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Removal of enzymatic and fermentation inhibitory compounds from biomass slurries for enhanced biorefinery process efficiencies

Raghu N. Gurram [a], Saurav Datta [b], Yupo J. Lin [b], Seth W. Snyder [b], Todd J. Menkhaus [a,*]

[a] Department of Chemical and Biological Engineering, South Dakota School of Mines and Technology, Rapid City, SD 57701, USA
[b] Process Technology Research, Energy Systems Division, Argonne National Laboratory, Argonne, IL 60439, USA

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ABSTRACT

Within the biorefinery paradigm, many non-monomeric sugar compounds have been shown to be inhibitory to enzymes and microbial organisms that are used for hydrolysis and fermentation. Here, two novel separation technologies, polyelectrolyte polymer adsorption and resin-wafer electrodeionization (RW-EDI), have been evaluated to detoxify a dilute acid pretreated biomass slurry. Results showed that detoxification of a dilute acid pretreated ponderosa pine slurry by sequential polyelectrolyte and RW-EDI treatments was very promising, with significant removal of acetic acid, 5-hydroxymethyl furfural, and furfural (up to 77%, 60%, and 74% removed, respectively) along with >97% removal of sulfuric acid. Removal of these compounds increased the cellulose conversion to 94% and elevated the hydrolysis rate to 0.69 g glucose/L/h. When using *Saccharomyces cerevisiae* D_5A for fermentation of detoxified slurry, the process achieved 99% of the maximum theoretical ethanol yield and an ethanol production rate nearly five-times faster than untreated slurry.

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1. Introduction

Biorenewable fuels such as ethanol, butanol, biodiesel, and biogasoline have received considerable attention as potential replacements for petroleum-derived products. Recent research and development for producing renewable products has concentrated on utilizing abundant lignocellulosic biomass as opposed to conventional agricultural crop-based raw materials such as cornstarch and sugar cane. However, due to the recalcitrant nature of the lignocelluloses, a pretreatment step is required during the biochemical conversion to disrupt its complex structure and release polymeric sugars prior to enzymatic hydrolysis. Dilute acid pretreatment has shown to be effective technique for disrupting the protective lignin barrier and increasing the accessibility of cellulose to enzymes (Mosier et al., 2005; Yang and Wyman, 2008). However, using harsh pretreatment conditions to help improve the eventual glucose yields, partially degrades the cellulose and hemicellulose derived sugars, as well as the lignin polymer, to soluble chemicals that inhibit enzymatic hydrolysis and fermentation; thus jeopardizing the rates and yields that can be obtained within the biomass-to-biorenewables process (Tucker et al., 2003).

Depending on the severity of the pretreatment conditions (defined as the combination of time, high temperature and low pH used) and the nature of the raw material used (grasses, hardwoods, softwoods, etc.), the final concentration of inhibitors can vary greatly. The first set of inhibitors generated from the breakdown of cellulose and hemicellulose are acetic acid (from de-acetylation of hemicellulose fraction), furfural (from thermal degradation of pentose sugars), and 5-hydroxymethyl furfural (HMF, from degradation product of hexose sugars). When the severity factor increases, other inhibitors may be formed such as levulinic or formic acid (further degradation products of furfural and HMF), additional aldehydes such as 4-hydroxybenzaldehyde and phenols including catechol and vanillin are formed from the lignin fraction (Klinke et al., 2004; Palmqvist and Hahn-Hägerdal, 2000). Inhibition effects of these compounds towards the growth rates and product yields during fermentation with various microorganisms have been investigated (Liu et al., 2005; Pampulha & Loureiro-Dias, 1990; Zaldivar and Ingram 1999).

Several methods have been explored to reduce the effects of inhibitory compounds on downstream enzymatic and fermentation operations. In some cases, the solid material following dilute acid pretreatment (containing cellulose, lignin, and some hemicellulose) has been separated from the liquor and washed prior to enzymatic hydrolysis to remove the inhibitors trapped within the cellulose-rich solids (Cantarella et al., 2004). However, this increases the need for additional process water and complicates the overall refinery operations. Therefore, other methods have been tested for removing acetic acid, furfural, HMF, and other potential inhibitory compounds from lignocellulosic

* Corresponding author. Address: Department of Chemical and Biological Engineering, South Dakota School of Mines and Technology, 501 East St. Joseph Street, Rapid City, SD 57701, USA. Tel.: +1 605 394 2422; fax: +1 605 394 1232.
E-mail address: Todd.Menkhaus@sdsmt.edu (T.J. Menkhaus).

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hydrolysates, including alkali treatment to precipitate inhibitors (Martinez et al., 2001), evaporation of inhibitors, sulfite addition to reduce the toxicity (Larsson et al., 1999), phenoloxidaselaccase to degrade the phenolic compounds (Jonsson et al., 1998), biological abatement to detoxify furans (Nichols et al., 2010), activated carbon (Mussatto and Roberto 2004), ion exchanges (Nilvebrant et al., 2001) have also been used to adsorb the inhibitors using chromatographic supports. While the removal of inhibitory compounds has led to more efficient product formation, the challenge of detoxification is the increased processing costs [e.g., for fermentation of a pentose-rich hydrolysate with recombinant *E. coli*, it was estimated that detoxification via overliming constituted 22% of the total ethanol production cost (Von Sivers and Zacchi, 1995)] and additional operations within the biorefinery that inevitably leads to loss of the valuable sugars, or even additional inhibitory compound formation. For example, overliming can result in major sugar degradation (Horváth et al., 2005), with subsequent reduction in fermentation yields. Sugar losses also were observed within an ion exchange process (26% sugar loss) and after treatment with *T. reesei* to degrade phenolics (35% sugar loss) (Larsson et al., 1999).

Flocculation by polyelectrolytes can be an alternative method to remove inhibitory compounds either before or after the enzymatic hydrolysis. Polethyleneimine (PEI) is a soluble secondary amine cationic polymer, commonly used as a flocculating agent to precipitate cellular debris and other insoluble solids. It has been evaluated for removal of suspended solids from biomass slurries (Burke et al., 2011) and more recently Carter et al. (2011 a, b) studied the efficiency of PEI to remove furfural and HMF from clarified pre-enzymatic hydrolysis liquor. It was shown that 88.3% and 66.4% of furfural and HMF, respectively, could be reversibly removed through a Mannich reaction sequence. However, in the same study, it was shown that sulfate and chloride ions interfered with the removal of acetic acid using PEI, and thus polyelectrolyte adsorption was not feasible for reducing acetic acid concentrations.

