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Scientific Note

Measurement of the Inhibitory Potential and Detoxification of Biomass Pretreatment Hydrolysate for Ethanol Production

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ABSTRACT

The Microtox assay represents a rapid, accurate, and reproducible method for determining general microbial toxicity. This assay was used to evaluate the relative toxicity of a variety of hydrolysate samples derived from dilute-acid and alkaline biomass pretreatment. Toxicity is elicited from biomass degradation products, such as furfural, hydroxymethyl furfural, and acetic acid, generated during pretreatment. Microtox results indicate that the pretreatment samples examined ranged from 9 to 71 toxicity units (TU). Correlations of TU and sample absorbance at several wavelengths were evaluated for all sample series. Sample TU values best agreed with absorbance at 230 nm, but the unsatisfactory fit suggests that absorbance should not be used as an absolute measure of sample toxicity.

Microtox data for pretreatment hydrolysate samples were correlated with the inhibition experienced by the ethanologenic yeast, *Saccharomyces cerevisiae* strain D_5A, during the simultaneous saccharification and fermentation (SSF) process of pretreated biomass. None of the alkaline pretreatment conditions produced inhibition during SSF (data not shown). However, the acid pretreatment conditions did produce a wide range of inhibitory and noninhibitory hydrolysates. In general, fermentation was inhibited for acid-pretreated hydrolysate samples with values exceeding 45 TU. Preliminary studies that focused on reducing hydrolysate sample toxicity (detoxification) indicate that adding perlite and zeolite had little effect. However, the use of charcoal, a universal flocculent, or ion-exchange resins significantly reduced sample toxicity, holding promise for the efficient bioconversion of pretreated biomass to ethanol. Moreover, the developed toxicity measurement assay can quickly monitor the quality of the pretreatment process. In this way, biomass conversion operation processes can be reliably controlled at the pilot and commercial scales.

Index Entries: Microtox; toxicity assay; ethanol fermentation; dilute-acid hydrolysis; detoxification.

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INTRODUCTION

Numerous pretreatment technologies have been developed for solubilizing portions of biomass feedstocks before simultaneous saccharification and fermentation (SSF) *(1)*. In general, these treatments effectively enhance enzymatic cellulose conversion rates and yields by opening new surface areas for hydrolytic enzyme attack. However, solubilized sugars undergo further degradation reactions, especially under acidic/high-temperature conditions, thus creating unfermentable (and possibly toxic) compounds. Inhibitory compounds produced during dilute sulfuric acid pretreatment of biomass include acetic acid, furfurals, and numerous aromatic compounds. The identification and potential toxicity levels of suspected inhibitors from acid pretreatment processes were recently reviewed by McMillan *(2)*. Indeed, laboratory experiments conducted with selected pretreatment hydrolysates have demonstrated complete inhibition of yeast cell growth and ethanol production.

A rapid biological assay has been developed to determine the relative level of toxicity for waste waters and aqueous extracts (Microtox assay, Microbics Corporation, Carlsbad, CA). This assay measures the light output of luminescent indicator bacteria beforehand and after they are challenged by a sample of unknown toxicity. The degree of light loss (the indication of metabolic inhibition in the test organisms) indicates the sample's degree of toxicity. This type of assay is well suited to determining gross levels of toxicity, which may be a result of multiple variables.

In the present study, the Microtox assay was employed to determine the relative toxicity of pretreatment hydrolysate samples prior to ethanol fermentation. Microtox data were compared to those of sample absorbance and actual fermentation performance. It was also used to examine the efficacy of a variety of methods to detoxify a representative pretreatment hydrolysate sample before it was used in fermentation.

