Biobutanol production by *Clostridium acetobutylicum* using xylose recovered from birch Kraft black liquor

Rasika L. Kudahettige-Nilsson [a], Jonas Helmerius [a], Robert T. Nilsson [a], Magnus Sjöblom [a], David B. Hodge [a,b,c,d], Ulrika Rova [a,*]

[a] Division of Chemical Engineering, Luleå University of Technology, SE-971 87 Luleå, Sweden
[b] Department of Chemical Engineering & Materials Science, Michigan State University, USA
[c] Department of Biosystems & Agricultural Engineering, Michigan State University, USA
[d] DOE Great Lakes Bioenergy Research Center, Michigan State University, USA

HIGHLIGHTS

- Hardwood Kraft black liquor was successfully fractionated and hydrolyzed into xylose.
- Active carbon was effective to remove inhibitors in the hydrolyzate.
- Xylose recovery was 99–100% during active carbon detoxification.
- ABE production reached 1.8–2.1 g/L in xylose recovered from Kraft black liquor.

ARTICLE INFO

ABSTRACT

Acetone–butanol–ethanol (ABE) fermentation was studied using acid-hydrolyzed xylan recovered from hardwood Kraft black liquor by CO_2 acidification as the only carbon source. Detoxification of hydrolyzate using activated carbon was conducted to evaluate the impact of inhibitor removal and fermentation. Xylose hydrolysis yields as high as 18.4% were demonstrated at the highest severity hydrolysis condition. Detoxification using active carbon was effective for removal of both phenolics (76–81%) and HMF (38–52%). Batch fermentation of the hydrolyzate and semi-defined P2 media resulted in a total solvent yield of 0.12–0.13 g/g and 0.34 g/g, corresponding to a butanol concentration of 1.8–2.1 g/L and 7.3 g/L respectively. This work is the first study of a process for the production of a biologically-derived biofuel from hemicelluloses solubilized during Kraft pulping and demonstrates the feasibility of utilizing xylan recovered directly from industrial Kraft pulping liquors as a feedstock for biological production of biofuels such as butanol.

1. Introduction

Growing energy demands coupled with limited resources of petroleum and environmental concerns have generated new interest in production of fuels from renewable biomass, such as residuals of agricultural crops and lignocellulosic waste from the forest industry. Relative to ethanol, butanol is superior in energy content, has lower volatility and hygroscopicity and is also less corrosive to existing infrastructure (Wu et al., 2013). Although commercialization of *n*- and *iso*-butanol is ongoing (e.g. Cobalt and Green Biologics using engineered *Clostridia* sp. and Gevo and Butamax by engineered yeast or *Escherichia coli*), three main challenges remain to be solved if biobutanol is to become a major counterpart in the bioenergy market. This includes optimizing feedstock utilization, reaching theoretical maximum yields of butanol and minimizing energy consumption during separation and purification (Tracy et al., 2012).

One important driver for a biobased economy is the exploitation of the biorefinery concept (Kamm and Kamm, 2004) where maximum value can be derived from the biomass through the generation of multiple products and the effective use of process integration. Chemical pulp mills are current examples of biorefineries that can convert lignocellulosic biomass into energy, pulp, cellulose derivatives, tall oil, etc. One strategy for mills to counteract competition from tropical countries using fast-growing raw materials is to further expand the product portfolio into additional value-added products. Cellulose is the primary fraction utilized for

chemical pulp production, while lignin, hemicellulose and extractives could be considered as by-products in the process. Currently lignin and a portion of the hemicellulose, and the pulping inorganics end up in the black liquor which is concentrated and burnt in a recovery boiler to recover the chemicals and to supply energy for a large fraction of the mills process steam requirements. Considering lignin has double the heating value as polysaccharides, the hemicellulose fraction solubilized during alkaline pulping represents an underutilized resource in many mills.

One option to enhance the value of the hemicellulose is to recover this fraction prior pulping by using hot-water extraction (Borrega et al., 2013; Helmerius et al., 2010). However, despite high yields of xylan, the removal of hemicellulose from birch wood chips prior Kraft cooking has a negative impact of some pulp properties affecting the quality of the paper. If a decrease in pulp strength properties cannot be accepted, another option is to recover the xylan fraction from the black liquor. Acidification of alkaline pulping liquors precipitates lignin as well as any hemicellulose present in the liquor (Stoklosa and Hodge, 2012). Technologies for recovery of lignin from Kraft liquors via CO_2 acidification has been the subject of pilot and demonstration-scale processes (Kouisni et al., 2014; Zhu et al., 2014) with proposed process of generating fuels, materials, and chemicals from the lignin as well as the opportunity for "de-bottlenecking" capacity-limited recovery-boilers. Hemicelluloses are typically degraded to hydroxy acids during Kraft pulping (Kakola et al., 2007), although oligomeric xylan from hardwoods may be more resistant to alkaline pulping than the glucomannans which are the predominant softwood hemicelluloses due to the protection of glucuronic acid substitutions against end-wise alkaline degradation (Sjostrom, 1977), and as such, these xylans may offer an opportunity for recovery and utilization as a feedstock for bioconversion.

The subject of this study was to assess the feasibility of employing xylan recovered from hardwood Kraft black liquor as a feedstock for the production of butanol as well as a lignin co-product. A precipitate from CO_2-acidified industrial Kraft black liquor (pulped under conditions which may be optimized for xylan recovery) was first characterized and the dilute acid hydrolysis of the xylan in this precipitate was investigated with respect to xylose yield and inhibitor generation. Acetone butanol ethanol (ABE) fermentation utilizing *Clostridium acetobutylicum* ATCC 824 was conducted using these xylose hydrolyzates to assess the impact on key fermentation metrics including rates, yields, and titers. To this end, we have compared cell biomass production, solvent (acetone, butanol and ethanol) production, acid (acetic acid and butyric acid) production, product yields, inhibitor content and decomposition of *C. acetobutylicum* cultures in pure hydrolyzate, detoxified hydrolyzate, these hydrolyzes at various dilutions and semi-defined P2 medium with xylose as the only carbon source.