Resin-wafer electrodeionization (RW-EDI) is one of several processes than can be used to remove organic and mineral acids from solution, an alternative to reduce the overliming cost. RW-EDI has been used extensively for production of boiler grade water from impaired sources, high fructose corn syrup desalination, desalination of glycerol, production and recovery of organic acids (Arora et al., 2007), and is even being explored for capture of carbon dioxide from flux gas. In conventional EDI, a commercial electrodialysis (ED) stack is modified by packing ion exchange resins in the diluate chamber to enhance ion transport. In RW-EDI, the ion exchange resins are molded into a porous "resin wafer" that provides superior performance in ion transport, pH control, and solution flow control. pH can be electrochemically controlled, enabling selective removal of acids or other charged species based on the isoelectric point (pI). At low conductivity, RW-EDI offers a significant decrease in power consumption compared to ED. In comparison to conventional ion exchange columns, RW-EDI does not have to be regenerated with stoichiometric amounts of acids/bases. Rather, in situ regeneration of the resin beads in EDI takes place by water-splitting due to the applied electric field.

Here we explore PEI polyelectrolyte adsorption along with RW-EDI for removal of soluble inhibitory compounds from dilute acid pretreated biomass slurry. Following different combinations of detoxification, enzymatic hydrolysis and fermentation were completed. Three different enzyme dosages were studied using both washed and unwashed solids along with PEI and/or RW-EDI treated liquor samples. Statistical analyses were used to evaluate the primary and secondary effects of individual compounds and their synergistic inhibition potential on both enzymatic and fermentation efficiency.

2. Methods

2.1. Raw material

Ponderosa pine wood (35% moisture) was kindly provided by Baker Timber (Rockerville, SD) in sawdust form. The saw dust was sieved to obtain a particle size of <850 μm and this was used for all experiments. The composition of the pine wood, as determined by using chemical analysis and testing laboratory analytical procedures (LAP) – LAP-002, LAP-003, LAP-004, LAP-014, LAP-015 developed at the National Renewable Energy Laboratory (NREL), was 36.5% glucan, 20.9% xylan, mannan, and galactan (combined fraction), 7.3% acid soluble lignin, 30.5% acid insoluble lignin, 1.6% ash, 1.8% ethanol extractive, and 1.4% water extractive. Except high glucan (45.1%) and low total lignin (27.7%), similar composition was reported for spruce (Bosch et al., 2010) and pine (Hamelinck et al., 2005), respectively.

2.2. Dilute acid pretreatment

All pretreatment experiments were performed using a 4-L stirred vessel Parr reactor (Parr Instrument Company, Moline, IL) equipped with an internal cooling jacket, along with temperature and agitation control. Slurry was prepared according to NREL LAP 007 with 10 dry wt.% pine and 1 w/v% H_2SO_4. Pretreatment was performed at 160–165 °C, at 120–150 PSI, and 100 RPM stirring for 30 min, as previously described in more detail by Burke et al. (2011). The calculated combined severity factor for these operating conditions was 2.39. After cooling to room temperature, the slurry was neutralized from pH ~1.3 to pH 5.0 by addition of NH_4OH. Due to the limited size of the reactor, the pine wood was pretreated in batches of 3 L and the slurries obtained were mixed to form one large batch to ensure continuity between processing operations. The pretreated slurry was stored at 4 °C until further analysis and testing.

2.3. PEI treatment

After dilute acid pretreatment, the material was separated into a solid fraction and liquid fraction (pre-hydrolysate) with a 0.2 μm nylon filter (Millipore, Billerica, MA) by vacuum filtration. The resulting solids were used either directly for "unwashed solids" experiments, or quadruple washed with 0.5 L of water per 100 g of solids (wet weight) while filtering to obtain "washed solids" for testing. Initially, Polyethyleneimine (PEI; 50% w/v, 60,000 average molecular weight, Sigma Aldrich) was added to 10 mL of liquid filtrate in varying molar equivalents to determine the optimal dosage as previously described (Carter et al., 2011 b). The optimum PEI dosage of 26 g/L, which represented six molar equivalents of PEI to total known furan inhibitor concentrations (data not shown), was added to 4 L filtrate and mixed with a magnetic stirrer to ensure homogeneous separation. The resulting mixture was passed through a 10 kDa molecular weight cutoff ultrafiltration membrane (Millipore, Danvers, MA) to remove polymer containing the adsorbed inhibitory compounds. Due to the addition of PEI, the pH of the permeate from the ultrafiltration process was adjusted from pH 7.8 to pH 5.0 by addition of sulfuric acid. All samples were analyzed for sugar and inhibitors concentration by High Performance Liquid Chromatography (HPLC), as discussed below.

2.4. RW-EDI treatment

The pre-hydrolysate liquor was subjected to RW-EDI treatment using a EUR2B-10 ED-stack supplied by Ameridia Corporation. The Neosepta cation exchange (CMX), anion exchange (AMX) and

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bipolar membranes (BP) were also procured from Ameridia Corporation. The PEI-treated pre-hydrolysate liquor was used as it is, whereas, the untreated pre-hydrolysate liquor was permeated through a 65 KDa hollow fiber membrane prior to RW-EDI. In a typical run, 2 L of pre-hydrolysate liquor was treated in batch mode by RW-EDI consisting of six cell pairs (195 cm^2 surface area, 0.25 cm thickness of each wafer). A flow rate of 200 ml/min and current density of 50 amp/m^2 were applied. The pre-hydrolysate liquor was passed through the diluate chamber (ion-out), where under the influence of the applied electric field ionic species moved outward from the diluate chamber, were transported across the ion exchange membranes and collected in the concentrate chamber (ion-in).

2.5. Enzymatic hydrolysis

The liquid (liquor) following different treatments (PEI, RW-EDI, PEI and RW-EDI, or no detoxification) were added back to the saved "washed" or "unwashed" solids in the same ratio as the original pretreated slurry. The commercial second-generation cellulase cocktail CellicCTec (supplied by Novozymes A/S, Bagsvaerd, Denmark) was used in all enzymatic hydrolysis experiments. A dosage of 3.4 g enzyme/100 g cellulose (3.4%) was added to the slurry for the majority of experiments. However, two lower dosages of 1.7% and 0.8% were used to evaluate the RW-EDI and PEI–RW-EDI treated pre-hydrolysate with washed solids to evaluate if less enzyme dosing could be used. In all cases, enzymatic hydrolysis was carried out at 50 °C in 500 mL Erlenmeyer flasks placed in a shaker at 200 RPM for 72 h. One-half milliliter samples were drawn at regular intervals of 3 h to monitor glucose concentration. All of the hydrolysis experiments were run in duplicate with 250 ml of slurry.

