

# Acetone-butanol-ethanol Fermentation from Different Pulp and Paper Manufacturing Process Side-streams

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The pulp and paper industry produces a diverse range of side-streams from multi-stage processes, but these remain underutilized despite their high potential for use as biofuels. This study investigated acetone-butanol-ethanol (ABE) fermentation by *Clostridium acetobutylicum* DSM 1731 from the side-streams of three different stages of the pulp and paper manufacturing process (PI, PII, and PIII). Biomass specimens with and without water washing were pretreated with 0.2% H<sub>2</sub>SO<sub>4</sub> at 180 °C for 10 min, followed by enzymatic hydrolysis, to obtain fermentable sugars. The results showed that the produced ABE solvent concentrations were 12.8 g/L, 5.2 g/L, and 6.3 g/L from PI, PII, and PIII, respectively. The butanol yields of PI, PII, and PIII were 0.25, 0.18, and 0.19 g/g sugars, respectively. Among the tested side-streams, PI was shown to have potential as a feedstock for butanol production without prewashing prior to dilute acid pretreatment, enzymatic hydrolysis, and microbial fermentation.

*Keywords:* Acetone-butanol-ethanol (ABE) fermentation; Butanol; *Clostridium acetobutylicum*; Hydrolysis; Pretreatment; Pulp and paper manufacturing process side-streams

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## INTRODUCTION

Global environmental concerns, rising energy demand, and the need to reduce greenhouse gas emissions are driving the search for new opportunities in the energy sector. In particular, biofuel production derived from the microbial fermentation of low-cost feedstocks, such as agricultural residues and industrial wastes has been shown to be a promising alternative to petroleum-based fossil fuels. Biofuel production from pulp and paper manufacturing process side-streams has been considered as one possible solution to these challenges. Paper mill sludge contains a high content of lignocellulose and has a well-dispersed structure, which catalyzes butanol and ethanol production (Kang *et al.* 2010; Gogoi *et al.* 2018).

Pulp and paper manufacturing involves five major processes: mechanical, chemical, chemo-mechanical, thermo-mechanical pulping, and papermaking (Ashrafi *et al.* 2015). Side-streams and waste from pulp and paper mills can be divided into four main categories, namely, (i) rejects, (ii) de-inked sludge, (iii) primary sludge, and (iv) secondary sludge (Gottumukkala *et al.* 2016). In this study, PII and PIII are primary and secondary sludges, whereas PI is the rejects from pre-hydrolysis stage before kraft pulping (Fig.1). In 2007, global production of paper and board was estimated at approximately 384 million tons (Pätäri *et al.* 2011), and USA and Japan generated nearly 5 million tons of paper mill sludge annually (Boshoff *et al.* 2016). In 2018, the Finnish pulp and paper industry

produced about 19 million tons of pulp, paper and board, whereas fiber sludge, biosludge and primary sludge generated about 3.9 million tons, and approximately 68,000 tons of pulp and paper mill waste as landfill (FFI 2018; Hassan *et al.* 2019). Moreover, the Waste Act in Finland allows for the valorization of the side-streams from pulp and paper industry as by-products (FFI 2018).

The prospective of the pulp and paper manufacturing process side-streams for biogas (Gottumukkala *et al.* 2016), butanol (Guan *et al.* 2016), ethanol (Boshoff *et al.* 2016), and lactic acid (Marques *et al.* 2008) production has received some attention. Bio-based butanol (C<sub>4</sub>H<sub>9</sub>OH) is an advanced biofuel and is a potential feedstock for various chemicals, as well as an important intermediate in chemical synthesis with a wide range of industrial uses (Dürre 2008; Tashiro *et al.* 2013; Yang *et al.* 2013). It has been proposed, therefore, as the next generation of alternative biofuels, due to its unique properties that are superior to ethanol (*i.e.*, blending efficiency with gasoline at any percentage, high energy content, high hydrophobicity, less corrosiveness, and low vapor pressure) (Dürre 2008; Tashiro *et al.* 2013; Karimi *et al.* 2015). Biochemically, butanol is produced from sugars by acetone-butanol-ethanol (ABE) fermentation with *Clostridium acetobutylicum*, with a typical product ratio of ABE 3:6:1 (Yang *et al.* 2013). However, the production of biobutanol from pulp and paper manufacturing process side-streams may require the ABE process integration to the processes in the existing pulp and paper mill. Potential sugar-containing side-streams originate from different stages of the process. The present research focused on side-streams that are currently unutilized (Fig.1).

The kraft pulping process involves an alkaline pretreatment as a carbohydrate reaction (Saville 2011). The benefits of alkali pretreatment include the removal of lignin and the improvement of cellulose digestibility. However, drawbacks include the high cost of the alkali and the large amount of wastewater produced, which limit its application (Rydholm 1965; Talebnia *et al.* 2010). On the other hand, dilute sulfuric acid pretreatment has been applied to a wide range of lignocellulosic biomass, resulting in high solubility of hemicellulose, altering lignin, and enhancing the availability of cellulose for subsequent enzymatic hydrolysis (Kumar *et al.* 2009). It has been shown that a pre-handling step to the pretreatment processes, such as prewashing and de-ashing, would enhance the efficiency of pretreatment and increase the hydrolysis yield for bioethanol and biobutanol production (He *et al.* 2014; Guan *et al.* 2016; Huang *et al.* 2016). In many pretreatment studies, the prewashing step seems to be an optional operation, since the ash content in lignocellulose biomass can absorb more solvent than lignocellulose fibers (He *et al.* 2014; Tang *et al.* 2019). Therefore, water washing is usually applied to remove the soil or air dust portions of the ash (extractable ash) in order to increase the pretreatment efficiency (He *et al.* 2014).

Phillips *et al.* (2013) earlier proposed that much of the equipment used in the pulp and paper mill manufacturing process, *e.g.*, wood chipping, digesting, mechanical refining, and evaporation, can be used for pretreatment operations, since they may already be present in the prospective pulp mills. Paper sludge with a high ash content has been reported to have an additional advantage for butanol fermentation, while other studies have found that kraft paper mill sludge requires a partial removal of ash to enhance its enzymatic digestibility in ABE fermentation using *C. acetobutylicum* (Yang *et al.* 2013; Gottumukkala *et al.* 2016; Guan *et al.* 2016). There is a considerable debate between the benefit of water washing to remove ash, and the advantage of a high ash content in pulp and paper manufacturing process side-streams as a feedstock in butanol production. Moreover, evaluation of prewashing effects on pretreatment or on the enzymatic hydrolysis

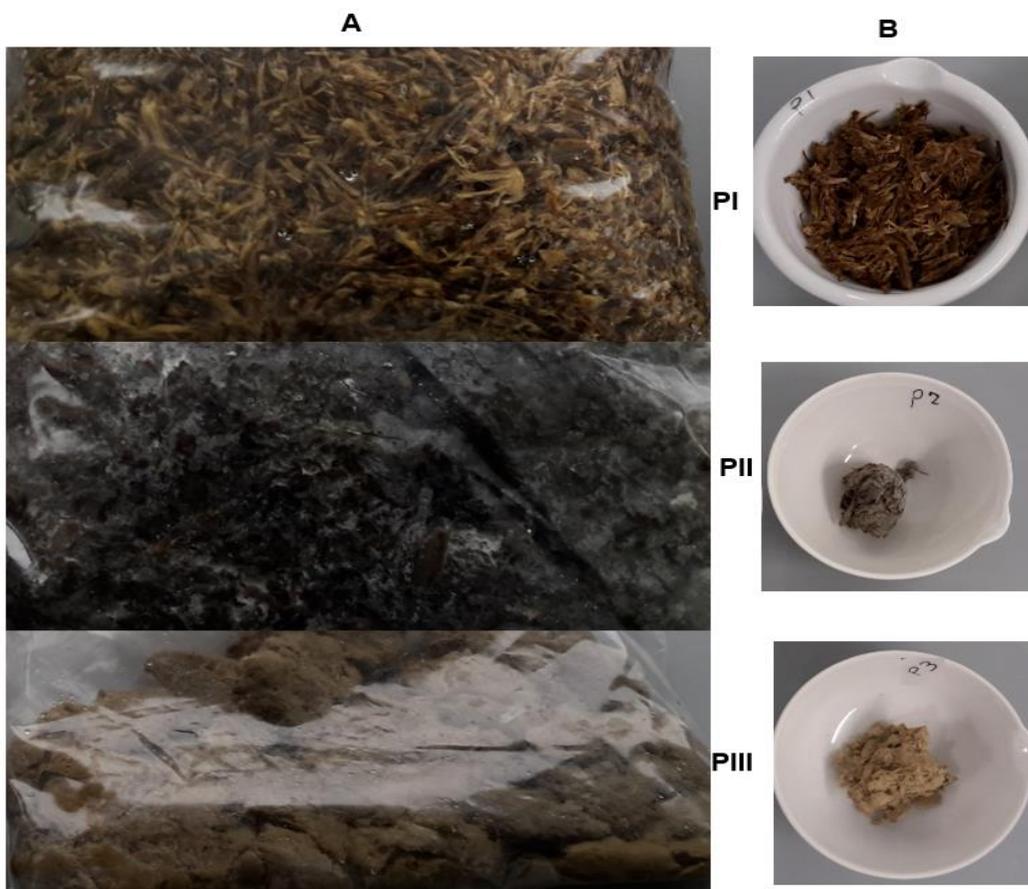
of pulp and paper manufacturing process side-streams from different pulping processes has been rarely reported (Guan *et al.* 2016).

Therefore, the aim of this study was to characterize different pulp and paper manufacturing process side-streams and their chemical composition, and to test them with and without prewashing before pretreatment, enzymatic hydrolysis, and ABE fermentation.