2. Methods

2.1. Organisms, media and culture conditions

C. acetobutylicum ATCC 824 strain was obtained from the DSMZ culture collection (Germany) and was cultured using sterile clostridial nutritive medium (CNM, from Fluka Analytical) in an anaerobic chamber (Coy Laboratory Products Inc., Michigan, United States) at 37 °C for 18–20 h. Stock cultures, harvested at an OD_{600} of 1.5–2 , were stored in 15 % glycerol at −80 °C.

2.2. Birch Kraft black liquor fractionation and detoxification

The overall process is schematically described in Fig. S1. CO_2 precipitated filter cake (50–60% dryness) from birch Kraft black liquor was supplied from Smurfit Kappa, Piteå, Sweden, using the LignoBoost CO_2 precipitation process with minor modification (Zhu et al., 2014). The birch Kraft black liquor was produced using standard commercial conditions and contained 30% dry solids. Importantly, this liquor was withdrawn from the upper section of the digester corresponding to the initial stage of the Kraft delignification process (Fig. S1). The consequences of this are that substantial lignin removal has not yet occurred and xylan degradation is not concurrently as high as later in the cook. Therefore, the black liquor used in this study has high xylan contents and xylan–lignin ratios relative to other liquors from other stages of the Kraft cook (data not shown). To generate soluble monomeric xylose, the precipitate was subjected to hydrolysis using dilute sulfuric acid under conditions corresponding to a combined severity factor (CSF) (Chum et al., 1990) corresponding to 1.5,1.2 and 0 (Table 1). After evaluating the effect of hydrolysis severity on the resulting xylose yield, hydrolysis at a $log_{10}CSF$ of 1.5 was used for the further experiments (Table 1). $Ca(OH)_2$ was used to set pH 7 of the hydrolyzates. Hydrolyzates contained mainly xylose as the carbon source, which was the targeted substrate for the fermentation experiments. Activated carbon (AC) detoxification was conducted for each hydrolyzate using 5% (w/w) AC powder (ColorSorb G5, Jacobi Carbons, Kalmar, Sweden) at 60 °C at 200 rpm for 2 h, as previously described (Hodge et al., 2009).

2.3. Growth and fermentation experiments

For all scale of fermentations, the cultivation medium was anaerobically inoculated with 10% (v/v) *C. acetobutylicum* stock culture, cultivated under anaerobic conditions at 37 °C for 16–18 h or until an OD_{600} of 1.5–2 was obtained. Mini scale fermentation of *C. acetobutylicum* was performed in 50 mL falcon tubes containing 25 mL of semi-defined P2 medium consisting of (g/L): xylose as control, 30; yeast extract, 1.0; ammonium acetate, 2.2; KH_2PO_4, 0.5; K_2HPO_4, 0.5; $MgSO_4·7H_2O$, 0.2; $MnSO_4·7H_2O$, 0.01; $FeSO_4·7H_2O$, 0.01; NaCl, 0.01. The medium was sterilized at 121 °C for 20 min; thereafter different dilutions of un-treated hydrolyzate and detoxified hydrolyzate and 1 g/L yeast extract where added as filter sterilized (0.2 μm). The fermentations were done in three replicates from where 1.0 mL samples were taken every day up to 6 days for analytical measurements. Batch fermentations where conducted in 1 L stirred bioreactors (Applikon® Biotechnology, The Netherlands). Inoculation level was 10% (v/v) after 16–18 h growth at 1.5–2 OD_{600} nm. The working volume of the fermenter was 400 mL and prior inoculation the medium was sparged with N_2 to ensure anaerobic condition. Fermentation was performed at 37 °C and 100 rpm with pH controlled to 5.1 using automatic addition of 4.5% NH_4OH. 10 mL samples were taken for further analysis during every 24 h up to 3 days in sterile P2 medium with xylose

Table 1
Yields from Kraft lignin precipitate after acid hydrolysis in different CSF and corresponding pH levels.

Components	$Log_{10}CSF = 1.5$ pH 0.9 (%)	$Log_{10}CSF = 1.2$ pH 1.2 (%)	$Log_{10}CSF = 0$ pH 2.5 (%)
Xylose	18.4	3.6	2.5
Acetic acid	1.8	2.3	nd
Formic acid	1.1	nd	nd
ASL	2.3	1.4	nd
HMF	0.3	1	nd
Furfural	<0.01	<0.1	nd
AIL	63.2	70.7	72.4
Ash	12.9	nq	nq

$Log_{10}CSF$ was calculated (Chum et al., 1990) based on pH and the fixed reaction conditions 121 °C and 60 min. ASL: acid soluble lignin; AIL: acid insoluble lignin; nd: not detected; nq: not quantified.

(control) and 10 days in filter sterilized and detoxified hydrolyzate. All batch fermentation experiments were duplicated and averages of parameters detected were reported.

2.4. Sugar, solvent, acid and inhibitor analysis

The xylan content of the recovered precipitates were determined as previously described (Sluiter et al., 2008) with adaptation (Stoklosa and Hodge, 2012). Xylose of the hydrolyzate and the other growth media used in this study were analyzed using a HPLC system (Perkin Elmer) equipped with an ion exchange column (BioRad Aminex HPX-87P, 300 mm × 7.8 mm) maintained at 80 °C by a column oven and refractive index (RI) detection. 20 μL of sample was injected into the system with an auto-injector, using distilled water as the mobile phase with a flow rate of 0.6 mL/min during analysis. Acids: formic, levulinic, acetic and butyric; solvents: acetone, butanol and ethanol; inhibitors: furfural and HMF were analyzed using the same HPLC system (Perkin Elmer) but equipped with an organic acids analysis column (BioRad Aminex HPX-87H, 300 mm × 7.8 mm) maintained at 65 °C, RI detection and 5 mM H_2SO_4 as the mobile phase with a flow rate of 0.6 mL/min with injection volume of 20 μL.

2.5. Lignin, phenolics and ash (elements) analysis

Acid soluble lignin (ASL) and acid insoluble lignin (AIL) were analyzed as described previously (Sluiter et al., 2008) and phenolics were analyzed using the Folin–Ciocalteu assay with vanillin as a standard (Hodge et al., 2009). The elementary analysis of the extraction residues (ash fraction) was carried out by an accredited laboratory (ALS Scandinavia AB).