2.6. Fermentation

Saccharomyces cerevisiae D_5A, obtained from NREL, was used in the ethanol fermentation experiments. One mL of frozen yeast concentrate was pre-cultured for 16 h at 37 °C in yeast peptone dextrose (YPD) media containing 20 g/L glucose, 20 g/L peptone and 10 g/L yeast extract. Enzymatic hydrolysate was vacuum filtered through a 0.2 μm vacuum filter and the solid free hydrolysate was supplemented with additional nutrients according to Tanner (2007); 10 g/L KH_2PO_4, 20 g/L $MgSO_4{\cdot}7H_2O$, 4 g/L $CaCl_2{\cdot}2H_2O$, 200 mg/L $ZnSO_4{\cdot}7H_2O$, 20 mg/L $Na_2MoO_4{\cdot}2H_2O$, 200 mg/L $CoCl_2{\cdot}2H_2O$, 2 mg/L d-biotin, 5 mg/L p-aminobenzenoic acid, 5 mg/L nicotonic acid, 5 mg/L calcium pantothenate, 10 mg/L pyridoxine–HCl, 5 mg/L Thiamine–HCl, and 10 mg/L lactocide. If necessary, the pH was adjusted to 5.0 using H_2SO_4 or NH_4OH and then filtered through a 0.2 μm filter. Clear nutrient rich hydrolysate was inoculated with the 16 h pre-culture of D_5A at a 10% (v/v) level (0.4 g dry wt /L) aseptically. Fermentation was carried out in 250 mL duplicate batches on a rotary shaker, operated at 37 °C and 200 RPM. Sugar, ethanol and inhibitory compound levels were monitored by sampling at 2 h intervals using an HPLC, and the optical density was measured at OD_{600nm} using Beckman Coulter (Brea, CA) DU 640 UV/Vis spectrophotometer to estimate cell density.

2.7. HPLC analysis

Enzymatic hydrolysis and fermentation samples were filtered through 0.2 μm nylon syringe filters (Fisher Scientific, Pittsburgh, PA) to remove any suspended solids. In order to analyze for sugars, ethanol and inhibitory concentrations, clear filtrate (50 μL) was injected onto a heated Aminex ion exclusion column HPX-87H (Biorad, Hercules, CA). The HPLC system (Beckman Coulter, Brea, CA) was equipped with a refractive index (RI) detector (Model RI-1530) and the column was heated to 65 °C with a Timberline Instruments Model 105 column heater. Samples were eluted with flow rate of 0.6 ml/min using 5 mM H_2SO_4 as the mobile phase. The system was equipped with a Cation-H Refill Cartridge (Bio-Rad, Hercules, CA) as a guard column. Five standard concentrations of each compound being followed (sugars: glucose, xylose, arabinose, mannose and galactose (>99% pure, Fisher Scientific, Pittsburgh, PA), acetic acid, ethanol (200 proof), aldehydes: 5-hydroxymethyl furfural and furfural (Sigma Aldrich, St. Louis, MO) were prepared to calibrate the column for accurate analysis. Due to the separation using the HPX-87H column, xylose, mannose and galactose were combined into a single "peak" and analyzed as a combined concentration. All other species were separated as single compounds and the concentration analyzed individually.

2.8. Statistical analysis

Statistical analysis was carried out using a two-level factorial design (2^3) in the Design-Expert® program from Stat-Ease, Inc. (Minneapolis, MN). The order of significant effect (from most to least significant) for three primary treatments (PEI, RW-EDI, and solids washing) was chosen according to their rank and position above the *t*-value and Bonferroni lines in the Pareto chart. Using the analysis of variance (ANOVA) in the Stat-Ease statistical program, the effects of the wash, PEI, and RW-EDI treatments on enzymatic hydrolysis and fermentation associated performance parameters were evaluated. In a separate analysis, multiple variable regression was carried out using the Microsoft Excel data analysis package to see the individual effects of inhibitory compounds on the yields and rates of downstream processes (hydrolysis or fermentation) with a 95% confidence level. For this, the regression model used was $Y = b + b_1 \times [\text{Acetic acid}] + b_2 \times [\text{HMF}] + b_3 \times [\text{Furfural}]$, where Y was the performance parameter of enzymatic hydrolysis or fermentation, b was the intercept of the model when the theoretical inhibitory compound concentrations were all zero, and b_1, b_2, and b_3 were the coefficients of acetic acid, HMF and furfural, concentrations, respectively. In both statistical analyses the sign (positive or negative) of the model coefficients, and the *F* and *P*-values were used to describe the effect (positive or negative) and statistical significance of the various treatments and inhibitory compounds.

3. Results and discussion

3.1. Detoxification by PEI and EDI treatments

The pre-hydrolysate liquor after dilute acid pretreatment of Ponderosa pine wood had 9.68 g/L glucose, 9.24 g/L xylose, mannose and galactose (total combined concentration of these three sugars), 1.38 g/L arabinose, 15 g/L sulfuric acid, 7.32 g/L acetic acid, 1.12 g/L HMF, and 2.22 g/L furfural. Since there was 15 g/L sulfuric acid in the solution from the pretreatment step, the sulfate ions were expected to interfere with the removal of acetic acid using PEI, in accordance with previous studies (Carter et al., 2011 a, b). Therefore the RW-EDI process was used to remove both acetate and sulfate ions from PEI treated and untreated samples. It was found that RW-EDI was able to remove >97% of sulfate ions with up to 200 g/cm^2/h removal flux. It was also able to remove substantial amount (>77%) of acetate ions (removal flux of up to 100 g/cm^2/h) from all samples.

Following the various combinations of detoxifying operations, the concentration of different inhibitors in the pre-hydrolysate slurry varied considerably (Table 1). Even though the PEI and RW-EDI operations were successful in removing large quantities of inhibitory compounds, after re-combining the treated liquid

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Table 1
Inhibitor concentrations in the liquor following different treatments, and the liquor with unwashed or washed solids before enzymatic hydrolysis.

	Acetic acid (g/L)	HMF (g/L)	Furfural (g/L)
Only liquor			
Untreated	7.32	1.12	2.22
PEI treated	6.67	0.52	0.64
RW-EDI treated	1.78	0.76	1.12
PEI–RW-EDI treated	1.54	0.45	0.58
Liquor with unwashed solids			
Untreated	7.32	1.12	2.22
PEI treated	6.98	0.66	0.89
RW-EDI treated	2.67	0.95	1.96
PEI–RW-EDI treated	2.25	0.59	0.81
Liquor with washed solids			
Untreated	6.26	0.83	1.28
PEI treated	5.97	0.47	0.59
RW-EDI treated	1.48	0.69	0.97
PEI–RW-EDI treated	1.08	0.38	0.53

with previously filtered but unwashed solids, the concentration of inhibitors was again elevated slightly (Table 1). It was observed that even after vacuum filtration the solids retained 15% of the pre-hydrolysate liquid (based on moisture content). The liquid within the solids contained high concentrations of inhibitors, and released them back into the PEI and RW-EDI treated liquor when mixed prior to the enzymatic hydrolysis step. Hence washing with 5.0 L distilled water per kg (wet) of pretreated solids for four consecutive times, was carried out. Washing of the pretreated solids removed 14%, 26%, and 42% of acetic acid, HMF and furfural, respectively.

