SIMULTANEOUS PRETREATMENT OF LIGNOCELLULOSE AND HYDROLYSIS OF STARCH IN MIXTURES TO SUGARS

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Mixtures of starch and lignocelluloses are available in many industrial, agricultural, and municipal wastes and residuals. In this work, dilute sulfuric acid was used for simultaneous pretreatment of lignocellulose and hydrolysis of starch, to obtain a maximum amount of fermentable sugar after enzymatic hydrolysis with cellulase and β-glucosidase. The acid treatment was carried out at 70-150°C with 0-1% (v/v) acid concentration and 5-15% (w/v) solids concentration for 0-40 minutes. Under the optimum conditions, obtained at 130°C, 1% acid, and 7.5% solids loading for 30 min, the starch was almost completely converted to glucose. However, the acid treatment was not successful for efficient hydrolysis of pure cellulose. A mixture of pine softwood and potato as representatives of lignocellulosic and starch components, respectively, were treated at the optimum conditions for acid hydrolysis of starch. The dilute-acid treatment resulted in 1.2, 60.5, and 23.6% hydrolysis of glucan, xylan, and mannan of pine wood and 67% of potato starch to fermentable sugars. After the acid treatment, the solid residue of the mixture was subjected to enzymatic hydrolysis. The enzymatic hydrolysis under the optimum conditions resulted in conversion of 76% of the glucan in the treated softwood. Therefore, using acid treatment of the mixture is a promising process for pretreatment of wood in addition to the hydrolysis of starch.

Keywords: Pretreatment; Dilute–acid hydrolysis; Lignocellulose; Starch; Biomass; Enzymatic hydrolysis

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INTRODUCTION

Bioethanol can be produced from sugars (e.g. in sugarcane), from starch (e.g. in cereal grains, potato, sweet potato, and cassava), and from cellulose-based materials (e.g. paper, cardboard, wood, and agricultural residues) (Tengborg 2000; Taherzadeh and Karimi 2007). Mixtures of lignocelluloses and starch are available in several different wastes and residuals, including compostable parts of municipal solid wastes, agricultural wastes such as cassava waste, and industrial food wastes e.g. olive pomace, apple pomace, and maize-food remains. Bioconversion of these materials to ethanol involves four major unit operations including pretreatment, hydrolysis, fermentation, and product separation or distillation (Demirbas 2005). In order to improve the hydrolysis rate and its efficiency, pretreatment is required to alter the macroscopic and microscopic size and structure of biomass as well as its chemical composition. Pretreatment affects the struc-

ture of biomass by solubilizing hemicellulose, reducing crystallinity, and increasing the available surface area and pore volume (Chandel et al. 2007). Dilute-acid hydrolysis is the most widely used pretreatment method. It can be used either as a pretreatment of lignocellulose for enzymatic hydrolysis, or as the actual method of hydrolysis to fermentable sugars. Dilute-acid pretreatment can be performed either with a short retention time (e.g. 5 min) at high temperature (e.g. 180°C), or at lower temperatures (e.g. 120°C) with a relatively long retention time (e.g. 30-90 min) (Taherzadeh and Karimi 2008).

The starch in grains such as corn or wheat are typically used for ethanol production. However, it is important not to overlook other components in the grains and also their stems, leaves, and straws as suitable source materials for ethanol production (Chisholm 2004). In order to make complex and rigid lignocellulosic component ready for the hydrolysis stage, usually harsh conditions are necessary in dilute-acid pretreatment. These conditions may further destroy monomeric sugars, produced from easily hydrolysable starch components, and produce inhibitors that would strongly reduce ethanol production during fermentation.

The main objective of this work was to find optimum mild conditions for dilute-acid hydrolysis pretreatment of lignocellulosic and starch mixtures, without their separation, allowing for maximum production of fermentable sugars and minimum destruction and conversion to inhibitors. Therefore, the effects of four effective variables including temperature, retention time, acid concentration, and solids loading on dilute-acid pretreatment were studied. Moreover, enzymatic digestibility of the pretreated lignocellulosic material after dilute-acid pretreatment under different conditions was also evaluated and optimized.

