous SSF work with lime-treated wheat straw, the hydrolytic enzymes and *Bacillus coagulans* - a pentose-converting lactic acid-producing bacterium - showed that the concentration of xylo-oligomeric sugars and xylose remain low (Maas et al., 2007a). This indicates that the presence of xylose monomers in the medium of the pilot-scale ethanol process inhibits the hydrolysis of soluble xylo-oligomeric sugars. Another possible explanation can be found in the fact that the used cellulase cocktail GC 220 is not optimized for hemi-cellulolytic activity and therefore lacks one of the essential enzymatic activity e.g. deacetylating and debranching enzymes or ß-xylosidase. In addition, the xylan-to-xylose conversion at pilot-scale SSF (24%) agrees relatively well with the yield obtained at laboratory scale SSF experiments (28%). Further optimisation of the enzymatic hydrolysis and fermentation step, can be realised by the use of a more efficiently hydrolytic enzyme cocktail and a pentose-

converting yeast resulting in a higher ethanol concentration and yield. In case a xylose-converting yeast will be used, an additional ethanol amount of approx. 1.4 kg (assuming conversion of 74% of xylan, which is the fraction that was present as monomer and oligomer after 53 h of incubation, and with an ethanol yield of 0.51 g/g) can be formed from this C5 sugar. Less dissolved substrate will be available for anaerobic digestion resulting in approx. 55% reduction of methane to ~0.5 kg.

Ethanol was upgraded via two separate distillation processes resulting in a distillate containing 90% wt ethanol. Tests showed that the ethanol distillate, in comparison to the ethanol fuel specifications of Detroit Diesel Corporation, seems suitable as transportation fuel.

The fermentation residue was separated in two fractions; one fraction containing mainly solids functioning as fuel for thermal conversion and a liquid fraction containing mainly soluble compounds serving as substrate for anaerobic fermentation to biogas. The liquid fraction has a moderate anaerobic biodegradability of 60% respective to the total COD content, and no signs of toxicity could be observed in the accumulation test. The relatively low value is largely a result of the high amount of solid organic matter in the liquid fraction, which was only partly degraded. More efficient removal of the solid matter would contribute to a significant reduction of the COD load. As the dissolved matter has a high anaerobic biodegradability, anaerobic treatment would then be an attractive way of processing this effluent for COD reduction and energy production in the form of methane gas.

The solid fraction of the distillation residue appeared to be suitable as fuel for thermal conversion, although it contains a relatively high ash concentration and relatively high concentrations of sulphur and calcium due to additions in the upstream processes. It is expected that the combustion behaviour will show a behaviour between the original feedstock wheat straw and (clean) wood. Based on the analysed residue composition there is some risk of smelt induced bed agglomeration. The risk of sinter induced bed agglomeration is less than for straw, because of the partial removal of potassium in the upstream processes.

Combustion in a circulating fluidized bed (CFB) was selected as the most suitable technology, due to the small particles size of the fuel (<several mm), moisture content (max. 50-60% after dewatering) and the required capacity (30 MW_{th} is considered the minimum scale for CFB). The combustion temperature is relatively low (800-900°C) to reduce the risk of sintering of the ashes in the bed. To reduce the risk of agglomeration it is furthermore possible to partly refresh the bed material during operation. It is expected that emission standards can be met in a full-scale process by standard flue gas cleaning technologies (FGD, de-NO_X, particle removal).

The combustion experiments (duration 4-7 h) showed no agglomeration and no extensive coating has been detected on the bed material that indicate agglomeration. Long-duration experiments are however necessary, to provide a more profound assessment of the fuel behaviour. With respect to fouling, high ash deposition rates were observed. The composition of both bottom ashes and fly ashes were determined. Most likely, both the bottom ashes and fly ashes can be utilised rather than landfilled. The main options are utilisation as building material and in some cases as fertilizer. More conclusive assessment of the potential utilisation of the ashes requires larger samples that are more representative for full scale, because the ashes from the pilot-scale test contain less realistic salt contents. Details on the assessment of ashes will be presented in a follow-up paper.

In conclusion, this paper describes the conversion of high dry matter content lime-treated wheat straw via SSF into bioethanol at pilot-scale. In comparison to conversions obtained by laboratory experiments, we can conclude that comparable fermentable sugar and ethanol yields were achieved at pilot-scale. Tests showed that the produced ethanol was suitable as transportation fuel. The side-streams were quantitatively analysed. The liquid fraction with mainly soluble components was valorised by anaerobic fermentation to biogas (methane) and sludge. The remaining solid fraction was a suitable fuel for conversion via combustion yielding thermal energy and inorganic ashes with decent perspective for being utilized rather than landfilled.

ACKNOWLEDGEMENTS

This research is supported with a grant of the Dutch Programme EET (Economy, Ecology, Technology) a joint initiative of the Ministries of Economic Affairs, Education, Culture and Sciences and of Housing, Spatial Planning and the Environment. The programme is managed by the EET Programme Office, SenterNovem. The authors thank Mirjam A. Kabel and Jacinta C. van der Putten for performing carbohydrate analysis and Richard G.M. Op den Kamp and Patrick F.N.M. van Doeveren for providing technical assistance with the pretreatment and the fermentation.

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CHAPTER 7

Lactic Acid Production from Lime-Treated Wheat Straw by *Bacillus coagulans*: Neutralization of Acid by Fed-Batch Addition of Alkaline Substrate

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ABSTRACT

Conventional processes for lignocellulose-to-organic acid conversion requires pretreatment, enzymatic hydrolysis and microbial fermentation. In this study, lime-treated wheat straw was hydrolyzed and fermented simultaneously to lactic acid by an enzyme preparation and *Bacillus coagulans* DSM 2314. Decrease of pH due to lactic acid formation was partially adjusted by automatic addition of the alkaline substrate. After 55 hours of incubation, the polymeric glucan, xylan and arabinan present in the lime-treated straw were hydrolyzed for 55, 75 and 80%, respectively. Lactic acid (40.7 g/l) indicated a fermentation efficiency of 81% and a chiral L(+)-lactic acid purity of 97.2%. In total 711 g lactic acid was produced out of 2706 g lime-treated straw, representing 43% of the overall theoretical maximum yield. Approximately half of the lactic acid produced was neutralized by fed-batch feeding of lime-treated straw, whereas the remaining half was neutralized during the batch phase with a $Ca(OH)_2$ suspension. Of the lime added during pretreatment of straw, 61% was used for the neutralization of lactic acid. This is the first demonstration of a process having a combined alkaline pretreatment of lignocellulosic biomass and pH control in fermentation resulting in a significant saving of lime consumption and avoiding the necessity to recycle lime.

INTRODUCTION

Lactic acid is used throughout the world in manufacturing of food, chemicals and pharmaceutical products. Recently, there is a lot of interest in biodegradable poly-lactic acid (PLA) which is an alternative to petrochemically derived plastic (Drumright et al., 2000). Chiral pure lactic acid is produced commercially by microbial fermentation of the carbohydrates glucose, sucrose, lactose and starch/maltose derived from feedstocks such as beet sugar, molasses, whey and barley malt (Narayanan et al., 2004). The choice of feedstock depends on its price, availability and on the respective costs of lactic acid recovery and purification (Datta et al., 1995; Vaidya et al., 2005).

As an alternative to these traditional feedstocks, lignocellulosic biomass is an inexpensive and widely available renewable carbon source that has no competing food value. Lignocellulose consists primarily of cellulose and hemicellulose; polymers build up of mainly hexose sugars and pentose sugars, which are embedded in a matrix of the phenolic polymer lignin. The main pathway to derive fermentable sugars from lignocellulose is through enzymatic hydrolysis by cellulolytic and hemicellulolytic enzymes. A mechanical and chemical pretreatment of the lignocellulose is required in order to reduce particle size, to modify and/or to remove the lignin and with that enhance the accessibility of the polysaccharides for enzymatic hydrolysis (Claassen et al., 1999). Various chemical pretreatments of biomass have been studied in research and development of lignocellulose-to-ethanol production technology (Mosier et al., 2005). One is the use of lime (calcium hydroxide) at relatively mild temperature conditions (Chang et al., 1998). Lime as pretreatment agent has promising potential because it is inexpensive, safe and its use hardly results in sugar degradation products such as furfural and hydroxymethyl furfural. Nevertheless, this alkaline pretreatment features a relatively high pH value (>10) of the treated biomass and, at these pH levels, the activity of common cellulolytic and xylanolytic enzymes, necessary for the depolymerization of (hemi)-cellulose, is negligible low. Therefore, lowering the pH is essential in order to achieve an efficient enzymatic hydrolysis of the polysaccharides. One approach to remove calcium hydroxide is by washing the lime-treated biomass prior to enzymatic hydrolysis (Chang et al., 1998); however, this leads to the use of high amounts of water. Another way to lower the pH of the pretreated material is by neutralizing calcium hydroxide with sulphuric acid. Yet this results in the formation of the low value by-product gypsum.

As an alternative improvement to these approaches, we propose to use the calcium hydroxide present in lime-treated biomass as neutralizing agent for organic acids produced in microbial fermentation processes. To examine this proposed concept, lime-treated wheat straw was added fed-batch wise during a SSF process in a 20L controlled stirred fermenter containing hydrolytic enzymes and *Bacillus coagulans* DSM 2314, a thermophilic

bacterium capable to convert both hexoses and pentoses homofermentative to L(+)-lactic acid (Otto, 2004; Patel et al., 2006). The objective of this research was to evaluate whether high alkaline-treated lignocellulosic biomass (without neutralization) can be used directly in a SSF process by (1) providing a carbon source for enzymatic hydrolysis and fermentation and (2) providing a source of alkali to control the pH in the fermentation process.

