0273-2289/90/2425—0275$02.40

Evaluation of Pretreated Herbaceous Crops for the Simultaneous Saccharification and Fermentation Process

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ABSTRACT

Three dilute acid pretreated herbaceous crops (Weeping lovegrass, *Eragrotis curvula;* Sericea lespedeza, *Lespedeza cuneata;* and switchgrass, *Panicum virgatum*) were evaluated in the simultaneous saccharification and fermentation (SSF) process for ethanol production with selected yeast strains and enzyme combinations. *Saccharomyces cerevisiae* (D_5A) was used in the fermentations as a single culture and in a mixed culture with β-glucosidase, producing *Brettanomyces clausenii* Y-1414. Cellulase enzyme was either employed alone or in combination with β-glucosidase. SSF's were run at 37°C for 8 d along with a saccharification study at 45°C under similar enzyme loadings. Both cultures achieved high ethanol yields and rates of hydrolysis at the higher enzyme loadings, and the mixed culture shows improvement over *S. cerevisiae* for the lower enzyme loadings without β-glucosidase supplementation. Of the three herbaceous crops tested, Weeping lovegrass excels in both rate and final ethanol yields, followed closely by Switchgrass, whereas the legume (*Sericea lespedeza*) trailed behind both grasses in conversion performance.

Index Entries: Simultaneous saccharification and fermentation, dilute acid pretreatment; herbaceous crops; cellulase; β-glucosidase.

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INTRODUCTION

The simultaneous saccharification and fermentation (SSF) process for conversion of cellulose into ethanol was first studied by Takagi et al. (*1,2*) over 10 years ago, and the process still shows great potential for economic production of ethanol. In light of our mounting environmental concerns, this high octane, clean burning fuel becomes even more important as an alternative to fossil fuels. The SSF process employs fermentative microorganisms in combination with cellulase enzyme to minimize accumulation of sugars in the fermenter. As a result, inhibition of the enzyme by its product sugars is reduced, and higher hydrolysis rates and yields are possible than for saccharification without fermentation (*2*). However, to produce ethanol from the SSF process that is competitive in price with the cost of petroleum-derived fuels, hydrolysis yields must be increased, enzyme loadings must be reduced, and ethanol production rates must be improved.

Yeast selection for SSF has been described in several publications (*2–8*). Some of this work involved the selection of thermotolerant yeasts (*6–8*), with the goal of using a yeast that can ferment at a temperature close to the optimal hydrolysis temperature for the cellulase enzyme, 45°C (*6*). However, although an increase in temperature can speed up the hydrolysis, loss of cell viability results in lower ethanol yields, and 37–40°C appears to be the best temperature for the SSF process (*6,8*).

Cellobiose fermenting yeast have also been studied because additional β-glucosidase activity can speed up the SSF reaction (*4,10–12*). The importance of end-product inhibition of the cellulase enzymes during cellulose hydrolysis has been modeled by Howell (*13*). Some publications discuss the advantage of the cellobiose fermenting yeasts in decreasing end-product inhibition of cellobiose to the cellulase enzyme (*14,15*). In general, *S. cerevisiae*, a strong ethanol tolerant glucose fermerter with a fast rate of fermentation, has been found to perform well if the enzyme preparation is high in β-glucosidase, whereas a mixed culture of *B. clausenii* and *S. cerevisiae* provides better yields, rates, and concentrations if the enzyme is lower in β-glucosidase.

Another important element in the SSF process is the choice of substrate. Several cellulose-containing substrates have been evaluated in the SSF process, including sugar cane bagasse, rice straw, wheat straw, wood fractions, and paper mill byproducts (*9,15–20*). Although these substrates are all potentially important, fast growing herbaceous crops may prove economically attractive as substrates for ethanol production in the future, and an important consideration is the acceptability of these crops for biological conversion to ethanol. Therefore, this project was undertaken to evaluate several promising herbaceous crops as substrates for the SSF process. Since high β-glucosidase activity has been shown to be important for high yields by Spindler et al. (*17*), the cellulase enzyme was used alone

and with β-glucosidase supplementation to establish the highest possible cellulose conversions.

