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The Application of Ultrasound in the Enzymatic Hydrolysis of Switchgrass

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Abstract In a series of experiments, untreated and ammonium hydroxide pretreated Klenow lowland variety switchgrasses are converted to reducing sugars using low-frequency (20 kHz) ultrasound and commercially available cellulase enzyme. Results from experiments using untreated and pretreated switchgrasses with and without ultrasound are presented and discussed. In untreated switchgrass experiments, the combination of ultrasound and enzymes resulted in an increase of 7.5% in reducing sugars compared to experiments using just enzymes. In experiments using ammonium hydroxide pretreated switchgrass, the combination of ultrasound and enzymes resulted in an increase of 9.3% in reducing sugars compared to experiments using just enzymes. Experimental evidence indicates that there is a synergistic effect from the combination of ultrasound and enzymes which lowers the diffusion-limiting barrier to enzyme/substrate binding and results in an increase in reaction rate. Scanning electron microscopic images provide evidence that ultrasound-induced pitting increases substrate surface area and affects reaction rate and yield.

Keywords Enzyme · Ultrasound · Switchgrass · Synergism · Pretreatment · Biofuels

Introduction

As global demand for fossil fuels steadily increases, there is an ever-increasing push for the development of alternative energy sources. The list of promising bioenergy crops encompasses a number of warm seasonal grasses including switchgrass which is considered

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among the most promising due to its perennial growth, high yield in poor-quality soil, ease of cultivation using conventional equipment, and improved soil conservation [1]. Furthermore, switchgrass offers reduced carbon emissions, lower fertilizer and pesticide requirements, and is not a food crop [2]. There is currently a plethora of research projects aimed at obtaining biofuel from switchgrass which have investigated numerous processing conditions including among others ammonia fiber expansion [3], steam explosion [4], dilute acid [5], or alkaline modification [6], all with the purpose of obtaining high yields of fermentable sugars which can be converted to ethanol. Many peer-reviewed articles have reported on efforts to overcome the inherent impediments to switchgrass hydrolysis, namely the rugged plant cell wall structure and crystalline structure of cellulose [7–9].

For the past several years, researchers at the Southern Regional Research Center in New Orleans, Louisiana has been investigating the application of ultrasonic energy as a means of improving the biofuels processing of corn stover, cotton lint, cotton trash, and sugarcane bagasse by enzymatic hydrolysis [10, 11]. This biofuel research is based on the theory that there is a synergistic effect when using a combination of ultrasound and enzymes that lowers the diffusion-limiting barrier between the macroscopic enzyme and the cellulosic substrate. According to the theory, during the collapse of the ultrasound-induced cavitation bubbles, powerful jet streams are created in the liquid media which act as a transport mechanism, lowering the diffusion-limiting barrier surrounding the substrate and allowing for an increase in the rate of enzyme–substrate binding. After enzymatic hydrolysis is complete, the ultrasound aids once again in the removal of hydrolysis products away from the substrate using a similar transport mechanism. There are many review articles published which corroborate the synergistic role of ultrasound in liquid–liquid-phase chemical reactions [12, 13], but the lowering of the diffusion barrier and the increase in the reaction rate also applies to solid–liquid-phase systems as reported for immobilized enzymes, drug delivery, and waste products [14–16]. A schematic diagram representing the effects of ultrasound on the enzymatic hydrolysis of switchgrass is depicted in Fig. 1.

The present paper focuses on the application of ultrasound in the enzymatic hydrolysis of untreated and ammonium hydroxide pretreated switchgrass and seeks to ascertain the nature of the synergistic effect between ultrasound and enzymes which results in the conversion of cellulose-containing switchgrass to reducing sugars. Several experiments were performed and several scanning electron microscopic (SEM) images were taken. The results are presented and discussed herein.