## EXPERIMENTAL

### Pulp and Paper Manufacturing Process Side-streams Materials

Three pulp and paper manufacturing process side-streams (PI, PII, and PIII) were investigated for butanol production by ABE fermentation using *C. acetobutylicum*. The three side-streams used in this study were acquired from two Finnish pulp, paper, and board mills, one using the mechanical pulping process and the other using the chemical pulping process, as shown in Fig. 1. The side-streams originated from different stages of kraft pulping and the mechanical pulping process of mixed species softwood biomass, such as rejects from pre-hydrolysis of the pulping process, and wastewater treatment sludge (Table 1). They also contained residues of cooking and clarifying chemicals, bark residues, and wood knots, for example.



**Fig. 1.** The samples used in this study: PI and PII from kraft pulping and PIII from mechanical pulping processes (A) Original samples (B) Dried samples

**Table 1.** Origin of the Studied Pulp and Paper Manufacturing Process Side-streams

Name of Pulp and Paper Process Side-streams	Origin: Pulp Process Stage	Sample Type
PI	Kraft pulping, pre-hydrolysis (Rejects)	Softwood, contains fiber materials, bark residues, wood knots, residues from wood handling
PII	Kraft pulping, final process sludge (Primary sludge)	Pressed sludge, contains fiber residues, cooking chemicals, traces etc.
PIII	Mechanical pulping, sludge from wastewater treatment (Secondary sludge)	Softwood, contains fiber residues etc.

## Methods

### *Pretreatment procedure*

Before selecting suitable pretreatment conditions for the tested samples, the PI samples were pretreated with water and two concentration gradients of sulphuric acid (H<sub>2</sub>SO<sub>4</sub>) 0.1 and 0.2% (w/v) at 180 °C and 200 °C for 10, 20, 30, 45, and 60 min (see supplementary information in the Appendix, Fig. S1). The pretreatment was carried out with two approaches: one set of pulp side-stream was unwashed, and the other set was washed according to their dry matter (DM) content (10% w/v) with 200 mL of distilled water before dilute acid pretreatment. The most suitable pretreatment indicated in Fig. S1 was ascertained to be 0.2% H<sub>2</sub>SO<sub>4</sub> at 180 °C for 10 min. Both sets of air-dried PI, PII, and PIII side-streams (10 g of dry weight) were pretreated with 0.2% H<sub>2</sub>SO<sub>4</sub> (w/v) at a ratio of 1:10 in a steel cylinder and heated to 180 °C under a 1 MPa pressure, and the temperature was maintained for 10 min. After the mixture had cooled to room temperature, it was separated using 125 mm diameter filter paper (Whatman 589/1, Schleicher and Schuell, Little Chalfont, UK). The pretreated liquid hydrolysate was stored at -20 °C until the carbohydrates and degradation products were analyzed. The solid fractions from the cylinder and filtrate were washed using 100 mL deionized water and stored at -20 °C for enzymatic hydrolysis. All pretreatments were carried out in duplicate.

### *Enzymatic hydrolysis*

Enzymatic hydrolysis was performed on four categories for all tested side-streams: (i) original sample (UN), (ii) washed samples without dilute acid pretreatment (W), (iii) unwashed- pretreated sample with dilute sulfuric acid (UWP), and (iv) washed-pretreated sample with dilute sulfuric acid (WP). Enzymatic hydrolysis was performed using a combination of a commercial cellulase preparation (CEL) Celluclast 1.5 L (Sigma-Aldrich, Saint Louis, MO, USA) (10 filter paper units (FPU)/g of DM),  $\beta$ -glucosidase (Novozyme 188, Novozymes A/S, Bagsværd, Denmark) (200 nkat/g DM) and endo-1,4-b-xylanase (XYL) from *Trichoderma longibrachiatum* (2g/100g DM) after 48 h of incubation at 50 °C with an agitation speed of 200 rpm. After enzymatic hydrolysis, the samples were boiled for 10 min to stop the hydrolysis process and were then centrifuged at 16128 g for 10 min. The supernatant was then collected for reducing sugar analysis with the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959). Sugar and acid concentrations were analyzed by nuclear magnetic resonance (NMR).

### *Microorganism culture preparation and ABE fermentation*

Freeze-stored (-70 °C) *C. acetobutylicum* DSM 1731 bacteria (DSMZ, Braunschweig, Germany) were activated in 50 mL of RCM media (Hirsch and Grinsted 1954) at 30 °C for 14 to 16 h. The inoculum was prepared by transferring 1 mL of actively growing culture into 50 mL of a sterilized pre-fermentation P2 medium supplemented with 30 g/L of glucose in a 125 mL screw-capped Pyrex bottle. The P2 medium contained filter-sterilized stock solution buffer: 50 g/L monopotassium phosphate (KH<sub>2</sub>PO<sub>4</sub>); 50 g/L dipotassium phosphate (K<sub>2</sub>HPO<sub>4</sub>); 220 g/L ammonium acetate (NH<sub>4</sub>CH<sub>3</sub>CO<sub>2</sub>); 20 g/L magnesium sulfate heptahydrate (MgSO<sub>4</sub>·7H<sub>2</sub>O); 1 g/L manganese sulfate monohydrate (MnSO<sub>4</sub>·H<sub>2</sub>O); 1 g/L ferrous sulfate heptahydrate (FeSO<sub>4</sub>·7H<sub>2</sub>O); 1 g/L sodium chloride (NaCl); 0.1 g/L para-aminobenzoic acid; 0.1 g/L thiamin; 0.001 g/L biotin, and 10 g/L of yeast extract (Yang *et al.* 2015). The culture was anaerobically incubated for 16 h at 37 °C before inoculation into ABE fermentation media. All experiments were done in duplicate.

Batch fermentations of all tested pulp side-streams were carried out in 100 mL serum bottles with 30 mL working volume that contained a mixture of P2 medium, RCM and enzymatic hydrolysate of the pulp side-streams. The initial pH of the fermentation broth was adjusted to 6.5 with calcium hydroxide before fermentation. The fermentation bottle was purged with nitrogen (N) gas for 10 min and crimp-sealed with a rubber stopper to maintain anaerobic conditions. The bottles were then autoclaved at 121 °C for 15 min. The ABE fermentation was started by inoculating the samples with 2 mL of *C. acetobutylicum* DSM 1731 culture. All the experiments were conducted in duplicate under strict anaerobic conditions. Aliquots (2 µL) of fermentation samples were taken at 0, 24, 48, 72, and 96 h after inoculation.

### *Chemical analyses*

Determination of the DM and ash contents of the side-streams was carried out according to the National Renewable Energy Laboratory (NREL) standard procedure (Sluiter *et al.* 2008a,b). The organic matter content, as measured by loss-on-ignition (LOI), was determined according to Kuokkanen *et al.* (2008). Total nutrients and heavy metal concentrations in the tested samples were determined by an inductively coupled plasma optical emission spectrometer (ICP-OES), model ICP-OES-IRIS Intrepid II XSP (Thermo Fisher Scientific, Waltham, MA, USA). Two reagents (concentrated 65% w/w nitric acid (HNO<sub>3</sub>) and 30% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) were used in the preparation of the elemental samples. The dried samples were digested with 10 mL of concentrated HNO<sub>3</sub> and analyzed with a CEM Mars 5 microwave at 175 °C for 10 min. Total N concentration in the tested samples was measured by the Kjeldahl method (Kjeldahl 1883).

The chemical composition of the solid pulp samples was determined according to Hayes (2012). Liquid samples before and after pretreatment, enzyme hydrolysis and ABE fermentation were analyzed by proton nuclear magnetic resonance (<sup>1</sup>H NMR) spectroscopy for the solvents (acetone, butanol, ethanol), sugars (glucose, xylose, and arabinose), and other compounds (furfural, 5-hydroxymethylfurfural — HMF, formic acid, acetic acid, butyric acid, and lactic acid) (Yang *et al.* 2015). The original side-stream samples were first centrifuged at 5000 g for 10 min before the supernatant was sent for NMR analysis. The NMR spectra for quantification of these compounds were recorded on a 600 MHz Bruker NMR spectrometer, equipped with a CryoProbe™ (Bruker Prodigy TCI 600 S3 H&F-C/N-D-05 Z, Ettlingen, Germany) and an automatic cooled SampleJet robotic sample changer. These compounds were identified from routine two-dimensional proton-proton and proton-carbon correlated spectra. Prior to the NMR measurements, 200 µL of the

sample liquid was transferred to a 5 mm NMR tube, followed by addition of deuterium oxide (D<sub>2</sub>O, 275  $\mu$ L) and 3-(trimethylsilyl)-propionic -d<sub>4</sub> acid (25  $\mu$ L, 20 mM) in D<sub>2</sub>O as an internal standard of known concentration.

## RESULTS AND DISCUSSION

### Characterization of the Pulp and Paper Manufacturing Process Side-streams

The physical appearances of PI, PII, and PIII conformed with their process stages, *i.e.*, the dark brown color of PI was from the residue of pre-hydrolysis before kraft dissolving pulp, whereas PII's dark tone was from the waste stream of physical treatment after cooking, delignification, and dewatering, and the brown color of PIII was from the waste stream of biological treatment (Fig. 1). The PI sample was noticeably more basic (pH 10.1) than the PII (pH 7.3) and PIII (pH 7.4) samples (Table 2). This is reasonable because causticizing reagents are used in the pulping process, and the secondary sludge (PIII) is generated from a wastewater treatment process where the pH is nearly neutral before the wastewater is discharged to water bodies. The moisture contents of the PI, PII, and III samples were 77.3, 75.8, and 87.7%, respectively, which conforms to values for pulp mill primary sludge 67 to 77.1% (Simão *et al.* 2018). The high moisture content in the studied samples indicates that the incineration of these side-streams, as such, did not create any net energy (Pöykiö *et al.* 2018). The organic matter content, measured as LOI, was slightly greater in the PI sample compared to the PII and PIII samples (Table 2). The very high LOI values in these samples are also reasonable because these samples are still rich in cellulose, hemicellulose, and lignin, as well as the organic binders that originate from the pulping process (Pöykiö *et al.* 2018). As shown in Table 2, the ash content of PI (5.19%) was lower than PII (7.08%) and PIII (7.11%), but all our samples are comparable to the 6% ash content reported for generated paper mill waste by Méndez *et al.* (2009).