MATERIALS AND METHODS

Materials

The herbaceous crops employed in this study (Weeping lovegrass, *Eragrotis curvula;* Sericea lespedeza, *Lespedeza cuneata;* and Switchgrass, *Panicum virgatum*) were supplied to SERI through the coordination of the Biomass Production Program at Oak Ridge National Laboratory (ORNL), Oak Ridge, Tennessee. The fermentation yeasts used were *S. cerevisiae* (D_5A), a SERI strain genetically derived from Red Star Brewers Yeast and *B. clausenii* Y-1414 obtained from the Northern Regional Research Laboratory (NRRL), USDA, Peoria, IL. Chemicals were purchased from the Sigma Chemical Company, St. Louis, MO, and yeast extract and peptone growth media were ordered from Difco, Detroit, MI. Cellulase enzyme (150 L) was purchased from Genencor Inc., San Francisco, CA, and β-glucosidase (Novozyme-188) from NOVO Laboratories, Inc., Wilton, CT. Shaker flask, 250 mL Pyrex graduated vessels were used for the fermentations.

Methods

Shaker flask SSFs were carried out in 250 mL flasks outfitted with stoppers constructed to vent CO_2 through a water trap. These flasks contained 100 mL of fermentation broth and were agitated at 150 rpm in a shaker incubator at 37°C. A 1% yeast extract and 2% peptone (w/v) media was used with a subtrate loading of 7.5% (w/v) cellulose. A lipid mixture of ergosterol (5 mg/L) and oleic acid (30 mg/L) was added to the media for improved ethanol yield (22). Also, penicillin and streptomycin at 10 mg/L were used to minimize bacterial contamination. The inocula were grown in a shaker flask with YP media and 2% (w/v) glucose at 37°C, and a 1/10 (v/v) ratio of yeast culture to total volume of media was added to the fermentation. The substrate was autoclaved in fermentation flasks, and sterile media, lipids, antibiotics, and enzyme were added before the inoculum. For saccharification without fermentation (SAC), 20 mL vials were used on a rotary shaker incubator set to 45°C. Substrate loading of 7.5% cellulose, sterile water, enzyme, and the antibiotic tetracycline (40 μg/mL) were combined to a total volume of 10 mL. Also, detailed fermentations were employed to gather more data on the SSFs, which were performed as above, but analysis included several more parameters, as described below.

For both SSFs and SACs, the ethanol concentrations in the supernate were measured by gas chromatography using a Porapak Q80/100 column. The internal standard was 4% isopropanol. For some of the SSFs, a more detailed analysis was carried out where residual sugars (glucose and cel-

lobiose) were determined as glucose by incubation of the sample with 2 mg/mL almond extract β-glucosidase from Sigma for 1 h at 37°C, and total sugars were measured on the model 27 glucose analyzer from Yellow Springs Instruments, Yellow Springs, OH. Also, viable cell densities were measured as colony forming units (CFU) by plating serial dilutions on YPD or YPC plates.

Cellulase enzyme loadings of 7, 13, 19, and 26 IU/g cellulose substrate were used in the shake flask screening SSFs and for the SACs to span the range of activity previously shown to be important for SSFs. In this work, IU stands for international units of filter paper activity in μm glucose/min (*23*). β-glucosidase enzyme was also employed in these study at ratios of 1, 2, and 8 parts to 1 part cellulase, as measured by IU of β-glucosidase per IU of cellulase. The β-glucosidase activity was determined by *p*-Nitrophenyl-β-glucoside assays at a temperature of 37°C since this is the temperature for the SSFs. The activity of cellulase increases with increasing temperatures to an optimum at 45°C (*6,8*), the temperature selected for saccharification without fermentation studies. The IUPAC revisions of measured cellulase activities indicate that the level of β-glucosidase in an enzyme preparation may affect the results of the cellulase assay in filter paper units (*23*).

Herbaceous crops were treated as 500 g batches of Wiley milled (2 mm screen) substrate and pretreated with dilute sulfuric acid (0.5% v/v) in a 2 gal Parr reactor. The crops were pretreated at 140°C for 1 h with stirring at 185 rpm. After reaction, the slurries were washed several times with hot water in a large Buchner funnel lined with a linen sheet to bring the pH of ≈1.3 up to ≈4.5. These batches were combined, immediately placed in freezer storage bags, and stored at −20°C. Approximately 58% of the pretreated grasses dry wt was found to be cellulose, 40% lignin and acid insoluble ash, and 2% xylan. The pretreated legume gave a lower dry wt of cellulose at 45%, with 51% lignin and acid insoluble ash, and 2.5% xylan.