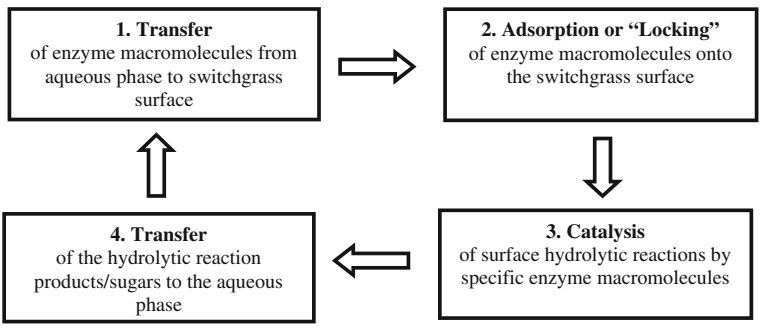

Fig. 1 Schematic diagram of the general stages of an enzymatic reaction on switchgrass

Experimental

Materials The switchgrass used for this study was a Klenow lowland variety harvested post-frost, dried at 50 °C, and ground in a cutting mill to pass through a 2-mm mesh.

Pretreatment Switchgrass (75 g) was mixed with 425 mL ammonium solution (8%, *w*/*v*) in a 316 SS tube reactor. The reactor was heated in a fluidized sand bath to 180 °C and reacted for 20 min before being quenched in an ice water bath. Temperature was monitored by using an inserted thermocouple. The reactor contents were transferred to a Pyrex container and dried at 50–60 °C to evaporate the ammonium. The contents of four such reactions were combined together.

Hexagonal Ultrasound Reactor Manufactured by Advanced Sonics Company, this medium-scale sonication reactor introduces ultrasound energy via six sets of identical transducers attached to the six sides of the hexagonal reaction chamber (volume ~ 4.0 L), thus ensuring uniform and controlled sonication of the sample. Deionized water (3.5 L) containing 1 mL of Triton-X 100 was added to the Hexagonal reaction vessel and degassed for 1 h at 50 °C using 20 kHz ultrasound frequency at 19 A of power. A 0.5-L stainless steel cylinder (63.5 mm diameter; 305 mm height) was then place into the degassed water, and 10 g of untreated or pretreated switchgrass was added, followed by 250 mL of prepared 0.1-M sodium acetate buffer solution. The reaction mixture was further degassed for 20 min at 50 °C using 20 kHz ultrasound frequency at 19 A of power. A 3-mL aliquot was removed at the beginning of the experiment prior to adding the cellulase enzyme, boiled for 6 min, centrifuged for 4 min at 4,000 rpm, decanted, and placed in a refrigerator at 2 °C. Accellerase 1500® (1.0 mL) supplied by Genencor was then added to the reaction vessel, the overhead stirrer was increased to 200 rpm, and the sonication continued at 20 kHz ultrasound frequency at 19 A of power. Aliquots (3 mL) were removed and processed as previously described at intervals of 15, 30, 60, 90, 120, 150, 180, 240, and 300 min. The experimental design is depicted in Fig. 2.

Analytical Methods

Moisture contents were determined by drying samples at 105 °C for 24 h. Samples used for moisture determinations were discarded and not used for further analysis.

Monosaccharides from compositional analysis were measured using high-performance liquid chromatography (HPLC). Samples were analyzed using a SpectraSYSTEM liquid chromatography system (Thermo Electron Corporation, San Jose, CA, USA) equipped with an automatic sampler, column heater, isocratic pump, refractive index detector, and computer-based integrator running Chromquest ver. 2.5 software (Thermo Electron Corporation). Samples were injected (20 μL) onto an organic acid column (Aminex HPX-87H Column, 300×7.8 mm, Bio-Rad Laboratories, Inc.) and eluted with 5 mM sulfuric acid at 0.6 mL/min and 65 °C.

Switchgrass contained the following carbohydrates (monosaccharide conversion): glucose=12.6 g/kg (glucose=12.6 g/kg), sucrose=13.3 g/kg (sucrose=13.3 g/kg), cellulose=354.0 g/kg (glucose=393.33 g/kg), xylan=226.0 g/kg (xylose=256.82 g/kg), arabinan=27.0 g/kg (arabinose=30.68 g/kg), and starch=11.0 g/kg (glucose=12.22 g/kg).