**Table 2.** Physical and Chemical Properties of the Studied Pulp and Paper Manufacturing Process Side-streams

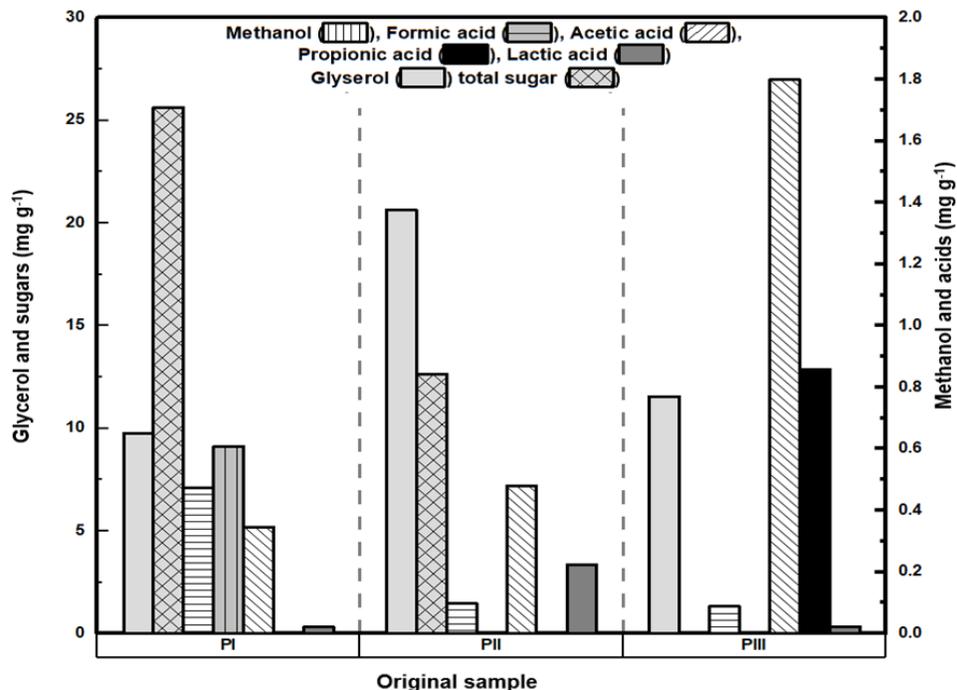
Property and Component	PI	PII	PIII
<b>pH</b>	10.1	7.3	7.4
<b>Moisture content (%)</b>	77.30	75.80	87.70
	(% dry basis)		
<b>Lost-on-ignition (LOI)</b>	94.81	92.92	92.89
<b>Glucan</b>	56.54	43.72	39.16
<b>Xylan</b>	6.82	4.01	6.26
<b>Mannan</b>	5.52	2.67	8.17
<b>Arabinan</b>	0.90	0.58	0.89
<b>Galactan</b>	1.11	0.99	1.71
<b>Rahmnan</b>	0.03	0.34	0.29
<b>Total sugars</b>	70.91	52.32	56.48
<b>Klason lignin</b>	19.10	19.15	24.87
<b>Acid soluble lignin (ASL)</b>	0.51	2.63	0.81
<b>Ash</b>	5.19	7.08	7.11
<b>Extractives</b>	7.50	10.05	7.71
Results of Klason lignin, ASL, extractives, and structural carbohydrates were measured according to Hayes (2012).			

The manufacturing processes in the pulp and paper industry produce variable amounts of side-streams and waste, *e.g.* primary and secondary sludges, all inherently different in their composition. Primary sludge (PII in the present study) and secondary wastewater treatment sludge (PIII in the present study) are rich in both organic and inorganic contents (Bajpai 2015; Gottumukkala *et al.* 2016). In contrast, the PI sample in this study is the reject from the pre-hydrolysis stage of the kraft pulping, in which the content of extractives (7.5%) was lower than in PII (10.05%) and PIII (7.71%) (Table 2). The previous studies reported that solid waste from softwood pulp mills contained high levels of wood extractives; however, pre-hydrolysis pulping removed 50% of extractives (Wegner *et al.* 2012), while the primary sludge adsorbed only 3.9 to 14% of the extractives at primary clarifier. By contrast, the reduction of extractives was over 90% in the pulp and paper mill effluents of all studied wastewater treatment plants (Kostamo *et al.* 2004).

In general, a side-stream such as PI is recycled back to the main stream for refining (Gavrilescu 2008). Furthermore, the chemical composition of the pulp and paper manufacturing process side-streams vary considerably from one industrial plant to another, depending on the wastewater cleaning technique applied, fresh water consumption, and the type of raw materials (Kuokkanen *et al.* 2008). Among our samples, PI exhibited the greatest sugar content; 63.3% (glucan 56.5% and xylan 6.8%), and the lowest lignin (19.1%) content (See PI in Table 2), which is in agreement with the sugar and lignin contents reported by Guan *et al.* (2016) and Gogoi *et al.* (2018) for paper mill sludge (64.4% and 16%, respectively). The sugar contents of PII (47.7%: glucan 43.7% and xylan 4%) and PIII (45.4%: glucan 39.2% and xylan 6.2%) (Table 2) are similar to those found in primary sludge (glucan 44.5% and xylan 9.9%) and recycled sludge from kraft paper mills (glucan 49.6% and 13.7%) (Kang *et al.* 2010). However, Gottumukkala *et al.* (2016) reported that the carbohydrate content of paper sludge can vary considerably (between 20% and 75%).

The supernatant derived from the original PI, PII, and PIII samples after centrifugation also contained weak organic acids and alcohols, such as acetic acid, formic acid, glycerol, and methanol (Fig. 2). The centrifuged supernatant from PI had the greatest total sugar content (25.63 mg g<sup>-1</sup>), while PII contained approximately 50% of the sugar content (12.65 mg g<sup>-1</sup>) observed in PI (see PI and PII in Fig. 2). In contrast, the centrifuged supernatant PIII sample did not contain free sugars. Glycerol, methanol, acetic acid, and lactic acid were found in all tested samples, whereas PI and PIII contained exclusively formic and propionic acids. The high glycerol contents found in all tested samples can originate from lipids in the manufacturing process. A previous study reported that biochemical compounds, such as proteins, carbohydrates, lipids, and inorganics are found in the activated sludge from the biological process in pulp and paper wastewater treatments (Rozich and Gaudy 1992).

Determination of inorganic compounds in the pulp and paper manufacturing process side-streams is important because they are indicative of the total and leachable trace element concentrations for the wastewater treatment plant (Kuokkanen *et al.* 2008). Also, the high concentration of a dominant inorganic component, CaCO<sub>3</sub>, in the paper sludge, can affect the enzymatic hydrolysis and cause viscosity issues at high solid loadings, but can benefit the butanol production and fermentation process (Yang *et al.* 2013, Gottumukkala *et al.* 2016).



**Fig. 2.** Chemical content of the supernatant from the original pulp and paper manufacturing process side-streams

The major organic components in paper sludge are cellulose fiber, lignin, and organic binders, while the main inorganic components are paper additives such as kaolinite (clay) and  $\text{CaCO}_3$  as well as heavy metals from the wood raw material (Kuokkanen *et al.* 2008). For example, kraft process side-streams such as lime mud, grits, and dregs contain calcium (Ca) as the predominant ion, together with barium (Ba), chromium (Cr), copper (Cu), lead (Pb), nickel (Ni) and zinc (Zn) in grits, magnesium (Mg), potassium (K), sodium (Na), Cr, manganese (Mn) and iron (Fe) trace elements in lime mud, and substantial amounts of Na, K, Mn, Mg, and Zn in the dregs (Simão *et al.* 2018). In this study, the primary (N, phosphorus (P), and K) and secondary plant nutrients (Ca, Mg, and sulfur (S)), and trace element concentrations (*e.g.* arsenic (As), boron (B), Cu, Mn, Fe, molybdenum (Mo), cadmium (Cd), cobalt (Co), Cr, Ni, Pb, titanium (Ti) and vanadium (V)) were compared with those recorded from paper mill sludge from the Stora Enso Oyj paper mill (in Oulu) and with pulp sludge from a wastewater treatment plant of the Finnish kraft pulp and linerboard mill (Table 3). The concentrations of Na and S in the PI and PII samples were high because they were collected from the kraft pulping sulfate process, and Na, in the forms of sodium sulfide ( $\text{Na}_2\text{S}$ ), sodium hydroxide (NaOH) and sodium sulfate ( $\text{Na}_2\text{SO}_4$ ), is present in cooking chemicals (Table 3). The PIII samples had the highest Ca content ( $18471 \text{ mg kg}^{-1}$ ) compared to the PI ( $1386 \text{ mg kg}^{-1}$ ) and PII ( $7471 \text{ mg kg}^{-1}$ ) samples (Table 3). This was much lower in comparison to the Ca content recorded in the paper mill sludge from the Stora Enso Oyj paper mill ( $194000 \text{ mg kg}^{-1}$ ), and from secondary sludge in a wastewater treatment plant in Finland ( $34900 \text{ mg kg}^{-1}$ ), because Ca is the most abundant mineral in wood (Kuokkanen *et al.* 2008; Pöykiö *et al.* 2018).

