Continuous Alkali-Cellulase Processing of Corn Stover to Glucose for Bioethanol

John J. Savarese

Preprocessing of biomass at or near the growing site has considerable advantages over transporting to distant fermentation refineries equipped to process cellulosic material. The alkali-cellulase (Alkcell) process converts biomass to glucose using materials and methods that can be implemented at or near a growing site. This study has shown that the Alkcell process can be configured to run continuously. The use of carriers to contain the biomass allows continuous movement along a treatment train starting with alkali pretreatment and ending with glucose release. Conditions for pretreatment with NaOH, washing, and pH adjustment have been determined. Immersion of the carriers in a cellulase bath at optimal temperature and duration follows. The carriers are then submerged in a large volume of buffer at pH and temperature that allows release of glucose. Finally, the residual solids are returned to the start of the process to be mixed with fresh biomass and the treatment cycle is repeated. The glucose solution can be concentrated locally to reduce volume and enhance transportation savings. The local operation can be done at farms or near regional centers prior to being transported to distant existing conventional fermentation facilities.

Keywords: Biomass conversion; On-farm bioconversion; Continuous Alkcell process; Alkali pretreatment; Cellulase hydrolysis; Glucose transportation; Glucose fermentation

Contact information: MobinolFuel Institute, a division of Mobinol Fuel Co., LLC, 119 Hunt Club Dr., Collegeville, PA 19426 USA; savaresephdmd@mobinolfuel.com

INTRODUCTION

According to experts, "For cellulosic ethanol to become a reality, biotechnological solutions should focus on optimizing the conversion of biomass to sugars" (Lynd *et al.* 2008). The need to process biomass rather than the cost of the biomass itself is a major economic factor in converting cellulosic materials to fuels such as ethanol (Blanch *et al*. 2011). While combining processing steps such as in simultaneous saccharification and fermentation that can improve economics, albeit with substantial technical difficulties, continuous processes for converting biomass to glucose and further to ethanol is perhaps a better cost reducer; however, little work has been done in this regard (Brethauer and Wyman 2010). One continuous biomass fractionation process for producing ethanol as well as other products has been demonstrated in the laboratory, and pilot plant work is ongoing (Kadam *et al.* 2009). However, this process requires sophisticated equipment and extreme operating conditions that can only be managed in a large dedicated bioreactor facility at a distance from growing fields.

Another roadblock to efficient and practical conversion of biomass to ethanol is the cost of transporting the cellulosic material from the farm gate to the refinery gate. It is estimated that a refinery producing 50 million gallons of ethanol annually would need 135 deliveries per day by truck of baled biomass (Yu *et al.* 2012). The social and

environmental impact in the area surrounding the facility and along the transport route due to engine emissions alone would be substantial. Transportation cost is estimated at nearly 40% of the delivered feedstock cost (Haque and Epplin 2012). Upwards of $100 is estimated for one truck transporting biomass a distance of 60 km from farm to refinery (Brechbill *et al.* 2011). An approach to reducing transportation cost could involve preprocessing locally at regional centers, but this is envisioned as being limited to mechanical preparation of the biomass such as cleaning, comminution, and packaging (Larson *et al.* 2010). A feedstock cooperative among farmers could provide economies in use of equipment and provide employment during seasons when the biomass is not grown.

Alkali-cellulose (Alkcell) processing is an approach to solving the transportation problem and the need to have an efficient conversion of biomass to glucose. Previous work has shown the practicality of local processing, which is processing at the growing site or at close regional centers. By such an arrangement, Alkcell processing of biomass can produce glucose that would then be processed at already existing grain fermenting facilities to produce ethanol for fuel. The Alkcell process has been studied at a laboratory scale using batch type operations (Savarese 2013a,b). However, for routine operation a continuous process will be the most efficient and cost effective to produce glucose. At the end of one processing cycle, residual solids would be recycled to yield more glucose. The glucose solution can be concentrated locally using membrane or thermal methods. In a continuous process design, the biomass is carried in containers that move through the stages of the process and then back to the start for recycling of residual solids and loading with fresh biomass.