Our results showed that detoxification by sequential PEI and RW-EDI treatments was very promising, with significant removal of acetic acid, HMF and furfural from the untreated crude pre-hydrolysate (77%, 60%, and 74% removed, respectively) (Table 1). These results compare very favorably to previous reports of detoxifying softwood biomass and dilute acid pretreatment using an alkali process (Persson et al., 2002).

3.2. Effect of reduced inhibitor concentration on enzymatic hydrolysis

The influence of PEI, RW-EDI and PEI–RW-EDI treated pre-hydrolysates on the cellulose conversion during enzymatic hydrolysis with a dosage of 3.4% dosage (g enzyme/g cellulose) using unwashed solids is shown in Fig. 1. For the untreated sample and unwashed solids (from here on referred to as "control"), the commercial second-generation enzyme cocktail produced 22.3 g/L of glucose (81.05% of maximum cellulose conversion) at slowest rate of 0.22 g glucose/L/h (Table 2), which, as expected, had the highest inhibitor concentrations (Table 1). This percentage of cellulose conversion with the high level of inhibitors present in the pre-hydrolysate was four times more than that reported from steam pretreated spruce that generated lower concentrations of inhibitor (acetic acid 5.7 g/L, HMF 1.0 g/L and Furfural 0.6 g/L) (Tengborg et al., 2001) when using a mixture of Celluclast 2 L and Novozyme 188. Similarly, an investigation by Garcia-Aparicio et al. (2006) reported only a 75% cellulose conversion with steam exploded barley straw that generated even less inhibitors (acetic acid 2.1 g/L, Furfural 0.7 g/L, HMF 0.2 g/L, and total phenolic compounds 0.23 g/L) when using a similar enzyme mixture as that studied by Tengborg et al. 2001. From these comparisons it suggests that the second generation CellicCTec had a higher tolerance to the potential inhibitors, but that there was still room for improvement.

Removal of 41% of the HMF and 60% of the furfural using the PEI treatment with unwashed solids increased the rate and percentage of glucose yield (Table 2). While 74% removal of acetic acid alone (with minimal HMF or furfural removal) could be accomplished using RW-EDI treatment only, the cellulose conversion and rate were only marginally improved compared to the untreated case and was not as effective as the PEI treated pre-hydrolysate (Table 2). This suggested that furans were the major inhibitory compounds during enzymatic hydrolysis, which was in accordance with findings obtained by other authors. Jing et al. (2009) reported cellulose conversion was reduced up to 5% and 10% when furfural and HMF were added individually at 3 g/L inhibitor concentration, but acetic acid had little effect. Another study by Szengyel and Zacchi (2000) reported 55% reduction of cellulase activity of *Trichoderma reesei* RU C30 yeast at 1.2 g/L furfural.

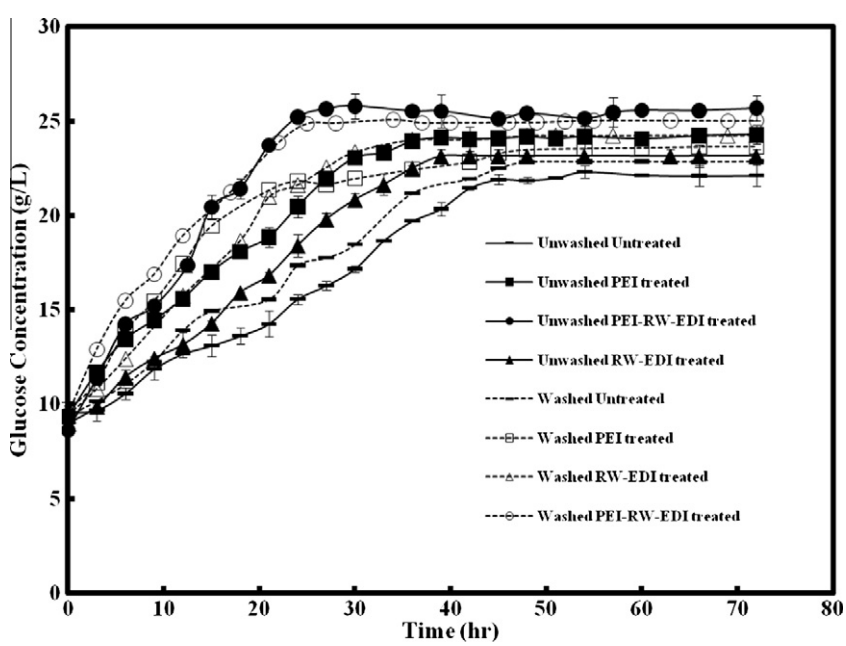

Fig. 1. Influence of PEI, RW-EDI, and PEI–RW-EDI treated pre-hydrolysate on enzymatic hydrolysis with washed and unwashed solids at 3.4% dosage (g enzyme/g cellulose). Error bars with less than 5% standard deviation and smaller than symbols are not shown.

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Table 2
Effect of inhibitor concentration on % glucose yield and rate of hydrolysis of different treatment after enzymatic hydrolysis at 3.4% dosage (g enzyme/g cellulose).

	% Glucose yield	Rate of hydrolysis (g glucose/L/h)
Unwashed untreated	81.1	0.22
Unwashed PEI treated	89.0	0.38
Unwashed RW-EDI treated	84.8	0.36
Unwashed PEI–RW-EDI treated	94.1	0.69
Washed untreated	81.4	0.30
Washed PEI treated	86.6	0.45
Washed RW-EDI treated	88.9	0.42
Washed PEI–RW-EDI treated	91.7	0.71

By reducing inhibitory concentrations of both organic acids and furans by sequential PEI and RW-EDI treatments, further increases in cellulose conversion (up to 94.1%) and rate (0.69 g glucose/L/h) were observed. The increase in rate and sugar yield with decreased inhibitory concentrations could have been either due to less enzyme-furan inhibitory complex formation or the slightly positive effect seen for acetic acid at low concentrations and at low pH (Jing et al., 2009; Szengyel and Zacchi, 2000).