Shaker flask results are reported as percent of maximum theoretical ethanol yields and do not account for substrate used for cell growth. Thus, the maximum expected ethanol yield is about 95%, assuming about 5% of the substrate is needed for cell growth. These calculations are based on the measured ethanol concentrations and a 56.7% theoretical ethanol yield conversion of cellulose to ethanol only. However, the saccharifications with cellulose are reported on the basis of percent of the maximum amount of sugars possible. Thus, comparison of the SSF and straight saccharification results must consider that subsequent fermentation of the sugars produced in the latter will also result in about a 5% loss to cell growth.

Although in previous work with Sigmacell-50 (*3,4,8*) we were able to measure the residual cellulose via a wash filtration method, this method for measuring residual cellulose in real substrates, such as pretreated herbaceous crops, was not effective because of lignin interference. Therefore, for our detailed analysis, an alternative method was developed that could estimate the residual cellulose by poisoning the yeast cells with

NaFl and allowing the excess enzyme to complete the saccharification from a given point in time of the SSF. The final glucose concentration is proportional to the cellulose concentration at the time of poisoning. Background measurements of glucose were subtracted that stem from the β-glucosidase enzyme itself and from some residual glucose not taken up by the cells at time of sample. At lower enzyme levels, additional cellulase and β-glucosidase were added to complete the saccharification.

The straight saccharification (SAC) yields are calculated as the amount of glucose produced compared to the potential glucose in the cellulose feed. The β-glucosidase activity of the Novo-188 cellobiase enzyme, used in supplementation for both saccharifications and SSFs, was determined by performing *p*-Nitrophenyl-β-glucoside assays at selected temperatures of 37, 40, and 50°C. It was found that at 37°C, the activity is 125 IU/mL, at 40°C, the activity is 200 IU/mL, and at 50°C, it is 500 IU/mL. Since the SSF's of herbaceous crops were performed at 37°C, the β-glucosidase activity was based on this temperature, whereas the IUs for the cellulase activity are measured at 50°C according to IUPAC procedures (*23*). Similarly, the saccharifications run at 45°C were supplemented with β-glucosidase, reflective of the 37°C assay.

The substrate level was limited to 7.5% cellulose because mixing problems were encountered at higher cellulose levels during preliminary SSF evaluations performed with *S. cerevisiae* on pretreated wheat straw in previous studies at higher substrate levels (*17*).

RESULTS

Table 1 illustrates the final yields for straight saccharification at 45°C for the three pretreated herbaceous crops at the selected cellulase loadings and supplementations with β-glucosidase for 250 mL shake flasks. The best overall rates of hydrolysis and final conversions to simple sugars were observed for Weeping lovegrass and Switchgrass. The highest enzyme loadings of 26 IU cellulase/g cellulose with an 8:1 ratio of β-glucosidase were necessary to achieve 70–73% conversion for these grasses, and the saccharifications took 5 d to achieve these yields. Only 40% of the cellulose remaining in the pretreated legume, Lespedeza, was saccharified under these conditions. It has been reported (*24*) that the legume required harsher conditions of pretreatment (180°C for 20 min using 0.5% H_2SO_4 v/v) to achieve the high conversion yields (ca. 70%) observed with the other two grasses.

Table 2 shows the 3-d rate of ethanol production for the herbaceous crops with *S. cerevisiae* and the mixed culture. Here, we observed near completion of fermentations with Weeping lovegrass at the higher enzyme loadings. Table 2 shows Weeping lovegrass to be considerably faster in rate and yield of fermentation over the 3-d period. As would be expected,

Table 1
Summary of Final (8 d) Percent Saccharification Yields for Acid Pretreated Herbaceous Crops with Selected Cellulase and β-Glucosidase Loadings at 45°C[a]

IU β-glucosidase: IU cellulase	0:1				2:1				8:1			
IU cellulase/g cellulose	7	13	19	26	7	13	19	26	7	13	19	26
Weeping lovegrass (*Erogrotis curvula*)	30	47	60	69	37	50	62	71	47	60	68	73
Switchgrass (*Panicum virgatum*)	32	45	53	61	38	46	51	62	47	56	64	70
Sericea lespedeza (*Lespedeza cuneata*)	8	12	15	19	8	12	17	20	9	17	24	39

[a]Saccharification yields are expressed in percents of theoretical conversion of celulose to glucose.