EXPERIMENTAL

Raw Material

The pure substances used for optimization of dilute-acid hydrolysis were potato starch (BDS Chemical Co., England) and cellulose (Avicel, Merck). The lignocellulosic and starchy materials used were a mixture of Russian pine wood chips and potato tubers. The wood was milled and screened to achieve the size of less than 0.8 mm. Then, it was washed several times with tap water and dried at 40 °C for two days. The potato was provided from Fereydan (Isfahan, Iran), peeled, and cut into 0.5 cm cubes.

Dilute-Acid Treatment

The hydrolysis apparatus was a high-pressure stainless steel (SS 316L) reactor with total volume of one liter. The reactor was equipped with a pressure indicator and a thermometer. Half of the reactor, 500 ml, was filled with the materials, and the reactor was placed in an oil bath for heating and hydrolysis at the desired temperature. The pretreatment operational variables studied in this work were temperature at 70-150 °C, sulfuric acid concentration between 0-1% (v/v), hydrolysis retention time of 0-40 min, and solids concentration of 50-150 g dry biomass/L. All of the hydrolysis experiments were performed in duplicates. After the pretreatment, the whole materials were centrifuged at 7000 rpm and filtered. Supernatants were collected and analyzed to

measure the monomeric sugars and fermentation inhibitory components.

A mixture of pine wood and potato was also treated with dilute acid. The solid residue was washed several times with tap water, and dried for two days at 40 °C before enzymatic hydrolysis. Oven dry at high temperatures was avoided in order to reduce the irreversible pore collapses effects of drying.

Enzymatic Hydrolysis

Enzymatic hydrolyses were performed with commercial cellulase (Celluclast 1.5L, Novozymes, Denmark) and β-glucosidase (Novozyme 188, Novozymes, Denmark). The cellulase had 70 FPU/ml activity measured according to Adney and Baker (1996), while the activity of the β-glucosidase was 220 IU/ml according to Ximenes et al. (1996).

The effects of the three variables of (a) cellulase loading (15 and 30 FPU/g dry mass), (b) β-glucosidase loading (30 and 60 IU/g dry mass), and (c) substrate loading (2, 5, and 10%w/v) on digestibility of pretreated residues were studied. Enzymatic hydrolyses were performed in 100 mL Erlenmeyer flasks, each containing 50 mL of 0.05M sodium citrate buffer (pH 4.8) in a shaking water bath at 45°C and 140 rpm for 72 h. After the hydrolyses, the liquid samples were centrifuged at 9000 rpm, filtered, and the supernatants were analyzed. All experiments were performed in duplicates, and the presented results are averages of the replications.

Analytical Methods

The moisture content of the biomass was measured by oven drying at 105 °C to obtain constant weights (Sun and Cheng 2005). The ash was determined by calcination of triplicate samples at 575 °C for 3 h (Sluiter et al. 2008a). Glucan, xylan, mannan, and lignin content of softwood were determined according to the method presented by Sluiter et al. (2008b). The cellulose fraction was determined according to the method presented by Rowell et al. (2005), and the hemicellulose content was determined according to the method presented by Viera et al. (2007). Starch content of potato was measured by polarimetry (Haase 2003).

The hydrolysates from dilute-acid and enzymatic hydrolysis were analyzed by high performance liquid chromatography (HPLC) equipped with UV/vis and RI detectors (Jasco International Co., Tokyo, Japan). Hydroxymethyl furfural (HMF) and furfural were analyzed on an Aminex HPX-87H column (Bio-Rad, Richmond, CA, USA) at 65 °C with 0.6 ml/min eluent of 5 mM sulfuric acid. Glucose, xylose, and mannose were analyzed on an Aminex HPX-87P at 80 °C with 1 ml/min eluent of deionized water. Glucose, xylose, and mannose were determined from RI chromatograms, while HMF and furfural were determined from UV chromatograms at 210 nm.