MATERIALS AND METHODS

Feedstock and pretreatment

Wheat straw was selected as lignocellulose model feedstock and was purchased from a farm in the Northeast of the Netherlands. The wheat straw was air dry (89.5% (*w/w*) dry matter) and ground through a 2-mm screen. The lime pretreatment was performed by filling two 15 l mixers (Terlet, The Netherlands), both with 1650 g ground wheat straw, 13 kg tap water and 165 g calcium hydroxide. This wheat straw suspension was heated and kept at 85°C for 16 hours under continuously stirring at 30 rpm. The lime-treated wheat straw (LTWS) suspension was subsequently cooled to 30°C, dehydrated by placing the LTWS in a cotton bag, and pressing the suspension using a manual piston press at pressure up to 9.7 kg/m^2. After dehydration, an amount of 11.45 kg LTWS with an average dry matter content of 27.0% (*w/w*) and pH 11.8 was obtained and served as substrate for further experiments. The chemical composition of LTWS was determined as described by van den Oever et al. (2003).

Enzyme preparation

The enzyme preparation GC 220 (Genencor-Danisco, Rochester, USA) containing cellulase, cellobiase and xylanase activity of 116, 215 and 677 U/ml, respectively, (Kabel et al., 2006) and was used for this study. The preparation had a specific gravity of 1.2 g/ml and contained 4.5 mg/ml glucose, 2.9 mg/ml mannose and 0.8 mg/ml galactose.

Micro-organism and pre-culture

The bacterium *Bacillus coagulans* strain DSM 2314 was used as lactic acid-producing micro-organism. Bacterial cells were maintained in a 10% (*w/w*) glycerol stock solution and stored at -80°C. Chemicals, unless indicated otherwise, were purchased from Merck (Darmstadt, Germany). Gelrite plates were prepared with medium containing (per liter) glucose, 10 g; Gelrite, 20 g (Duchefa, Haarlem, The Netherlands); yeast extract, 10 g (Duchefa); $(NH_4)_2HPO_4$, 2 g; $(NH_4)_2SO_4$, 3.5 g; BIS-TRIS, 10 g (USB, Ohio, USA); $MgCl_2.6H_2O$, 0.02 g and $CaCl_2.2H_2O$, 0.1 g. Glucose and Gelrite were dissolved in stock

solution A (4 times concentrated). The pH of this stock solution was adjusted to 6.4 with 2M hydrochloric acid and autoclaved for 15 min at 125°C. The remaining nutrients were dissolved in stock solution B (1.33 times concentrated) which was also adjusted to pH 6.4 with 2M hydrochloric acid but was filter sterilized (cellulose acetate filter with pore size of 0.2 μm, Minisart, Sartorius). After sterilization, the medium was prepared by combining stock solution A and B and Gelrite plates were poured. The bacteria were cultivated on Gelrite plates for 48 h at 50°C.

An isolated colony was used to inoculate a 100-ml broth with similar composition and preparation as described above, however without the addition of Gelrite. The culture was incubated statically for 24 h at 50°C and functioned as inoculum for a 1400-ml broth. This culture was incubated also statically for 12 h at 50°C and served as a 10% (*v/v*) pre-culture for the SSF experiments.

Simultaneous saccharification and fermentation

The SSF of LTWS was carried out in a 20L-fermenter (Applikon, Schiedam, The Netherlands) with pH and temperature control (biocontroller ADI 1020). At the start of SSF, the fermenter was filled with 6.0 kg tap water and 1400 g dehydrated LTWS (DM content of 27.0% (*w*/*w*)). The following nutrients were then added to the LTWS suspension: yeast extract, 150 g (Duchefa); $(NH_4)_2HPO_4$, 30 g; $(NH_4)_2SO_4$, 52.5 g; $MgCl_2.6H_2O$, 0.3 g and $CaCl_2.2H_2O$, 1.5 g. The LTWS suspension was then heated to 50°C and the pH was adjusted to 6.0 with 101 g 3M sulphuric acid (~ 30 g H_2SO_4).

The SSF process of LTWS to lactic acid consisted of three phases; I) the pre-hydrolysis phase of pre-loaded LTWS, II) the fed-batch phase with automatic feeding of LTWS from a screw feeder, and III) the batch phase with pH control by a calcium hydroxide suspension and no LTWS feeding. A schematic representation of the experimental set-up is shown in Figure 1. The pre-hydrolysis was initiated by addition of 40 ml enzyme preparation (88 mg enzyme/g DM substrate) to the LTWS suspension and was incubated for two hours at 50°C under continuously stirring at 250 rpm. The fed-batch phase was initiated by addition of 1500-ml pre-culture of *B. coagulans* DSM 2314 to the fermenter. The lactic acid produced by the bacteria was neutralized by the automatic addition of 8623 g dehydrated LTWS (DM of 27.0%) to the fermenter through a feeder (K-Tron Soder Feeders, Canada) and was regulated by the pH of the medium which was set at 6.0. Throughout the fed-batch phase, an amount of 280 ml of enzyme preparation (total enzyme loading of 98 mg/g DM substrate) was added proportional to the LTWS addition rate into the fermenter. During the batch phase, the pH was controlled at 6.0 by the addition of 20.0% (*w/v*) calcium hydroxide suspension. Samples were withdrawn for dry matter, substrate and (by)-product analysis.

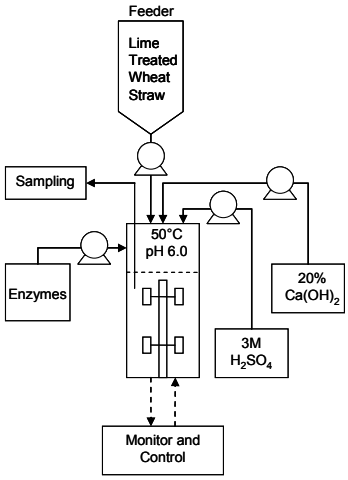

Figure 1. Schematic representation of the simultaneous saccharification and fermentation of lime-treated wheat straw to lactic acid.

Analytical methods

For the analysis of monomeric sugars, the fermentation broth samples were centrifuged (3 min at 17400×*g*), the pH of the supernatant was adjusted to 5.0 with barium carbonate using a pH-indicator (Bromophenolblue) followed by filtration of the liquid. The analysis was performed by high-performance anion-exchange chromatography using a Carbopack PA1 column (column temperature of 30°C) and a pulsed amperometric detector (ED50) (Dionex, Sunnyvale, CA). Prior to injection, the system was equilibrated with 25.5 mM NaOH for 10 min at a flow rate of 1.0 ml/min. For the separation of monomeric sugars, at injection the mobile phase was shifted to de-ionized water for 30 min. Post-column addition of sodium hydroxide was used for detection of the neutral monomeric sugars.

The determination of soluble oligomeric sugars was performed by centrifugation for 5 minutes at 3000 rpm (Centaur 2, Beun de Ronde, The Netherlands) of pre-weighed samples and freeze drying the supernatant overnight. Pellets were weighed, hydrolyzed with sulphuric acid and neutral monomeric sugars were determined according to the method as described by van den Oever et al. (2003). For the calculations, an average molecular weight of oligomers from glucan and xylan of 166 and 132 g/mol, respectively, were applied, resulting in a hydrolysis factor of 1.08 and 1.14, respectively.

For the analysis of insoluble polymeric sugars, samples of 25 gram were centrifuged for 5 min at 3000 rpm (Centaur 2, Beun de Ronde, The Netherlands), supernatant was removed and the pellet was washed by re-suspension in 25 ml fresh demineralised water following by a centrifugation step of 5 minutes at 3000 rpm (Centaur 2, Beun de Ronde, The

Netherlands). The sequence of re-suspension and centrifugation was repeated three times. After the last removal of the supernatant, the pellets were freeze dried overnight. The pellets were weighed (values used for dry matter (DM) calculation), polymeric material hydrolyzed with sulphuric acid and neutral sugars analyzed according to the method as described by van den Oever et al. (2003). For the calculations, a molecular weight of glucan and xylan of 162 and 132 g/mol, respectively, were applied and resulting in a hydrolysis factor of polymer to monomer of 1.11 and 1.14, respectively.

The analysis of organic acids was performed by high pressure liquid chromatography according to the procedure described by Maas et al. (2006). The chiral purity (%) of lactic acid was determined by derivatization of all lactates using methanol, after which both enantiomers of methyl lactate were separated on a chiral Gas Chromatography column and detected using a Flame Ionization Detector. The chiral purity was expressed as the area of the main enantiomer divided by the sum of areas of both enantiomers.

Calculations

The theoretical maximum lactic acid ($LA_{theor.max.}$ (g)) production was calculated according the following equation [Eq. 1].

$$LA_{theor\max} = DM_{substrate} * F_{polysacch.} * HF_{monosacch./\,polysacch.} * FF \qquad \text{[Eq. 1]}$$

Where $DM_{substrate}$ = the total Dry Matter of substrate LTWS (g); $F_{polysacch.}$ = Fraction polysaccharides per substrate (g/g); $HF_{monsacch./polysacch.}$ = Hydrolysis Factor of polysaccharides, incorporation of water results in 1.11 g hexose from 1.00 g glucan and 1.14 g pentose from xylan and arabinan (g/g) and *FF* = Fermentation Factor of 1.00 g lactic acid per g of monomeric sugar.