Reported here is an examination of several parameters crucial to a continuous Alkcell process. Several types of materials were tested as carriers, and their operating performance was evaluated. The initial stage of Alkcell processing is immersion of the biomass containing carrier in an alkali solution for sufficient time to achieve adequate solution of lignin and change in biomass structure to allow subsequent enzyme hydrolysis. Previous work has shown six hours to be adequate for the alkali (NaOH) pretreatment at T = 100 °C (Savarese 2013b). Before enzyme hydrolysis, the alkali treated biomass requires adjustment to pH=4-5, and this must be done with a wash process that allows continuous movement of the biomass carriers.

The carriers are then immersed in a cellulase bath. Minimal cellulase loading that allows sufficient binding to cellulose substrate is a goal that some have begun to study (Klein-Marcuschamer *et al.* 2012). An optimum temperature for adsorption has been explored with conflicting results (Kumar and Wyman 2009). To minimize production of glucose in the enzyme bath, small bath volumes and optimum temperature were evaluated. Also, the amount of cellulase in the enzyme bath was evaluated. These are critical conditions, since the cost of enzyme is a major economic factor (Sathitsuksanoh *et al*. 2013).

While it has been reported that the amount of biomass is not a factor in continuous fermentation of cellulosic biomass to ethanol (South *et al.* 1993), determination of an optimum biomass load would seem critical for an efficient and economical Alkcell operation, and this was examined. Furthermore, the potential to recycle the residual solids to yield additional glucose is possible with the Alkcell process, as previously shown (Savarese 2013a,b).

In the present report further evaluation of the processing of residual solids will be presented. Overall, this report provides new information that further characterizes methods and conditions for a continuous Alkcell process to convert biomass to glucose that can be performed locally or at nearby regional centers.

EXPERIMENTAL

Materials

Corn stover (CS) was used in these experiments since it is readily susceptible to Alkcell processing. The corn stover was harvested locally and shredded coarsely with an electric garden shredder (McCulloch Model MCS1400). To accommodate laboratory glassware, the shredded material was further comminuted with a kitchen blender to centimeter size particles. The cellulase used in these experiments was Accellerase 1500 (AC) supplied by Genencor. AC is composed of endoglucanase (2200-2800 CMC U/g) and beta-glucosidase (450-775 pNPG U/g).

Methods

The continuous Alkcell process was simulated under laboratory conditions as follows. CS was placed in containers of various materials. These biomass carriers were composed of paper, polyester fabric of 0.5 mm porosity, hard plastic cylinders with 1 mm slits around, and fiberglass mesh with 1 mm openings. In all experiments the carriers were immersed in 400 mL tap water in jars containing 1 g NaOH. The jars were placed in boiling water for 6 h. Following this pretreatment the carriers were rinsed in tap water and then immersed in a 100 mL enzyme bath of cellulase for 1 h at specified temperatures. The carriers were then removed, and excess cellulase bath liquid was allowed to drip back into the bath. This typically took several seconds. The carriers were then immersed in 500 mL citrate buffer 30 mM pH = 4.5, T = 60 °C and stirred gently with magnetic stirring bars at lowest setting. This was considered 0 h, and glucose was measured at this time and at 1, 3, and 24 h. Solids remaining after 24 h hydrolysis were dried and weighed to obtain the amount of residual solids.

Glucose was measured with a glucose meter and associated glucose oxidase assay strips (Reveal blood glucose meter). Validation with standard glucose solutions done in citrate buffer pH 4.5 at 40 °C demonstrated the accuracy, precision, and reliability of the method. According to the manufacturer, samples reading LO contained less than 20 mg/dL and were considered undetectable in this study. Samples reading HI, that is, above 600 mg/dL were diluted and rerun. Measurements were done in duplicate. HPLC is typically used in the laboratory to measure sugar levels; however, it is not suited for immediate determination of glucose levels as might be done with an Alkcell operation near a growing site.

A glass thermometer was dipped into the liquid to be assayed. When 40 °C was reached, the tip was touched to the assay strip. This method requires a drop or less of sample and is specific for glucose. It does not test positive for xylose or glucan polymers that may be released into the liquid phase by enzymatic hydrolysis.