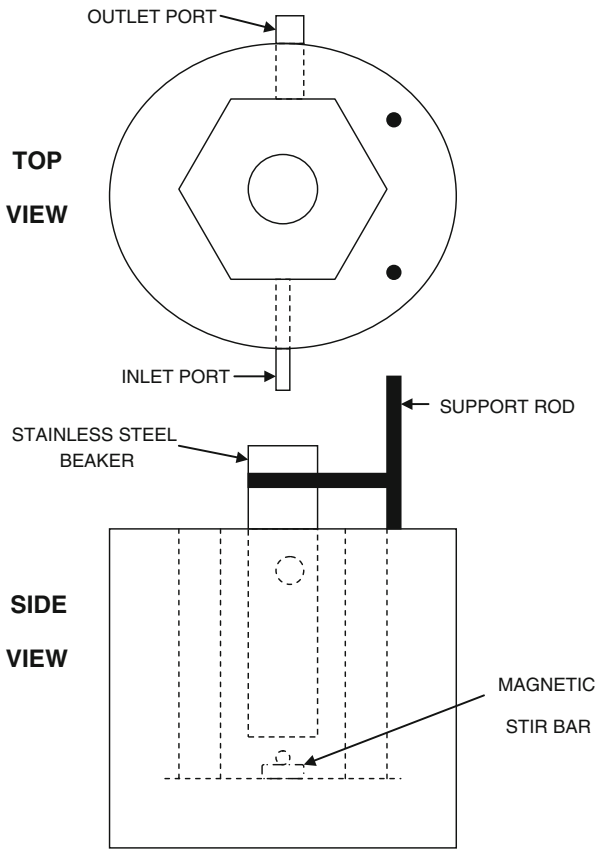

Fig. 2 Experimental design: overhead stirrer not shown

Other measured components were lipids=1.6 g/kg, crude protein=3.7 g/kg, Klasson lignin=187.0 g/kg, and mineral ash=24.1 g/kg. Values are means of replicates.

Starch was estimated following treatment with amylases [17]. Structural carbohydrates (cellulose, xylan, and arabinan), Klasson lignin, and ash were determined according to the analytical procedure of the National Renewable Energy Laboratory [18]. Lipid content was determined by exhaustive extraction with ether using a standard method [19]. Nitrogen content was determined by combustion, and crude protein concentration was estimated as Nx6.25 [20]. Soluble sugars were extracted by combining 200 mg alfalfa with 3.0 mL distilled water in a glass test tube and sonicating in an ultrasound bath for 30 min. Extracted sugars were measured using HPLC.

Structural Analysis of the Switchgrass Surface

SEM was used to observe the microstructure and the surface morphology of the treated and untreated switchgrass samples. The instrument was a Phillips XL 30 ESEM with the acceleration voltage set at 12 keV and a beam current of 0.5 nA. The samples were coated with a gold palladium alloy to provide a 200-Å gold palladium layer of thickness using a vacuum sputter coater. The switchgrass samples were examined at magnifications ranging from ×100 to ×2,500.

Reducing Sugar Analysis

All chemicals were obtained from Sigma-Aldrich Scientific Co.

DNS Reagent Sodium hydroxide (4.0 g, 100 mmol) and 3,5-dinitrosalicylic acid (DNS; 2.13 g, 9.32 mmol) were dissolved in 50 mL of deionized water. Sodium potassium tartrate tetrahydrate (67.1 g, 238 mmol) was dissolved separately in 125 mL of deionized water. The two solutions were combined and heated to dissolve all solids. Deionized water was then added to bring the total volume to 250 mL. The reagent was stored at 4 °C in a dark flask and was allowed to reach room temperature prior to use.

Sodium Acetate Buffer Preparation Acetic acid (12.01 g, 200 mmol) was dissolved in 1.8 L of deionized water and titrated to pH 5.0 with 50% NaOH solution at 25 °C, then diluted to 2 L with deionized water and used at 50 °C.