The efficiency of the enzymatic hydrolysis (%, *w/w*) was based on the amount of hydrolyzed polysaccharides (g) (calculated by the difference between initial amounts and analyzed insoluble amounts) divided by the amount of polysaccharides (g) initially present in the substrate. The fermentation efficiency (%, *w/w*) is expressed as the amount of lactic acid produced (g) divided by the amount of monomeric sugars consumed (g) by the bacteria. The overall efficiency of the SSF (%, *w/w*) was calculated by the amount of lactic acid produced (g) divided by the theoretical maximum amount of lactic acid (g) determined as described in Eq. 1.

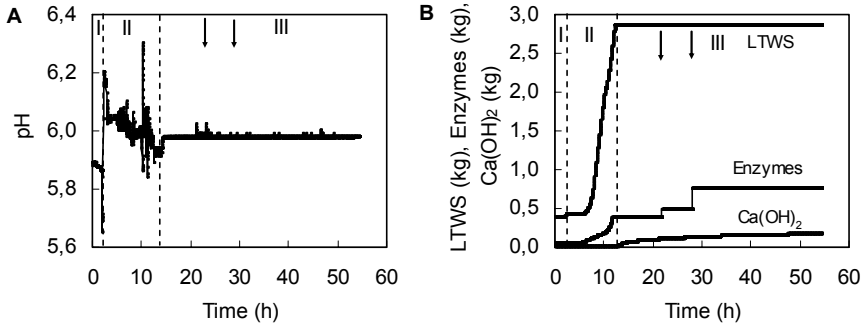

Figure 2. Control of pH (*A*) during simultaneous saccharification and fermentation of lime-treated wheat straw by commercial enzyme preparation GC 220 and *Bacillus coagulans* DSM 2314 (*B*). The areas between the dotted lines represent the pre-hydrolysis phase (I), the fed-batch phase (II) with pH control by addition of alkaline LTWS and enzymes, and the batch phase (III) with pH control by addition of $Ca(OH)_2$ suspension. Extra enzyme preparation GC220 was added at the times indicated by the arrows.

RESULTS

Simultaneous saccharification and fermentation of LTWS to lactic acid

The polysaccharide composition of the lime-treated wheat straw (LTWS) consisted mainly of glucan, xylan and arabinan of 33.0, 19.0 and 2.0% (*w/w*), respectively, whereas the remaining mass constituted of lignin, ash, extractives and uronic acids. Some of the soluble components in wheat straw were partially removed by the solid/liquid separation (dehydration) of the LTWS. The focus of this study was on the conversion of glucan, xylan and arabinan which are the predominant polysaccharides present in LTWS and accounted for 99.8% (*w/w*) of the total polymeric sugars. Previous work showed that the cellulase preparation GC 220, used for the saccharification of polysaccharides, functioned optimally at 50°C and pH 5.0 (Maas et al., 2007), whereas growth conditions for *Bacillus coagulans* DSM 2314 were 54°C and pH 6.5 (Otto, 2004). In this study, both the enzymatic hydrolysis and the fermentation occurred simultaneously in the same reactor at compromising conditions which were set at 50°C and pH 6.0.

The SSF of LTWS to lactic acid was studied in a 20L controlled stirred fermenter. Previous results showed that when this process was performed without a pre-hydrolysis of an initial amount of LTWS, the concentration of monomeric sugars was low and resulted, therefore, in relatively low lactic acid productivity. As a consequence, the fed-batch addition rate of the alkaline substrate to neutralize the produced lactic acid was low (results not shown).

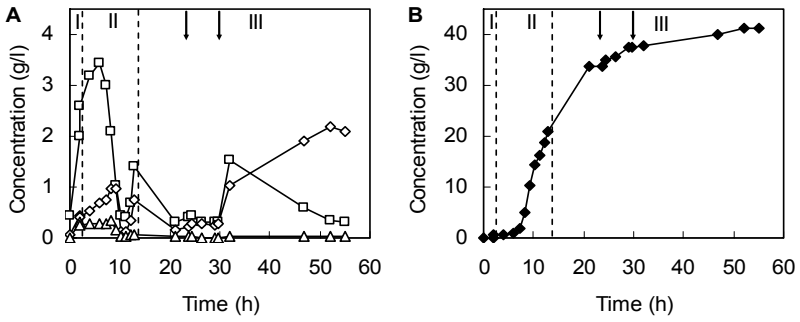

Figure 3. Profiles of glucose (□), xylose (◇), arabinose (△) (*A*) and lactic acid (◆) (*B*) in simultaneous saccharification and fermentation of lime-treated wheat straw by commercial enzyme preparation GC 220 and *Bacillus coagulans* DSM 2314. The areas between the dotted lines represent the pre-hydrolysis phase (I), the fed-batch phase (II) and the batch phase (III). Extra enzyme preparation GC220 was added at the times indicated by the arrows.

In order to start the fermentation with a substantial initial amount of fermentable sugars (> 2 g/l), a pre-hydrolysis of 378 g LTWS and enzyme preparation (88 mg per g DM LTWS) in approximately 6 liter volume at pH 6.0 for two hours was introduced. This resulted in glucose, xylose and arabinose concentrations of 2.0, 0.4 and 0.3 g/l, respectively (Fig. 3B).

The second phase (II) was initiated by introducing a 1500-ml pre-culture of *B. coagulans* DSM 2314. A minor amount of lactic acid produced in the pre-culture caused a slight pH decrease and was automatically neutralized by the addition of LTWS (Fig. 2A, B). After a lag phase of four hours, the dissolved oxygen concentration decreased rapidly from 100% to oxygen-limiting conditions of below 1% (results not shown). At that moment, a concentration of glucose, xylose and arabinose of 3.3, 0.7 and 0.3 g/l, respectively, was present (Fig. 3A). These sugars were consumed simultaneously where glucose was utilized faster than xylose and arabinose. Simultaneous with the consumption of these monomeric sugars, lactic acid was produced which was neutralized by the automatic addition of alkaline LTWS. By the addition of alkaline substrate throughout the fed-batch phase, the pH was maintained accurately at 6.0 ± 0.1 (Fig. 2A, B). At the end of phase II, a total amount of 10023 g dehydrated LTWS (~ 2706 g DM LTWS) and 320 ml of enzyme preparation was added to the fermenter. A lactic acid concentration of 20.5 g/l supernatant was detected (Fig. 3B), corresponding to a total of 342 g lactic acid. The chiral L(+) purity of lactic acid was determined at 99.4% which is similar to that obtained with xylose as sole carbon source (Otto, 2004).

At the end of phase I, a low acetic acid concentration was detected in the medium which increased to 1.5 g/l throughout phase II but, remained constant during phase III (results not shown). This indicates that acetic acid was most likely not a fermentation product formed

by *B. coagulans*. Acetic acid can be released upon solubilisation and hydrolysis of hemicellulose during chemical pretreatment (Palmqvist et al., 1999). By the dehydration procedure of the LTWS, part of the acetic acid was easily separated from the substrate by removing the press water. Apparently, a remaining amount of acetic acid was fed together with the substrate to the fermenter. Also, minor traces of other organic acids such as succinic acid and formic acid (<0.5 g/l) were detected in the fermentation broth.

Phase III was initiated by changing the pH control from the addition of alkaline LTWS to a 20% (*w/v*) calcium hydroxide suspension. To maintain the pH at 6.0, addition of calcium hydroxide suspension occurred relatively fast but shifted, however, after a few hours to a lower addition rate indicating a decline of the volumetric lactic acid productivity (Fig. 2B, 3B). To exclude limitation (e.g. by inactivation) of enzymes, an extra dosage of enzyme preparation (80 ml) was added to the fermenter after 23.5 h of incubation. This resulted immediately in a slight acceleration of the calcium hydroxide addition rate indicating an increased lactic acid productivity and limitation of enzymatic activity (Fig. 3B). Nevertheless, after 29.7 h of incubation, a decline of the calcium hydroxide addition rate was observed again. Therefore, a second extra dosage of the enzyme preparation (240 ml) was added and resulted this time in a slight accumulation of glucose and xylose of 1.5 and 1.0 g/l (Fig. 3A), respectively, indicating that microbial conversion instead of enzymatic hydrolysis was rate limiting. After 32 h of incubation, a lactic acid concentration of 37.1 g/l was obtained, with a chiral L(+)-lactic acid purity of 99.4%. Continuation of the SSF process to a total incubation period of 55 h resulted in a slightly increased lactic acid concentration of 40.7 g/l supernatant (~37.8 g lactic acid/kg fermentation broth) with an overall volumetric lactic acid productivity of 0.74 g/l/h. At this stage, a chiral L(+)-lactic acid purity of 97.2% was analyzed. This slight decline in lactic acid purity is possibly a result of infection with other undesired lactic acid-producing microorganisms. Since the substrate used was not sterile and also the chemical pretreatment and fermentation occurs in an open system under non-sterile conditions, microbial contamination throughout the SSF process is possible.

Conversion efficiency

The efficiency of the enzymatic hydrolysis of the polymeric material present in LTWS is shown in Figure 4. The insoluble polymeric fraction was determined at various time points throughout the SSF experiment. At the end of the pre-hydrolysis (2 h) of 378 g LTWS, 36% of the insoluble glucan (Fig. 4A), 55% of xylan (Fig. 4B) and 62% of arabinan (Fig. 4C) was converted to soluble saccharides including monomeric sugars and oligomeric sugars. After the fed-batch phase (13 h), 2706 g LTWS was added and resulted in a conversion of 42% of glucan, 57% of xylan and 63% of arabinan to products including soluble saccharides and lactic acid.