Statistical Analysis

In dilute-acid treatments, four parameters and each at five levels were chosen to be studied in order to investigate the optimum mild hydrolysis conditions, i.e. temperature (70, 90, 110, 130, 150 °C), time (0, 10, 20, 30, 40 min), acid concentration (0, 0.25, 0.5, 0.75, 1%), and solids concentration (50, 75, 100, 125, 150 g/L). Experimental design was performed using Response Surface Design, Central Composite Rotable Design (CCRD) (Lazic 2004; Deam 1999), which resulted in the 31 experiments

presented in Table 1. The software package Minitab® 15 was used for statistical modeling, analyses, and optimization of the experimental data. The effects of pretreatment temperature, retention time, acid concentration, and solids concentration on the yields of monomeric sugars, were analyzed using P and T tests. Second-order polynomial equations were used with the $P < 0.05$ significance level to predict the relationship of monomeric sugar yields in the dilute acid hydrolysate with temperature, retention time, acid concentration, and solids loading (Talebnia et al. 2008),

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} b_{ij} X_{ij} + e \quad (1)$$

where Y is the dependent or response variable to the model, which in this study is the glucose yield released in acid hydrolysis of pure starch and cellulose, X_i and X_j are the independent variables (factors), and b_i, b_{ii}, and b_{ij} are the measures of the X_i, X_i^2 and $X_i X_j$ effects, respectively. The variable $X_i X_j$ represents the first-order interactions between X_i and X_j. When the response data were obtained from the experiments, a regression analysis using the least-squares method was carried out to determine the coefficients of the response model, the standard errors, and significance (Talebnia et al. 2008).

RESULTS

Dilute-acid Hydrolysis

The hydrolyses of pure cellulose and starch were performed in a high-pressure reactor with 500 mL working volume. The operational variables were temperature (70-150 °C), sulfuric acid concentration (0-1%v/v), retention time (0-40 min), and solids loading (50-150 g dry biomass/L). The yields of glucose released and dry solids remaining after dilute-acid hydrolysis of pure substances are shown in Table 1. Second-order polynomial predictive equation coefficients, P-values, and T-values for estimation of glucose yield released from pure starch and cellulose (Avicel) are shown in Table 2. The established empirical models are appropriate in the range of temperature, sulfuric acid concentration, retention time, and solids loading used in this experiment.

Cellulose hydrolysis

In hydrolysis of pure cellulose, the most effective parameter was temperature (P=0.000), while acid concentration (P=0.084), time (P=0.278), and solids loading (P=0.946) did not show significant effects on yield of glucose formation (Table 2). The coefficients b_2 (-0.361) and b_4 (0.047) showed that time and solids loading were less effective. Moreover, as can be observed from X_{14} coefficient (b_{14}), there was no interaction between temperature and solids concentration in hydrolysis of cellulose. However, there was a significant interaction between time and acid concentration (b_{23}=0.207). The R^2 value for glucose yield was 80.73, which indicated that the model fit the data relatively well. The optimum condition determined for maximum production of glucose was 150 °C, 40 min, 1% acid concentration, and 150 g/L solids concentration. At this condition, approximately 3.3% of cellulose was converted into monomeric fermentable sugar.

Table 1. Experimental Design by CCRD Method for Studying the Effects of Temperature, Retention Time, Acid Concentration, and Solids Loading on the Yields of Released Glucose Produced and Dry Solids Remaining in Dilute-acid Pretreatment of Pure Substances.[a]