Dextrose–DNS Standardization One gram of dextrose was dissolved in a 100-mL volumetric flask containing deionized water. Six test tubes containing 0.0–1.0 mL of dextrose solution and 1.0 to 0.0 mL of deionized water were each combined with 1.0 mL of DNS reagent and placed in a boiling bath for six min, then cooled to room temperature in an ice bath. Each test tube was further diluted with 10 mL of deionized water, mixed thoroughly, and analyzed at 540 nm using a Milton Roy Spectronic 21. Absorbance versus concentration was plotted, and the standardized equation of the line (y=3.9073x) was determined according to Beer's law. All calculations of reducing sugar concentration derive from this equation.

Results and Discussion

A series of experiments with and without ultrasound were performed using untreated switchgrass and Accellerase 1500® enzymes, and the results are depicted in Fig. 3. In a comparison of experiments with and without ultrasound (Fig. 3, plots 1 and 3), the simultaneous application of ultrasound and enzymes resulted in an increase in the amount

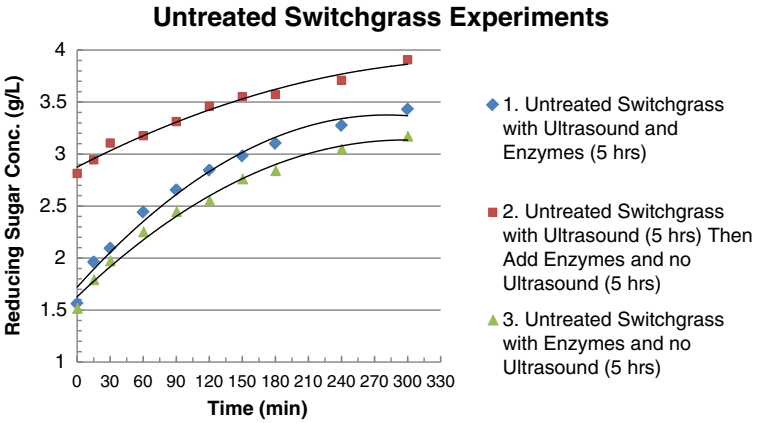

Fig. 3 Untreated switchgrass with/without ultrasound

of reducing sugars ranging from 5.7% to 10.2% at any one time point with an average increase of 7.8% at any one time point. The total yield of reducing sugar obtained after 5 h of simultaneous application of ultrasound and enzymes is 7.5% greater than in experiments without ultrasound (3.4 versus 3.2 g/L). The rate of enzymatic hydrolysis is also greater when ultrasound and enzymes are combined (plot 1) as opposed to when the experiment is performed using enzymes only (Fig. 3, plot 3).

Based upon the standard model of ultrasound, the classic explanation for the increase in the rate of enzymatic hydrolysis is the lowering of the diffusion-limiting barrier surrounding the switchgrass as was described in the introductory section of this paper. If this theory is true, then an experiment which uses ultrasound as a pretreatment method separately from enzymes (Fig. 3, plot 2) should not experience this ultrasound/enzyme synergism, and the rate of enzymatic hydrolysis should be lower. In Fig. 3, plot 2 depicts the data obtained when switchgrass was subjected to ultrasound pretreatment for 5 h, then enzymes were added, and the experiment continued for an additional 5 h without ultrasound. As can be seen from the plotted data, the rate of hydrolysis is lower. This confirms that there is indeed a synergistic effect from the combination of ultrasound and enzymes which results in an increase in the rate of enzymatic hydrolysis. When ultrasound and enzymes are used separately (Fig. 3, plots 2 and 3), the synergistic effect disappears and the rate of enzymatic hydrolysis is lower.

Figure 3, plot 2 reveals that simply by pretreating switchgrass with ultrasound for 5 h *separately* from enzymes, an additional 0.5 g/L (14%) more reducing sugars can be obtained compared to Fig. 3, plot 1 wherein ultrasound and enzymes were combined *simultaneously*. This is important from a processing point of view because this additional yield was produced using the same amount of energy (discounting the energy required to stir the reaction for an additional 5 h). The improvement in yield between the reaction conditions in Fig. 3, plots 2 and 3 was 0.7 g/L (23%), though the energy costs were lower in plot 3 due to the absence of the ultrasound.