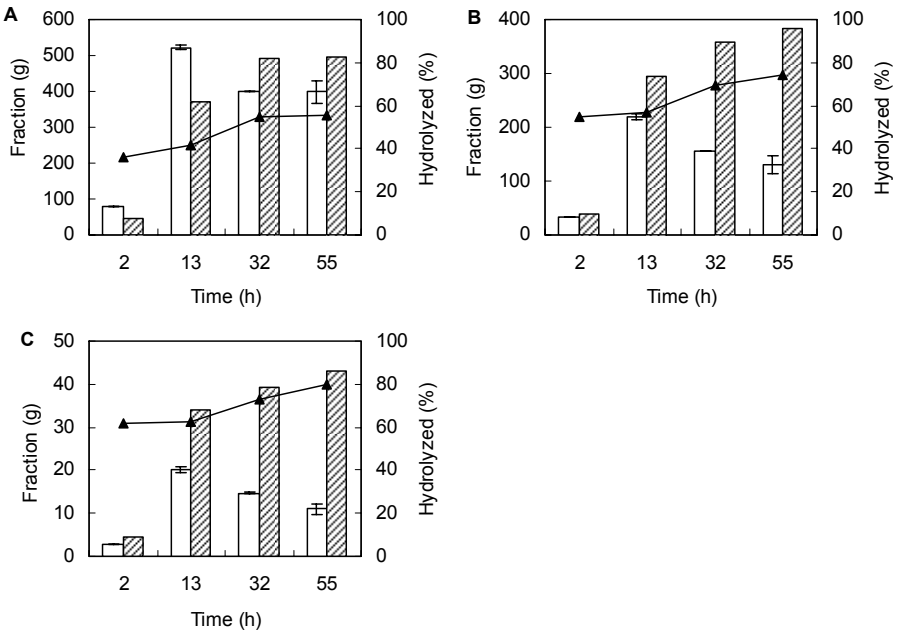

Figure 4. Insoluble fraction (□) and hydrolyzed soluble fraction (▨) (g) (calculated by the difference between initial amounts and analyzed insoluble amounts) of the polysaccharide glucan (*A*), xylan (*B*) and arabinan (*C*) at various time points during the simultaneous saccharification and fermentation of lime-treated wheat straw. Figure represents also the percentage of polysaccharide hydrolyzed into soluble products (▲). The error bars denote the deviation of duplicate analysis.

Between 13 and 32 h of incubation, further hydrolysis of the polymeric sugars was observed. However, during the last 23 h of the SSF, minor hydrolysis of the polysaccharides occurred and this corresponded with the decline in lactic acid productivity during this phase. After 55 h, 398 g of glucan, 130 g of xylan and 11 g of arabinan was still present as insoluble polymeric material. With these values, the hydrolysis efficiency of the initial glucan, xylan and arabinan present in LTWS were calculated as 55, 75 and 80%, respectively.

TABLE 1. Fate of polysaccharides[a] initially present in lime-treated wheat straw after 55 h of simultaneous saccharification and fermentation. Presented values are averages based on duplicate analytical measurements

Fraction	Percentage (% *w/w*)
Polysaccharides (insoluble)[b]	37
Oligosaccharides (soluble)	5
Monosaccharides (soluble)	3
Lactic acid (soluble)	43
Unaccounted (insoluble/soluble)[c]	13

[a]Total of glucan, xylan and arabinan [b]Part of the initial polysaccharides remained present as insoluble polysaccharides [c]Part of the initial polysaccharides was not recovered and therefore denoted as 'unaccounted'.

The monomeric sugars, derived from the LTWS, were partly converted to lactic acid (711 g) by *B. coagulans* and accounted for 81% (*w/w*) of the theoretical maximum, indicating the formation of other products such as microbial biomass and carbon dioxide. An overall conversion yield of 43% (*w/w*) of the theoretical maximum was calculated according to Equation 1. The fate of polysaccharides initially present in LTWS after 55 h of incubation is shown in Table I. A part of the polysaccharides present in LTWS, remained as insoluble polysaccharides (37% *w/w*) whereas a minor part was converted into soluble oligomeric (5% *w/w*) and monomeric (3% *w/w*) sugars. Another part of the initial polysaccharides present in the LTWS was not recovered in the form of saccharides or lactic acid and was therefore ascribed as 'unaccounted'.

Neutralization of acid by alkaline substrate

The lactic acid produced (342 g) during the fed-batch phase (II) was neutralized with alkaline pretreated wheat straw. During this phase, an amount of 2328 g LTWS was added to the fermenter. Together with this substrate, an amount of 230 g calcium hydroxide was added to the fermenter and accounted for a ratio of 0.67 g calcium hydroxide per g of lactic acid. The lactic acid (369 g) produced during the batch phase (III) was neutralized with 163 g calcium hydroxide resulting in a ratio of 0.44 g lactic acid per g calcium hydroxide.

DISCUSSION

Lignocellulosic feedstocks are considered as potential attractive substrates for the production of bulk chemicals. Pretreatment of biomass is required in order to break open the lignocellulosic matrix, an enzymatic hydrolysis is necessary for the hydrolysis of polymeric carbohydrates. The lime pretreatment has proven to enhance enzymatic digestibility of the polysaccharides present in lignocelluloses (Chang et al., 1998; Kaar and Holtzapple, 2000) and results, in comparison to other pretreatment routes, in minor inhibitor formation. However, prior to the enzymatic hydrolysis, it is essential to adjust the pH to a level optimal for enzymatic activity. In this study, the reduction of pH by washing or neutralization was omitted by using the alkaline character of LTWS in order to neutralize lactic acid produced by microbial fermentation during a SSF process.

The results showed that the largest part of the polysaccharides in LTWS was converted enzymatically and the resulting sugars were fermented simultaneously to mainly lactic acid by *B. coagulans* DSM 2314. Between 10 and 30 h of incubation, the bacteria utilized the monomeric sugars, as soon as they appeared in the medium, resulting in relatively low monomeric sugar concentrations (< 2 g/l). This indicates that throughout this period, the enzymatic hydrolysis was the rate-controlling step. The highest lactic acid productivity was

observed during the fed-batch phase and the initial hours of the batch phase and declined rapidly after approximately 20 hours of incubation, as shown in Figure 3B. An extra addition of enzyme preparation showed a slight improvement of the volumetric lactic acid productivity but shifted within a few hours again to a relatively low production rate. A second extra enzyme addition did not affect the lactic acid productivity significantly (Fig. 3B). This addition of new enzymes resulted in a modest liberation of hemicellulose sugars (xylose, arabinose) but no further hydrolysis of glucan occurred. This shows that the remaining glucan was too recalcitrant or not accessible for further hydrolysis, resulting in decreasing lactic acid productivity. Another possible explanation of the decreased lactic acid productivity is the inhibition of enzymes and/or bacteria by the increasing lactic acid concentration.

A lactic acid concentration of 40.7 g/l supernatant (~37.8 g lactic acid/kg fermentation broth) with a relatively high chiral purity was determined after 55 hours of incubation, corresponding to an overall lactic acid yield of 43% of the theoretical maximum. Moreover, the efficiencies of the enzymatic saccharification and the fermentation were both determined. These calculations showed that, based on residue analysis, at the end of the SSF process (55 h) 55% of the glucan, 75% of the xylan and 80% of the arabinan present in LTWS was enzymatically hydrolyzed which agree well with previously obtained results (manuscript in submission). In order to improve the yield it is necessary to decrease the recalcitrance or improve the accessibility of polymeric sugars in the LTWS by optimization of the pretreatment procedure. The concentrations of soluble monosaccharides and oligosaccharides in the medium were relatively low which can be expected in a SSF process. A fermentation yield of 81% was determined and is slightly better than the results obtained by Otto (2004) who reported the production of 35 g/l lactic acid from 50 g/l xylose as sole carbon source. Since no other soluble fermentation products were detected, the remaining 19% of the LTWS derived monomeric sugars were most presumably converted to bacterial biomass and some carbon dioxide during the aerobic part of the fermentation. Several process parameters can be listed for enhancement of the overall lactic acid yield and productivity such as improving the accessibility of polysaccharides by a more severe lime pretreatment, enzyme dosage, type of enzymes, *Bacillus coagulans* strain, size and growth phase of inoculum, pH gradient in SSF and in situ product removal of lactic acid. These issues will be subject of further study.

During the fed-batch phase (II) it was possible to counterbalance the pH decrease caused by lactic acid production by addition of the alkaline feedstock, showing that lime treatment can be combined well with the production of a wide range organic acids from lignocellulosic biomass. Throughout this phase, the ratio of calcium hydroxide in LTWS added per produced lactic acid was determined at 0.67 g/g. The theoretical stoichiometric neutralization of 1.00 g lactic acid requires 0.41 g calcium hydroxide. Therefore, only 61%

of the calcium hydroxide initially added to the wheat straw was used for lactic acid neutralization. On the other hand, throughout the batch phase (III), an alkaline/acid ratio of 0.44 g/g was calculated corresponding to 93% of the added calcium hydroxide suspension used for lactic acid neutralization. The low efficiency of the calcium hydroxide added with the LTWS for lactic acid neutralization during phase II has three possible explanations. Firstly, part of the calcium hydroxide could have been used during the chemical pre-treatment of the wheat straw such as the neutralization of acetic acid or other organic acids and/or irreversible binding to the lignin. Secondly, the calcium hydroxide might be released slowly from the insoluble wheat straw fibers and could therefore partly have been used for lactic acid neutralization in the fed-batch phase. Finally, besides lactic acid production, other acidification reactions could have contributed to the decrease of pH and therefore the demand of alkaline substrate. For instance the uptake and dissociation of the nitrogen source ammonium by the micro-organism into ammonia and protons (Guebel et al., 1992).