An increase in rates and yields were similarly observed when the PEI and RW-EDI treated pre-hydrolysate liquid was added to the washed pretreated solid fractions at an enzyme dosage of 3.4% (Fig. 1 and Table 2). With the washed solids, the rate of hydrolysis was comparatively higher for the combined PEI–RW-EDI treatment than each individual treatment as seen previously for the unwashed solids. The drawback to solids washing was that the glucose yield with the washed solids, especially for PEI and PEI–RW-EDI treated samples, was reduced by 2.4% compared to unwashed solids. This was likely due to the loss of soluble sugars within the entrapped liquid that was lost during washing; thus giving lower yields, but higher rates due to lower inhibitor concentrations.

The inhibitory hierarchy was analyzed by the two-level factorial design (Table 3) testing for the effects of different treatments (PEI, RW-EDI, and solids washing). It was found that a positive coefficient was calculated for each treatment relating to higher glucose concentrations and faster rate indicators after treatment, and negative coefficients were calculated for final inhibitory concentrations observed since there was removal of acetic acid, HMF, and furfural. High significance, as displayed by large *F* and low *P*-values are shown in Table 3. From this analysis there was not a single treatment that always provided the largest impact; for at least one performance indicator each treatment was found to be the most valuable in improving its value.

Multiple variable regression analysis was tested for the main effects of inhibitory compound concentrations on enzymatic hydrolysis (as shown in Table 4). While the model did not allow for secondary interaction effects to be analyzed due to limited data, it was found that all coefficients in the regression model were negative (since lower inhibitory compound concentrations led to higher glucose yields and faster rates). Similar to the effects of the different treatments, each specific inhibitor was more dominant toward some factors and less dominant toward others. It is likely that there are complex interactions taking place between all inhibitors, with many synergistic impacts taking place. Similarly, because the *R*-squared values are generally low for each model of the performance indicators, it is likely that there are many other inhibitors that are contributing to the observed phenomena (especially phenolic compounds) that were not measured here.

Table 3
Statistical analysis of wash, PEI, and RW-EDI treatment effects on inhibitory concentrations and their interaction effects on enzymatic hydrolysis and fermentation associated performance parameters. (Two-level factorial design in Design-Expert® from Stat-ease, Inc. considering A: Wash, B: PEI, and C: RW-EDI).

	Significant effect order	ANOVA			
		Coefficients	*F*-value	*P*-value	*R*-squared
Acetic acid	C	−2.38	11520.71	<0.0001	0.9997
	A	−0.55	623.01	<0.0001	
	B	−0.18	66.75	0.0012	
HMF	B	−0.11	800.63	<0.0001	0.9967
	A	−0.071	326.56	<0.0001	
	C	−0.035	77.41	<0.0001	
Furfural	B	−0.45	75.03	0.0010	0.9682
	A	−0.31	36.27	0.0038	
	AB	0.17	10.49	0.0317	
Glucose yield	B	0.027	27.42	0.0034	0.9041
	C	0.031	19.72	0.0068	
Rate of hydrolysis	B	0.099	1830.27	<0.0001	0.9993
	C	0.091	1532.95	<0.0001	
	BC	0.059	661.03	0.0001	
	A	0.018	59.36	0.0045	
Cell yield	A	0.019	95.34	0.0002	0.9562
	B	0.007	13.29	0.0148	
Rate of glucose consumption	B	0.72	138.20	0.0003	0.9811
	A	0.34	39.12	0.0033	
	C	0.38	30.49	0.0053	
Ethanol yield	A	0.047	240.67	0.0001	0.9878
	B	0.025	66.67	0.0012	
	AB	0.013	16.67	0.0151	
Rate of ethanol production	B	0.28	71.82	0.0034	0.9801
	A	0.25	54.98	0.0051	
	C	0.11	11.08	0.0447	
	AB	0.11	10.10	0.0502	
Final ethanol titer	A	1.07	16.25	0.0100	0.8331
	B	0.78	8.74	0.0317	

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Table 4
Values of the statistically significant regression coefficients and *t* statistic obtained in the excel multiple regression analysis for all the performance parameters of enzymatic hydrolysis and fermentation.

Coefficient	Glucose yield		Rate of hydrolysis		Cell yield		Rate of glucose consumption		Ethanol yield		Rate of ethanol production		Final ethanol titer	
	Value	*t*	Value	*t*	Value	*t*	Value	*t*	Value	*t*	Value	*t*	Value	*t*
Intercept	0.957	15.220	0.952	5.105	0.108	3.020	5.119	11.011	0.585	6.710	2.497	7.413	15.832	8.622
Acetic Acid	−0.008	−1.464	−0.027	−1.623	0.000	−0.035	−0.051	−1.236	0.004	0.483	0.009	0.291	−0.026	−0.160
HMF	−0.002	−0.009	−0.756	−1.085	−0.067	−0.501	−3.528	−2.030	−0.382	−1.171	−3.736	−2.966	−10.740	−1.565
Furfural	−0.042	−0.477	0.122	0.463	−0.001	−0.010	−0.009	−0.013	0.069	0.564	0.846	1.779	2.204	0.851
F-value	3.539		6.556		2.007		36.509		2.813		13.567		5.081	
P-value	0.0001		0.0069		0.0392		0.0004		0.0026		0.0018		0.0009	
R-squared	0.726		0.831		0.60		0.965		0.678		0.911		0.792	

3.3. Effect of enzyme dosage on RW-EDI and PEI–RW-EDI treated enzymatic hydrolysis

Three different enzyme dosages of 0.8%, 1.7% and 3.4% (g enzyme/g cellulose) were tested on RW-EDI and PEI–RW-EDI treated pre-hydrolysates with washed solids (Table 5 and Figs. 2 and 3). With only organic and mineral acids removed through RW-EDI process, an increase in dosage from 0.8% to 3.4% resulted in increase of hydrolysis rate by three-fold and a 30% increase in glucose conversion. In the case of PEI–RW-EDI treated hydrolysis, with an accompanying major reduction of all inhibitor concentrations, higher sugars yields and rates than RW-EDI treated hydrolysis alone were observed (Table 5). At the 1.7% enzyme dosage with PEI–RW-EDI treatment, the rate of hydrolysis was almost equal to the untreated hydrolysis with a 3.4% dosage, but the final yield was lower for the lower enzyme dosage. One possible explanation is that due to less active enzymes to convert available cellulose to glucose at low enzyme concentrations, the active enzyme at 1.7% became quickly saturated by inhibition or adsorption to residual lignin, whereas with the 3.4% dosage, even though the rate was slower, due to higher concentration of inhibitors, there was still enough active enzyme to convert more cellulose to glucose in the long run. The rate of glucose production and glucose yields were roughly linear compared to the percentage of enzyme dosage for both RW-EDI and PEI–RW-EDI treated enzymatic hydrolysis, indicating that the positive effect from adding more enzyme could be easily predicted. These experiments reveal that even with lower inhibition levels the enzyme dosage needed to remain elevated to achieve accelerated rates and high overall conversion of cellulose.