In an effort to better understand the above results and the effect of ultrasound on untreated switchgrass, several SEM were taken before and after ultrasound and enzymatic treatments. The images obtained are shown in Fig. 4a–c.

The first SEM image in Fig. 4a is of untreated switchgrass before ultrasound or enzymatic application. Debris ranging in size from 1 to 10 μm, and parallel striations in the fibrous material are visible in the image. There is a slight unevenness in the surface and cracks are apparent. In the SEM image in Fig. 4b, the switchgrass has been subjected to 5 h of ultrasound treatment *with no added enzyme* using the hexagonal reactor (20 kHz, 2,000 W, 19 A). In comparing the images in Fig. 4a, b, the debris has been removed in Fig. 4b, leaving a smooth surface with only trace quantities of particles less than 1 μm in size. Of special interest is the appearance of several perforations in the surface of the untreated switchgrass. These perforations do not appear at regular intervals across all the switchgrass surface and range in size from 1 to 2 μm. This "pitting" phenomena associated with ultrasound is not new [21], but this is the first observation we are aware of that has been reported in relation to switchgrass after the application of ultrasound. Some regions of untreated switchgrass in Fig. 4b are thoroughly perforated while adjacent regions are free of perforations. The explanation for this goes back to the fundamentals of ultrasonic cavitation and the difference between cavitation in a homogeneous and a heterogeneous solution. In a homogeneous solution, there is an even distribution of cavitation spheres throughout the solution due to the uniformity of density. However, in a heterogenous solution, the introduction of particles in solution creates a non-uniform density distribution which causes a localized acoustic streaming of solvent jets in response to the collapse of the cavitation

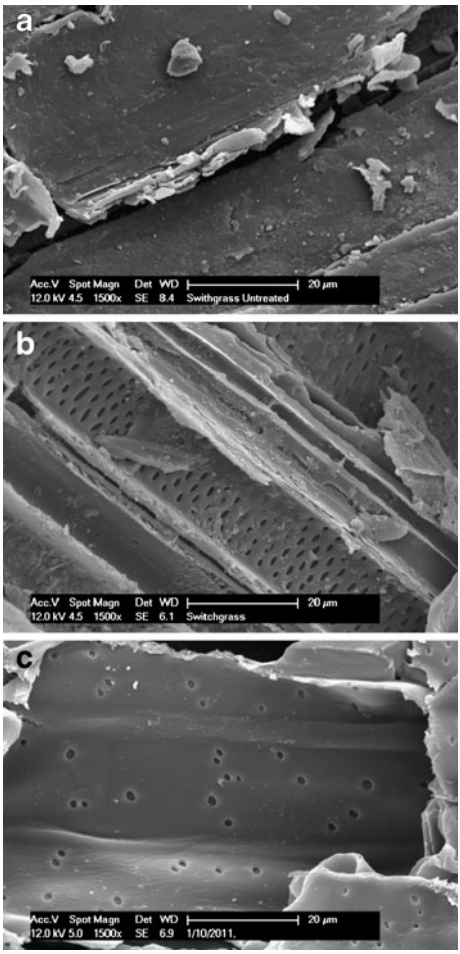

Fig. 4 Untreated switchgrass (×1,500) **a** no ultrasound, no enzyme; (**b**) with ultrasound (20 kHz, 2,000 W, 19 A), no enzyme; and **c** with ultrasound (20 KHz, 2,000 W, 19 A), with enzyme

bubbles. The result is some areas of the switchgrass are heavily perforated while others are left intact. In Fig. 4c, the switchgrass has been subjected to 5 h of *simultaneous* ultrasound and enzymatic processing, and all but the smallest particles have been swept away as was observed in Fig. 4b. Once again, the perforations appear to be 1–2 μm in size and are unevenly dispersed across the surface area of the switchgrass. Based upon these images, it is possible that the perforations in the untreated switchgrass expose greater substrate surface area and offer a partial explanation for the observed rate of enzymatic hydrolysis.