Variables				Cellulose		Starch	
Temperature (°C)	Time (min)	Acid (%v/v)	Solids Concentration (g/l)	Glucose (mg/g DS)	Remainder (%)	Glucose (mg/g DS)	Remainder (%)
110	20	0.5	150	0.23	97.2	69.6	0.7
130	30	0.25	125	2.21	93.8	581.1	0.2
90	30	0.25	75	0	97.9	0.6	54.1
110	40	0.5	100	0.6	97.6	197.4	0.7
110	20	0.5	100	0.29	97.6	72. 7	0.6
150	20	0.5	100	31.43	78.1	655. 6	0.5
130	30	0.25	75	3.18	94.3	776.9	0.8
110	20	0.5	50	0.36	97.6	83.3	0.3
90	10	0.75	125	0	97.8	0.7	67.1
130	10	0.75	75	4.49	92.6	705.9	0.2
110	20	0.5	100	0.32	97.8	71.1	0.6
130	10	0.75	125	4.33	92.7	672. 8	0.3
90	10	0.75	75	0	97.9	0.5	63.2
110	20	0	100	0	98.8	0	87.1
110	0	0.5	100	0.09	98.4	7.0	9. 7
110	20	1	100	0.51	98.3	228.9	0.6
70	20	0.5	100	0	99.5	0	86.4
130	30	0.75	125	10.55	92.2	609.4	0.8
110	20	0.5	100	0.31	98.0	71.7	0.5
90	30	0.75	125	0.08	97.5	4.2	16.7
130	10	0.25	125	1.65	94.1	479.7	0.7
110	20	0.5	100	0.33	98.0	70.3	0.5
90	10	0.25	75	0	99.3	0.3	82.8
130	30	0.75	75	7.38	93.0	960.5	0.3
110	20	0.5	100	0.35	98.6	71.3	0.5
110	20	0.5	100	0.37	97.7	72.3	0.5
130	10	0.25	75	2.72	95.1	479.9	0.6
110	20	0.5	100	0.39	98.8	70.3	0.5
90	30	0.75	75	0.11	99.4	7.3	9.6
90	30	0.25	125	0	98.9	1.2	42.6
90	10	0.25	125	0	99.0	0.3	84.2

[a] Data are averages of two replicates.

Table 2. Second-order Polynomial Predictive Equations Used for the Effect of Temperature, Time, Acid Concentration, and Solids Loading on the Yields of Glucose from Pure Components. X_1, X_2, X_3, and X_4 are symbols for temperature, time, acid concentration, and solids loading. X_{ij} represents the first order interactions between X_i and X_j.

Term*	Cellulose			Starch		
	Coefficient	P-value	T-value	Coefficient	P-value	T-value
Constant	100.359	0.675	0.422	1736.15	0.03	2.151
X_1	-1.917	0.000	9.627	-37.88	0.000	15.252
X_2	-0.361	0.278	1.099	-15.14	0.027	2.283
X_3	-23.963	0.084	1.767	-1017.93	0.014	2.554
X_4	0.047	0.946	0.068	0.73	0.163	-1.418
X_{11}	0.009	0.000	8.989	0.23	0.000	5.677
X_{22}	-0.003	0.4390	-.0781	0.37	0.030	2.243
X_{33}	-5.293	0.405	-0.841	638.48	0.19	2.430
X_{44}	-0.001	0.420	-0.813	0.05	0.070	1.852
X_{12}	0.003	0.243	1.182	0.18	0.107	1.646
X_{13}	0.210	0.051	2.000	7.76	0.084	1.767
X_{14}	0.000	0.906	0.119	-0.07	0.107	-1.645
X_{23}	0.207	0.331	0.983	-4.95	0.575	-0.564
X_{24}	0.001	0.685	0.409	-0.13	0.148	-1.470
X_{34}	0.050	0.553	.598	-1.92	0.588	-0.546
$R^{2\,a}$		80.73			86.57	

[a] Coefficient of determination.
[*] X_1= Temp (ºC), X_2= Time (min), X_3= %Acid, and X_4= Solids concentration (g/l)

The values in optimum condition were selected as the hold values, and the effects of two parameters were plotted each time, while other two parameters were constant in hold values (Fig 1a-f). Increasing temperature from 130 °C to 150 °C greatly enhanced the glucose production (about 185%), and this would confirm the importance of the temperature effect on the glucose production reaction (Fig 1a, 1b, & 1d). However, the increasing trend by temperature elevation was more than that by acid concentration (Fig 1a). According to Fig 1a, in the absence of acid, the glucose production from cellulose started at a temperature of 130 °C. However, in the presence of 1% sulfuric acid, glucose formation was started at a lower temperature (100°C). As can be observed in Fig 1d, the graph is almost symmetrical and constant with respect to solids loading. When the acid concentration was kept constant, the amount of glucose was increased slightly with time. However, if temperature and acid concentration were constant at their optimum values (temperature 150 °C; acid concentration 1%), then the yield of released glucose would be in maximum range of 25-35 mg/g DS for all values of time and solids loading (Fig 1f).