The PI samples exhibited lower inorganic element concentrations compared to the PII and PIII samples, with the exception of Na and K. A previous study reported that primary sludge is composed of small amounts of K, Mg, Na and several toxic metals, and exhibits high C:N ratios (150 to 250), while secondary sludge, in contrast, has a low C:N

ratio (5 to 30) (Simão *et al.* 2018). Furthermore, the heavy metal concentrations (As, Cd, Cr, Cu, Hg, Ni, Pb, Zn) in the PI and PIII samples were lower than the maximum permissible content for Finnish fertilizer products (MMM (24/2011) 2013). However, the Cd concentration ( $3 \text{ mg kg}^{-1}$ ) in the PII samples exceeded the  $1.5 \text{ mg kg}^{-1}$  limit for Finnish fertilizer products, and elevated N and P concentrations were also found in the PII samples. Most probably, the collected PII sample is a primary sludge from the final process of kraft pulping, where nutrients, such as N and P, dissolved organic compounds and colloidal particles are still in the residue (Pöykiö *et al.* 2018). The authors suggest that PI could be a potential feedstock for butanol production due to its high sugar, low lignin, and low ash contents.

**Table 3.** Comparison of Element Contents between the Studied Pulp and Paper Manufacturing Process Side-streams and Pulp and Paper Mill Sludge from Other Finnish Pulp and Paper and Board Industry

Elements	In this study: Finnish Pulp Mills (Kraft and Mechanical Pulping Processes)			Stora Enso Oyj, Oulu Mill, Finland <sup>A</sup>	Wastewater Treatment Plant of Finnish Kraft Pulping and Linerboard Mill <sup>B</sup>	
	PI	PII	PIII	Paper mill sludge	Primary sludge	Secondary sludge
	mg kg <sup>-1</sup> (dry matter)					
Aluminium, Al	<LQD	2558	3081	NA	1910	8940
Arsenic, As	<LQD	<LQD	<LQD	<2.0	<3.0	4.6
Boron, B	<LQD	13	<LQD	NA	<4.0	68
Calcium, Ca	1386	7471	18471	194000	4970	34900
Cadmium, Cd	0	3	0	0.3	0.5	4.8
Cobalt, Co	<LQD	1	0	<1.0	<1.0	2.8
Chromium, Cr	8	75	32	8.0	19	49
Copper, Cu	1	12	16	6.5	6.3	20
Iron, Fe	52	3611	1065	850.0	1930	6280
Mercury, Hg	<LQD	<LQD	<LQD	NA	<0.04	<0.04
Potassium, K	1456	886	299	120.0	<200	2490
Lithium, Li	<LQD	<LQD	<LQD	NA	NA	NA
Magnesium, Mg	0.32	0.85	1.22	1300	540	3830
Manganese, Mn	128	1242	35	54.6	79.0	6330
Molybdenum, Mo	<LQD	1	<LQD	NA	<1.0	4.7
Sodium, Na	13145	1940	502	360.0	550	13500
Nickel, Ni	3	7	5	3.0	8.6	32
Phosphorus, P	21	2351	674	79.0	320	5660
Lead, Pb	1	5	2	6.5	<3.0	6.0
Sulphur, S	3490	6411	1296	530.0	2730	20100
Antimony, Sb	<LQD	<LQD	<LQD	NA	<3.0	<3.0
Selenium, Se	<LQD	<LQD	<LQD	NA	<3.0	<3.0
Silicon, Si	54	573	513	NA	NA	NA
Titanium, Ti	<LQD	44	6	461.0	53	90
Vanadium, V	7	19	7	<1.0	NA	NA
Tungsten, W	<LQD	<LQD	<LQD	NA	NA	NA
Zinc, Zn	17	247	16	11.9	110	490
N	1000	26000	6000	NA	4000	42600

PI: Reject from kraft pulping, PII: Primary sludge from kraft pulping, PIII: Secondary sludge from mechanical pulping, LQD: lowest quantitative determinable concentration, <sup>A</sup> Data from (Kuokkanen *et al.* 2008), <sup>B</sup> Data from (Pöykiö *et al.* 2018)

## Sugar Extraction with Dilute Sulfuric Acid Pretreatment

In this study, washed and non-washed steps were performed prior to dilute acid pretreatment (0.2% H<sub>2</sub>SO<sub>4</sub> at 180 °C for 10 min) as alternative approaches to determine whether the studied side-streams require a prewashing step to increase pretreatment efficiency. Water washing and de-ashing are usually applied to remove ash, clay, CaCO<sub>3</sub>, impurities, and cooking chemical residues from the pulping process, in order to increase the pretreatment and hydrolysis yield for bioethanol and biobutanol production (He *et al.* 2014; Guan *et al.* 2016; Huang *et al.* 2016).

The pH values of the PII and PIII samples were almost similar in the washed and unwashed samples before 0.2% H<sub>2</sub>SO<sub>4</sub> pretreatment, and were close to the mean pH (7.09) of paper mill sludge (Abdullah *et al.* 2015). Both PI and PII side-streams were collected from the kraft pulping process (using NaOH and Na<sub>2</sub>S), but nevertheless, the pH of the unwashed PI was strongly alkaline (pH 10.1) (Table 4). However, the pH was the same as the values recorded in wastewater generated from a kraft mill (Ashrafi *et al.* 2015). The high pH of paper mill sludge is caused by the presence of causticizing reagents used in the pulping process and/or CaCO<sub>3</sub> used in the paper finishing process (Abdullah *et al.* 2015). The change in pH values in the unwashed PI, PII, and PIII samples (4.9, 4.8, and 4.6, respectively) after dilute acid pretreatment, compared to the original pH of these samples before acid pretreatment (10.1, 7.3, and 7.4, respectively) was presumably indicative of neutralization of the samples, rather than a pretreatment effect on the tested side-stream materials.

A key advantage of the pulp and paper manufacturing process side-streams is that the crystalline structure of cellulose has been disrupted during the papermaking process, which enhances enzymatic hydrolysis. However, there are several inherent disadvantages. For example, an ash content of >50% in paper mill sludge harms enzyme hydrolysis as it interferes with pH control (Kang *et al.* 2010; Boshoff *et al.* 2016). Ash in paper mill sludge is composed of inorganic salts, of which CaCO<sub>3</sub> is the predominant component. The CaCO<sub>3</sub> can dramatically increase the pH of the pulp sludge suspension in water or buffer solution. Therefore, ash in paper sludge should be removed by washing in order to improve glucan digestibility (Guan *et al.* 2016). The ash contents in our PI, PII, and PIII samples were slightly reduced (to 2.25%, 6.18%, and 6.75%, respectively) after a one-cycle water wash. A previous study discovered that the ash content of unwashed kraft paper mill sludge could be as high as 32.7%, although, the ash content was observed to decrease to 25.1% and 6.1%, and the glucan content was observed to increase to 55.5% and 71.8%, after two and seven cycles of water washing, respectively (Guan *et al.* 2016). However, some studies suggest that the high ash content in pulp and paper sludge can play a positive role in ABE fermentation; *Clostridia* evinced increased sugar utilization to produce higher butanol yields in the presence of CaCO<sub>3</sub> in pulp mill sludge (Yang *et al.* 2013; Gottumukkala *et al.* 2016).

The prehydrolysate from wood is a very complicated mixture of different sugars that cannot be simulated just by pure glucose; for example, hemicellulose sugars and lignin can be obtained either in the liquid or remain in the solid part that has the potential to be used for high-value products (Galbe and Zacchi 2007; Karimi *et al.* 2015). However, the prehydrolysate of the studied samples was different from that of wood, as they had already experienced pretreatment under the pulping processes. Furthermore, this study only focused on the cellulosic sugars left in the pulping processes side-streams. Table 4 represented that the prehydrolysate of the PI unwashed sample (330 mg g<sup>-1</sup>) contained an elevated amount of reducing sugars compared to the washed sample (242.5 mg g<sup>-1</sup>),

whereas the opposite situation was found in the PII and PIII samples. In practice, sugars were not found in either the unwashed samples or in the washed prehydrolysate PII and PIII liquid samples. Thus, it would seem that dilute acid pretreatment does not convert hemicellulose to monomeric sugars. Instead, inhibitory compounds, such as furfural and HMF, are formed. This finding is reasonable because dilute H<sub>2</sub>SO<sub>4</sub> is usually mixed with biomass to hydrolyze hemicellulose and to continuously break down xylose and other sugars to form furfural (Kumar *et al.* 2009). However, the washed prehydrolysate PI liquid sample contained monosaccharide sugars (0.04 mg g<sup>-1</sup> glucose, 10.75 mg g<sup>-1</sup> xylose, 5.07 mg g<sup>-1</sup> arabinose, and 0.17 mg g<sup>-1</sup> mannose) after dilute acid pretreatment (Table 4).