3. Results and discussion

3.1. Characterization and hydrolysis of xylan-rich precipitate

The CO_2-precipitated xylan and lignin from black liquor derived from the Kraft pulping of silver birch (*Betula pendula*) was first characterized for its response to dilute sulfuric acid hydrolysis to monomeric xylose (Fig. S1). The average composition of the recovered precipitate (filter cake) on the dry weight basis is xylan 15.3% (corresponding to 17.4% xylose), acetic acid 2.6%, ASL 12.0%, AIL 52.1%, ash 15.2% and unquantified 2.8%. Variability in the components in the recovered precipitate is expected as the liquor composition is subjected to seasonal variation of the feedstock and its processability. These challenges are reduced by the feedstock preparation as well as tuning of the impregnation and cooking parameters. The substantial quantity of sugars in the filter cake represents an opportunity for both recovery and utilization. Previous work demonstrated that alkali-solubilized sugar oligomers preferentially precipitate at higher pH than lignin (Stoklosa et al., 2013) suggesting that sequential CO_2 precipitation could be employed to selectively enrich and recover xylan from lignin-containing liquors. The high recovery of xylan and remaining acetic acid (2.6%) is the result of withdrawing the liquor from the upper section of the digester corresponding to the early stages of the cook. The recovered xylan-rich precipitate was subjected to dilute acid hydrolysis at different combined severity factor (CSF) levels (Table 1), and resulted in a liquor mainly composed of xylose and lignocellulose-derived inhibitory compounds (acetic acid, phenols, HMF and furfural) and ash. The CSF is a term that integrates changes in temperature, time and acidity into a single parameter, which facilitates comparisons of different conditions (Chum et al., 1990). Based on our data, both xylose content (18.4%) and lignocellulose-derived inhibitory compounds were considerably higher for hydrolysis at a $\log_{10}$CSF of 1.5 than other $\log_{10}$CSF (Table 1). Complete hydrolysis was achieved at $\log_{10}$CSF of 1.5 with no remaining oligomeric xylose (data not shown) as reported on xylose as monomer 28.4% and xylose as oligomer 0% corn stover hydrolyzate at $\log_{10}$CSF 1.48 (Lloyd and Wyman, 2005). In addition, the ash (12.9%) was found at $\log_{10}$CSF of 1.5 hydrolysis condition (Table 1). As a result, this hydrolyzate from recovered Kraft black liquor solubles is a unique hardwood hydrolyzate from residual biomass derived from the pulp and paper industry in Sweden, consisting of only xylose as the sugar substrate (Tables 1 and 2). Analysis revealed differences in the content of xylose and other components according to the initial composition and batch of hydrolyzate (H1, H2, H3 and H4; Table 3).

Table 2
Composition of hydrolyzates derived from Kraft black liquor precipitate.

	Xylose (g/L)	Acetic acid (g/L)	Levulinic acid (g/L)	Formic acid (g/L)	HMF (g/L)	Furfural (g/L)	Phenolics (g/L)
H1	34.5 ± 0.3	3.3 ± 0.05	0	2.00 ± 0.02	0.63 ± 0.02	0.02	4.0 ± 0.10
H1 + AC	34.3 ± 0.1	3.3 ± 0.05	0	2.00 ± 0.01	0.30 ± 0.01	0	0.8 ± 0.01
H2	40.2 ± 0.1	3.9 ± 0.01	0	2.60 ± 0.01	0.79 ± 0.02	0.04	5.3 ± 0.20
H2 + AC	40.2 ± 0.1	3.9 ± 0.05	0	2.60 ± 0.04	0.39 ± 0.01	0	1.0 ± 0.05
H3	28.9 ± 0.1	2.8 ± 0.01	0	1.56 ± 0.01	0.52 ± 0.01	0.05	3.7 ± 0.20
H3 + AC	28.5 ± 0.1	2.8 ± 0.01	0	1.56 ± 0.01	0.32 ± 0.01	0	0.9 ± 0.05
H4	20.5 ± 0.1	2.0 ± 0.01	0	1.63 ± 0.01	0.49 ± 0.01	0.05	2.9 ± 0.10
H4 + AC	20.3 ± 0.2	2.0 ± 0.02	0	1.63 ± 0.02	0.27 ± 0.01	0	0.6 ± 0.04

AC: activated carbon treated.

Table 3
ABE fermentation efficiency of *C. acetobutylicum* ATCC 824 in Kraft liquor-derived xylose hydrolyzates and xylose substrate.

Treatments	Initial sugar conc. (g/L)	Sugar utilization			ABE (g/L)	ABE yield[a] (g/g)	Total acids (g/L)	Acid yield (g/g)	ABE production efficiency[b] (%)
		(g/L)	(%)	(g/L/h)					
Xylose (P2 media)	29.4 ± 0.3	28.0	95	0.39	9.4	0.34	1.5	0.05	87
Hydrolyzate 50% (H2 + AC)	18.6 ± 0.4	17.5	93	0.07	2.2	0.13	5.5	0.31	33
Hydrolyzate 100% (H3 + AC)	24.4 ± 0.2	22.8	93	0.1	2.8	0.12	5.1	0.22	31
Hydrolyzate 100% (H4 + AC)	17.7 ± 0.4	16.9	95	0.07	2.2	0.13	5.2	0.31	33

[a] Actual ABE yield g/g, after xylose: 3 days, and hydrolyzates:10 days of fermentation.
[b] Efficiency of ABE production %, = (actual ABE yield/experimentally reported maximum ABE yield) * 100. Experimentally reported maximum ABE yield after 6 days of fermentation (0.39 solvents/xylose) by *C. beijerinckii* BA101 (Qureshi et al., 2008).