3.4. Effect of reduced inhibitor concentration on fermentation

Fig. 4 represents the general time course profile of various compounds (sugars, inhibitors and metabolites e.g., ethanol) during the fermentation of an untreated hydrolysate using *S. cerevesiae* D5A. This served as the control fermentation to analyze the capability of *S. cerevesiae* to produce ethanol in the presence of relatively high inhibitory compound concentrations. Even with these relatively high inhibitory levels, the yeast consumed glucose and produced ethanol, albeit at lower rates. The ability to ferment and produce ethanol is likely because of the high initial cell mass used in this evaluation, which is consistent with Larsson et al. (2000). The prolonged lag phase and relatively low cell mass produced here has been explained as a synergistic effect of acetic acid, HMF, and furfural, which was observed previously by Palmqvist et al., 1999.

Our D_5A strain was able to metabolize both furfural and HMF, likely to furfuryl alcohol and 5-hydroxymethylfufurly alcohol, respectively, and not to ethanol, which are much less toxic to cells (similar observations were reported by Liu et al., 2005). The HMF conversion rate was observed to be much slower than furfural, which is likely due to lower membrane permeability of HMF as described in the other publication (Palmqvist and Hahn-Hägerdal, 2000). The acetic acid concentration remained constant throughout the fermentation with minimal variations. The combination of xylose, galactose, and mannose concentrations was also reduced during the fermentation. It is well known that *S. cerevesia* cannot consume pentose sugars like xylose and galactose; hence the decreasing trend can be explained as the consumption of mannose. Similar observations were reported by Luo et al. 2010 (yeast strain: *S. cerevesia* D5A) and Tian et al. 2010 (yeast strain: *S. cerevesia* Y5), which showed consumption of mannose in the absence of glucose. It can be seen in Fig. 4 that the mannose (as part of the "xylose" peak) concentration was reduced significantly after glucose was completely consumed, and when the concentration of HMF and furfural had been reduced. The ability to utilize mannose in the absence of glucose, HMF, and furfural is important to maximizing product yield from lignocellulosic derived fermentable sugars.

The effect of reduced inhibitory compound concentrations resulting from different treatments on fermentation is shown in Figs. 5 and 6. The associated performance parameters (product yield and titer, rates of glucose formation and ethanol production, and yield of cell mass) are summarized in Table 6. In general, with a decrease in any or all inhibitor concentrations, there was a concomitant increase in the yield of ethanol, final ethanol concentration, higher cell density, faster rate of glucose consumption, and ethanol production. However, depending on the final concentration of the inhibitors, and whether or not other inhibitors were present, the cell growth, final product titer, and rates of substrate consumption and product formation followed a complicated pattern. In some cases there were threshold limits for a single inhibitor that seemed to control at least one performance indicator, while in other cases there was a clear interaction between several inhibitors that influenced the fermentation efficiency.

Table 5
Comparison of % dosage effect on yield and rate of hydrolysis for washed RW-EDI and PEI–RW-EDI treated hydrolysate.

Dosage (g enzyme/g cellulose)	RW-EDI treated		PEI–RW-EDI treated	
	% Glucose yield	Rate of hydrolysis (g glucose/L/h)	%Glucose yield	Rate of hydrolysis (g glucose/L/h)
3.4%	88.93	0.42	91.66	0.71
1.7%	67.32	0.21	74.11	0.29
0.8%	57.78	0.13	65.93	0.22

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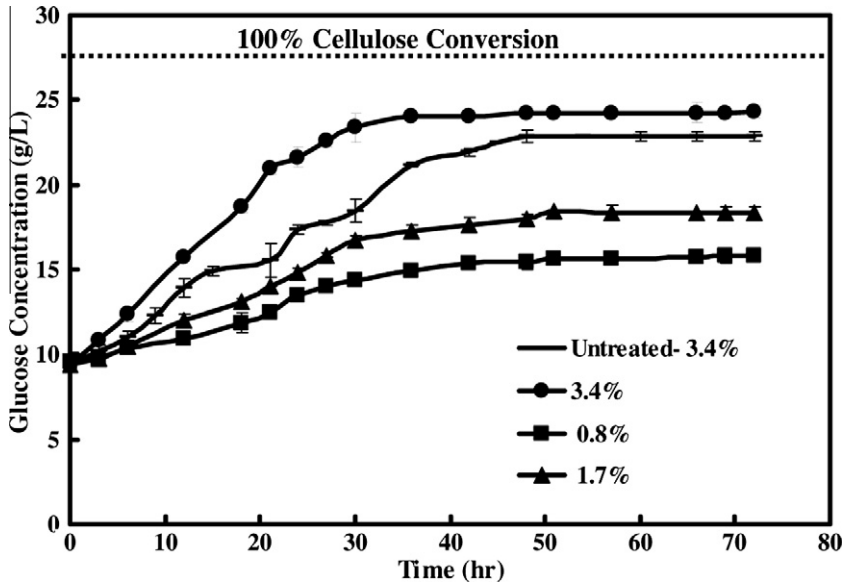

Fig. 2. Enzymatic hydrolysis of RW-EDI treated biomass slurry with washed solids at different % dosage (g enzyme/g cellulose). Error bars with less than 5% standard deviation and smaller than symbols are not shown.