**Table 4.** Comparison of Composition and pH values of the Liquid Prehydrolysate of Unwashed and Washed PI, PII, and PIII Before and After Dilute Acid Pretreatment (0.2% H<sub>2</sub>SO<sub>4</sub> at 180 °C for 10 min)

Parameters	Unwashed prior to Acid Pretreatment			Washed prior to Acid Pretreatment		
	PI	PII	PIII	PI	PII	PIII
pH before pretreatment	10.1	7.3	7.4	7.4	6.9	7.2
pH after pretreatment	4.9	4.8	4.6	5.6	4.3	4.6
Sugars, furans and acids in liquid prehydrolysate			(mg g <sup>-1</sup> ) *			
Reducing sugars	330.0	127.5	35.0	242.5	153.8	129.2
			(mg g <sup>-1</sup> ) **			
Glucose	0.00	0.00	0.00	0.04	0.00	0.00
Xylose	0.00	0.00	0.00	10.75	0.00	0.00
Arabinose	0.00	0.00	0.00	5.07	0.00	0.00
Mannose	0.00	0.00	0.00	0.17	0.00	0.00
Furfural	0.87	0.62	0.44	1.36	1.05	0.52
HMF	0.10	0.70	0.16	0.28	1.15	0.19
Formic acid	3.28	0.00	1.37	1.87	1.34	1.34
Acetic acid	1.08	0.00	5.65	0.00	0.00	2.38

\* Results measured by DNS method (Miller 1959); \*\* Results measured by NMR

Previous studies reported that hemicellulose fractions (termed as prehydrolysate) of pine chips containing a large amount of mannose oligomers were mixed with kraft pulp mill sludge for the production of lactic acid and ethanol (Kang *et al.* 2012; Shi *et al.* 2015). Mendes *et al.* (2014) studied the conversion of a residual stream of primary sludge to bioethanol, in which CaCO<sub>3</sub> neutralization pretreatment was performed with water, spent acid from the mill, carbonated water, and different organic and inorganic acids. To our knowledge, the previous researchers did not analyze the sugar contents in the prehydrolysate of pulp and paper mill sludges. Probably, the hemicellulose fraction in the kraft pulping process is released into black liquor, which is combusted to recover chemicals and to generate energy. On the other hand, kraft pulping is the best-suited pretreatment for softwoods; however, the various pretreatment methods give different types of products. The studied samples are the processed lignocellulosic biomass in kraft pulping and mechanical pulping. Notably, they do not require chemical pretreatment, detoxification, and pH control (Guan *et al.* 2016). Worldwide, kraft pulping is the dominating process for producing chemical pulp due to the properties of its pulp fibers to produce paper, board,

dissolving pulp, and other applications. However, the properties of pulps depend not only on wood material but also on the pulping process, the extent of delignification, and the bleaching sequences (Rahman *et al.* 2017). In kraft cooking, two types of carbohydrate reaction occur: a peeling and an alkaline pretreatment, in which the latter one tends to have a marked effect on lignin due to disruption of ester bonds between lignin and xylan, some removal of hemicellulose, and changes to the less crystalline solid residue of cellulose (Saville 2011). The previous research found that the hot-water extraction could extract approximately 15% of the chip mass and remove a significant amount of xylan from the chips, or kraft cooking time can be reduced from 120 min to <30 min at 165 °C (Amidon *et al.* 2008). The studied sample PI has a high expectation of having the best ABE yield due to its original process stage, which involved autohydrolysis.

### Enzymatic Hydrolysis of Untreated and Pretreated Side-streams

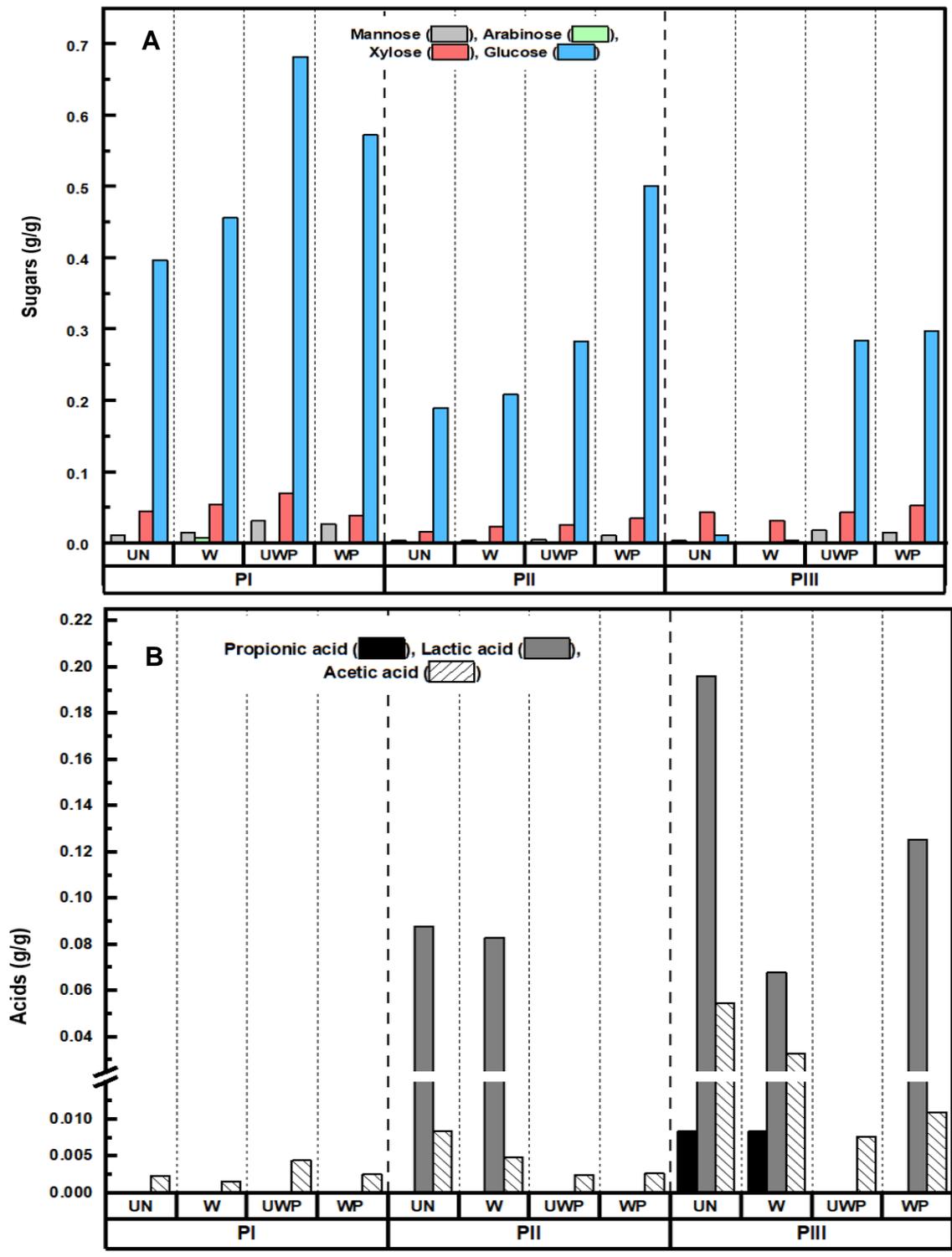
Enzymatic digestion of lignocellulosic materials requires suitable DM loading, which involves the synergistic interaction of a group of different enzymes: endoglucanases and cellobiohydrolases, as well as a variety of other enzymes, including xylanases and mannanases. In this study, the side-streams were subjected to washing and non-washing before dilute acid pretreatment and enzymatic hydrolysis. Therefore, the unwashed and washed PI samples were preliminarily hydrolyzed with different DM loadings, and with different enzymes (see supplementary, Fig. S2A, S2B, and S2C).

All side-streams (PI, PII, and PIII) were proceeded by 7% DM loading with a CEL+XYL enzyme combination. Enzymatic hydrolysis was conducted under four types of reaction conditions: (i) untreated (UN) as a control, (ii) washed only (without dilute acid pretreatment) (W), (iii) unwashed- pretreated sample with dilute sulfuric acid (UWP), and (iv) washed-pretreated sample with dilute sulfuric acid (WP). According to the results of the 48-h enzymatic hydrolysis, all the raw pulp side-streams in this study could produce sugars without acid pretreatment. However, the sugar concentration was found to be higher after dilute acid pretreatment in all raw samples.

The PI hydrolysate exhibited lower glucose yields in both the UN and W reactions, compared to UWP and WP, prior to pretreatment with 0.2% H<sub>2</sub>SO<sub>4</sub>. The glucose differences are illustrated in Fig. 3A, which shows that the highest glucose yield (0.68 g/g) for PI was achieved in UWP, whereas WP yielded the highest glucose concentration (0.50 g/g) in PII. Dilute acid pretreatment was required for PIII, as glucose concentrations in UN and W were 0.011 g/g and 0.004 g/g, respectively, whereas glucose concentrations in the UWP and WP samples were 0.28 g/g and 0.29 g/g, respectively, as represented by the right part of Fig. 3A.

As shown in Fig. 3B, all PI hydrolysates contained only acetic acid, while lactic acid and acetic acid were found in the control (UN) PII and PIII samples and in the washed (W) PII and PIII samples. Propionic acid was found in the UN and W PIII samples. Moreover, the untreated (UN) PIII sample contained less sugars and generated more lactic acid (0.19 g/g) (see Fig. 3B). Furthermore, propionic acid was only found in the supernatant of the original PIII sample and in the enzymatic hydrolyzed PIII sample (see Fig. 2 and Fig. 3B).

The UWP PI sample (0.68 g/g) had a greater sugar concentration than the WP PI sample (0.57 g/g), which showed that prewashing was not needed for the PI samples. This result indicates that enzymatic hydrolysis seemed to work better in the unwashed PI samples because prewashing can remove soluble carbohydrates and, consequently, decrease the sugar yield.



Untreated and pretreated pulp side-streams after 48 h enzymatic hydrolysis

**Fig. 3.** Enzymatic hydrolysis of pulp and paper manufacturing side-streams, PI, PII and PIII: untreated as control (UN), washed without dilute acid pretreatment (W), unwashed (UWP) and washed (WP) prior to pretreatment with 0.2% H<sub>2</sub>SO<sub>4</sub> at 180 °C for 10 min. (A) Sugars concentration, (B) Acids concentrations

It can be concluded that enzyme hydrolysis of a pulp side-stream such as PI, does not require prewashing, and that all the tested samples in this study displayed better results with dilute acid pretreatment, especially sample PIII.