Table 2
Summary of Ethanol Yields in SSF's after 3 d for Pretreated Herbaceous Crops at 37°C for Selected Cellulase and β-Glucosidase Loadings[a]

IU β-glucosidase: IU cellulase	0:1				2:1				8:1			
IU cellulase/g cellulose	7	13	19	26	7	13	19	26	7	13	19	26
Yeast	*S. cerevisiae*											
Weeping lovegrass	37	44	54	63	51	60	65	66	58	67	74	85
Switchgrass	30	41	49	50	49	52	57	62	52	62	66	68
Sericea lespedeza	16	19	22	25	22	25	28	31	23	31	42	44
Yeast	Mixed culture[b]											
Weeping lovegrass	49	56	58	67	58	64	68	72	60	72	80	80
Switchgrass	38	48	52	55	56	57	61	66	55	62	66	74
Sericea lespedeza	22	23	24	25	24	25	31	32	27	41	43	43

[a]Ethanol yields are calculated as percents of theoretical yield of ethanol from cellulose.
[b]Mixed culture = *Saccharomyces cerevisiae* and *Brettanomyces clausenii*.

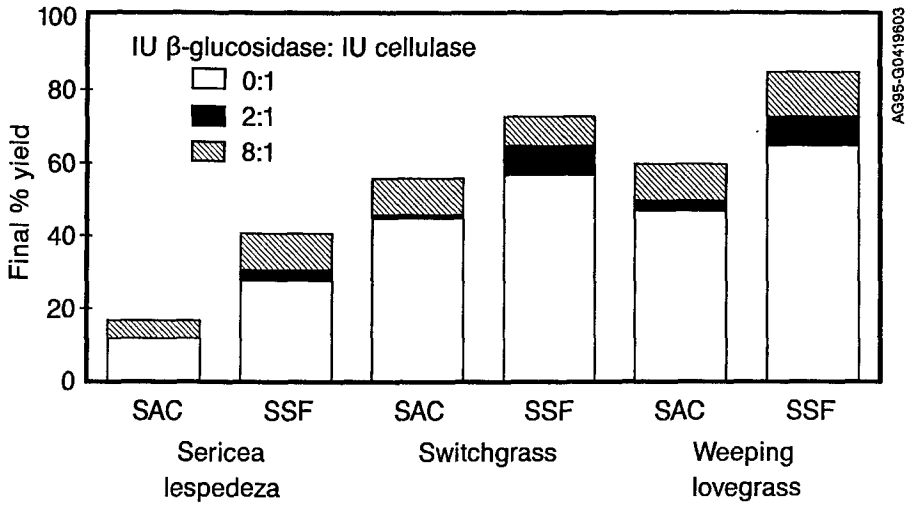

Fig. 1. Comparison of saccharification (SAC) and simultaneous saccharification and fermentation (SSF) of herbaceous crops at 13 IU cellulase/g cellulose (7.5%) and various β-glucosidase loadings. The SSF with the mixed culture of *S. cerevisiae* and *B. clausenii* was performed at 37°C, whereas the SAC was performed at 45°C. The bars indicate the percent final saccharification yields for SAC and final percent of theoretical ethanol yield for SSF.

the mixed culture shows improvement over *S. cerevisiae* in rate of ethanol production, with exception of the highest enzyme loadings.

Figure 1 compares the final SAC sugar yields with SSF ethanol yields at 13 IU cellulase/g cellulose and various β-glucosidase loadings. SSF yields are consistently higher despite being penalized by their small loss of sugars to support yeast growth and maintenance (*see* Methods section). Although the saccharifications were run at 45°C, β-glucosidase ratios are reflective of the activity measured at a temperature of 37°C. Therefore, the β-glucosidase activity in saccharification was higher than indicated for supplementation. This is why we see our greatest difference between SAC and SSF at the zero β-glucosidase addition level. Even with this increased β-glucosidase activity in the SAC, SSF still outperforms at all enzyme loadings and β-glucosidase supplementation.