We succeeded in achieving xylose titers in the range of 20–40 g/L for each hydrolyzate (Table 3). Additionally, increasing xylose levels results in a proportional increase in the inhibitory components in the recovered hydrolyzates (Table 3) as reported (Hodge et al., 2009). All hydrolyzates contained levels of weak acids that would be expected to inhibit bacterial growth (Table 3). Levulinic acid was not detected in any hydrolyzates (Table 3), and as the black liquor precipitates contained primarily pentoses the HMF was consequently low (Table 1). Formic acid and levulinic acid arise as acid-catalyzed hydrolysis and dehydration of polysaccharides (Jonsson et al., 2013). Total furanics were low while phenolics were significantly higher 2.9–5.3 g/L (Table 3). In a recent study using liquor from pulping of hardwoods, recovered polysaccharides and lignin was further hydrolyzed with sulfuric acid (Lu et al., 2013) and released primarily xylose and glucose as well as inhibitors g/L (acetic acid 2.95, formic acid 0.27, furfural 0.34, HMF 0.08, levulinic acid 1.22 and phenolics 0.01). The growth and solvent production in Clostridium beijerinckii BA101 has been found to be substantially reduced by the inhibitory compounds in a hydrolyzate derived from dilute acid pretreated corn fiber (Ezeji et al., 2007). Furthermore, ABE fermentation (C. acetobutylicum) of Pinus radiata hydrolyzates have been shown to require detoxification with stream stripping accompanied by AC to enable successful fermentation (Maddox and Murray, 1983).

According to a recent assessment, the pretreatment processing cost of cellulosic biomass for large-scale biofuel production is high relative to the cost of the available feedstock, and development of cost-effective technologies to obtain fermentable sugars from lignocellulosic biomass is urgently needed (Jang et al., 2012). Removal of inhibitors can be achieved by a number of approaches that include individual or combined physical, chemical and biological strategies. The degree of detoxification depends on the chemical structure of inhibitors, but in general removal of aldehyde-containing inhibitors (e.g. furfural, HMF, phenolic aldehydes) can be achieved by over-liming (Xie et al., 2012) although drawbacks include substantial build-up of inorganics in process water streams, while other detoxification approaches include adsorption using ion-exchange resins or AC and biological treatment (Cho et al., 2009). To evaluate the removal efficacy of the inhibitory compounds in the hydrolyzate, activated carbon treatment was conducted and the data demonstrated 76–81% removal of phenolics (Table 3). The decreases of HMF by 38–52% is in agreement with previously reported results (Hodge et al., 2009) where activated carbon treatments of softwood dilute acid hydrolyzate showed significant decreases of phenolics in the range of 86–98% and was effective at removing furans. Nevertheless, the reactions of lignin-derived phenolic compounds are more complex and interpretation of results strongly depends on the quantification methods (Hodge et al., 2009). Activated carbon has shown to be effective in removing furfural (80%), HMF (87.9%), levulinic acid (99.9%) and phenolic compounds (99.9%) with increased butanol production by 40% using hardwood-derived hydrolyzate (Lu et al., 2013). Based on our data, xylose and weak acids (acetic and formic) in hydrolyzates were not removed by activated carbon treatment (Table 3), in partial agreement with previous observations showing that activated carbon adsorption of birch wood hydrolyzates do not affect the sugar (glucose, xylose and arabinose) content but slight removal of acetic and formic and total removal of levulinic acids (Lu et al., 2013). In contrast, stream stripping accompanied by active carbon detoxification of P. radiata wood hydrolyzate resulted in unacceptable sugar losses (Maddox and Murray, 1983). Dilute acid hydrolysis of the recovered lignin-xylan precipitates resulted in enriched acid-insoluble lignin (63.2–72.4%; Table 1). Potential integration and conversion of lignins into other chemicals or polymers production make this type of fractionation process of an industrial byproduct (black liquor) more profitable. Industrial sectors (e.g. pulp and paper, wood processing, biodiesel production) could evolve into advance biorefineries via valorization of low-value byproducts (Koutinas et al., 2014). Chemical product diversification and valorization using industrial waste and byproducts in existing industrial plants would be able to reduce current utilization of petroleum for chemical production by 7% (Koutinas et al., 2014).

3.2. Growth behavior of C. acetobutylicum in Kraft liquor-derived hydrolyzate

Solventogenic clostridia are particularly well adapted for fermenting sugars derived from lignocellulose, including fermentation of hexoses, pentoses, cellobiose, as well as polymeric xylan and decrystallized cellulose (Lee et al., 1985). Additionally, they can grow on a broad range of substrates containing many growth inhibitors formed during the pretreatment and hydrolysis (Green, 2011). In the growth experiments, different dilution of pure hydrolyzate, detoxified with activated carbon (H1 and H1 + AC, Table 2) and P2 medium with xylose 30 g/L as control, C. acetobutylicum showed a typical growth pattern (Fig. 1). A significant difference in biomass production was observed using either non-detoxified or detoxified hydrolyzate (Fig. 1A and B). C. acetobutylicum cultures exhibited a 2 day lag phase using 10% and 25%, and 3 and 4 days lag time for 50% and 100% non-detoxified hydrolyzate respectively (Fig. 1A). Based on the data, a full-strength, un-detoxified hydrolyzate culture could promote growth up to an OD_{600} of 0.12 while a biomass concentration corresponding to values for OD_{600} of 1.8, 2.0 and 0.9 was obtained when using 50%, 25% and 10% diluted cultures respectively during 6 days cultivation (Fig. 1A). Moreover, higher levels of lignocellulose-derived inhibitors, corresponding to g/L; acetic acid 3.3, formic acid 2.0 and phenolics 4.0 levels, probably further explains the significant growth reduction in 100% non-detoxified hydrolyzate (H1, Table 2) than for diluted cultures (Fig. 1A), detoxified hydrolyzate and control (Fig. 1B). This is in agreement with reported results for ABE fermentation using untreated and undiluted hardwood pulp hydrolyzate which revealed a 50.8% reduction of ABE efficiency relative to the control (Lu et al., 2013).The slower growth, when using non-detoxified 10% hydrolyzate culture can be attributed to the lower xylose content relative to the 25% and 50% cultures. The slow growth of non-detoxified 50% hydrolyzate with respect to non-detoxified 25% could be explained by growth inhibition by lignocellulose-derived inhibitors (Fig. 1A).