### **ABE Fermentation from Pulp and Paper Manufacturing Process Side-streams**

The unwashed hydrolyzed (UWP) PI, PII, and PIII samples were selected for all the ABE fermentations conducted in this study, and slurries were supplemented with 30 g/L of glucose, minerals, and vitamins for microbial growth. In ABE fermentation, glucose is more preferable than other reducing sugars because of the higher efficiency in sugar-specific mechanisms, including transcriptional regulation of transport, and the number of metabolism genes is far higher than in other carbon sources (Jiang *et al.* 2014). The addition of glucose to the lignocellulosic hydrolysate is a common practice to increase the total and individual sugar concentrations in the fermentation medium.

In this study, a similar concentration of glucose was added to the hydrolyzed samples, although the original reducing sugar contents of PI, PII, and PIII were different (65.8 g/L, 33.5 g/L, and 38.5 g/L, respectively). The aim was to investigate the fermentability of the pulp and paper manufacturing process side-streams by glucose uptake at different concentrations.

ABE fermentation by *C. acetobutylicum* can be divided into biphasic stages: acidogenesis (rapid cell growth and an acid production stage), and solventogenesis (solvent production stage, in which the produced acids are consumed by the cells) (Yang *et al.* 2013; Jiang *et al.* 2014). These stages are significantly affected by pH, and by the intra- and extra-cellular environment (Maddox *et al.* 2000; Yang *et al.* 2013). Normally, acid production starts with the exponential phase of cell growth (within the initial 24 h period), while solvent production commences after 24 h.

During the fermentation with the PI side-stream, acetic acid with a maximum acid concentration equivalent to 3.94 g/L was produced within the initial 24 h period and the concentration remained unchanged at the end of the fermentation process. Butyric acid was produced predominately after 48 h, reaching a maximum acid concentration of 1.86 g/L at 72 h, while 5.1 g/L of butanol produced at 24 h increased to a maximum yield 8.96 g/L at 72 h, thereby demonstrating the correlation between butyric acid and butanol production, as indicated in Fig. 4A. This finding agrees with previous studies, in which a high level of acetic acid production (5.9 g/L) did not significantly affect solventogenesis and butanol production (Qureshi *et al.* 2008). However, the initiation of solventogenesis correlates strongly with the concentration of undissociated butyric acid, at a minimum of 1.5 g/L (Monot *et al.* 1984; Ibrahim *et al.* 2017).

Fermentation with low glucose consumption in both PII (6.7 g/L) and PIII (4.9 g/L) samples was immediate (at 0 h) and butanol production was slowly increased to 4.38 g/L and 4.91 g/L, respectively, by 72 h. Low ABE fermentation rates in PII and PIII may have resulted from the fact that acids were produced more at the beginning of the process; a high butyric acid concentration (2.55 g/L) was especially detected in PII during the initial 24 h fermentation period (shown at the left Fig. 4B). At the right Fig. 4B, an initial acidogenic phase between 0 and 24 h of fermentation was shown, during which glucose was consumed rapidly at the beginning of the first acidogenic phase, but the consumption rate of xylose was much slower. This finding was incompatible with the previous report of Pang *et al.* (2016), in which the glucose had suddenly increased after 24 h by adding a feeding fermentation medium.

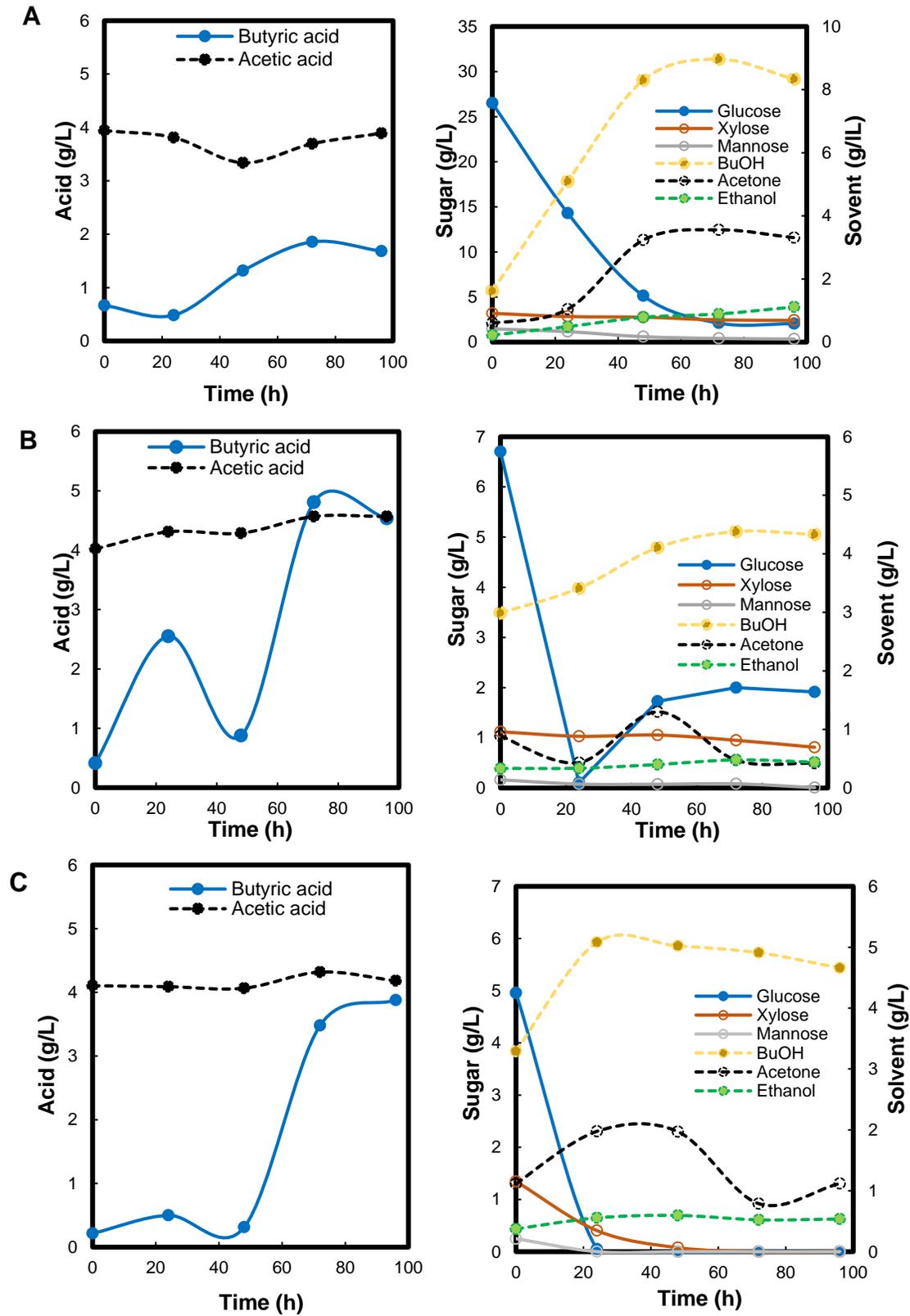


Fig. 4. ABE fermentation by *C. acetobutylicum* DSM 1731 using enzymatic hydrolysate of pulp and paper manufacturing process side-streams for 96 h: (A) PI, (B) PII, (C) PIII

Therefore, the authors expressed it as an abnormal finding in typical research of ABE fermentation. Probably, PII experienced an “acid crash” or “acid flash” phenomena. An acid crash occurs under uncontrolled pH fermentation and results in the cessation of glucose uptake, acid, and butanol production. In contrast, an acid flash causes a weak fermentation in the solventogenic phase due to excessive acids or low pH (Maddox *et al.* 2000; Yang *et al.* 2013). Furthermore, all the sugars were not fully utilized in the PII sample, thereby indicating that the cells might have experienced an inhibitory effect during butanol production. A previous study reported that 7 and 10.5 g/L of butanol were potent inhibitors of the growth of the organism and led to 50% inhibition of xylose and glucose incorporation into cell materials (Ounine *et al.* 1985).

At the log phase of cell growth, PI, PII, and PIII yielded 4.30 g/L, 6.86 g/L, and 4.59 g/L of total acids, respectively. The high concentration of acid production in all samples could have originated from the original side-streams. PII contained the greatest amount of total acids, and glucose uptake in PII ceased immediately after 24 h. The sugar remained unconsumed (1.91 g/L remained after 96 h), resulting in low solvent production (5.2 g/L). This finding agrees with a previous report that a threshold amount of butyric acid initiates solvent production (Yang *et al.* 2013). However, an excess of acetic and butyric acid can be produced in the batch fermentation of *C. acetobutylicum* without pH control and subsequently causes a failure of transition from the acidogenic to the solventogenic phase, and the cessation of glucose utilization (Maddox *et al.* 2000). This finding also agrees with Ibrahim *et al.* (2015), who found that fermentation with an initial sugar concentration of <40 g/L yielded more acids than solvents during a 24 h fermentation period, and also inhibited cells after 48 h fermentation. Over 28% of the glucose in PII was not consumed due to the potential “acid flash” phenomenon rather than “acid crash”, because total solvent production and the fermentation process were completed early in this experiment. During the initial 24 h fermentation period, glucose, xylose, and mannose were quickly consumed in the PIII sample; however, butyric acid production by *C. acetobutylicum* reached a plateau of 3.48 g/L at 72 h (Fig. 4C).

