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Pilot-Scale Fermentation of Aqueous-Ammonia-Soaked Switchgrass

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Abstract Aqueous-ammonia-steeped switchgrass was subject to simultaneous saccharification and fermentation (SSF) in two pilot-scale bioreactors (50- and 350-L working volume). Switchgrass was pretreated by soaking in ammonium hydroxide (30%) with solid to liquid ratio of 5 L ammonium hydroxide per kilogram dry switchgrass for 5 days in 75-L steeping vessels without agitation at ambient temperatures (15 to 33 °C). SSF of the pretreated biomass was carried out using *Saccharomyces cerevisiae* (D_5A) at approximately 2% glucan and 77 filter paper units per gram cellulose enzyme loading (Spezyme CP). The 50-L fermentation was carried out aseptically, whereas the 350-L fermentation was semiaseptic. The percentage of maximum theoretical ethanol yields achieved was 73% in the 50-L reactor and 52–74% in the 350-L reactor due to the difference in asepsis. The 350-L fermentation was contaminated by acid-producing bacteria (lactic and acetic acid concentrations approaching 10 g/L), and this resulted in lower ethanol production. Despite this problem, the pilot-scale SSF of aqueous-ammonia-pretreated switchgrass has shown promising results similar to laboratory-scale experiments. This work demonstrates challenges in pilot-scale fermentations with material handling, aseptic conditions, and bacterial contamination for cellulosic fermentations to biofuels.

Keywords Switchgrass · Pilot scale · Aqueous ammonia soaking · Ethanol production · Simultaneous saccharification and fermentation (SSF)

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Introduction

Recovery of nutrients from biorefineries and recycling them back to crop fields can significantly improve the sustainability of biofuel production and can improve the overall energy balance of cellulosic ethanol systems [1]. Advanced biorefinery designs usually integrate biological and thermochemical processes in which the unfermented portion of the biomass is thermally converted to produce additional fuels as well as heat and energy to drive the conversion processes. In theory, nutrient cycles can be closed by capturing the plant nutrients that are concentrated in ash and as gaseous ammonia produced during thermal conversion of the fermentation residue and by recycling them to crop production fields where feedstock are grown [1]. To test this theory, quantities of fermentation residue sufficient to feed a pilot-scale gasifier were required. The target gasifier located at Iowa State University requires approximately 10 kg of dry fermentation residue to achieve the steady-state operation required for consistent data generation [2]. Thus, one of the objectives of this study was to develop pilot-scale fermentation protocols that would generate a sufficient amount of fermentation residue for future gasification studies.

Pilot-scale fermentation experiments using steam-exploded aspen and corn fiber have been reported previously in the literature [3–5]. De Bari et al. [3] reported achieving 79% of theoretical ethanol yields from steam-exploded aspen in helical-stirred 10- and 50-L pilot-scale bioreactors. Schell et al. [4] described an ethanol plant design which can continuously process a lignocellulosic feedstock at a rate of 900 kg/day (dry weight) and they evaluated the equipment operation in the ethanol plant and generated performance data using dilute-acid-treated corn fiber. The authors have also discussed significant operational problems such as settling of solids during fermentation, difficulty in mixing, and bacterial contamination. In their second study, the authors [5] presented information on subsequent fermentation experiments and identified the primary source of contaminating microorganisms as *Lactobacillus* bacteria in the main fermentors. These papers provided valuable information on pilot-scale ethanol production from lignocellulosics; however, to our knowledge, there are no articles in the literature on pilot-scale ethanol production from dedicated energy crops such as switchgrass or pilot-scale fermentation studies using aqueous ammonia pretreatment.

Ethanol production from switchgrass has been the focus of different studies [6–10]; however, all of these studies were performed at the bench scale. Therefore, the second objective of the study was to show that our previously proposed bench-scale simultaneous saccharification and fermentation procedure [6] can be successfully scaled up.

Materials and Methods

Switchgrass samples were collected from mature stands of the Cave-in-Rock cultivar while dormant (early spring) in Chariton, IA. Dry switchgrass was ground to an average size of 5–6 mm in a tub grinder by the Biomass Energy Conversion Center, Nevada, IA, USA. The compositions of the switchgrass (before and after pretreatment) were determined by Iowa State University, Department of Agronomy using the ANKOM method (ANKOM Technol. Corp., Fairport, NY, USA) as described by Vogel et al. [11]. Untreated switchgrass (starting material; dry weight bases) contained 32% cellulose, 31% hemicellulose, 4.4% acid detergent lignin, 27% Klason lignin and 0.7% ash. Klason lignin values were determined as explained by Isci et al. [6].