Unlike sugars derived from sugarcane and corn starch used in first-generation ethanol processes, dilute acid pretreatments of lignocellulose do not produce a clean liquor of fermentable sugars, but rather a complex mixture of toxic compounds that inhibits microbial growth, including furans, phenolics and aliphatic acids (Hodge et al., 2009). Although dilution of the hydrolyzate is an effective method to reduce the concentration of inhibitors, the reduced sugar content will have a negative effect on the ABE titer. In order to have a cost-effective lignocellulose fractionation process, the final sugar concentration has to be substantially higher. However, higher sugar concentrations in hydrolyzates correspond to higher amounts of inhibitors that pose a challenge for microbial fermentation which may require either a detoxification of the hydrolyzate or the use of microorganisms evolved or engineered to grow in the presence of these compounds. To determine the effect of detoxification of hydrolyzate on the growth of C. acetobutylicum, cultures using hydrolyzate (H1 + AC, Table 2), detoxified hydrolyzate, where grown in anaerobic chamber for 6 days. The cultures were sampled daily. Cell biomass production reported typical growth pattern without a lag phase (Fig. 1B) in both 75% and 50% strength hydrolyzates while undiluted, detoxified feedstock resulted in only 1 day lag time, indicating that possible
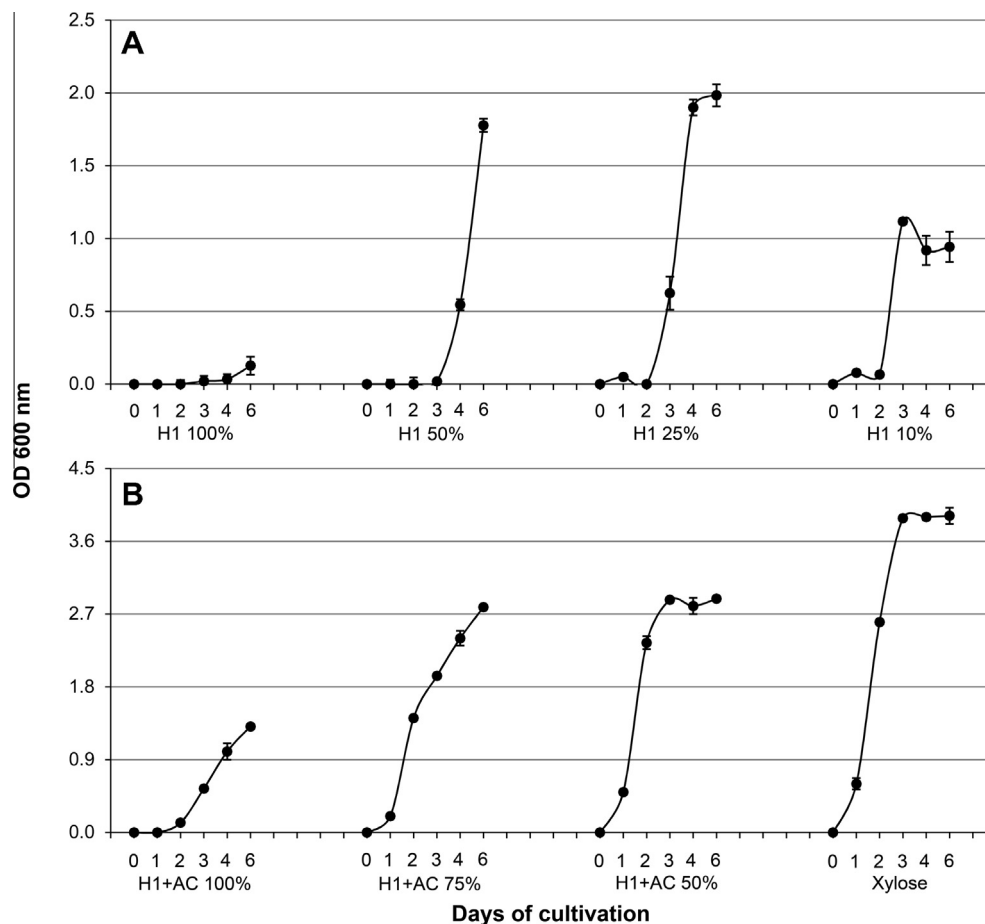
Fig. 1. Growth behavior of *C. acetobutylicum* ATCC 824 in hydrolyzate. Biomass (OD$_{600}$ nm) production in (A) Pure hydrolyzate (H1, Table 2). (B) Detoxified (H1 + AC, Table 2) hydrolyzate and P2 media with xylose (30 g/L) substrate as control in the anaerobic chamber at 37 °C without pH control and shaking. Dilution was conducted with di-ionized water. The data represent the mean ± sd from three biological replicates.

dilution was also needed for hydrolyzate subsequent the activated carbon treatment. Cell biomass production was rapid and higher in 50% activated carbon treated hydrolyzate than using 75% during 24 hours of culturing (Fig. 1B). This further indicates the strong impact of inhibitors rather than the availability of sugars for microbial growth. Cell biomass production (OD$_{600}$ of 1.2, 2.8, and 2.9) using detoxified hydrolyzate at 100%, 75% and 50% strength were significantly improved (Fig. 1B) with respect to the non-detoxified hydrolyzate (Fig. 1A). Moreover, wild-type *C. acetobutylicum* demonstrated adaptation for growth inhibitors by producing cell biomass without lag phase when using detoxified (50–75% hydrolyzate) and only 1 day lag time (100% hydrolyzate) with a considerable amount of inhibitors g/L; acetic acid 3.3, formic acid 2.0 and phenolics 0.75 (H1 + AC, Table 2). This data further stresses the degree of toxicity of phenolic compounds for clostridial growth as more severe than other weak acids, hence activated carbon treatment primarily removes phenolic and furans from the hydrolyzate (Table 2). Phenolic acids and aldehydes at the level of 1 g/L, including *p*-coumaric acid, ferulic acid, 4-hydroxy benzoic acid, vanillic acid, syringaldehyde and vanillin in lignocellulosic hydrolyzates have been shown to inhibit the cell growth by 64–75% in *C. beijerinckii* NCIM 8052 without the production of butanol (Cho et al., 2009). It should be highlighted that the activated carbon treatment was highly selective for inhibitor removal versus sugar removal as less than 1% of the sugar was lost while HMF removal ranged from 38% to 52%, furfural removal was 100%, and phenolics removal ranged from 76% to 81% (Table 2). While 5% (w/w) AC loading on the hydrolyzate may be considered high for a

commercial process, there are opportunities for optimization of the detoxification processes as well as approaches for recovery of furans and phenolics from the adsorbent for further product valorizations.