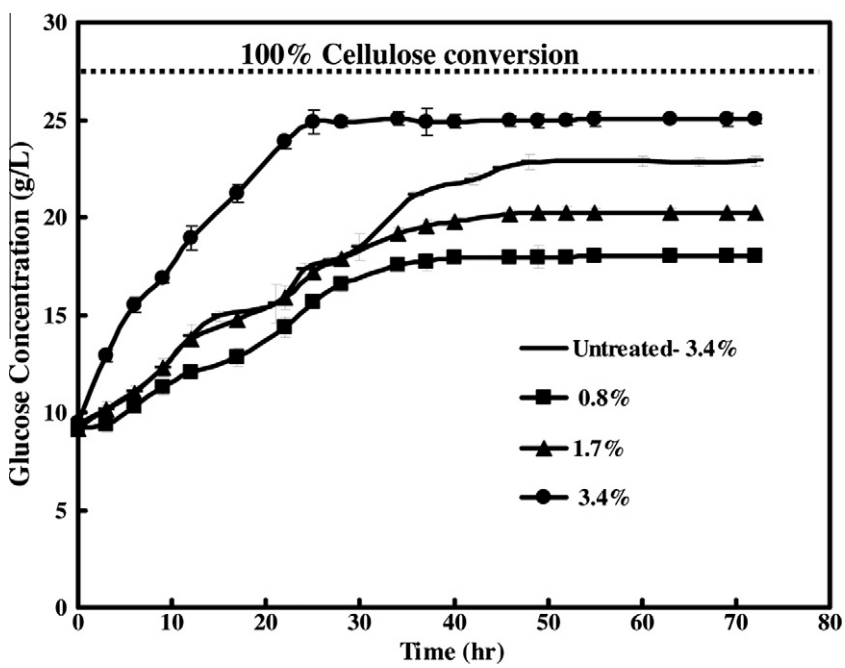

Fig. 3. Influence of PEI–RW-EDI treated pre-hydrolysate on enzymatic hydrolysis with washed solids at different % dosage (g enzyme/g cellulose). Error bars with less than 5% standard deviation and smaller than symbols are not shown.

Fermentation of unwashed PEI-only treated enzymatic hydrolysate (with reduced levels of HMF and furfural, as shown in Table 1) produced ethanol and consumed sugars much faster (<12 h) than the control (20 h) as a result of higher cell density, but had an approximately equal final ethanol titer (9.15 g/L, Table 4). This observation suggested that HMF and furfural were potential growth inhibitors, which is in agreement with Tofighi et al. (2010), who reported a decrease in dry cell weight from 0.6 to 0.31 g/100 mL when 4 g/L furfural was added to the fermentation medium. In another study, by Rachma et al. (2010), a decrease in glucose consumption was observed from 100% with no HMF to 34% when 1 g/L HMF was added. Statistical analysis also showed the clear negative effect of HMF and Furfural on cell growth due to the negative coefficients and significant *t*, *F* and *P*-values (Table 4).

Fermentation of an RW-EDI-only treated hydrolysate, with 63% of the original acetic acid removed (Table 1), resulted in an ethanol titer and rate of glucose consumption that was in between the untreated control and PEI-only treated fermentation (Table 6). This, again, is likely due to the cell growth inhibition of furans at high concentration and its antagonistic interaction with acetic acid, even at low concentrations (Palmquvist et al., 1999).

As seen in Figs. 5 and 6 and Table 6, when all of the potential inhibitors were reduced by sequential PEI and RW-EDI treatments, an increase in cell density (1.6 dry wt g/L) was achieved along with faster consumption of glucose, faster rate of ethanol production and

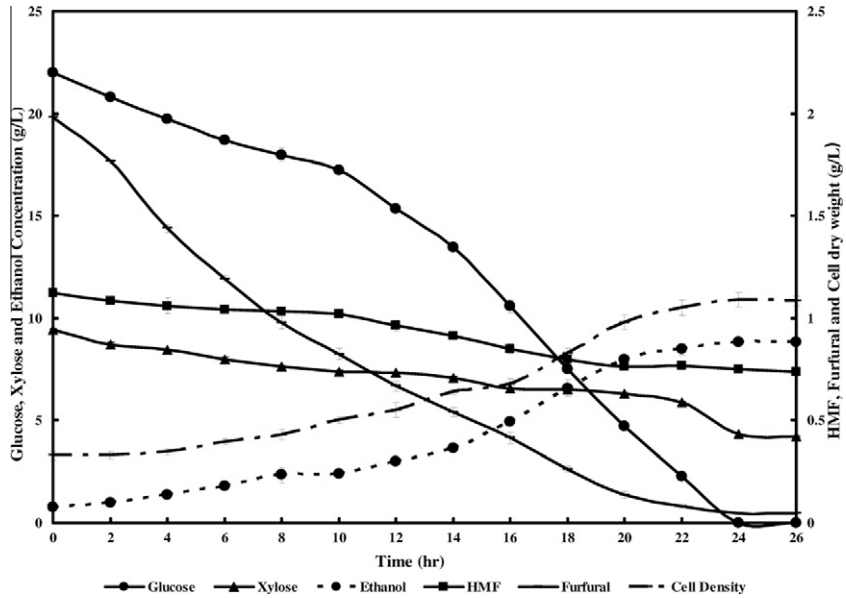

Fig. 4. Influence of untreated enzymatic hydrolysate on fermentation by *S. cerevesia*. (Xylose curve is combination of xylose, galactose, and mannose). Error bars with less than 5% standard deviation and smaller than symbols are not shown.

higher final titer of ethanol (3.2, 1.0, and 10.9 g/L, respectively). The synergistic inhibition of cell growth and function was clearly removed only when all inhibitory compounds were reduced.

Similar performance trends were observed within the fermentation when using washed solids (Fig. 6). Removal of inhibitors by washing of the solids prior to enzymatic hydrolysis had significant impact on glucose consumption and ethanol production rates, as well as ethanol and cell mass yield (Table 6) during fermentation. Nearly a two-fold increase in cell mass yield and rate of glucose consumption were seen when using washed versus unwashed solids when no additional treatments were performed. Ethanol yield from PEI-only treated and PEI–RW-EDI treated with washed solids reached almost 99% of the maximum theoretical value (0.51 g ethanol/g glucose), with final ethanol titer of 13 g/L. These high yields may include the co-fermentation of mannose discussed previously. The higher ethanol yields were also due to minimal sugar losses during the detoxification methods applied here (PEI and RW-EDI) compared to the alternative biological abatement technique studied by Nichols et al. 2010 who reported a 9.2% reduction in final ethanol yield resulting from consumption of sugar by the *Coniochaeta ligniaria* used for detoxification. Our final ethanol yields seen here for Ponderosa pine wood hydrolysate detoxified with PEI and RW-EDI treatments are much higher than the ethanol yields for a softwood hydrolysate detoxified with various ion exchange resins and baggase detoxified with overliming, which were reported to range from 0.17 to 0.46 g/g (Nilverbrant et al., 2001) and 0.1 to 0.33 g/g (Martinez et al., 2001), respectively.