MATERIALS AND METHODS

Pretreatment Hydrolysates

Pretreatment hydrolysate samples were prepared using a mixed waste paper (white office paper, newsprint, coated paper magazines, and cardboard) feedstock. Before pretreatment, this feedstock was knife milled to pass a 3.1-mm (⅛-in.) rejection screen. Parameters including temperature, time, and acid addition are described in Table 1 for batch dilute sulfuric acid pretreatment. Additional pretreatments were performed under alkaline conditions using a temperature range of 25–120°C, 0–4 h, and 0–2 wt% sodium hydroxide. All pretreatments were conducted at 5% (wt/vol) paper solids using a 2-L (.53 gal) Parr stirred reactor constructed from Carpenter 30 cb3. Following pretreatment, hydrolysate samples were filtered through white linen to separate solids from liquids for individual analysis. Separated solids were stored wet and were generally in the range of 20–30% total solids. Liquid and solids fractions were stored at 4°C (39.2°F) until use.

Microtox Analysis

Before Microtox analysis, hydrolysate samples were removed from cold storage and allowed to reach room temperature. For dilute-acid pretreatment samples, the pH was adjusted to 7.0 by adding a commercial grade of solid hydrated lime (Snowflake Lime, Ash Grove Cement Company, Overland Park, KS). Although a

Table 1
Dilute-Acid Pretreatment of Mixed Paper Feedstock

Sample	Temperature, °C	Time, min	Sulfuric acid, wt%
1375-001-01	200 (392°F)	5	0.5
1375-001-02	200 (392°F)	20	0.73
1375-001-03	180 (356°F)	5	0.73
1375-001-04	200 (392°F)	5	0.73
1375-001-05	180 (356°F)	20	0.73
1375-001-06	190 (374°F)	12.5	0.616
1375-001-07	180 (356°F)	20	0.5
1375-001-08	190 (374°F)	12.5	0.616
1375-001-09	180 (356°F)	5	0.5
1375-001-10	200 (392°F)	20	0.5

rapid pH adjustment was observed when the lime was added, the samples slowly readjusted to a lower pH. For alkaline pretreatment samples, the pH was adjusted to 7.0 by adding 2*M* hydrochloric acid. To provide the necessary time for pH equilibration, hydrolysate samples were allowed to stir at room temperature for 2 h before final pH adjustment. The samples were then transferred to 50-mL (1.69-oz.) disposable centrifuge tubes and centrifuged at room temperature for 15 min at 3500 rpm (1400*g*) (Centra 4, International Centrifuge, Needham Heights, MA). The samples were then filtered using sterile 115-mL (3.89 oz.) capacity filter units equipped with a 0.45-μm membrane (Gelman Sciences, Ann Arbor, MI). For samples of known high toxicity or those with a substantially dark appearance, a primary dilution was made (generally 1:10) using distilled water. The Microtox analysis was performed as previously described (*3*) using a model 500 unit equipped with a microcomputer to gather and analyze data. Microtox software (version 6.1) was used to calculate for effective concentration 50 (EC_{50}) and toxicity units (TU) of the samples. Phenol at 90 mg/L in distilled water was used as a standard and analyzed daily to ensure the accuracy of the assay.

Hydrolysate Absorbance Measurements

The absorbance of each sample was analyzed following a 1:100 dilution with distilled water using a Hewlett Packard 8451A Diode Array Spectrophotometer. Samples were assayed at 208, 230, and 274 nm, using quartz cuvets.

Hydrolysate Detoxification

Activated charcoal (Darco-g-60, MCB Manufacturing Chemist, Cincinnati, OH), ion exchange resin (RG501 -XS, Bio-Rad Laboratories, Hercules, CA), flocculent (Universal Flocculent 1002, Nalco Chemical, Denver, CO), zeolite (U.S. Zeolites Inc., Golden, CO), and perlite (Horticulture Perlite, Premier Brands, New Rochelle, NY) were examined for their ability to reduce hydrolysate sample toxicity and color. In general, test materials were added as a dry powder to the pH-adjusted hydrolysate sample at varying concentrations (wt/vol). The sample was then mixed using a magnetic stirrer for 2 h at room temperature. Before analysis, the treated hydrolysate samples were filtered using 115-mL (3.89-oz.) filter units (0.45-μm membrane).