The results in this paper show that it is possible to use lignocellulosic materials for the production of lactic acid. Lignocellulosic biomass is a relatively inexpensive substrate and this affects feedstock costs for lactic acid production positively. Nevertheless, in comparison to the traditional relatively 'clean' feedstocks with well defined composition, using heterogenic lignocellulosic substrates will require a more intensified down stream processing (DSP) to recover and purify the lactic acid from the complex fermentation broth. The costs of feedstock materials and operational costs of the DSP contribute considerably to the overall production costs of lactic acid (Åkerberg and Zacchi, 2000). Whether the cost decrease of using lignocellulosic feedstocks outweighs the potential increasing costs of DSP was not analyzed at the moment.

In summary, lime-treated wheat straw was converted into L(+)-lactic acid by *B. coagulans* throughout a simultaneous saccharification and fermentation process at 20L bench-scale. The pentose and hexose sugars derived from the polymeric material were utilized simultaneously by *B. coagulans* resulting in a final lactic acid concentration of 40.7 g/l supernatant which accounted for 43% (*w/w*) of the theoretical yield. To our knowledge, this is the first paper demonstrating a process having a combined alkaline pretreatment of lignocellulosic biomass and pH control in organic acid fermentation resulting in a significant saving of lime consumption and avoiding the necessity to recycle lime.

ACKNOWLEDGEMENTS

This project is supported with a grant of the Dutch Programme EET (Economy, Ecology, Technology) a joint initiative of the Ministries of Economic Affairs, Education, Culture and Sciences and of Housing, Spatial Planning and the Environment. The programme is run by the EET Programme Office, SenterNovem. Patrick F.N.M. van Doeveren and Jeroen J.C.F. van Bon (AFSG, Wageningen, The Netherlands) are gratefully acknowledged for technical assistance.

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Summary and concluding remarks

The limited availability of fossil fuel sources, fluctuating energy prices, increasing world-wide energy demands, and anticipated climate changes caused by greenhouse gasses are important reasons for finding alternative sources for the production of energy and bulk chemicals. A major strategy to meet the increasing energy demand and to reduce the emission of the anthropogenic greenhouse gas CO_2 is by large-scale substitution of fossil fuel sources derived energy and chemicals by renewable sources such as wind, solar, water, geothermal energy and biomass. Of these sources, plant material or biomass is one of the main contributors to provide the global energy demand in the near future. Moreover, in contrast to the CO_2-cycle of fossil fuel utilization, the short CO_2-cycle of biomass utilization results in less accumulation of CO_2 in the atmosphere.

Fermentation processes can be used to convert biomass into energy carriers such as ethanol, butanol and hydrogen, and bulk chemicals such as lactic acid. The carbohydrates present in biomass serve as substrate for microbial conversion. Carbohydrates can be divided in three groups: the simple sugars (*e.g.* sucrose), the storage sugars (*e.g.* starch) and the structural sugars (*e.g.* cellulose). Due to their molecular structure, the simple sugars and storage sugars are relatively easily fermentable to products. Nevertheless, this type of substrates are also suitable for various food and feed applications resulting in competing claims and consequently increasing prices of agricultural raw materials, food and feed. These kinds of crops might therefore be less attractive as substrate for the large-scale production of biofuels and bulk chemicals.

In contrast to these crops, lignocellulosic materials are widely available, relatively inexpensive, more sustainable in terms of CO_2-emissions and non-competitive to food applications, but require a more intensive pretreatment to release fermentable sugars. Lignocellulose composes of a complex structure of mainly cellulose, a linear polymer of β-1,4 linked glucose residues; hemi-cellulose, a hetero-polymer of pentoses and hexoses; and lignin, an aromatic polymer. Lignocellulosic feedstocks typically contain 55 to 75% (*w/w*) carbohydrates; however, the chemical composition depends on plant species, age and cultivation conditions.

The conventional lignocellulose-to-product conversion consists of several process steps, namely a physical and/or chemical pretreatment, a chemical and/or enzymatic hydrolysis, a fermentation and a product recovery or down stream processing (DSP). In general, the pretreatment aims to remove or modify lignin and hemi-cellulose, reduce cellulose cristallinity and increase the porosity of the substrate in order to enhance the accessibility of

enzymes for hydrolysis. Physical, chemical, biological and combined pretreatments are developed; however, all have their own characteristics depending on the reaction conditions, and chemical composition and structure of the feedstock. Throughout pretreatment processes, components can be released from the lignocellulosic biomass (*e.g.* acetic acid from acetylated hemicellulose) or can be produced due to too severe reaction conditions (*e.g.* furfural from xylose and 5-hydroxymethylfurfural from glucose) and can potentially inhibit microbial activity during fermentation processes. Polymeric sugars function as substrate for hydrolysis by cellulases and hemi-cellulases, and the resulting monosaccharides serve as carbon source for microbial conversion. The characteristics of the fermentation step, the substrate and medium composition, type of micro-organism and the fermentation product determine the choice of product recovery or down stream processing technologies.

The aim of the research described in this thesis was to study the conversion of lignocellulose-derived carbohydrates into the bio-fuel ethanol and the bulk chemical lactic acid. Wheat straw is an abundantly available lignocellulosic agro-industrial side stream in the Netherlands and was used as model substrate. The mild-temperature lime treatment was chosen as representative method for mild-temperature treatments. Enzymatic hydrolysis and fermentation of pretreated substrates were studied with regard to productivities and yields. For ethanol production we used the wild-type bakers' yeast *Saccharomyces cerevisiae* and for lactic acid fermentation the fungus *Rhizopus oryzae* and the bacterium *Bacillus coagulans*. Special attention has been paid to the conversion of xylose into lactic acid by *R. oryzae* and process integration ranging from the pretreatment and the hydrolysis steps to the fermentation step.

In *Chapter 2*, the effects of lime ($Ca(OH)_2$) treatment at mild-temperature on wheat straw under various conditions were described. This alkaline treatment has gained interest because of perceived benefits compared to other treatments including minor inhibitor formation. Results showed that this treatment did not lead to significant solubilisation of xylan or lignin. The efficiency of the treatment was validated by hydrolysis experiments with commercial available cellulolytic and hemi-cellulolytic enzymes and subsequently by ethanol fermentations with *S. cerevisiae*. The optimal reaction conditions were determined at 0.1 g $Ca(OH)_2$ per g biomass and 16-20 h of incubation at 85°C. Furthermore, a simple washing procedure of the lime pretreated substrate was performed. This resulted in reduction of the acetic acid concentration in hydrolysates, without losing valuable fermentable sugars, improving the fermentability at pH between 4.0 and 5.0. Enzymatic hydrolysis under optimal conditions showed that lime pretreatment results in hydrolysis of 93% of glucan and 81% of xylan to monomeric sugars, a three to four fold improvement when compared to water treatment alone.

Chapter 3 describes the conversion of xylose, abundantly present in lignocellulosic hydrolysates, to lactic acid by the filamentous fungus *R. oryzae*. Lactic acid has received a lot of attention due to the application of optically pure lactic acid as biodegradable plastic poly-lactic acid (PLA), which is a biobased replacement of plastics derived from petrochemical materials. The ten tested strains of *R. oryzae* produced lactic acid from xylose as sole carbon source with yields varying between 0.41 and 0.71 g/g and the formation of some by-products such as xylitol, ethanol, glycerol, carbon dioxide and fungal biomass. In artificial media containing glucose and xylose, the fungus showed bi-phasic or diauxic growth where first glucose was consumed completely followed by the utilization of xylose by a lower consumption rate. In a lime-treated wheat straw hydrolysate, the fungus utilized the sugars in a similar diauxic fashion.

In *Chapter 4*, it was demonstrated that *R. oryzae* strain CBS 112.07 produces lactic acid only under growing conditions. Deprivation of nutrients, such as nitrogen (N), which are essential for fungal growth, resulted in cessation of lactic acid production. In case of complete xylose utilization, a lower C/N ratio (61/1) in the medium is required in comparison to complete glucose utilization (C/N of 201/1), caused by higher fungal biomass yields. Furthermore, the oxygen transfer rate influenced the xylose consumption rate significantly whereas the utilization rate of glucose was less affected. Both the growth-associated lactic acid formation and the oxygen dependency were related to the fact that *R. oryzae* utilizes xylose via the so-called two-step reduction/oxidation route. The consequences of these effects for *R. oryzae* as potential lactic acid producer were discussed.

In the conventional lignocellulose-to-product process, the enzymatic hydrolysis step and fermentation step occur separately at their respective optimal reaction conditions (SHF). By this approach, the release of sugars such as glucose inhibits the cellulolytic system throughout hydrolysis. As an alternative, both the hydrolysis and fermentation can occur simultaneously in one reactor at compromising conditions and is known as simultaneous saccharification and fermentation (SSF). In this system fermentable sugars are converted, as soon as they become available, by the micro-organism resulting in less inhibition of hydrolysis caused by sugars. In *Chapter 5*, the conversion of lime-treated wheat straw, with dry matter content of 10.9% (*w/w*), to ethanol by SHF and SSF routes with commercial cellulase preparation and the yeast *S. cerevisiae* was described. Our results showed that the highest glucan-to-glucose conversion of 56% and xylan-to-xylose conversion of 67% were found at an enzyme concentration of 169.1 mg per g substrate, pH 5.0 and 4.5 and 50°C. Acetic acid (2.7 g/l) present in the lime-treated wheat straw seemed to inhibit ethanol formation during SSF, especially at low pH of 4.0. By applying a washing step prior to SSF, the acetic acid concentration could be reduced to 0.4 g/l resulting in improved fermentability. The SHF and SSF route showed, at optimized reaction conditions, comparable volumetric ethanol productivities (0.25 g/l/h) and glucan-to-ethanol yields of

55% after 56 hours of incubation. In addition, xylan was converted slightly better by the SHF in comparison to the SSF route.