Cellulase enzyme (Spezyme CP, lot no:301-05330-206) was provided by Genencor International (Palo Alto, CA, USA) and had an activity of 60 filter paper units (FPU) per milliliter, measured using standard procedures [12]. The yeast (*Saccharomyces cerevisiae* D_5A) was supplied by National Renewable Energy Laboratory and preserved at 4 °C after freeze drying with 20% nonfat dry milk.

Soaking in ammonium hydroxide, which was first studied by Kim and Lee [13] as a biomass pretreatment method, was performed to enhance subsequent enzymatic hydrolysis of switchgrass. It has been shown at the laboratory scale [6] that the process partially removes lignin and hemicellulose, while preserving cellulose fraction of the biomass. To test the performance of the pilot-scale pretreatment and fermentation, 4 kg of dry switchgrass was soaked in 20 L reagent-grade (29.5 wt.%) ammonium hydroxide (Fisher Scientific Inc., Hanover Park, IL, USA) for 5 days in 75-L vessels at the Iowa State University Livestock Environment Building and Research Center (LEBRC) near Boone, IA, USA, during the summer of 2007 [14]. The soaking ratio of 5 L aqueous ammonium hydroxide per kilogram dry switchgrass was selected based on bench-scale experiments [6]. The vessels were operated without any agitation at ambient temperatures (15 to 33 °C). The ammonia-soaked switchgrass from this first trial was used in the 50-L fermentation immediately after the soaking period. The design and performance of the pretreatment vessels have been described in detail elsewhere [14]. From the subsequent soakings, approximately 80 kg of wet aqueous-ammonia-soaked switchgrass was generated and stored at −20 °C for the 350-L fermentation and thawed during the 3 days before the fermentation. Pilot-scale simultaneous saccharification and fermentation (SSF) was performed following established procedures [15].

Fermentation Design

Extrapolating bench-scale experiments suggested that approximately 12–20 kg of ammonia-pretreated switchgrass (60–100-kg wet switchgrass) would need to be fermented to produce the 6–10 kg of residual. One steeping vessel run generated sufficient pretreated switchgrass for the 50-L fermentor, which allowed to directly scale-up to the 50-L fermentation. Performing a 50-L fermentation seven times with pretreated fresh switchgrass was considered; however, in order to produce a homogeneous residue, gain experience at larger scales, and save time, we chose to work at 350 L. The size of pretreatment vessel used required that pretreated switchgrass be stored by freezing until sufficient material could be generated to perform the 350-L fermentation.

The 50-L Fermentation

Approximately 13.3 kg of wet switchgrass (80% moisture content) was generated from an initial trial of pilot-scale ammonia soaking, which contained 48% cellulose, 23% hemicellulose, and 22% Klason Lignin. The average solid content of the wet switchgrass was determined by drying six switchgrass samples (20 g each) taken from different locations of the pretreatment vessel at 60 °C for 3 days.

A 50-L steam-jacketed fermentor (Fig. 1, Biostat U-50, B. Braun Biotech (Sartorius), Allentown, PA, USA) was loaded with 13.3-kg wet switchgrass (which corresponds to 2.4% (*w*/*v*) cellulose concentration), 1% (*w*/*v*) yeast extract (Ardamine Z, Indianapolis, IN,