Ethanol Fermentation of Pretreatment Hydrolysates

SSF experiments were performed with acid and alkaline slurry samples as previously described *(4)*. Acid-pretreated hydrolysate liquid was pH adjusted with solid NaOH to 6.5–7.0. Alkaline-pretreated hydrolysate liquid was pH adjusted with 96% phosphoric acid to 4.0. Non-pH-adjusted wet solids were then combined with hydrolysate to achieve a 10% solids loading and a slurry pH of 5.0. Dry nutrients were added so as not to dilute further the hydrolysate sample. Bactopeptone (2 g) and yeast extract (1 g, both from Difco, Detroit, MI) were added to each growth flask. The sample flasks were then autoclaved for 30 min. The flasks were then inoculated with a 10% vol/vol culture of nonhydrolysate adapted *Saccharomyces cerevisiae* strain D_5A and 10 filter paper units/g of cellulosic material of a liquid cellulase preparation (Econase CE, Enzyme Development Corporation, New York, NY). The flasks were incubated at 38°C and 150 rpm using a New Brunswick rotary shaker. Water traps were fitted to each growth flask to vent CO_2 and maintain anaerobic conditions. Whole slurry samples were analyzed every 24 h for free sugars and end products, including ethanol.

Before analysis, slurry samples were centrifuged to remove solids. The supernatant was further filtered using 0.45-μm syringe filters (Gelman Sciences) before HPLC analysis. A Hewlett Packard HPLC (model 1090) equipped with an organic acid column (HPX-87H, Bio-Rad) was used to quantify glucose, cellobiose, glycerol, acetic acid, and ethanol.

Ethanol produced after 7 d of fermentation was compared to the theoretical ethanol yield calculated from the initial six carbon sugars in the substrate.

RESULTS

Two series of pretreatment hydrolysate samples including dilute sulfuric acid pretreatment (#1375-001) and alkaline pretreatment (#1375-004) were evaluated for toxicity. These hydrolysates were stored at 4°C (39.2°F), and the dark components of the liquor impregnated the plastic storage container. The dark color of the hydrolysates correlated well with a strong "phenol-type" odor. In the case of the dilute sulfuric acid hydrolysate samples, pH readjustment was required over time because storage of the sample caused a continual pH drop.

The toxicity of the pH-adjusted and filtered hydrolysate samples was determined using the Microtox assay. The toxicity of each sample was evaluated at least twice (generally on separate days). The data for sample toxicity, expressed in average TU, are given for each series in Tables 2 and 3. In general, the toxicity within a given series varies significantly. Overall, the sample toxicity for the #1375 set ranged from 9 to 71 TU. The toxicity level of the hydrolysate samples may be compared to that of phenol at 90 mg/L, which exhibits a toxicity of 3–4 TU.

Because the toxic nature of the hydrolysate samples may be attributed to a variety of pretreatment by-products, including furfurals and lignin degradation products (i.e., *p*-hydroxybenzaldehyde, *p*-coumaric acid, and vanillic acid), the absorbance optima for these compounds were used to correlate with sample toxicity. Sample absorbance was evaluated for each experimental series at 208, 230, and 274 nm. The correlation of sample TU and absorbance is described in Figs. 1 and 2. In general, the best fit occurs with the correlation of TU and absorbance at 230 nm. However, all correlations of TU and absorbance demonstrate multiple outliers.