The fermentation results show that acid and solvent can be produced at 0 h because cell growth can happen during inoculation, before the start of the ABE fermentation process. Theoretically, when the glucose concentration decreases, more acids are expected to re-assimilate for butanol production. Thus, acetic acid in the PIII sample remained unchanged throughout the fermentation process, while glucose was completely consumed, and acetone, butanol, and ethanol were produced. This finding explains why an acetic acid concentration of approximate 4 g/L did not affect ABE fermentation. Although a linear correlation has been found between butanol and undissociated butyric acid concentrations (Monot *et al.* 1984; Hüsemann and Papoutsakis 1988), the presence of butyric acid in the medium after 48 h cannot enhance the production of butanol. This assumption is in agreement with a previous report, where butyric acid was required to start solventogenesis within 24 h and glucose utilization was stopped after 24 h without controlling pH (Yang *et al.* 2013). As a result, PIII produced 5.08 g/L butanol at 24 h and 4.91 g/L at 72 h, although its initial glucose yield was very low.

Table 5 documents results of several studies, showing that the ABE yields (0.22 to 0.38 g/g sugars) from the samples are comparable to other paper mill sludge values (0.24 to 0.29 g/g sugars) and lignocellulosic biomass substrates, such as barley straw (0.28 g/g sugar) and sugarcane bagasse (0.33 g/g sugars). A previous study reported that the final ABE solvent concentrations from paper sludge were 5 to 34% higher than that from dilute acid pretreated wheat straw (Guan *et al.* 2016).

**Table 5.** Comparison of ABE Fermentation and Butanol Production from the Studied Pulp and Paper Side-streams and Other Biomass Sources

Feedstock	Pretreatment Condition	Enzymatic Hydrolysis Condition	Strain and Fermentation Method	BuOH (g/L)	BuOH (g/g Sugar)	ABE (g/L)	ABE (g/g Sugar)	References
PI	0.2% H <sub>2</sub> SO <sub>4</sub> , 180 °C, 10 min	cellulase 10 FPU, xylanase 0.02%, 50 °C, 48 h	<i>C. acetobutylicum</i> DSM 1731, BF	8.33	0.25	12.76	0.38	This study
PII				4.33	0.18	5.20	0.22	
PIII				4.66	0.19	6.32	0.26	
Paper mill sludge	15% NaOH, 140 °C, 60 min	nd	<i>C. sporogenes</i> NCIM 2337, BF	nd	nd	0.559	nd	(Gogoi <i>et al.</i> 2018)
Recycled kraft paper mill sludge	None	10 FPU/g-glucan	<i>C. acetobutylicum</i> , SSF	6.8 - 9.7	nd	10.6 - 17.1	0.24 - 0.29	(Guan <i>et al.</i> 2016)
Sugarcane bagasse	1% NaOH, 60 °C, 72 h	cellulase 10 FPU, $\beta$ - glucosidase 160, CMCase 90, xylanase 4600, $\beta$ -xylosidase 18, 60 °C, 64 h	<i>C. acetobutylicum</i> GX01, FBF	14.17	0.22	21.11	0.33	(Pang <i>et al.</i> 2016)
Barley straw	1.5% H <sub>2</sub> SO <sub>4</sub> , 121 °C, 60 min	cellulase 10 FPU, xylanase, surfactants (PEG and Tween), 50 °C, 48 h	<i>C. acetobutylicum</i> DSM 1731, BF	7.9	0.2	10.8	0.28	(Yang <i>et al.</i> 2015)
Rice straw	75% ethanol, 1% H <sub>2</sub> SO <sub>4</sub> , 180 °C, 30 min	cellulase 25 FPU g <sup>-1</sup> , $\beta$ - glucosidase 4 IU g <sup>-1</sup> , 45 °C, 72 h	<i>C. acetobutylicum</i> NRRL B-591, BF	7.1	nd	10.5	nd	(Amiri <i>et al.</i> 2014)
Switch grass	1% NaOH	Cellic CTec 2 cellulase 15 FPU, 50 °C, 72 h	<i>C. saccharobutylicum</i> DSM 13864, SHF	13.0	nd	22.7	0.40	(Gao <i>et al.</i> 2014)
Wheat straw	1.6% NaOH, 2% H <sub>2</sub> O <sub>2</sub> , 35 °C, 24h	$\beta$ -glucosidase 60, CMCase 119, xylanase 1554, 45 °C, 72 h	<i>C. beijerinckii</i> P260, BF + Glc	12.33	0.23	22.17	0.42	(Qureshi <i>et al.</i> 2008)

BF: Batch fermentation; FBF: Fed-batch fermentation; SSF: Simultaneous saccharification fermentation; SHF: Separate hydrolysis and fermentation; Glc: glucose; nd: no data available

In this study, PI yielded 12.76 g/L of butanol, while PII and PIII yielded 5.20 g/L and 6.32 g/L, respectively. (Table 5). The xylose utilization by *C. acetobytylicum* DSM 1731 was low in PI and PII, with 2.42 g/L and 0.81 g/L of xylose, respectively, remaining at the end of fermentation. This finding is consistent with Pang *et al.* (2016), who reported that 11 g/L of xylose remained unconsumed (using *C. acetobytylicum* GX01) after 72 h fermentation. In particular, the PI result is comparable to a previous study of recycled kraft paper mill sludge, in terms of solid loading (6.3 to 7.4%), enzyme loading (10 FPU/g-glucan), and ABE yield (10.6 to 17.1 g/L) (Guan *et al.* 2016).

The studied side-streams were from three different pulping processes, in which the quantity will diverge, affecting the feasibility of utilizing for butanol production. In general, a Finnish pulp (kraft) and linerboard mill generates about 4000 tonnes of primary sludge and 3000 tonnes of secondary sludge annually (Pöykiö *et al.* 2018). In the pulp and paper industry, kraft pulp mill is the primary candidate to be transformed into an integrated biorefining system. Recently, dissolving pulps have been produced by acid sulfite pulping or by pre-hydrolysis kraft pulping. The removal of hemicelluloses from wood and pulp is a recent technological advancement towards kraft-based dissolving pulp producing mills. The dissolving pulp process requires the production of high-purity cellulose (90 to 99%) with low content of hemicellulose (<4%) and traces of other components (Kumar and Christopher 2017). Therefore, the full utilization of different pulp side-streams requires either combination of feedstocks or adding new operations in the future, where an ABE plant would be installed next to the kraft pulp mill.

## CONCLUSIONS

1. Based on the acetone-butanol-ethanol (ABE) fermentation yield results, the unwashed PI side-stream could be a potential feedstock for ABE fermentation, with an ABE yield of 0.38 g/g sugar, in comparison to the unwashed PII and PIII side-streams, 0.22 g/g and 0.26 g/g, respectively.
2. This study showed that washing did not influence sugar recovery in the PII and PIII prehydrolysate liquid samples. However, the prehydrolysate liquid of the washed PI sample contained sugars solubilized during the pretreatment when hemicellulose-derived monomeric sugars were released into the liquid fraction (prehydrolysate).
3. The chemical compositions of the studied PI, PII, and PIII side-streams were similar, with the exception of PI, which contained the highest sugar content and the lowest lignin and ash contents. Therefore, the enzymatic hydrolysate of the unwashed PI side-stream resulted in the highest sugar concentration (0.68 g/g). Moreover, the unwashed PI side-stream was found to be the best choice among the studied samples for bio-based butanol production, with a butanol yield of 0.25 g/g sugar.

## ACKNOWLEDGMENTS

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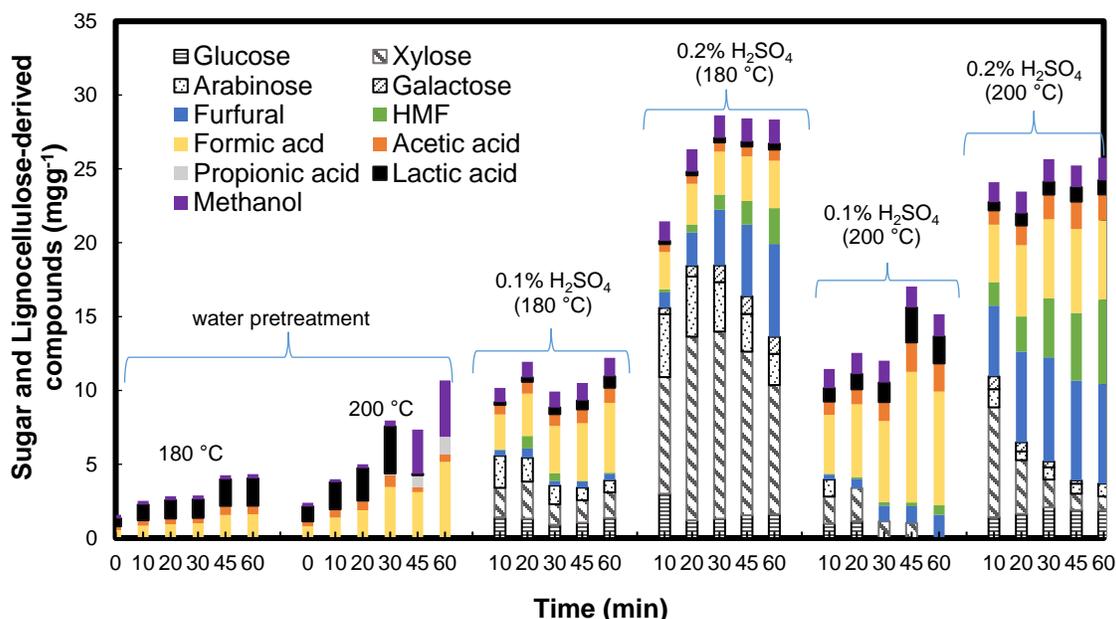
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## SUPPLEMENTARY MATERIAL

Following an assessment of suitable pretreatment conditions for the studied side-streams, PI was selected for further testing because of its potential chemical composition and high total sugar content. It was pretreated under a range of conditions with water: 0.1% and 0.2% H<sub>2</sub>SO<sub>4</sub> at 180 °C and 200 °C for 10, 20, 30, 45, and 60 min (Fig. S1). The applied pretreatment was not excessively strong because it would lead to sugar loss through the degradation of hemicellulosic and cellulosic sugars to furans, such as furfural, HMF (5-hydroxymethylfurfural), and to organic acids, such as formic acid and levulinic acid (Karimi et al. 2015). In this study, undesired inhibitory compounds, such as furfural, HMF, acetic acid, and formic acid, were released during the water and dilute acid pretreatment. These sugar degradation products, present in the pretreated hydrolysate liquid fraction, were formed at high temperatures (200 °C) or during an extended pretreatment time (30, 45, and 60 min) (Fig. S1). The main interest here was to produce butanol from the cellulosic sugars of the pretreated pulp and paper manufacturing side-streams, and therefore pretreatment with 0.2% H<sub>2</sub>SO<sub>4</sub> at 180 °C for 10 min was selected for all studied samples (*i.e.*, PI, PII, and PIII).