3.3. C. acetobutylicum fermentations

Lignocellulose offers potential as an abundant renewable resource with great promise for ABE fermentation. It contains about 20–40% hemicellulose with D-xylose as major constituent in dicots and presents a challenge to the biological conversion to liquid biofuels at high yields, titers, and productivities. To understand the xylose fermentation ability and efficiency of recovered hydrolyzate, growth (OD$_{600}$) and xylose content in bioreactor batch cultivation of *C. acetobutylicum* using detoxified hydrolyzate (100% and 50% strength) and P2 (xylose, control) media were analyzed. The results clearly showed that *C. acetobutylicum* thrived in all media, showing normal growth pattern without any lag phase (Figs. 2 and 3). Nevertheless, growth in P2 media (control) was rapid (3 days, Fig. 2) compared to hydrolyzate-grown cultures (10 days, Fig. 3). Moreover, bacterial growth for hydrolyzate (50% H2 + AC and 100% H4 + AC) showed comparable biomass production (OD$_{600}$ of 2) during 10 days of cultivation, i.e. both having comparable initial amount of sugars and inhibitors (Fig. 3). Additionally, a clear correlation could be observed between xylose utilization and growth for both the control (Fig. 2) and the hydrolyzate-grown cultures (Fig. 3). Xylose utilization and consumption rate were 95% and 0.39 g/L/h respectively in the control over 3 days
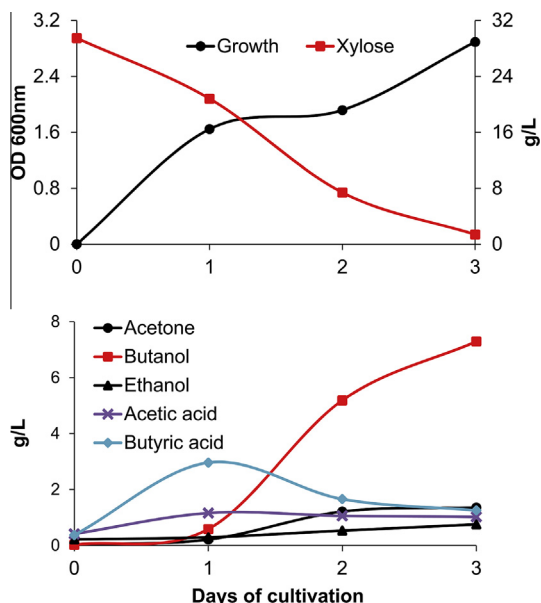
Fig. 2. Xylose utilization and ABE fermentation. Growth and metabolites profiles in batch fermentation of *C. acetobutylicum* ATCC 824 in P2 media with xylose substrate with pH controlled at 5.1 using 4.5% NH4OH.

rates (1.09, 1.12 g/L/h). This clearly demonstrates the effect of the inhibitors on the xylose utilization rate. Interestingly based on our data, xylose utilization of *C. acetobutylicum* using hydrolyzate and control was comparatively higher in our batch fermentations (Table 3) than recently published, where the sugar conversion of *C. beijerinckii* CC101 was 85.6% in control medium (mixed glucose and xylose) and 64.1% in active carbon detoxified mixed sugar birch wood hydrolyzate, grown in serum bottles and 63.9% in non-detoxified 70% wood hydrolyzate in batch fermentation (Lu et al., 2013). This data clearly confirm the value of these hydrolyzates as an alternative substrate for ABE fermentation, among other abundant and renewable lignocellulosic biomass resources.