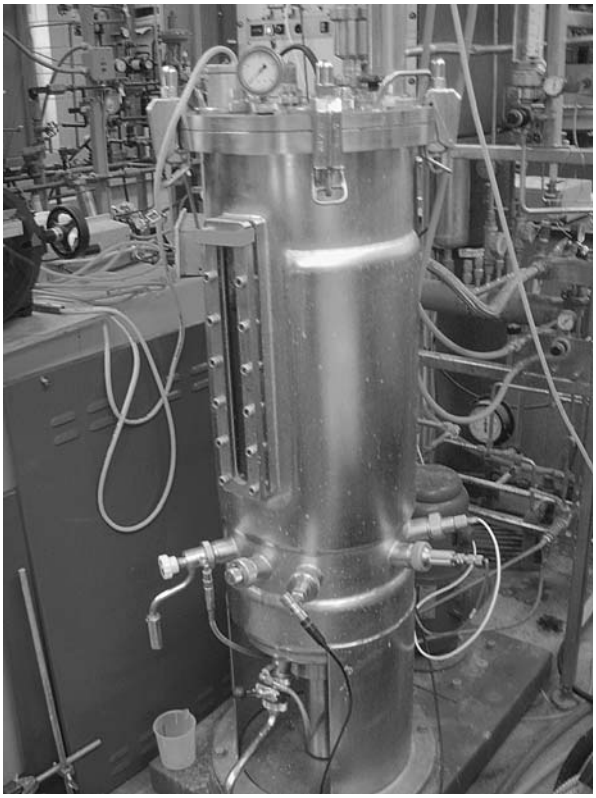

Fig. 1 Biostat U-50, 50-L fermentor

USA), 2% (*w*/*v*) peptone (Difco Laboratory, Detroit, MI), 0.05-M citrate buffer (pH 4.8), and deionized water added to make a working volume of 50-L then sterilized at 121 °C for 20 min. The 50-L fermentor was equipped with three Rushton-type impellors, which operated at 130 rpm during fermentation. Once the fermentation media cooled down to 35 °C, the inoculum and enzyme (77 FPU/g cellulose) was added aseptically. The 1-L *S. cerevisiae* D_5A inoculum was prepared in 2-L shake flasks with 1% (*w*/*v*) yeast extract (Ardamine Z), 2% (*w*/*v*) peptone (Difco Laboratory), and 5% (*w*/*v*) dextrose (Fisher Scientific Inc.) at 35 °C with shaking at 170 rpm for 24 h and was inoculated with one freeze-dried culture vial (2×10^9 cells per milliliter).

The 350-L Fermentation

Approximately 80 kg of wet switchgrass (80% moisture content) was generated by ammonia steeping in 75-L vessels by steeping for 5 days at a liquid to solid ratio of 5 L aqueous ammonia per kilogram dry switchgrass [14]. The pretreated switchgrass contained 45% cellulose, 23% hemicellulose, and 23.5% Klason lignin. The solid content and fiber content of the pretreated material were determined as explained above.

A 350-L fermentor (Fig. 2, Model PTT, Walker Stainless Equipment Co., New Lisbon, WI, USA) was loaded with approximately 80 kg of thawed wet switchgrass with a moisture content of 80% (~2% *w*/*v* cellulose), 1% (*w*/*v*) yeast extract, 2% (*w*/*v*) peptone, and 0.05-M

Fig. 2 350-L fermentor, Model PTT

citrate buffer (pH 4.8). A semiaseptic method was used, such that first yeast extract, peptone, water, and buffer were sterilized in the tank using steam jackets and then unsterilized switchgrass was added incrementally over 24 h. Specifically, approximately one third of the switchgrass (~27 kg) was added at times 0, 5, and 24 h, which allowed substrate thinning via the cellulase (77 FPU/g cellulose). Incremental addition of wet switchgrass was done because the material was dense and clumpy, and we risked damaging the reactor impeller if all biomass were added at once. The single 30-cm-diameter, three-blade axial flow impeller was operated at 200 rpm throughout the SSF process.

The 10-L *S. cerevisiae* D_5A inoculum was prepared in 20-L fermentor (Bioflo 500, New Brunswick Scientific, Edison, NJ, USA) with 1% (*w*/*v*) yeast extract (Ardamine Z), 2% (*w*/*v*) peptone (Difco Laboratories), and 5% (*w*/*v*) dextrose (Fisher Scientific Inc.) at 35 °C for 24 h with 250 rpm agitation and 5–10 L/min air flow. The inoculum was aseptically transferred into 350-L fermentor at time 0. Enzyme (77-FPU/g cellulose) was added at the same time from the top of the fermentor using sterile containers based on the final concentration of treated switchgrass.