With the optimized reaction conditions, the SSF process converting lime-treated wheat straw, with high dry matter content (~20% *w/w*), to ethanol was up-scaled to pilot-scale (100-l) and is described in *Chapter 6*. After 53 hours of incubation, an ethanol concentration of 21.4 g/l was detected, corresponding to a 48% glucan-to-ethanol conversion of theoretical maximum. The xylan fraction remains - probably due to inability of yeast to convert pentoses - mostly in the soluble oligomeric form (52%) in the fermentation broth. After the distillation of ethanol, the quality of ethanol was assessed for its usage as transportation fuel. The remaining distillation residue was separated in two fractions: the 'solid fraction' containing mainly solid particles serving as fuel for thermal conversion via combustion, and the 'liquid fraction' containing mainly soluble organic compounds functioning as substrate for anaerobic biogas production. Based on the experimental values, 16.7 kg lime-treated wheat straw could be converted to 1.7 kg ethanol, 1.1 kg methane, 4.1 kg carbon dioxide, ~3.4 kg compost and 6.6 kg solid lignine rich residue. The lignin-rich residue contains a Higher Heating Value of 13.4 MJ thermal energy per kg (dry basis).

In the SSF wheat straw-to-ethanol production process, it is essential to adjust the pH after lime treatment from ~10 to ~5 prior to using the substrate for enzymatic hydrolysis and fermentation. In an integrated lactic acid production process (*Chapter 7*), the alkaline substrate was used to neutralize the lactic acid produced and maintain the pH at 6.0 in the reactor. The lime-treated wheat straw was hydrolyzed and fermented simultaneously to lactic acid by a commercial enzyme preparation and the thermophilic bacteria *B. coagulans* DSM 2314. Thus, in this experimental set-up, the alkaline substrate has a dual function: to provide fed-batch wise a carbon substrate for lactic acid production and to neutralize the lactic acid produced. After 55 hours of incubation, the polymeric fractions glucan, xylan and arabinan were hydrolyzed for 55, 75 and 80%, respectively. A lactic acid concentration of 40.7 g/l was detected corresponding to a fermentation efficiency of 81%. Approximately half of the produced lactic acid was neutralized by the lime present in the substrate. The process integration of the alkaline pretreatment of wheat straw and the pH control throughout lactic acid production resulted in reduction of lime usage and therefore lactic acid production costs.

In conclusion, the results described in this thesis show the conversion of C5-sugars and C6-sugars present in wheat straw into ethanol or lactic acid in processes consisting of a mild-temperature alkali pretreatment, hydrolysis using commercial enzymes and fermentation. It was shown from the pilot-scale ethanol production process that it is technically feasible to valorize side streams such as lignin and soluble organic components,

by combustion and anaerobic digestion. Integration of the enzymatic hydrolysis and fermentation of lime-treated wheat straw into ethanol resulted in similar performance with regard to productivity and yield as obtained with the separate hydrolysis and fermentation. The advantages of simultaneous saccharification and fermentation – mainly prevention of product inhibition of the hydrolysis step – outweighed the compromised sub-optimal reaction conditions. Developing (hemi-)cellulolytic enzyme preparations and/or micro-organisms that are able to perform well under similar reaction conditions will further improve the integrated lignocellulose-to-product performance.

The advantage of process integration was demonstrated even better by combining lactic acid production with the alkali pretreatment. This indicates the importance of process integration which can lead to the reduction of chemical usage, processing time and overall production costs. Thus, optimization of the lignocellulose-to-product process requires an integrated approach parallel to improving single isolated steps.

Samenvatting

De beperkte beschikbaarheid van fossiele brandstofbronnen, fluctuerende energieprijzen, de mondiaal stijgende vraag naar energie en de klimaatveranderingen veroorzaakt door broeikasgassen zijn belangrijke redenen voor het zoeken naar alternatieve bronnen voor de productie van energie en bulkchemicaliën. Het grootschalig vervangen vanuit fossiele brandstoffen verkregen energie en chemicaliën door hernieuwbare bronnen zoals wind, zon, water, geothermische energie en biomassa is een belangrijke strategie om de emissie van het antropogene broeikasgas CO_2 te reduceren en te voorzien in de stijgende vraag naar energie. Van deze hernieuwbare bronnen is biomassa of plantenmateriaal een belangrijke grondstof voor de wereldwijde energieproductie in de nabije toekomst. In tegenstelling tot de CO_2-cyclus van fossiel brandstofgebruik is de CO_2-cyclus van biomassagebruik relatief kort en resulteert in geen of minder CO_2-ophoping in de atmosfeer.

Biomassagrondstoffen kunnen via fermentatieprocessen worden omgezet in energiedragers zoals ethanol, butanol en waterstof en bulkchemicaliën zoals melkzuur. De in biomassa aanwezige koolhydraten dienen als substraat voor de microbiële omzetting. Koolhydraten kunnen worden verdeeld in drie categorieën, te weten: eenvoudige suikers (bijv. sucrose), suikers die gebruikt worden als reservestof (bijv. zetmeel) en suikers die onderdeel zijn van de plantstructuur (bijv. cellulose). De eenvoudige suikers en reservesuikers zijn door hun moleculaire structuur relatief gemakkelijk te fermenteren naar gewenste producten. Dit type substraat is echter ook zeer geschikt voor voedseltoepassingen waardoor de energie- en voedselmarkt om de grondstof zullen concurreren met stijgende de grondstof- en voedselprijzen als gevolg. Daarom zijn dit type substraten mogelijk minder geschikt voor de grootschalige productie van biobrandstoffen en bulkchemicaliën.

In tegenstelling tot de eerder genoemde substraten zijn lignocellulose materialen beschikbaar in grote omvang, relatief goedkoop, hernieuwbaar in termen van CO_2 emissie en niet-competitief voor voedseltoepassingen. Echter, het gebruik van lignocellulose vereist intensievere voorbehandelingen om fermenteerbare suikers vrij te maken. Lignocellulose bestaat uit een complexe structuur van voornamelijk cellulose, een lineair polymeer opgebouwd uit glucose eenheden gebonden via ß-1,4 verbindingen; hemicellulose, een heteropolymeer opgebouwd uit pentoses en hexoses; en lignine, een aromatisch polymeer. Lignocellulose materialen bevatten 55 tot 75% (*m/m*) koolhydraten, waarbij de chemische samenstelling afhankelijk is van de plantensoort, de leeftijd en de groeicondities.

De conventionele omzetting van lignocellulose naar producten bestaat uit verschillende processtappen te weten: fysische en/of chemische voorbehandeling, chemische en/of enzymatische hydrolyse, fermentatie en isolatie/purificatie van het product. De doelstelling van een voorbehandeling is het verwijderen of modificeren van de lignine en de hemicellulose fractie, het reduceren van de cellulose kristalliniteit en het verhogen van de porositeit van het substraat om zo de toegankelijkheid voor hydrolytische enzymen te vergroten. Verschillende fysische, chemische en biologische voorbehandelingen (en combinaties hiervan) van lignocellulose zijn ontwikkeld, echter, allen met voordelige- en nadelige eigenschappen die afhankelijk zijn van de reactiecondities, chemische samenstelling en structuur van het substraat. Gedurende een voorbehandeling van lignocellulose kunnen verbindingen vrijkomen (bijv. azijnzuur van de geacetyleerde hemicellulose) of kunnen verbindingen worden geproduceerd door te stringente reactiecondities (bijv. furfural uit xylose en 5-hydroxymethylfurfural uit glucose) en deze kunnen de microbiële activiteit tijdens de fermentatie remmen. De polymere koolhydraten functioneren als substraat voor hydrolyse door cellulases en hemicellulases waarna de resulterende monomere suikers dienen als koolstofbron voor microbiële omzetting. De eigenschappen van de fermentatiestap, de samenstelling van het substraat en medium, het type micro-organisme en het fermentatieproduct bepalen de keuze van product isolatie- en purificatietechnologieën.

Het doel van het in dit proefschrift beschreven onderzoek was het bestuderen van de omzetting van uit lignocellulose verkregen koolhydraten naar de biobrandstof ethanol en het bulkchemicalie melkzuur. Tarwestro is in Nederland een beschikbare agro-industriële reststroom en werd in dit onderzoek gebruikt als lignocellulose modelsubstraat. De calciumhydroxidebehandeling bij relatief milde temperatuur werd beschouwd als representatieve methode voor de milde temperatuur behandelingen. De enzymatische hydrolyse en fermentatie van het voorbehandelde substraat werden bestudeerd met betrekking tot de productiviteit en opbrengst. Het wildtype bakkersgist *Saccharomyces cerevisiae* werd gebruikt voor de ethanolproductie en de schimmel *Rhizopus oryzae* en de bacterie *Bacillus coagulans* voor de productie van melkzuur. Extra aandacht werd besteed aan de omzetting van xylose naar melkzuur en de procesintegratie van de voorbehandelingstap en de hydrolysestap tot en met de fermentatiestap.

In *hoofdstuk 2* werden de effecten van calciumhydroxide voorbehandeling bij milde temperatuur op tarwestro onder verschillende condities bestudeerd. Deze alkalische voorbehandeling heeft interesse verworven dankzij enkele voordelen ten opzichte van andere voorbehandelingen zoals een lage productie van inhibitoren. De resultaten laten zien dat deze voorbehandeling nauwelijks resulteert in oplossen van xylaan en lignine. De efficiëntie van de voorbehandelingen werd gevalideerd door middel van hydrolyse experimenten met commercieel verkrijgbare cellulolytische en hemicellulolytische enzym

preparaten gevolgd door ethanol fermentaties met *S. cerevisiae*. De optimale condities waren 0.1 g calcium hydroxide per g biomassa voor 16 tot 20 uur incubatie bij 85°C. Daarnaast werd een relatief eenvoudige wasprocedure uitgevoerd. Deze methode resulteerde in een reductie van de azijnzuurconcentratie, zonder verlies van waardevolle fermenteerbare suikers, en verbetering van fermenteerbaarheid bij pH waarden tussen 4.0 en 5.0. Onder optimale condities vertoonde de enzymatisch hydrolyse een omzetting van glucaan en xylaan naar monomere suikers van respectievelijk 93 en 81% hetgeen een drie tot viervoud verbetering is in vergelijking tot een voorbehandeling met alleen water.