Starch hydrolysis

In starch hydrolysis, treatment temperature (P=0.000), time (P=0.027), and acid concentration (P=0.014) showed significant influence on glucose production, and temperature was the most effective parameter. This result could also be concluded from the coefficients b_4=0.73 and b_{44}=0.05. Similar to cellulose hydrolysis, in starch hydrolysis there was no interaction between temperature and solids concentration (b_{14}=0). The R^2 value for glucose yield was 86.57%, which indicated that the model fit the data well. The optimum conditions determined by statistical analysis were 130 °C, 30 min, 1% acid concentration, and 75 g/L solids loading. In this condition of operation, approximately 99% of the starch was converted into monomeric fermentable sugar. The values corresponding to the optimum condition were selected as hold values, and each time effects of two parameters were plotted, while the other two parameters were kept constant as hold values (Fig 2). Glucose content of the hydrolysate decreased by increasing the solids loading (Fig 2d, 2e & 2f). In all figures, in which one of the variables was temperature, the glucose yield graph was almost symmetrical respect to another variable (Fig 2a, 2b &2d). However, if temperature and acid concentration were constant in their optimum value (130 °C; 1% acid concentration), then the yield of released glucose was in a maximum range of 600-1000 mg/g DS for all values of time and solids concentration (Fig 2f). If the variables of temperature and time were fixed at optimum values, then the range of released glucose yield increased to 200-1000 mg/g DS (Fig 2e).

Dilute acid treatment of lignocellulose and starch mixture

The chemical composition of the pine wood was analyzed, and results are shown in Table 3. The starch content of potato was 72% on a dry weight basis, and no cellulose was detected in the potato. According to the results obtained from acid hydrolysis of pure substances, starch can be almost completely converted into glucose under optimum conditions of 130°C, 30 min, 1% acid concentration, and 75 g/L solids content. In order to prevent further destruction of monomeric sugars, the potato slices and wood chips were treated under the optimum conditions achieved for pure starch hydrolysis. Under these conditions, the glucose, xylose, and mannose yields from wood treatment by dilute acid were 5.1, 37.4, 30.3 mg/g DS respectively, while no HMF and furfural were detected. Therefore, 1.2, 60.5, and 23.6% of the glucan, xylan, and mannan of the wood, respectively, were converted into monomeric sugars during the acid treatment. The remaining solids after the pretreatment of the wood, i.e. the pretreated wood, contained 60.5, 1.84, 8.95, and 20.71% of glucan, xylan, mannan, and lignin, respectively.

The glucose yield was 671 mg/g potato starch, and the HMF yield was 3.8 mg/g DS for potato pretreatment; thus 67% of potato starch was converted into glucose, and the amount of HMF produced from potato was only 0.38%.

Enzymatic Hydrolysis

The solid residues from pretreatment of the mixture of wood and potato under the optimum conversion conditions for starch hydrolysis, were subjected to enzymatic hydrolysis. The treated solids contained 60.5% w/w glucan based on the dry weight. It was hydrolyzed by cellulase and β-glucosidase. The hydrolysis of the solid residue was carried out by 15 or 30 FPU/g cellulase and 30 or 60 IU/g β-glucosidase loading at 45 °C

for 72 h. Three different solids loadings of 2, 5, and 10% (w/v) were applied. The results are summarized in Table 4.