Theoretical ethanol yields were calculated as follows based on the maximum (51%) conversion of glucose into ethanol by yeast [6].

$$\text{Theoretical ethanol yield}\,(\%) = \frac{\text{Ethanol produced}\,(\text{g})\,\text{in reactor}}{\text{Initial sugar}\,(\text{cellulose})\,(\text{g})\,\text{in reactor} \times 0.511} \times 100$$

At the end of each fermentation, the biomass fermentation residue remaining in the fermentors was pumped into containers and screened through 2-mm fiberglass mesh (charcoal fiberglass, New York Wire) and the captured solids were dried at 60 °C for 3 days.

Analytical Procedure

Samples were taken at times 0, 4, 8, 24, 48, and 72 h from 50-L fermentation and at times 0, 5, 24, 28, 48, 72, 96, 120 h from 350-L fermentation. The samples were analyzed for sugars (cellobiose, glucose, and xylose), ethanol, and organic acids by high-performance liquid chromatography (Varian ProStar 210) with a refractive index detector (Varian 355 RI). A MetaCarb 87P column (water as a mobile phase, flow rate of 0.4 mL/min, column temperature of 80 °C, and injection volume of 20 μL) was used for sugar analysis, while a Bio-Rad 87H column (0.01 N sulfuric acid as a mobile phase, flow rate 0.6 mL/min, column temperature 65 °C, and injection volume of 20 μL) was used for determination of ethanol and organic acid concentrations.

Results and Discussion

In our previous study [6], we showed that ammonia soaking at room temperature is an effective method for removing Klason lignin while conserving the cellulose fraction and enhancing the subsequent SSF of switchgrass at laboratory scale. Therefore, ammonia soaking at room temperature was also selected as the pretreatment method for these pilot-scale fermentation experiments. Approximately 30–35% weight loss was observed from the initial pilot-scale ammonia soaking of switchgrass. The details of the compositional changes of switchgrass have been reported elsewhere [14]. The focus of the current paper was to generate large amounts of biomass fermentation residue sufficient for gasification and to demonstrate that SSF of ammonia-treated switchgrass is feasible at pilot scale under nonaseptic conditions.

It was also reported earlier that at higher enzyme loadings ethanol production was not greatly influenced by pretreatment intensity [6]. Therefore, the experiments reported in this paper used relatively high enzyme loading (77-FPU/g cellulose).

Figure 3 shows sugar and ethanol concentrations over time for the 50-L fermentation. A set of standard SSF trajectories similar to those previously obtained at laboratory scale [6] were observed. The ethanol concentration increased slowly during the first 24 h of fermentation, with a more rapid increase between 24 and 48 h. A rapid decrease in glucose concentration was simultaneously observed, indicating that yeast cells began to utilize glucose and convert it into ethanol effectively after 24 h. In a laboratory-scale fermentation performed under similar conditions [6], the ethanol concentration reached its peak at 24 h when sugar was depleted and remained constant thereafter. The slower response observed at pilot scale could be due to scale-up-induced changes in mixing, shear forces, and mass transfer and/or it might reflect the lower inoculum concentrations used in the 50-L fermentation. In bench scale, freeze-dried inoculum was used and each fermentation flask (100-mL working volume) contained approximately 2×10^7 cells. On the other hand, the inoculum of the 50-L fermentation had an absorbance at 620 nm of 0.5, which corresponds to a cell concentration of 0.23 g/L (dry weight) [16]. It might have taken longer for the number of cells to reach a level where glucose consumption equaled or exceeded the rate of

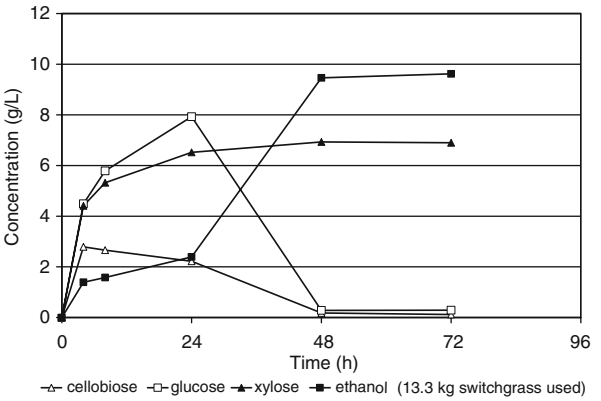

Fig. 3 Time courses of sugars and ethanol concentrations for 50-L SSF of ammonium-hydroxide-steeped switchgrass (5 L/kg ammonia soaking for 5 days, 2.4% (*w*/*v*) cellulose, and 77-FPU/g cellulose)

enzymatic hydrolysis. De Bari et al. [3] have reported that doubling inoculum yeast concentration from 3 to 6 g/L produced nearly identical ethanol concentrations after 48 h in pilot-scale fermentations.