A series of experiments with and without ultrasound were performed using ammonium hydroxide pretreated switchgrass and Accellerase 1500® enzymes. The results are depicted in Fig. 5. In sharp contrast to the series of experiments with untreated switchgrass, there is considerably less amount (0.3–0.4 g/L) of reducing sugar present in pretreated switchgrass at the beginning of the experiments before the addition of enzymes or ultrasound. This highlights one drawback of the ammonium hydroxide pretreatment process, namely the loss of soluble reducing sugars found in untreated switchgrass. This loss amounts to 1.1–1.2 g/L or 13–15% of the total amount of reducing sugars produced in this series of experiments. At the conclusion of this series of experiments, the pretreated switchgrass which was exposed

to ultrasound and enzymes *simultaneously* produced 9.3% more reducing sugars than pretreated switchgrass which was exposed to ultrasound and enzymes *separately*. Once again, as was the case with untreated switchgrass, the combination of ultrasound and enzymes produced a synergistic effect and resulted in the lowering of the diffusion-limiting barrier to enzyme/substrate binding and an increase in the rate of enzyme hydrolysis. Even with the loss of 13–15% of reducing sugars due to the ammonium hydroxide pretreatment process, the experiment using pretreated switchgrass, Accellerase 1500®, and ultrasound (Fig. 5, plot 1) still produced 8.8 g/L of reducing sugar in 5 h. This compares to 3.4 g/L produced in experiments with untreated switchgrass under similar conditions (Fig. 3, plot 1) in which the reducing sugar is not lost. This represents a 256% increase in reducing sugar production. For experiments without ultrasound, 8.0 g/L of reducing sugar was produced by the hydrolysis of pretreated switchgrass, compared to 3.2 g/L of reducing sugar in untreated switchgrass. This amounts to a 251% increase in reducing sugar production when ammonium hydroxide pretreatment is used and ultrasound is not. Thus, even with the 13–15% loss of reducing sugars due to the ammonium hydroxide pretreatment process and when comparing pretreated versus untreated switchgrass in experiments with and without ultrasound, there is a 5% increase in yield when ultrasound is used (256−251=5%).

An experiment was performed using ammonium hydroxide pretreated switchgrass in which the pretreated switchgrass was exposed to 5 h of ultrasound, followed by enzyme addition, and stirred for an additional 5 h without ultrasound (Fig. 5, plot 2). The purpose of this experiment was to investigate the synergistic properties of ultrasound and enzymes as they apply to ammonium hydroxide pretreated switchgrass. Again, according to theory, if there is an ultrasound/enzyme synergism which lowers the diffusion-limiting barrier to enzyme/substrate binding, then the experimental conditions as outlined should result in the loss of a synergistic effect and should result in a lower hydrolysis rate since the ultrasound and enzymes are being used separately. What the experiment shows, however, is that the exact opposite occurs, at least as the initial rate of hydrolysis is concerned. From Fig. 5, it is apparent that the 5 h of ultrasound pretreatment in the absence of enzymes (plot 2) actually resulted in an increase in the initial rate of hydrolysis when compared to when ultrasound and enzymes were combined simultaneously (plot 1). However, after the initial 15 min, the plots of the two reaction conditions gradually merge and the final yield is the same.