Table 2
Microtox Analysis of Pretreatment Hydrolysate Samples #1375-001

	Assay date	Dilution	Total TU	Sample
001-01	3-22-94	1:20	42.6	42.6 ± 0.7
	3-24-94	1:10	43.3	
	3-29-94	1:10	41.9	
001-02	3-22-94	1:20	62.7	64.4 ± 5.4
	3-23-94	1:20	68.0	
	3-24-94	1:10	69.2	
	3-29-94	1:10	57.5	
001-03	6-22-94	1:2	33.4	33.6 ± 0.3
	6-23-94	1:4	33.8	
001-04	6-24-94	1:20	73.6	70.6 ± 4.3
	6-27-94	1:20	67.5	
001-05	6-24-94	1:10	52.6	53.6 ± 1.4
	6-27-94	1:10	54.6	
001-06	6-24-94	1:20	53.9	47.1 ± 6.1
	6-27-94	1:10	45.5	
	9-20-94	1:10	42.0	
001-07	6-24-94	1:10	47.2	46.9 ± 0.4
	6-27-94	1:10	46.6	
001-08	3-22-94	1:20	44.5	45.5 ± 2.5
	3-24-94	1:10	43.7	
	3-29-94	1:10	48.4	
001-09	3-22-94	1:20	20.6	21.1 ± 1.6
	3-23-94	1:10	22.2	
	3-24-94	1:2	19.1	
	3-29-94	1:2	22.6	
001-10	3-22-94	1:20	59.2	62.9 ± 3.2
	3-24-94	1:10	65.2	
	3-29-94	1:10	64.2	

Figure 3 describes the relationship of overall (ethanol) yield and sample TU. In general, the data indicate a sigmoidal curve in which hydrolysate sample TU values in excess of 45 result in severe toxicity to the yeast. In some samples with low TU values, ineffective pretreatment reduced cellulose availability to the hydrolytic enzyme and thus resulted in suboptimal overall yields.

Although some level of sample toxicity may be tolerated in ethanol fermentations, many of the pretreatment hydrolysate samples were demonstrated to be toxic to yeast. The ability to reduce the level of sample toxicity to one that yeast can tolerate was examined using a variety of adsorbents. Adsorbents were added to hydrolysate sample #1375-001-08 at two concentrations (except for the flocculent). The effects on sample toxicity of adding adsorbents are described in Fig. 4. These data indicate that only the flocculent, charcoal, and ion-exchange resins substantially reduced the sample toxicity.

Table 3
Microtox Analysis of Pretreatment Hydrolysate Samples #1375-004

Sample	Assay date	Dilution	Total TU	Average TU
004-01	7-1-94	1:10	32.9	35.2 ± 2.1
	7-5-94	1:5	35.8	
	7-5-94	1:5	37.0	
004-02	7-5-94	1.2	9.8	9.3 ± 0.7
	7-5-94	—	8.8	
004-03	7-1-94	1:10	50.2	52.2 ± 2.0
	7-5-94	1:10	54.2	
	7-5-94	1:10	52.3	
004-04	7-5-94	1:2	9.4	9.0 ± 0.6
	7-6-94	—	8.6	
004-05	7-6-94	1:10	39.6	35.6 ± 3.8
	7-6-94	1:2	32.1	
	7-7-94	1:5	35.0	
004-06	7-6-94	1:10	41.4	39.8 ± 1.4
	7-7-94	1:5	39.0	
	7-7-94	1:5	39.1	
004-07	7-6-94	1:5	15.3	15.2 ± 2.0
	7-7-94	1:2	17.2	
	7-7-94	1:2	12.4	
	7-11-94	1:2	16.0	
004-08	7-7-94	1:10	35.9	37.3 ± 2.0
	7-11-94	1:5	38.7	
004-09	3-24-94	1:2	9.5	10.2 ± 0.7
	3-29-94	1:2	10.3	
	3-29-94	—	10.8	
004-10	3-22-94	1:20	52.6	52.3 ± 2.7
	3-29-94	1:20	49.8	
	3-30-94	1:10	55.9	
	3-30-94	1:10	50.7	

DISCUSSION

Owing to the complex nature of inhibitors produced during pretreatment of biomass and cellulosic wastes, standard methods, such as solution absorbance, are ineffective at predicting the relative toxicity that may be experienced by a fermentation microorganism. The Microtox assay represents a rapid, reproducible, EPA-approved method for evaluating the relative toxicity of a variety of environmental samples, including pretreatment hydrolysates. In this study, pretreatment hydrolysate sample toxicity varied from 9 to 71 TUs. Correlating hydrolysate sample toxicity with absorbance demonstrates the best fit with readings at 230 nm, although substantial line fit variation occurred within series. All correlations of TU and absorbance demonstrate multiple outliers, which may indicate that the toxic byproducts from the pretreatment process are heterogeneous and vary greatly with changes in the pretreatment process. If true, this condition makes assumptions of sample toxicity using absorbance at 230 nm relatively inaccurate.