The theoretical ethanol yield of the 50-L fermentation was 73%, which was similar to the results (60–72%) obtained from laboratory-scale fermentations [6]. No lactic acid and acetic acid production was observed throughout the 50-L fermentation which indicates that a successful sterilization was achieved.

Xylose concentrations (7 g/L) observed in the 50-L fermentation are similar to those observed in laboratory-scale fermentations (6.79 g/L in 100-mL fermentation) as well and reflected hemicellulase activity in the cellulase enzyme used. Xylose concentrations at this level could possibly inhibit ethanol production [13].

Sugar, ethanol, and organic acid concentrations over time in the 350-L fermentation are presented in Fig. 4. The rate of ethanol production was observed to fluctuate significantly over the first 48 h, which corresponded to the incremental addition of pretreated switchgrass into the reactor. The ethanol concentration at 72 h was approximately 25% lower than that of the 50-L fermentation. This was likely due to bacterial contamination by heterofermentative lactic acid bacteria producing lactic acid and acetic acid, as evidenced by a continuous increase in both acid concentrations beginning at 24 h. The lactic acid and acetic acid concentrations reached to 9.75 and 6.85 g/L, respectively, at 120 h; these concentrations are reportedly inhibitory to yeast growth and ethanol production [17]. Schell

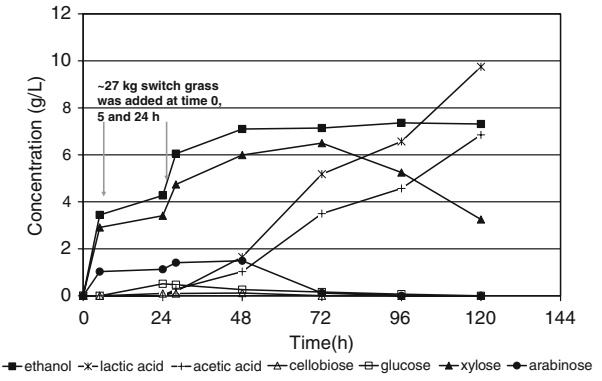

Fig. 4 Time courses of sugars, ethanol, and acid concentrations for the 350-L SSF of ammonium-hydroxide-steeped switchgrass. The total amount of wet switchgrass loaded was approximately 80 kg (5 L/kg ammonia soaking for 5 days, ~2% (*w*/*v*) cellulose, and 77-FPU/g cellulose)

et al. [4, 5] also reported decrease in ethanol production due to inhibitory effect of the relatively high organic acid concentrations on yeast performance.

Unlike 50-L fermentation, cellobiose and glucose concentrations never exceeded 0.5 g/L throughout the fermentation, which signified that as soon as cellulose was hydrolyzed into glucose it was being consumed by yeast and/or contaminating bacteria. Arabinose was completely utilized by bacteria after 48 h. Schell et al. [5] have also identified the contaminating microorganisms as different stains of *Lactobacillus* in their fermentation broth which consumed arabinose readily. After the depletion of arabinose, xylose concentrations started to decrease as the organic acid concentrations continued to increase which proves that the contaminating bacteria was able to consume different sugars.

A gradual decrease in pH from 5.0 to 4.3 was observed between 48 and 120 h of fermentation due to organic acid production which also was an indication of contamination (no pH control was employed). The corn dry-grind ethanol industry usually observes 2 to 3-g/L lactic acid production in 60 h of fermentation which also indicates lactic acid bacterial growth (Dr. Anthony L. Pometto, personal communication, November 27, 2007). The lactic acid concentration in our experiment was approximately 3 g/L at 60 h, which shows that a similar pattern was being followed. Since it would be extremely difficult to produce lignocellulosic ethanol aseptically at industrial scale, ways to keep contamination levels at minimum must be determined in detail before scaling up. In industrial fermentations, the most common method to control contaminations is based on the antibiotics virginiamycin and penicillin [18, 19]. Schell et al. [5] have also showed that the lactic acid bacteria contaminations can be controlled by antibiotic virginiamycin in industrial-scale lignocellulosic fermentations. In addition, modified microorganisms that can ferment all of the available sugars (xylose, arabinose, etc.) into ethanol could be used to compete with the contaminating microorganisms.