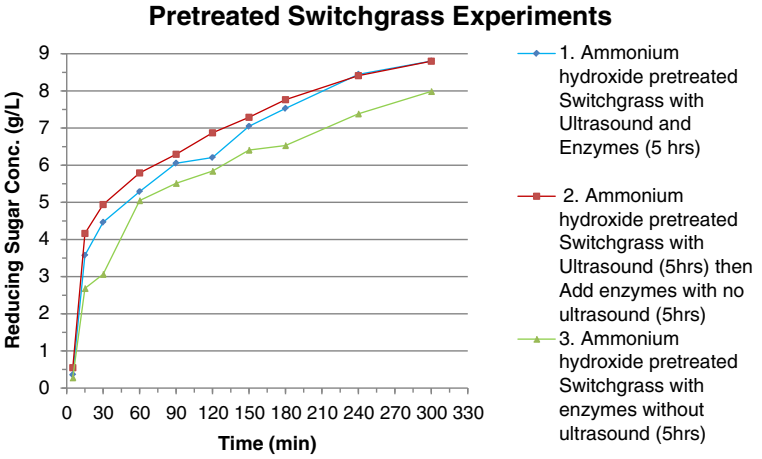

Fig. 5 Ammonium hydroxide pretreated switchgrass

A possible explanation for the above results is that the ammonium hydroxide pretreated switchgrass which was exposed to 5 h of ultrasound prior to enzyme addition had a greater surface area for enzyme binding than the switchgrass which was subjected to ultrasound and enzymes simultaneously. The effect of the ultrasound treatment was to break the switchgrass into smaller and smaller particles, thereby creating greater surface area for enzyme hydrolysis to occur. In this case, the greater surface area initially resulted in a higher rate of enzymatic hydrolysis, but as the two experiments progressed, the exposed surface areas in the two experiments became equal, thus resulting in similar overall reaction rates and final yields. The effects of ultrasound on ammonium hydroxide pretreated switchgrass must therefore be different than its effects on untreated switchgrass, since the application of ultrasound resulted in unique profiles for each.

In an effort to further explain these experimental results, two SEM images (Fig. 6a, b) were taken of ammonium hydroxide pretreated switchgrass. Figure 6a depicts the ammonium hydroxide pretreated switchgrass as it appears prior to exposure to ultrasound and Accellerase 1500® enzymes. The switchgrass material shows the pretreatment effects of exposure to 180 °C temperature and an 8% ammonium hydroxide solution and appears brittle and disrupted with extensive debris. The post-experimental effects of the simultaneous exposure to ultrasound and Accellerase 1500® enzymes on the surface of pretreated switchgrass can be seen in Fig. 6b. Except for small portions on the middle right and on the lower left side of Fig. 6b, the entire surface of the pretreated switchgrass appears dessicated and no longer smooth when compared to Fig. 6a. What is especially interesting is the absence of perforations in the pretreated switchgrass as was observed in untreated switchgrass (Fig. 4c). At this juncture, the explanation for the absence of perforations in ammonium hydroxide pretreated switchgrass is speculative and needs further investigation.

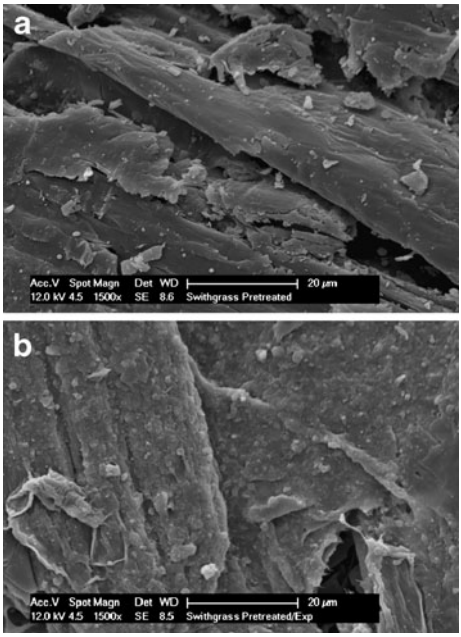

Fig. 6 Ammonium hydroxide pretreated switchgrass (×1,500) **a** no ultrasound, no enzyme and **b** with ultrasound, with enzyme

Conclusion

Under the set of experimental conditions outlined in this paper, there is evidence of a synergistic effect from the combination of ultrasound and enzymes in experiments with untreated or ammonium hydroxide pretreated switchgrass. The synergistic effect results in the lowering of the diffusion-limiting barrier to enzyme/substrate binding and increases the overall reaction rate. While the simultaneous use of ultrasound and enzymes did result in an improved yield of reducing sugars and an increased rate of hydrolysis in both pretreated and untreated switchgrass, based upon SEM images and experimental evidence, this higher yield and increased rate of reaction is due in part to an increase in surface area of the switchgrass caused by the cavitation effects of ultrasound. The experiments performed in this paper indicate that ultrasound pretreatment of switchgrass should be further explored in biofuel processing.

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