Table 3 compares the final (8 d) ethanol yields for SSF with different substrates at selected enzyme loadings and yeast combinations run in 250 mL shaker flasks. Just as for the straight saccharification, the rates and yields are consistently higher for Weeping lovegrass and Switchgrass compared to the legume, Sericea lespedeza.

Figure 2 presents the ethanol yields for the highest and lowest enzyme loadings for Weeping lovegrass with both *S. cerevisiae* and the mixed culture. At the low enzyme loading of 7 IU cellulase/g cellulose without β-glucosidase, we see that the mixed culture provides better conversion of cellulose to ethanol. At the highest enzyme loading of 26 IU cellulase/g cellulose with 8:1 IU β-glucosidase:IU cellulase, the results are very close,

Table 3
Summary of Final Ethanol Yields (8 d) in SSF's for Pretreated Herbaceous Crops
Run at 37°C for Selected Cellulase and β-Glucosidase Loadings[a]

IU β-glucosidase: IU cellulase	0:1				2:1				8:1			
IU cellulase/g cellulose	7	13	19	26	7	13	19	26	7	13	19	26
Yeast	*S. cerevisiae*											
Weeping lovegrass	47	57	64	75	56	67	72	74	63	71	85	89
Switchgrass	41	52	59	61	54	58	63	68	56	70	81	84
Sericea lespedeza	18	22	27	30	26	29	34	36	28	33	44	52
Yeast	Mixed culture[b]											
Weeping lovegrass	62	65	70	77	67	73	81	82	71	85	88	86
Switchgrass	49	57	63	67	61	65	69	76	62	73	83	87
Sericea lespedeza	25	28	33	33	28	31	39	46	29	41	49	50

[a]Ethanol yields are calculated as percents of theoretical yield of ethanol from cellulose.
[b]Mixed culture = *Saccharomyces cerevisiae* and *Brettanomyces clausenii*.

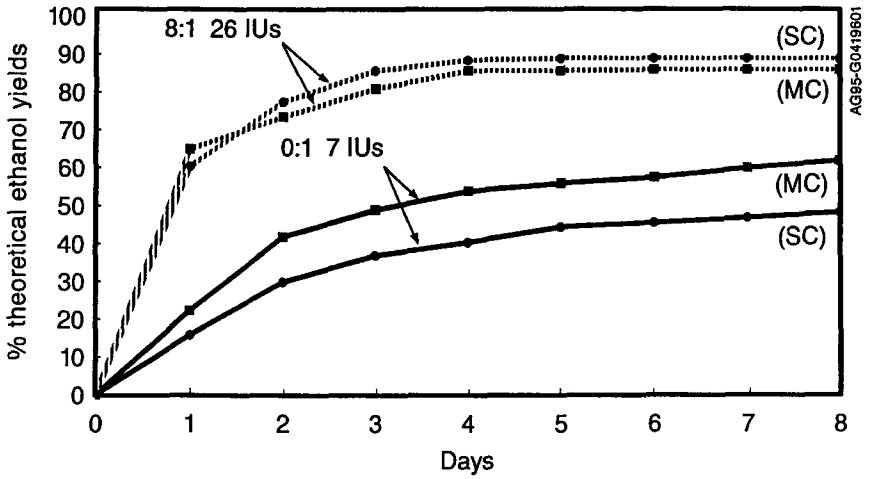

Fig. 2. Percent theoretical ethanol yields with *S. cerevisiae* alone (SC) and in mixed culture (MC) with *B. clausenii* at 37°C for 7.5% cellulose from Weeping lovegrass at the lowest enzyme loading without β-glucosidase and at the highest enzyme loading with an 8:1 ratio of β-glucosidase to cellulase (IU/IU).

with the single culture of *S. cerevisiae* giving a slightly higher ethanol yield. The other interesting result in this figure is that the rate is slower (≈8 d) for the lower enzyme loadings, whereas only 4–5 d are required to reach final ethanol yields at the higher enzyme loadings.