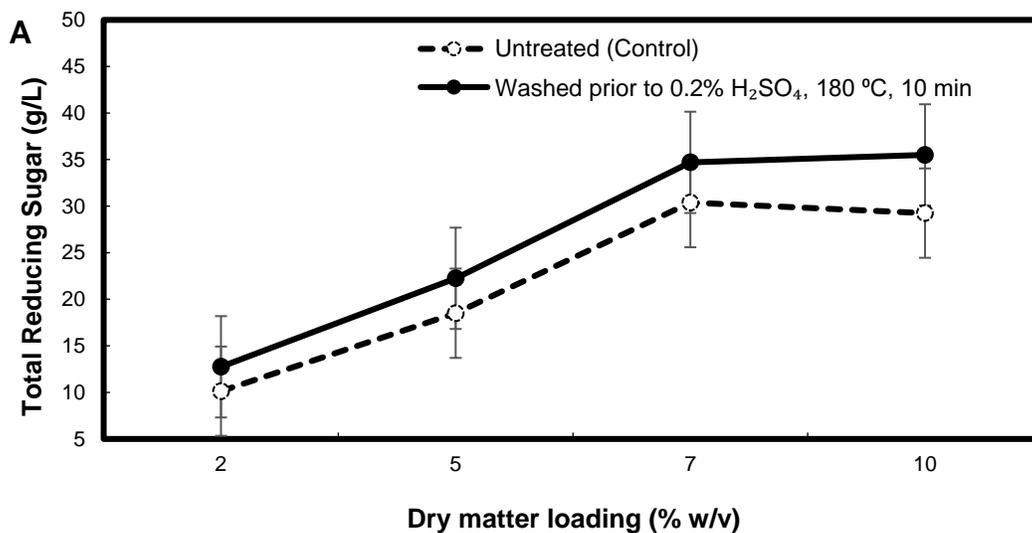
**Fig. S1.** Sugar and inhibitor contents in the prehydrolysate liquid fraction of the PI side-stream under different pretreatment conditions: water, 0.1% and 0.2% H<sub>2</sub>SO<sub>4</sub> at 180 °C and 200 °C

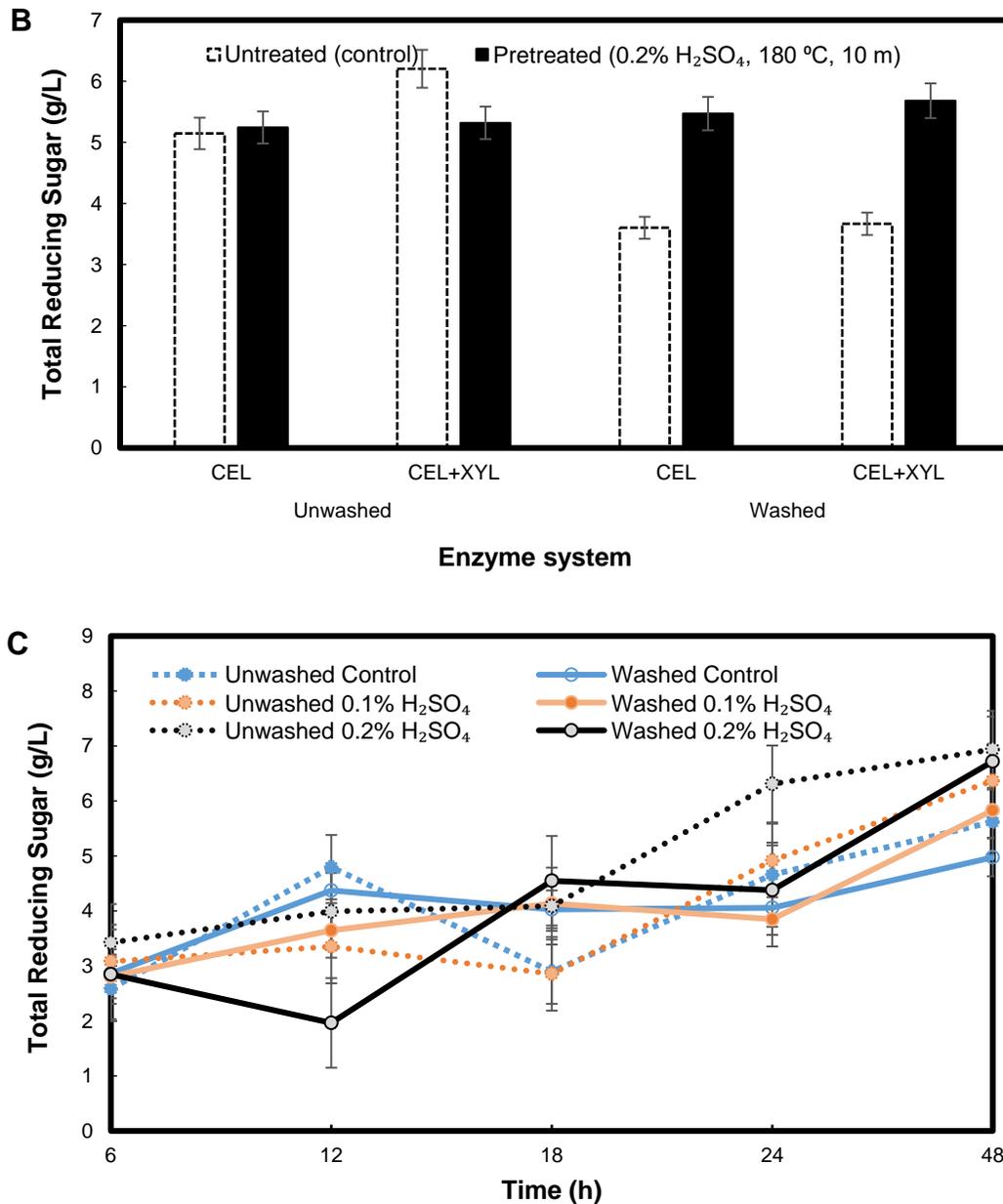
To determine a suitable DM loading for subsequent enzymatic hydrolysis and fermentation, PI was hydrolyzed (with a range of DM loadings: 2%, 5%, 7%, and 10%) for 48 h under two variable conditions: untreated and washed prior to 0.2% sulfuric acid at 180 °C. When the DM loading reached 7%, the sugar concentration in the PI washed sample pretreated with 0.2% H<sub>2</sub>SO<sub>4</sub> at 180 °C rose from 22.3 g/L to 34.7 g/L (Fig. S2A). From an economic point of view, 7% DM loading for fermentation is favorable, as an almost similar sugar concentration is produced at 7% (34.7 g/L) and 10% (35.5 g/L) in the washed sample at 180 °C. Therefore, 7% DM loading was chosen for enzymatic hydrolysis of all the tested samples (*i.e.*, PI, PII, and PIII).

Enzymatic digestion of lignocellulosic materials requires synergistic interaction of

a group of different enzymes: cellulase, endoglucanases, and cellobiohydrolases, as well as a variety of hemicellulases, such as xylanases and mannanases. Total reducing sugars following hydrolysis with cellulase (CEL) were 5.15 g/L and 3.6 g/L for the PI unwashed and the washed control, respectively, and were 5.24 g/L and 5.32 g/L, respectively, for the 0.2% H<sub>2</sub>SO<sub>4</sub> pretreatment (Fig. S2B). By adding 2 g/100 g DM xylanase (CEL+XYL), the glucose concentrations in the unwashed and the washed control PI samples increased to 6.2 g/L and 3.7 g/L, respectively, whereas the samples with 0.2% H<sub>2</sub>SO<sub>4</sub> pretreatment were 5.47 g/L and 5.68 g/L, respectively (Fig. S2B). The results showed that the synergistic interaction of xylanase and cellulase enzymes improved the hydrolysis of cellulose accessibility by removing the hemicellulose shields that cover the cellulose.

In this study, the raw pulp and paper manufacturing process side-streams were subjected to a washing process before dilute acid pretreatment and enzymatic hydrolysis. However, the enzymatic hydrolysate of the PI unwashed sample with 0.2% H<sub>2</sub>SO<sub>4</sub> pretreatment exhibited the highest glucose concentration (6.94 g/L) at 48 h (Fig. S2C).





**Fig. S2.** Enzymatic hydrolysis of PI side-stream after 48 h (A) Sugars recovery in different dry matter loading; (B) Comparison of sugar concentration between CEL (Celluclast 1.5 L and Novozyme 188) and CEL+XYL (Xylanase); (C) Kinetic reducing sugar production under three different conditions: Control (untreated), unwashed and washed prior to pretreatment 0.1% and 0.2% H<sub>2</sub>SO<sub>4</sub>, 180 °C for 10 min. (Error bars represent one standard deviation.)