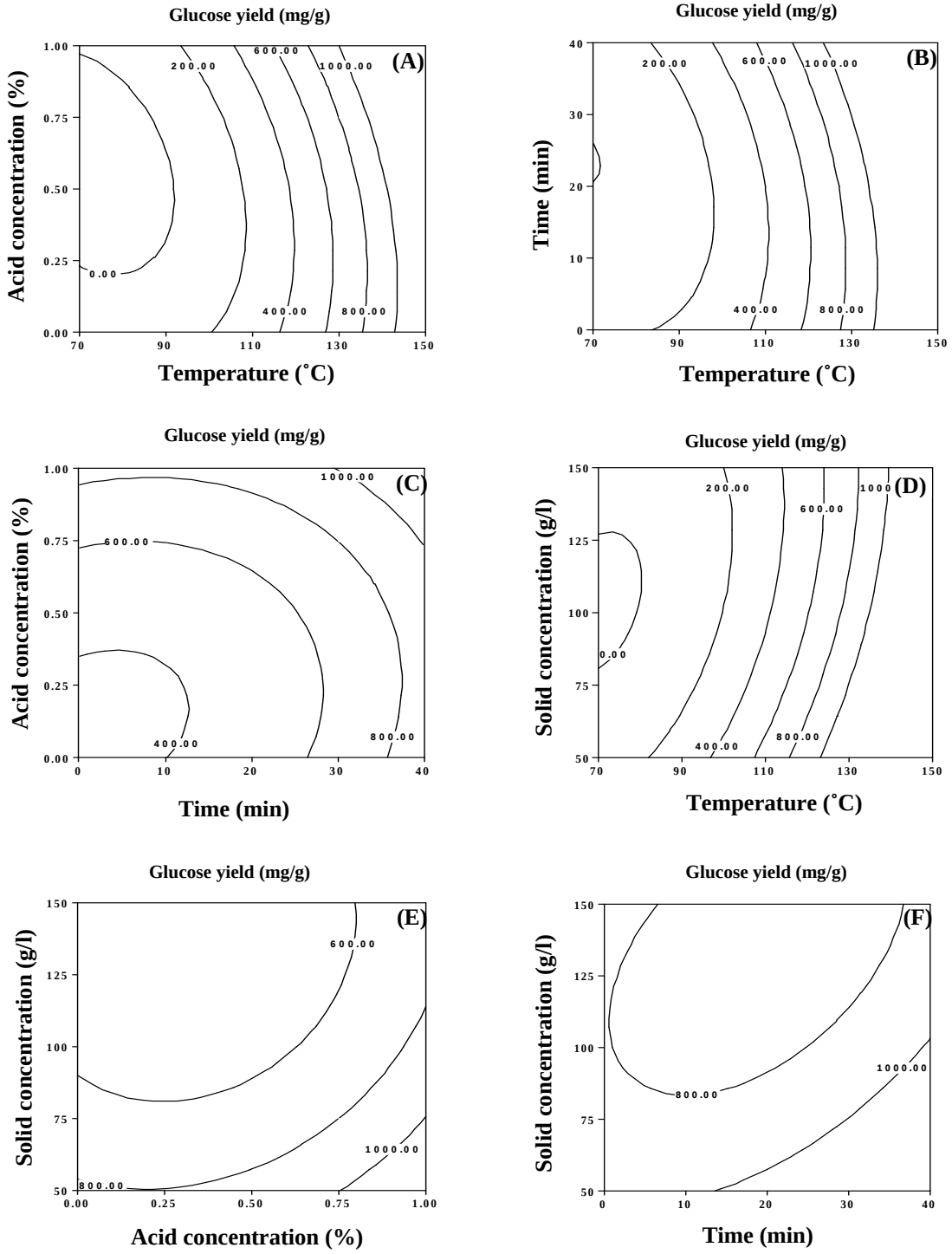

Fig 1. Effect of temperature, time, acid concentration, and solids concentration on dilute acid treatment of starch

Table 3. Chemical Composition of Pine Wood

Components	Native pine wood (% of dry weight)
Glucan	43.68
Xylan	6.19
Mannan	12.87
Cellulose	46.22
Hemicellulose	26.78
Lignin	27.60
Ash	0.75

Table 4. Results of Enzymatic Hydrolysis of Pretreated Wood under Optimum Conditions[a] (130°C, 30 min, 1% acid concentration, and 75 g/L solids loading)

Solids Concentration (%w/v)	Cellulase Loading (FPU/g Dry Mass)	β-glucosidase Loading (IU/g Dry Mass)	Glucose Production Yield (mg/g)[b]
2	15	30	436
2	15	60	499
2	30	30	576
2	30	60	762
5	15	30	381
5	15	60	447
5	30	30	602
5	30	60	669
10	15	30	184
10	15	60	226
10	30	30	341
10	30	60	515

[a] Data are means of two replicates.
[b] Calculated based on the percentage of theoretical yield [produced glucose/ (1.111* biomass (g/l) *F], where F is cellulose fraction in biomass and 1.111 is the hydration factor. No glucan was detected in the potato tubers.