Hoofdstuk 3 beschrijft de omzetting van xylose, aanwezig in lignocellulose hydrolysaten, naar melkzuur door de filamenteuze schimmel *R. oryzae*. Melkzuur krijgt tegenwoordig veel aandacht vanwege de toepassing om optisch zuiver melkzuur te verwerken tot biologisch afbreekbaar plastic poly-melkzuur en is een hernieuwbare vervanging van plastic verkregen uit petrochemische materialen. Tien *R. oryzae* stammen werden getest op melkzuurproductie uit xylose met opbrengsten variërend tussen 0.41 en 0.71 g/g en de vorming van de bijproducten xylitol, ethanol, glycerol, koolstofdioxide en schimmelbiomassa. In kunstmatige media met glucose en xylose vertoonde de schimmel bifasische groei of diauxie waarbij glucose eerst werd geconsumeerd gevolgd door de consumptie van xylose met een lagere snelheid. In een calciumhydroxide voorbehandelde tarwestro hydrolysaat werd een vergelijkbaar resultaat verkregen.

In *hoofdstuk 4* werd aangetoond dat *R. oryzae* stam CBS 112.07 alleen melkzuur produceert onder groeiende condities. Het limiteren van nutriënten, zoals stikstof (N) welke noodzakelijk is voor schimmelgroei, resulteert in een beëindiging van melkzuurproductie. In geval van volledig xylose verbruik is een lagere C/N verhouding (61/1) in het medium vereist in vergelijking tot de volledige consumptie van glucose (201/1) en wordt veroorzaakt door een hogere schimmelbiomassa opbrengst. De xylose consumptiesnelheid werd significant beïnvloed door de zuurstof diffusiesnelheid terwijl de glucose consumptiesnelheid nauwelijks werd beïnvloed. Zowel de groeigeassocieerde melkzuurproductie als de zuurstofafhankelijkheid werden beiden gerelateerd aan het feit dat *R. oryzae* xylose verbruikt via de zogenaamde tweestap reductie/oxidatie route. De consequenties van deze effecten voor *R. oryzae* als melkzuurproducerend micro-organisme werden bediscussieerd.

In het conventionele lignocellulose-naar-product proces worden de enzymatisch hydrolyse stap en de fermentatie stap gescheiden onder optimale reactiecondities uitgevoerd (SHF). Echter, tijdens de hydrolyse veroorzaakt de vorming van suikers zoals glucose remming op het cellulolytische systeem. Als alternatief kunnen de hydrolyse en fermentatie simultaan in één reactor en onder compromitterende reactiecondities uitgevoerd worden en dit proces staat bekend als simultane saccharificatie en fermentatie (SSF). In dit systeem worden de suikers zo gauw als ze vrijkomen, omgezet door het micro-organisme

met als gevolg minder inhibitie van de hydrolyse door de suikers. In *hoofdstuk 5* werd de omzetting van calciumhydroxide voorbehandelde tarwestro met droge stof gehalte van 10.9% (*m/m*) naar ethanol via SHF en SSF routes met commerciële cellulase preparaten en gist *S. cerevisiae* bestudeerd. Onze resultaten lieten zien dat de hoogste omzetting van glucaan naar glucose van 56% en van xylaan naar xylose van 67% wordt behaald met een enzymdosering van 169.1 mg per g substraat, pH 5.0 en 4.5 en 50°C. Het aanwezige azijnzuur (2.7 g/l) in calciumhydroxide voorbehandelde tarwestro bleek vooral bij lage pH van 4.0 de ethanolproductie gedurende SSF te remmen. Door het toepassen van een wasstap werd de azijnzuurconcentratie gereduceerd tot 0.4 g/l en dit resulteerde in een verbeterde fermenteerbaarheid. De SHF en SSF route vertoonden onder optimale reactie condities een vergelijkbare volumetrische ethanol productiviteit (0.25 g/l/h) en glucaan naar ethanol omzetting van 55% na 56 uur incubatie. Het xylaan werd iets beter omgezet bij de SHF in vergelijking tot de SSF route.

Met de geoptimaliseerde reactie condities werd het SSF proces van calciumhydroxide voorbehandelde tarwestro met hoge droge stof gehalte (~20% *m/m*) naar ethanol opgeschaald naar pilot-schaal (100-l). Dit wordt beschreven in *hoofdstuk 6*. Een ethanolconcentratie van 21.4 g/l, hetgeen correspondeert met 48% van het theoretische maximum, werd gedetecteerd na 53 uur incubatie. Een groot deel van de xylaan fractie blijft in oligomere vorm (52%) aanwezig in het medium, waarschijnlijk door het onvermogen van de gist om pentoses om te zetten. Na distillatie van het ethanol werd de kwaliteit beoordeeld voor het gebruik als transportbrandstof. Het overgebleven distillatie residu werd gescheiden in twee fracties, een 'vaste fractie' welke voornamelijk vaste delen bevatte en diende als brandstof voor thermische conversie, en een 'vloeibare fractie' welke voornamelijk opgeloste organisch componenten bevatte en functioneerde als substraat voor anaerobe biogas productie. Gebaseerd op experimentele data werd 16,7 kg calcium hydroxide voorbehandelde tarwestro omgezet in 1,7 kg ethanol, 1,1 kg methaan, 4,1 kg koolstofdioxide, ongeveer 3,4 kg compost en 6,6 kg lignine rijk residu. Het lignine rijk residu heeft een calorische waarde van 13,4 MJ thermische energie per kg (op droge stof basis, HHV waarde).

In het SSF proces is het noodzakelijk de pH van het voorbehandelde tarwestro aan te passen van ±10 naar ±5 alvorens het te gebruiken als substraat voor enzymatische hydrolyse en fermentatie. In een geïntegreerd proces (*hoofdstuk 7*) werd het alkalische substraat gebruikt om het geproduceerde melkzuur te neutraliseren en zo de pH in de reactor op 6.0 te houden. Het calciumhydroxide voorbehandelde tarwestro werd simultaan gehydrolyseerd en gefermenteerd naar melkzuur door een commercieel enzym preparaat en de thermofiele bacterie *B. coagulans* DSM 2314. In deze experimentele opzet had het alkalische substraat een tweeledige functie te weten koolstofbron voor melkzuurproductie en om het geproduceerde melkzuur te neutraliseren. De polymere fracties glucaan, xylaan

en arabinaan werden voor respectievelijk 55, 75 en 80% gehydrolyseerd na 55 uur incubatie. Een melkzuurconcentratie van 40.7 g/l werd gedetecteerd en komt overeen met een fermentatie efficiëntie van 81%. Ongeveer de helft van het geproduceerde melkzuur werd geneutraliseerd met het calciumhydroxide aanwezig in het alkalische substraat. Procesintegratie van de alkalische voorbehandeling van tarwestro en de pH controle gedurende de melkzuurproductie resulteerde in een afname van het calciumhydroxide gebruik gevolgd door reductie van de melkzuurproductie kosten.

Tot besluit, de resultaten in dit proefschrift beschrijven de omzetting van C5-suikers en C6-suikers aanwezig in tarwestro naar ethanol of melkzuur in processen bestaande uit een alkalische voorbehandeling bij milde temperatuur, een hydrolyse met commerciële enzymen en een fermentatie. Eveneens werd aangetoond bij een ethanol productie proces op pilot schaal dat het technisch haalbaar is om bijstromen zoals de lignine fractie en de fractie met opgeloste organische componenten om te zetten via verbranding en anaerobe vergisting. Het integreren van de enzymatische saccharificatie en de fermentatie van calciumhydroxide voorbehandelde tarwestro resulteerde in vergelijkbare prestaties met betrekking tot productiviteit en rendement behaald in het separate hydrolyse- en fermentatieproces. De voordelen van simultane saccharificatie en fermentatie, voornamelijk het voorkomen van product inhibitie tijdens de hydrolyse stap, compenseren de suboptimale compromitterende reactiecondities. De ontwikkeling van (hemi-)cellulolytische enzympreparaten en/of micro-organismen welke optimaal functioneren bij dezelfde condities zal leiden tot verdere optimalisering van de geïntegreerde lignocellulose omzetting.

Het voordeel van procesintegratie werd nog beter gedemonstreerd door het combineren van melkzuurproductie met alkalische voorbehandeling. Hierbij werd het belang van procesintegratie duidelijk aangetoond en kan leiden tot de afname van het chemicaliën gebruik, de procestijd en daarmee de productiekosten. Optimalisatie van processen waarbij lignocellulose materialen worden omgezet naar specifieke producten vereist naast het optimaliseren van afzonderlijke processtappen een geïntegreerde strategie.

Dankwoord

Na een vijftal jaren hard werken is mijn proefschrift af! Graag wil ik iedereen bedanken die heeft bijgedragen aan mijn promotietijd. Een aantal personen wil ik hierbij in het bijzonder noemen.