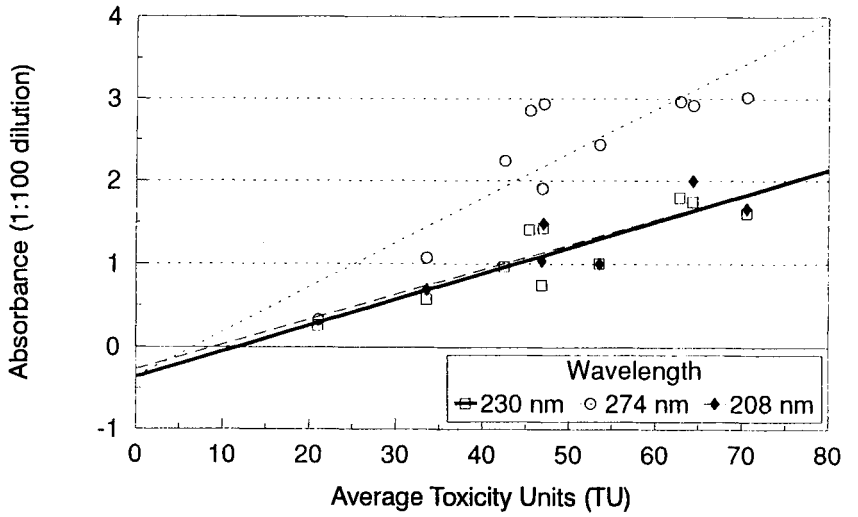

Fig. 1. Correlation of dilute sulfuric acid hydrolysate TU and sample absorbance. Microtox TU was as described in Table 2. Absorbance measurements were performed on samples diluted 1:100 with distilled water.

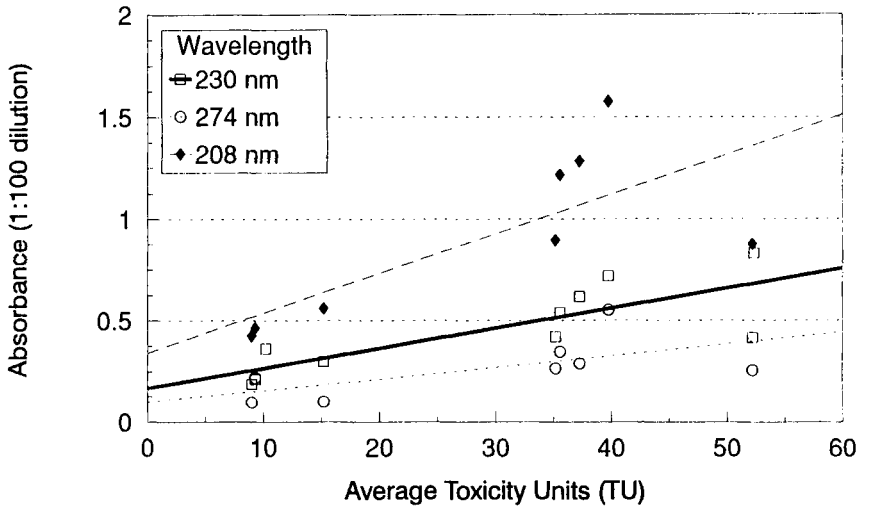

Fig. 2. Correlation of alkaline hydrolysate TU and sample absorbance. Analysis was performed as described in Fig. 1.