Figure 3 presents the concentrations measured for a detailed SSF fermentation conducted with *S. cerevisiae* in triplicate. A cellulase loading of 26 IU/g cellulose were used along with an 8:1 ratio of β-glucosidase supplementation. The ethanol concentration continually increased as cellulose concentration dropped, whereas glucose initially increased to about 20 g/L and then quickly dropped to less than 1 g/L. Both the ethanol yield and cellulose used correlate closely although the cellulose consumption is slightly higher owing to an assumed 5% loss of substrate to cell growth. The cell viability first increases and the declines from 5×10^7 to 2.5×10^7 CFUs. It is worth noting that *S. cerevisiae* shows sustained viability throughout the fermentation. The ethanol yields for the detailed fermentations correspond closely to the results shown in the shaker flask SSFs of Tables 2 and 3. Once again, *S. cerevisiae* exhibits a high rate of conversion at 26 IU cellulase/g cellulose suppmented with a 8:1 β-glucosidase-to-cellulase ratio. Similar results would be expected with the mixed culture.

CONCLUSIONS

Without β-glucosidase supplementation, the overall rates and yields were better for the mixed culture than *S. cerevisiae* owing to the additional β-glucosidase activity associated with *B. clausenii*. However, *S. cerevisiae* performed slightly better with substantial β-glucosidase addition, appar-

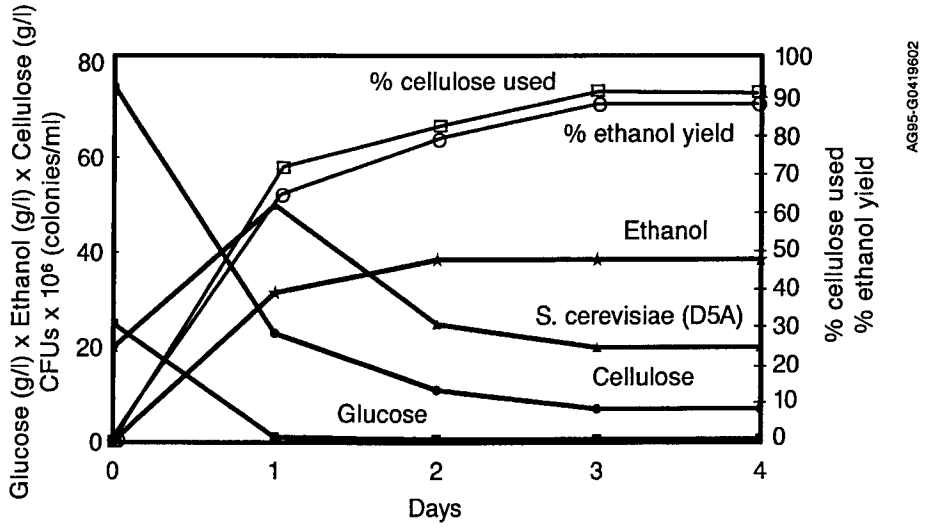

Fig. 3. Detailed fermentation study with Weeping lovegrass at an enzyme loading of 26 IU cellulase/g cellulose and an 8:1 ratio β-glucosidase to cellulase (IU/IU). SSF was performed at 37°C with *S. cerevisiae* and 7.5% cellulose.

ently owing to the higher ethanol tolerance. These results point out the necessity to provide high β-glucosidase levels to prevent accumulation of the powerful inhibitor cellobiose. Thus, the use of mixed cultures of a cellobiose-fermenting organism with an ethanol-tolerant strain or supplementation of the enzyme broth with β-glucosidase will continue to be desirable to obtain high yields until a cellulase enzyme is developed that has higher β-glucosidase activity.

From our data, we can conclude that Weeping lovegrass and Switchgrass respond well to dilute acid pretreatment, show fast enzyme hydrolysis rates, and give high ethanol yields through fermentation by selected yeast. The legume, Sericea lespedeza, did not respond as well to the dilute acid pretreatment conditions used for the other two grasses, and the conversion of cellulose to sugars or ethanol was much less for this substrate. However, it may be possible to increase the yields by selection of a different pretreatment condition.

ACKNOWLEDGMENTS

We would like to acknowledge the Oak Ridge Biomass Production Program and D. Parrish, P. Sullivan, and S. Nagle from the Virginia Polytechnic Institute and State University for providing us with the herbaceous crops. Also thanks to R. Torget and P. Werdene from the Solar Energy Research Institute for their dilute acid pretreatment work with these crops. This resarch was supported by funds from the Ethanol from Biomass Program of the DOE Division of Biofuels and Municipal Waste Technology Division.

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