The dilute acid treatment was able to significantly improve the enzymatic hydrolysis of the softwood, since only 15% of the glucan in the native pine wood could hydrolyse when subjected to enzymatic hydrolysis with 30 FPU/g cellulase and 60 IU/g glucosidase with 2% solids loading (data not shown). Increasing the enzymes loading resulted in increased cellulose conversion and glucose production from the acid-treated wood (Table 4). On the other hand, increasing the solids loading from 2% to 10% decreased the conversion of cellulose to glucose. More precisely, cellulose conversion reduction from 2% to 5% solids loading was much less than that from 5% to 10% solids loading. The best condition was hydrolysis of 2% solids with 30 FPU/g cellulase and 60 IU/g glucosidase, which resulted in the conversion of 76.2% cellulose in the wood to glucose.

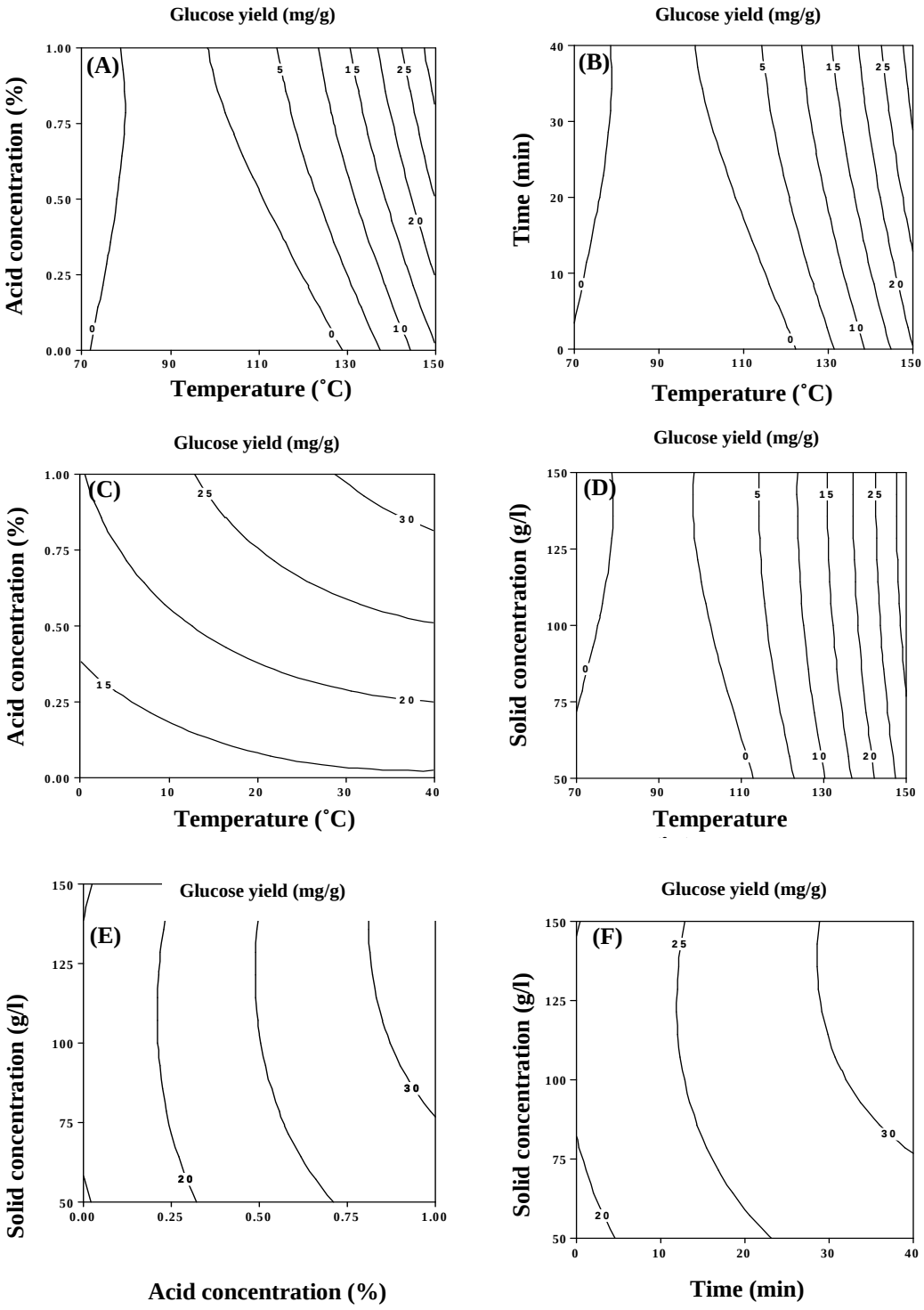

Fig 2. Effect of temperature, time, acid concentration, and solids loading on dilute acid treatment of cellulose.

DISCUSSION

Waste biomass mixtures, which usually contain both lignocellulosic and non-cellulosic materials, represent a source of carbohydrates that can be fermented into fuels and chemicals (Blaschek and Ezeji 2007). In many cases, it is not practical to separate the cellulosic and non cellulosic materials, e.g. the mixture of lignocelluloses and starch materials in agricultural residuals and compostable part of municipal solid wastes.

Potato is among the main food crops in many countries in Europe and Asia. Waste potato is available in huge amounts in the related food producing industries, and also as a part of municipal solid wastes.

Starch can be hydrolyzed much more easily than cellulose by acid hydrolysis, since cellulose is a straight chain polymer without coiling or branching, which contributes to its high crystallinity (Campo et al. 2006). However, the results of the current work showed that the lignocelluloses can efficiently be pretreated for enzymatic hydrolysis by acid hydrolysis in which the starch can be efficiently hydrolyzed. Therefore, using the acid treatment of the mixture is an interesting process for pretreatment of wood beside complete hydrolysis of starch.