To investigate the ABE fermentation ability and the efficiency of Kraft liquor-derived xylose hydrolyzate as a carbon source, growth and metabolites profiles of *C. acetobutylicum* bioreactor batch cultivation using detoxified hydrolyzate (50%, 100%) and P2 media (xylose, control) were analyzed. When using hydrolyzate (50%, 100%) *C. acetobutylicum* were able to produce a total solvents concentration of 2.2 g/L, 2.75 g/L and 2.15 g/L respectively (Fig. 3 and Table 2) during 10 days of cultivation, while the control xylose media resulted in 9.4 g/L (Fig. 2, Table 3) during 3 days of cultivation. In addition, ABE yield was significantly higher, 0.34 g/g in control than for the hydrolyzate-grown cultures 0.13–0.12 g/g (Table 3). Comparable ABE yields for hydrolyzate-grown cultures further demonstrate, as explained earlier, all having nearly similar levels of xylose and inhibitors. Therefore, the complex physiological impact of inhibitors in hydrolyzate during ABE fermentation of *C. acetobutylicum* should be further stressed. All hydrolyzate batch fermentations in our study were conducted 10 days to facilitate maximum production of solvents by providing sufficient residence time for re-assimilation of acids into solvents (Green, 2011). The
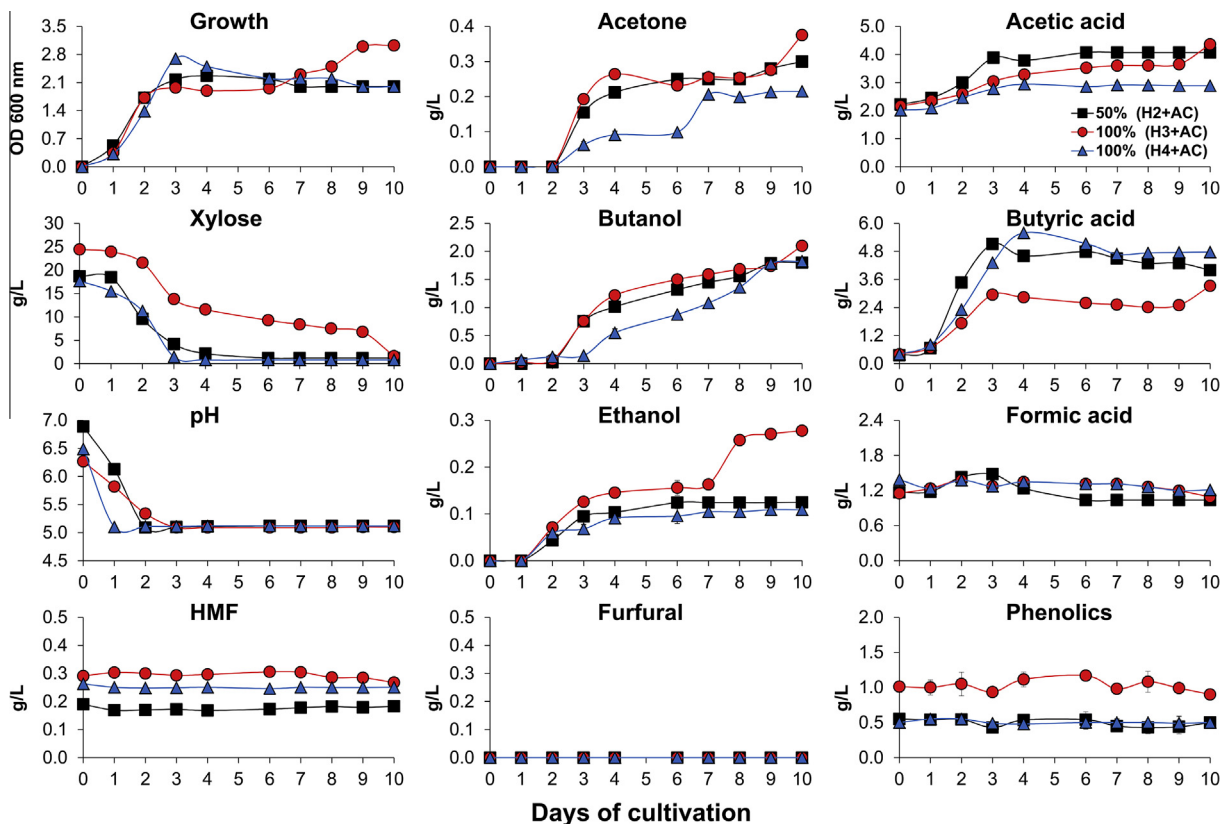
and 93–95% and 0.07–0.1 g/L/h respectively in hydrolyzate-grown cultures over 10 days (Table 3). This data is in agreement with previously reported results (Jaros et al., 2012) where glucose and xylose utilization rates (0.72, 0.86 g/L/h) were lower with higher initial acetate (26.3 g/L) in media with respect to control culture

Fig. 3. ABE fermentation of xylose hydrolyzates. Growth and metabolites profiles in batch fermentation of *C. acetobutylicum* ATCC 824 in detoxified hydrolyzate with dilution. pH was controlled at 5.1 using 4.5% NH4OH.

total acids (butyric and acetic) concentration were higher in (50%, 100%) hydrolyzate-grown cultures, 5.5 g/L, 5.12 g/L and 5.24 g/L in respect to the control 1.5 g/L (Table 3). Although as earlier mentioned, xylose in all media was utilized at similar efficiency, acid re-assimilation was weak in hydrolyzate cultures with acid/sugar 0.31, 0.22 and 0.31 g/g (Table 3) during the 10 days of cultivation with respect to control 0.15 g/g during 3 days of cultivation. Detoxified hydrolyzate is not a pure sugar feedstock as it contained weak acids (acetic and formic), furans and phenolics (Table 2), while acids and solvents generated during the ABE fermentation may further enhance the inhibitory effect for acid re-assimilation and conversion into solvents. In the literature, C. acetobutylicum fermentation of five different sugars mixture (g/L, mannose 12, xylose 6, galactose 5.5, glucose 4 and arabinose 2.5) simulating commercial softwood sulfite liquor, solvent productivity 0.36 g/g and sugar utilization efficiency 96% were obtained (Wayman and Yu, 1985). Moreover, ABE production of C. beijerinckii from fermentation of 25 g/L glucose and 25 g/L xylose was 9.9 g/L and 9.6 g/L respectively, suggesting the same sugars utilization efficiency including a solvents/sugar ratio of 0.39 g/g (Qureshi et al., 2008). Solvent production efficiency of control and hydrolyzate-grown cultures were 87% and 31–33% respectively (Table 3), with respect to a solvent production of xylose media corresponding to 0.39 g/g (Qureshi et al., 2008). This further demonstrates the feasibility of hardwood xylan solubilized during Kraft pulping as an alternative renewable carbon source for biobutanol production.

Acetic and butyric acids formed during acidogenic phase may be re-assimilated during solventogenesis, but the degree of re-assimilation depends on the strain used and the pH of the medium (Patakova et al., 2013). Optimum solvent production in genetically modified C. acetobutylicum strain 824ccpA was reported at pH 5, close to the pKa values of acetate (4.76) and butyrate (4.81) (Ren et al., 2010). In our study, we also controlled pH at 5.1 (Fig. 3) in batch fermentation using 4.5% NH_4OH and that might facilitate solvent production even in hydrolyzate-grown cultures which has considerable different forms of inhibitors as explained earlier (Table 2). The levels of formic acid, HMF and phenolics in all hydrolyzate-grown cultures remained unchanged during the cultivation with the production of ABE solvents and acids (Fig. 3). This data is supported by the finding that isolated mixtures of Clostridium spp. were able to produce butanol from agricultural wastes when different pretreatment and fermentation methods were used (Cheng et al., 2012), separate hydrolysis and fermentation (SHF) of rice straw resulted in 2.93 g/L butanol, combination of SHF with simultaneous saccharification and fermentation (SSF) of rice straw 2.92 g/L, bagasse SHF 1.95 g/L and bagasse SHF-SSF 2.29 g/L. C. acetobutylicum was able to produce maximum butanol concentrations of 3 g/L from 10% fresh domestic organic waste (DOW, a mixture sugars, acid soluble and insoluble lignin and uronic acids) and 4.2 g/L from 10% DOW hydrolyzate during 120 h of cultivation (Lopez-Contreras et al., 2000). In contrast to the positive effect of acetic, butyric and lactic acids on butanol production, an intensive adverse effect of formic acid, which accumulates to 0.5–1 mM in C. acetobutylicum DSM 1731 during glucose fermentation with uncontrolled pH has been demonstrated (Wang et al., 2011). Undissociated acids enter the cell through diffusion over the cell membrane and then dissociate due to the natural cytosolic pH that leads to decrease in the intercellular pH and the cause of cell death (Jonsson et al., 2013). As C. acetobutylicum naturally has a weak acid (acetic and butyric) assimilation mechanism that avoids detrimental effects at pH 5.1, considerable amounts of solvents can be produced. Possibly, the detrimental effect of formic acid (pKa 3.75) could be avoided in our batch fermentation by pH control at 5.1, which remains the acid in the dissociated form.