Allereerst mijn begeleiders. Mijn promotor Gerrit Eggink, ik wil je bedanken voor de mogelijkheid die ik heb gekregen om promotieonderzoek uit te voeren, het gestelde vertrouwen en de kritische blik waarmee je me hebt begeleid. Ik heb veel van onze discussies geleerd.
Mijn co-promotor Ruud Weusthuis verdient veel dank! Ruud, onze (werk)besprekingen waren voor mij in vele opzichten leerzaam en inspirerend. Je wist me op bepaalde momenten af te remmen of juist te stimuleren en gaf me vertrouwen wanneer het daar even aan ontbrak. Eveneens verbaasde ik me regelmatig hoe snel je mijn manuscripten had voorzien van waardevolle feedback. Ik heb je betrokkenheid en enthousiaste begeleiding enorm gewaardeerd!

Rob, interne projectleider en naaste collega, met erg veel plezier heb ik met jou aan de experimenten, rapportages en presentaties gewerkt. Van ons congresbezoek in de Verenigde Staten heb ik enorm genoten. Ik ben ervan overtuigd dat het resultaat van ons teamwerk in belangrijke mate heeft bijgedragen aan mijn proefschrift; ik ben zeer blij dat je mijn paranimf bent!
Ed, jouw inbreng en betrokkenheid heb ik erg gewaardeerd. Bedankt voor de correcties op het inleidende hoofdstuk van mijn proefschrift. Patrick, Richard en Jeroen, dank voor jullie inzet bij de uitvoering van de ‘grote schaal experimenten’. Jacinta en Jan-Gerard, zeer veel dank voor jullie hulp bij de oneindig vele suikeranalyses.

De medewerkers, AIO’s en stagiaires van AFSG, met in het bijzonder de Bioconversie groep, wil ik bedanken voor de leuke werksfeer, de leerzame werkbesprekingen, de geslaagde groepsuitjes en de getoonde interesse in mijn onderzoek.
Labcollega’s Hetty en Bwee, het was plezierig om met jullie op het laboratorium te werken.
Mijn vroegere kamergenoot Marcia, je Brabantse gezelligheid, optimisme en goede adviezen zorgden mede voor een prettige start van mijn promotieonderzoek; super dat je mijn paranimf wilt zijn!

Mijn andere kamer- en lotgenoten Floor en Joost, bedankt voor de prettige periode op kantoor, het delen van de zogenaamde schrijffrustraties en de gesprekken over het wel en wee van het AIO bestaan.

Graag dank ik iedereen van de sectie Proceskunde. De werkbesprekingen en discussies waren leerzaam en interessant. De AIO-reis naar Denemarken en Zweden was een zeer geslaagde activiteit waar ik met veel plezier aan terugdenk. In het bijzonder dank ik René Drissen en Rik Beeftink voor de prettige samenwerking; de gezamenlijke begeleiding van enkele studenten was voor mij een leerzame ervaring. Leuk dat onze samenwerking heeft geleid tot een tweetal manuscripten (waarvan al één gepubliceerd). Henk Schols en Mirjam Kabel, collega's bij de leerstoelgroep Levensmiddelenchemie, wil ik danken voor de prettige samenwerking. Mirjam, bedankt voor je hulp bij de vele analyses, het meedenken over interessante experimenten en je bijdrage aan de verschillende manuscripten.

Gedurende mijn promotieperiode heb ik een aantal studenten en buitenlandse gastmedewerkers mogen begeleiden. Arianne, Juliën en Iris, bedankt voor jullie inzet en bijdrage. Mireille and Sela, thank you very much for your contributions!

Mijn promotieonderzoek maakte deel uit van een 4-jarig project waarbij intensief werd samengewerkt met industrieën en onderzoeksinstituten. Ik dank de projectpartners Purac biochem, Royal Nedalco, Shell Global Solutions International, ECN, TNO en de WU-groepen Proceskunde en Levensmiddelenchemie voor de prettige samenwerking en bijdrage in de vorm van co-auteurschappen. In het bijzonder dank ik Jan de Bont en Hans Reith voor de coördinatie van het project.

Naast het doen van promotieonderzoek was er zo nu en dan tijd voor ontspanning in de vorm van sport. Hetty, Truus, Pieternel en Rob, bedankt voor de sportieve maar vooral ook gezellige tennispartijen!

Patrick, naast een prettige collega werd je al snel een goede vriend. Ik wil jou en Ilse bedanken voor jullie interesse, betrokkenheid en vriendschap. Hopelijk volgen er nog vele visuitjes!

Mijn goede vrienden Sander, Karen, Jos, Astrid, Joost, Kim, Ad en Marjolein, bedankt voor jullie steun en belangstelling in mijn onderzoek en bij het schrijven van het proefschrift. Dat we met z´n allen nog veel gezellige avonden, verjaardagen en vriendenweekeinden mogen beleven.

Hoewel ik regelmatig mocht uitleggen waar ik nu precies mee bezig was, had mijn familie en schoonfamilie voortdurend belangstelling in de vorderingen van mijn onderzoek. Yvonne, Niels, Henny, Marcel en Marco, en andere familieleden, veel dank voor jullie interesse.

Mijn ouders. Pap en mam, jullie hebben gedurende mijn studie evenals het promotieonderzoek altijd vertrouwen in mij gehad. Jullie stimulans en optimisme zijn ontzettend belangrijk voor mij geweest.

En natuurlijk Saskia, jouw liefde, steun en begrip was van onschatbare waarde. Mede dankzij jou is dit avontuur tot een goed einde gebracht. Laten we genieten van de vrije tijd die we nu samen krijgen.

Ronald

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CONFERENCE PROCEEDINGS

Bakker, R.R., R.J.A. Gosselink, R.H.W. Maas, T. de Vrije, E. de Jong, J.W. van Groenestijn, J.H.O. Hazewinkel. 2004. Biofuel production from acid-impregnated willow and switchgrass. 2nd World Conference on Biomass for Energy, Industry and Climate Protection, Rome: 1467-1470

PATENT

Bakker, R.R., R.H.W. Maas, E. de Jong, R.A. Weusthuis et al. 2007. *Mild alkaline pretreatment and simultaneous saccharification and fermentation of lignocellulosic biomass into organic acids*. P6016586EP.

Training activities

DISCIPLINE SPECIFIC ACTIVITIES

Courses
Advanced Course on Microbial Physiology and Fermentation Technology (BODL, 2005)
Biomassa: Kans of Bedreiging? (WBS, 2006)
Bioreactor Design and Operation (WGS, 2006)

Meetings
27th Symposium on Biotechnology for Fuels and Chemicals (USA, 2005)
5th Biomass PhD Day Wageningen University and Research Centre (2005)
2nd Symposium Graduate Research School Biotechnological Sciences Delft Leiden (2006)
11th Netherlands Biotechnology Congress (2006)
3rd International Conference on Renewable Resources and Biorefineries (Belgium, 2007)

GENERAL COURSES

Career Perspectives (WGS, 2006)
Professional Communication Strategies (WGS, 2006)

OPTIONALS

Preparing PhD research proposal (2005)
Brainstorm Week Food and Bioprocess Engineering (2005)
PhD Study Tour Denmark and Sweden (2006)
Meetings (2005-2007)

Curriculum Vitae

Ronald Hubertus Wilhelmus Maas was born in Maarheeze on the 25th of July 1977. In 1997 he started the study Microbiology at Fontys Hogeschool in Eindhoven. The study was finished with a thesis performed at the former Agrotechnological Research Institute (ATO) in Wageningen. After obtaining the Bachelor of Science degree in January 2001, he became a Research Assistant in the Paper and Fibre Technology group at ATO. From January 2003 till September 2007, he performed a PhD research at the Food and Bioprocess Engineering department at Wageningen University and the Bioconversion department of Agrotechnology and Food Sciences Group (AFSG, Wageningen UR), under supervision of Prof. Dr. G. Eggink and Dr. R.A. Weusthuis. The results obtained within this research, focusing on the microbial conversion of lignocellulose-derived carbohydrates into bioethanol and lactic acid, are written in this thesis.

Ronald Hubertus Wilhelmus Maas werd geboren op 25 juli 1977 te Maarheeze. In 1997 begon hij de studie Microbiologie aan de Fontys Hogeschool te Eindhoven. De opleiding werd afgerond met een afstudeeronderzoek uitgevoerd bij het toenmalige Instituut voor Agrotechnologisch Onderzoek (ATO) te Wageningen. In januari 2001 behaalde hij het ingenieursdiploma. De twee daaropvolgende jaren werkte hij als onderzoeksassistent binnen de afdeling Papier- en vezeltechnologie (ATO). Van januari 2003 tot september 2007 deed hij onderzoek als promovendus bij de groep Levensmiddelen- en bioproceskunde aan de Wageningen Universiteit en de afdeling Bioconversie van Agrotechnology and Food Sciences Group (AFSG, Wageningen UR), onder begeleiding van prof. dr. G. Eggink en dr. R.A. Weusthuis. De resultaten behaald binnen het onderzoek, gericht op de microbiële omzetting van koolhydraten uit lignocellulose naar bioethanol en melkzuur, staan beschreven in dit proefschrift.

The research described in this thesis was performed at the business unit Biobased Products, Agrotechnology and Food Sciences Group, Wageningen University and Research Centre, The Netherlands. This research was part of a joint project with the departments Food and Bioprocess Engineering and Food Chemistry of Wageningen University, Purac biochem, Royal Nedalco, Shell Global Solutions International, ECN and TNO, which was financially supported by the Dutch Ministries of Economic Affairs, Education, Culture and Science and of Housing, Spatial Planning and the Environment through the Programme EET (Economy, Ecology and Technology). Co-financing was provided by Program 412 Green Resources of the Dutch Ministry of Agriculture, Nature and Food Quality.