The pretreated switchgrass from the first pilot-scale steeping trial was used for the 50-L fermentation which generated approximately 0.8-kg dry switchgrass residue at the end of fermentation. Based on this data, it was decided to repeat the pilot-scale soaking eight times which was expected to generate 6.4-kg fermentation residue. However, because of the problems encountered during this first pilot-scale pretreatment experiment, a design change was made [14]. The redesigned steeping system, however, generated only 4.5-kg dry fermented switchgrass residue after fermentation in the 350-L fermentor. The difference between the amount of residue recovered and the predicted amount of fermentation residue was most likely due to the loss of fine particles during washing of pretreated switchgrass in the redesigned steeping system. It could also be attributed to a technical problem encountered on the first trial of the 350-L fermentation. As a consequence of mixing problems, the pretreated biomass was transferred back to containers from the fermentor before inoculation, which resulted in some loss of switchgrass. It was unknown exactly how much biomass was lost during the transfer. The agitation problem was overcome in the second 350-L fermentation trial by loading the fermentor with water initially and then gradually adding the switchgrass. Schell et al. [4] have also reported mixing difficulties in the first run of a 9,000-L fermentor due to settling of solids. For their second run, the authors started the fermentor with sterile water which thinned the broth enough to allow adequate mixing.

Due to uncertain loss of biomass in the 350-L fermentation as mentioned above, estimates have been made of the possible theoretical ethanol yields achieved (Table 1).

The lower limit (conservative case) was calculated assuming the same amount of biomass was recovered from the redesigned pilot-scale soaking vessels as in the first

Table 1 Mass flow of the process including theoretical ethanol yield estimations (all of the values are presented on dry basis).

	50 L	Conservative case 350 L	Medium case 350 L	Best case 350 L
Switchgrass before pretreatment (kg)	4[a]	32[a]	32[a]	32[a]
Switchgrass after pretreatment (kg)	2.7[a]	21[c]	16[c]	15[c]
Cellulose in pretreated switchgrass (kg)[b]	1.3	9.6	7.2	6.8
Ethanol yield (%)[b]	73	52	70	74
Switchgrass left after fermentation (kg)	0.8[a]	4.5[a]	4.5[a]	4.5[a]

[a] Measured values

[b] Calculated values (percent ethanol yields are calculated based on the total cellulose in the fermentor using the formula presented in "Materials and Methods" section)

[c] Estimated values

soaking design trial and there were no biomass loss from the 350-L fermentor. In that case, 21-kg pretreated biomass (2.6-kg dry pretreated switchgrass recovered from first steeping vessel and in total eight 75-L aqueous ammonia pretreatment was performed) should be available for ethanol conversion. Based on this assumption, the theoretical ethanol yield was calculated as 52% for the most conservative case. However, it was clear from lower amount of fermentation residue generation (~4.5 kg) that a significant amount of biomass was lost, which meant a better conversion rate was achieved in reality. Based on the biomass residue recovered from both the 50- and 350-L fermentations, it could be estimated that originally there were 15-kg dry pretreated switchgrass for 350-L fermentation, which corresponds to a theoretical ethanol yield of 74%. If the total biomass loss from the process (including both the pretreatment and fermentation) was assumed to be the half of the initial switchgrass, the theoretical ethanol yield would have been 70%. The last two estimates are more likely to be close to real values, which are also similar to laboratory-scale ethanol yields [6].

Conclusion

Simultaneous saccharification and fermentation of ammonia-soaked switchgrass was scaled up successfully with minor contamination and mixing problems. Better techniques to control the bacterial contamination and to improve mixing of wet biomass in the fermentor need to be studied for further scaling up trials. The theoretical ethanol yields achieved were between 52% and 74% which were similar to laboratory-scale results. Due to loss of biomass during the washing step of the pretreatment and the first trial of the 350-L fermentation, a lower amount of fermentation residue was generated than expected.

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