Among the studies of ABE fermentation that have been reported using lignocellulosic feedstocks and other renewable feedstock alternatives, very few studies have been reported for wood hydrolyzate (Table 4). Relative to hydrolyzates derived from herbaceous biomass including dedicated bioenergy grasses (miscanthus, switchgrass, reed canary grass) and agricultural residues (corn, rice and sugarcane), wood hydrolyzates often contain a considerable amount of inhibitory compounds (e.g. phenolics) that would be the reason for low solvent productivity even in detoxified hydrolyzate (Table 4). A number of studies have investigated the potential of woody biomass for bio-production of fuels and chemicals such as lactic acid, 2,3-butanediol, butanol, fumaric and succinic acid as recently reviewed by Koutinas et al. (2014). Biochemical conversion of woody biomass constitutes a promising but also a complex valorization method for byproducts stream and mainly includes alternative processing options. Valorization methods for byproducts streams via alternative processing technologies in the forest products industry needs to be further developed. Nevertheless, in this study we have fractionated a unique wood liquor, as a residue of pulp and paper production which only contains xylose as the carbon source and use this as an substrate for biobutanol production. This is to our knowledge, the first detailed report on utilizing

Table 4
Production of ABE from xylose and other substrates.

Ref.	Microorganism	Substrates	Acetone (g/g)	Butanol (g/g)	Ethanol (g/g)	ABE yield (g/g)
This work	C. acetobutylicum ATCC 824	Xylose	0.05	0.26	0.03	0.34
		50% hydrolyzate (H2 + AC)	0.02	0.1	0.01	0.13
		100% hydrolyzate (H3 + AC)	0.01	0.1	0.01	0.12
		100% hydrolyzate (H4 + AC)	0.01	0.1	0.01	0.12
Qureshi et al. (2008)	C. beijerinckii BA101	Xylose	0.1	0.26	0.02	0.39
		Glucose	0.16	0.24	0.02	0.39
		ETCFH[a]	–	0.26	–	0.35
		Detoxified SACFH[b]	0.11	0.27	0.01	0.39
Huesemann et al. (2012)	C. acetobutylicum ATCC 824	SWE[c] (glu and mannitol)	0.03	0.12	0.01	0.16
Ounine et al. (1983)	C. acetobutylicum ATCC 824	Xylose	0.07	0.19	0.02	0.28
		Arabinose	0.08	0.18	0.03	0.29
		Glucose	0.07	0.23	0.02	0.32
Lu et al. (2013)	C. beijerinckii CC101	WPH[d] 50% (sugar mix)	0.06	0.31	0.01	0.38
		WPH 60% (sugar mix)	0.08	0.27	0.01	0.36
		WPH 70% (sugar mix)	0.07	0.25	0.01	0.33
		WPH (sugar mix)	0.08	0.19	0.01	0.29

[a] ETCFH: enzyme treated corn fiber hydrolyzate.
[b] SACFH: sulfuric acid treated corn fiber hydrolyzate.
[c] SWE: sea weed extract.
[d] WPH: wood pulp hydrolyzate.

xylose wood hydrolyzate for ABE fermentation of *C. acetobutylicum* with a total solvent yield up to 0.13 g/g sugar. Butanol concentration and yield from *P. radiata* mixed sugar hydrolyzate using different detoxification methods (de-coloration and steam stripping: 1.6 g/L and 0.09 g/g; anion exchange: 2.7 g/L and 0.08 g/g; cation exchange: 1.8 g/L and 0.06 g/g and combination of anion and cation exchange: 5.7 g/L and 0.17 g/g) were demonstrated (Maddox and Murray, 1983). ABE fermentation by *C. beijerinckii* (Table 4) has been demonstrated in wood hydrolyzates (Lu et al., 2013) and enzyme and sulfuric acid treated corn fiber hydrolyzates (Qureshi et al., 2008). In a holistic perspective, the hydrolyzate fermentation results reveled that *C. acetobutylicum* can be utilized as an effective ABE biocatalyst for the alternative renewable substrate with further improvements as adaptation and genetically modification incorporated with fed-batch fermentation.

The maximum yield obtained in study was used in a techno-economic analysis using the modeling software Aspen Plus, employing integration of both lignin separation and ABE fermentation in full-scale pulp and paper production (Mesfun et al., 2014). As explained earlier, the toxic nature of the hydrolyzate resulted in low yield of butanol, causing the estimated cost of one tonne of butanol from this process to be rather high 5.56 kUSD compared to the current market price (Mesfun et al., 2014). This cost was obtained with integrated acetone and ethanol production and a lignin selling price of 30 USD/MWh. Actions to improve ABE yield using this unique substrate (only xylose) are challenging as the detoxification requirements might be higher than for other substrates containing glucose (Bellido et al., 2014). However composition of inhibitors is then very different in this substrate, opening for alternative detoxifications or valorization.

4. Conclusions

The presented results in this work demonstrate the feasibility of biobutanol production from hardwood Kraft liquor-derived xylan as an alternative renewable substrate by *C. acetobutylicum* ATCC 824. Further work to improve product yield for the transfer to commercial application are in progress. Xylan and lignin were recovered from industrial hardwood Kraft black liquor by precipitation, which in addition to xylose as the main carbon source also contained considerable amounts of inhibitors. Therefore, alternative hydrolysis methods not generating inhibitors (e.g. enzymatic) can be considered. Conversion of the xylan to furfural might be another alternative path to valorization of this unique substrate.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biotech.2014.11.012.

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