Statistical analysis results also showed that washing of the solids prior to enzymatic hydrolysis can improve cell yield, rate of glucose consumption, ethanol yield, rate of ethanol production and final ethanol titer with higher significance (Table 3). From the multiple variable regression analysis (Table 4), it can be seen that HMF was a negative inhibitor to all fermentation performance indicators, whereas a small positive effect was observed in the case of acetic acid and furfural. This observed result may be due to the consumption of furfural that is achieved with low acetic acid concentrations, thus improving the cell, ethanol yield. However, the relatively low regression model *R*-squared values suggest variance sensitivity and multiple interaction effects in a system that contains a wide spectrum of inhibitory compounds, including lignin derived inhibitors. Similar conclusions regarding the negative synergistic impact were drawn in a recent investigation by Franden et al. 2009. It was shown the individual inhibitors of acetic acid, HMF, furfural and soluble lignin did not slow organism growth. However the combined inhibitors resulted in almost no growth of *Zymomonas mobilis*. Hence a clear elucidation of the inhibitory interaction effects of these degradation products is needed to improve the efficiency of biomass-to-ethanol process with optimum conditions of pretreatments and selective methods of detoxification.

Table 6
Effect of inhibitor concentration on ethanol yield, cell yield and rate of glucose consumption of different treatment during fermentation by *S. cerevesiae*.

	Yx/s (g dry cell/g glucose)	Rate of glucose consumption (g/L/h)	Ethanol yield (g ethanol/g glucose)	Rate of ethanol production (g/L/h)	Final ethanol titer (g/L)
Unwashed untreated	0.034	0.92	0.34	0.34	8.85
Unwashed PEI treated	0.045	2.43	0.37	0.59	9.15
Unwashed RW-EDI treated	0.038	1.45	0.36	0.53	9.39
Unwashed PEI–RW-EDI treated	0.046	3.20	0.38	0.98	10.86
Washed untreated	0.062	1.88	0.42	0.60	10.25
Washed PEI treated	0.088	2.95	0.50	1.45	12.65
Washed RW-EDI treated	0.076	2.40	0.42	0.83	10.90
Washed PEI–RW-EDI treated	0.087	3.84	0.49	1.52	13.00

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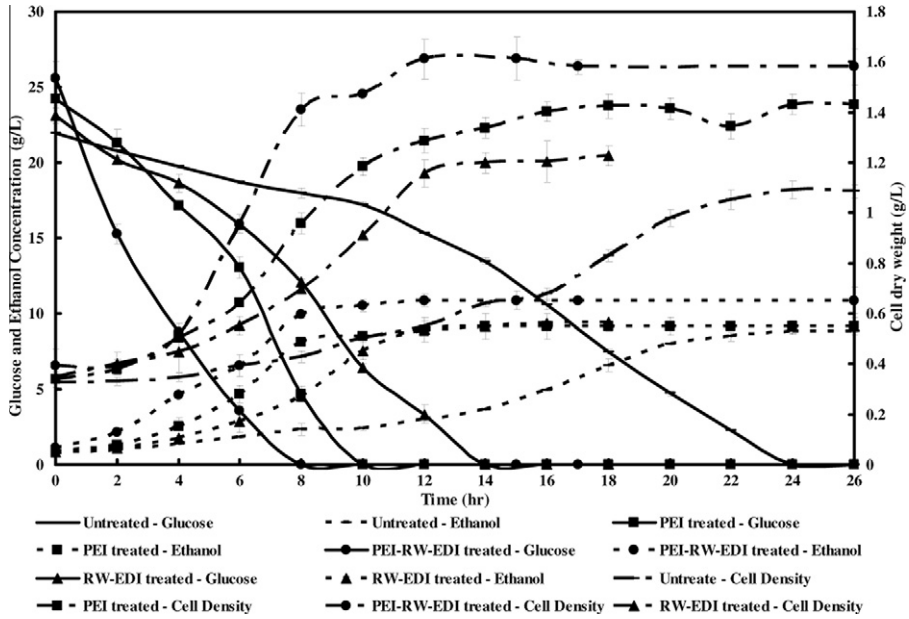

Fig. 5. Influence of PEI, RW-EDI, and PEI–RW-EDI treated enzymatic hydrolysate on fermentation with unwashed solids. Error bars with less than 5% standard deviation and smaller than symbols are not shown.

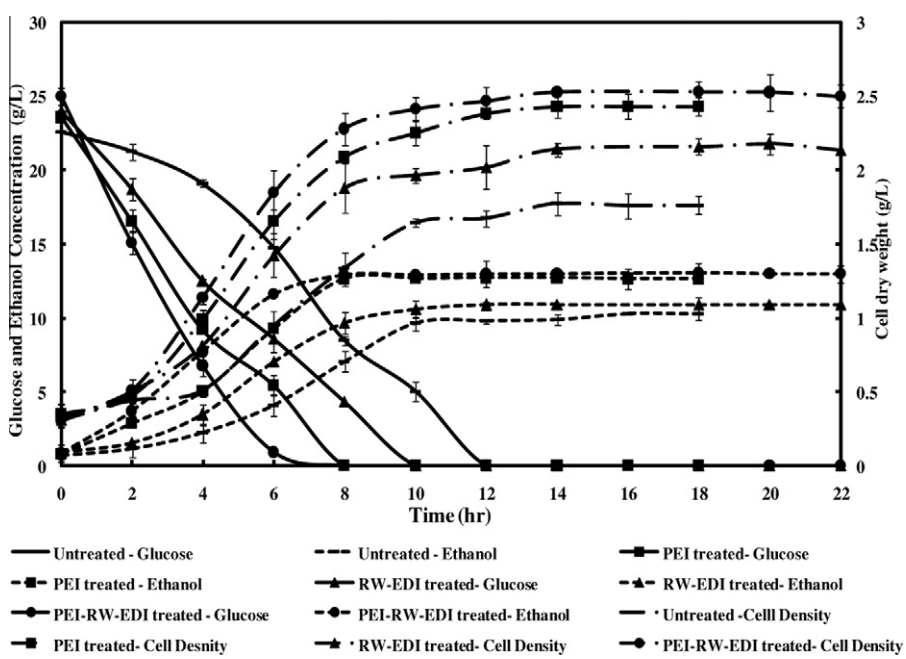

Fig. 6. Influence of PEI, RW-EDI, and PEI–RW-EDI treated enzymatic hydrolysate on fermentation with washed solids Error bars with less than 5% standard deviation and smaller than symbols are not shown.

4. Conclusions

A substantial reduction in the concentration of enzymatic hydrolysis and/or fermentation inhibitory compounds has been achieved by sequential polyelectrolyte adsorption followed by RW-EDI, without the need for overliming to remove sulfuric acid. Detoxification improved cellulose conversion to 94% after only 27 h, and allowed the fermentation to achieve 99% of the theoretical ethanol yield within 6 h. Combined, all of these results suggest that detoxification by PEI and RW-EDI treatments could greatly reduce the costs within the lignocellulosic biorefinery industry through higher yields (glucose conversion and product formation) and rates (hydrolysis and fermentation) as part of the biochemical transformation process.

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