The proposed utility of the Microtox assay is to predict the level of toxicity that may be tolerated by a specific fermentation catalyst, under the defined parameters with which this study was performed, i.e., feedstock type, pretreatment protocols, and fermentation catalyst. Although some level of adaptation to toxic components of pretreatment hydrolysates may be anticipated, this study identifies an approximate limitation of 45 TU for effective fermentation under the defined conditions.

Many adsorbents were chosen for study, and the Microtox assay used to determine their effectiveness on reducing overall toxicity. Perlite and zeolite

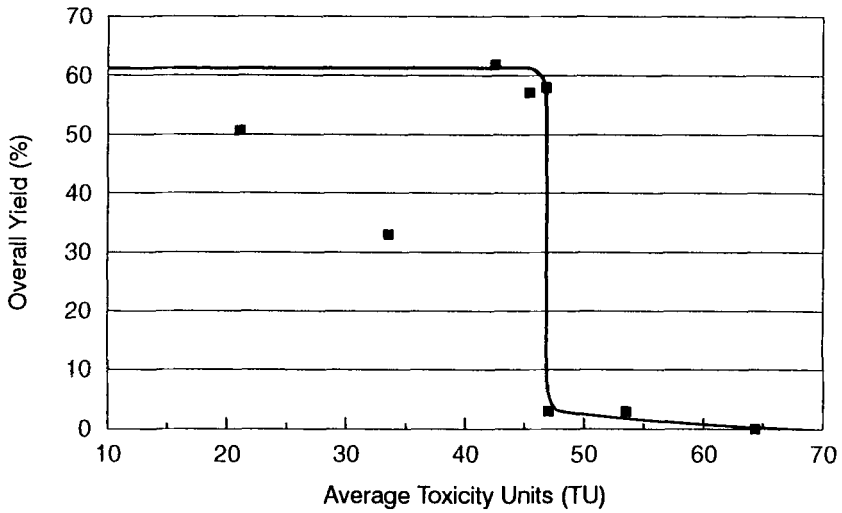

Fig. 3. Comparison of dilute-acid pretreatment sample SSF performance and sample toxicity. Overall yield was determined as described in Materials and Methods.

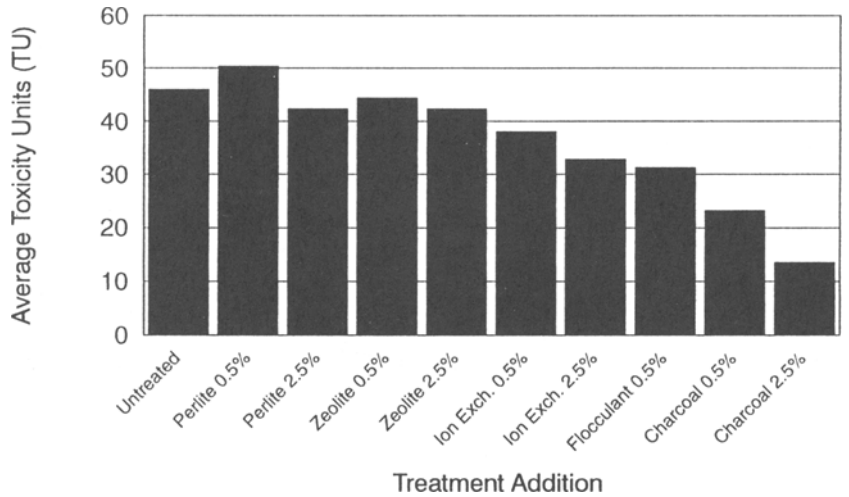

Fig. 4. Changes in measured TU of dilute-acid hydrolysate sample #1375-001-08 following treatment with potential detoxifying agents.

appeared to be relatively ineffective in reducing hydrolysate sample toxicity. However, applying activated charcoal, universal flocculent, or ion-exchange resins substantially reduced hydrolysate toxicity. Additional work will identify alternative detoxifying methods that can provide low cost and minimal obstacles to downstream processing.

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