Dilute-acid hydrolysis of lignocellulosic and starchy materials may result in sugars and other by-products in some serial and parallel reactions (Karimi et al. 2006; Gupta et al. 2009):

Glucan (Cellulose or starch) → Oligosaccharides → Glucose → HMF → Levulinic acid

The acid hydrolysis of hemicellulose may lead to monomeric sugars and furans (Palmqvist and Hahn-Hägerdal 2000; Lee et al. 1999; Zeitsch 2000):

Hemicellulose → Oligosaccharides → Sugars (xylose; arabinose; glucose; mannose; galactose) → Furfural and HMF→ Carboxylic acids

The yields of these reaction products are affected by the hydrolysis parameters such as acid concentration, temperature, retention time, and solids concentration. Among these variables, the effect of temperature is more significant in both cellulose and starch hydrolyses (Pan et al. 2006). Increases in temperature result in hemicelluloses degradation (Nguyen et al. 2009), while a temperature around 190°C was reported as giving maximum conversion of hemicelluloses to monomeric sugars (Tasic et al. 2009). The pretreatment of wood at 130°C in the current work resulted in hydrolysis of a part of the hemicellulose, e.g. hydrolysis of about 60% of xylan. On the other hand, hemicellulose surrounds the cellulose and can protect the cellulose from enzymatic hydrolysis; therefore, the acid hydrolysis can be considered as an effective pretreatment method prior to enzymatic conversion of cellulose to glucose. This could be the reason for the increase in conversion of glucan from 15% for untreated to 76% for the wood treated with dilute acid at 130°C and 1% acid for 30 min. The present results are in line of the optimum results which were obtained in previous studies (e.g. Sun and Cheng 2005).

CONCLUSIONS

It is possible to efficiently hydrolyze a mixture of lignocellulose and starch materials without separation, using dilute-acid treatment followed by enzymatic hydrolysis. Dilute-acid treatment under mild conditions, e.g. 130 °C, 30 min, 1% acid, and 75 g/L solids loading, can be used for hydrolysis of starch and pretreatment of lignocellulose. The rest of the solids from dilute acid treatment, which mainly contains cellulose, can efficiently convert into glucose by enzymatic hydrolysis using cellulase and β-glucosidase.

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