RESULTS AND DISCUSSION

Effect of Temperature on Cellulase Adsorption Using Paper Carriers

Paper carriers might be useful because they are easily replaceable and they provide an additional source of cellulose using recycled paper. Using paper carriers, the effect of enzyme bath temperature on adsorption of cellulase to alkali pretreated CS was measured in terms of the amount of glucose produced.

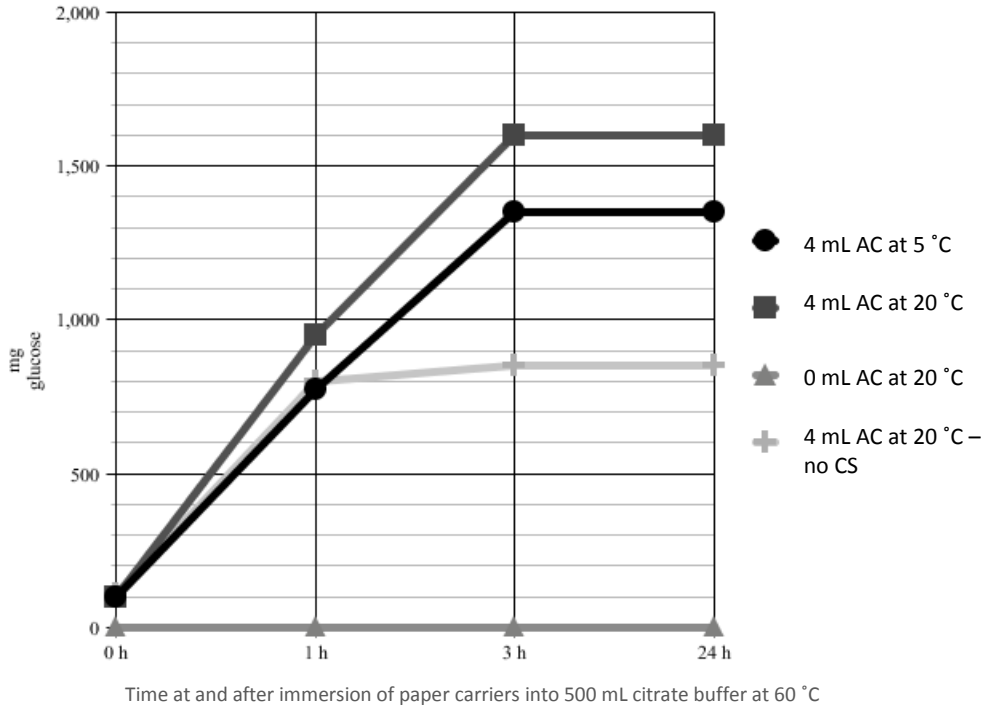

Fig. 1. Time course of total glucose produced from 5 g of NaOH pretreated CS following 1 h exposure to 4 mL AC in 100 mL citrate buffer pH = 4.5 at 5 °C and 20 °C. The CS was contained in paper carriers throughout Alkcell processing. Two controls used no AC or no CS with the paper carriers. Following AC exposure, the carriers were allowed to drip dry and were then immersed in 500 mL citrate buffer beginning at 0 h.

The paper carriers weighed 5 g, as did the CS. In Fig. 1, 0 h was the time of immersion of the carriers into the citrate buffer. Two controls were run, one with no CS in the paper carrier and the other with no enzyme. Without AC, no glucose was produced. With the paper carrier alone and without CS, about half as much glucose was produced following AC at 20 °C compared with CS. The glucose yield following AC at 5 °C was about 15% less than following AC at 20 °C. Also, glucose production peaked at 3 h in each run, confirming previous results that also showed the actual glucose production from CS was 60% of theoretical (Savarese 2013a). Therefore, the overall time required for one cycle of the Alkcell process is 6 h for NaOH pretreatment plus 1 h for AC adsorption plus 3 h for glucose production. The total of 10 h makes a continuous Alkcell process practical for daily operation and one that can be done at or near the farm since the process uses materials and methods easily employed locally.

Effect of Temperature on Cellulase Adsorption Using Polyester and Plastic Carriers

To further evaluate the effect of enzyme bath temperature on adsorption of cellulase to the pretreated CS, the experiment in Fig. 1 was repeated at 5 °C, 10 °C, 20 °C, 40 °C, and 60 °C. Results are shown in Figs. 2a and 2b.

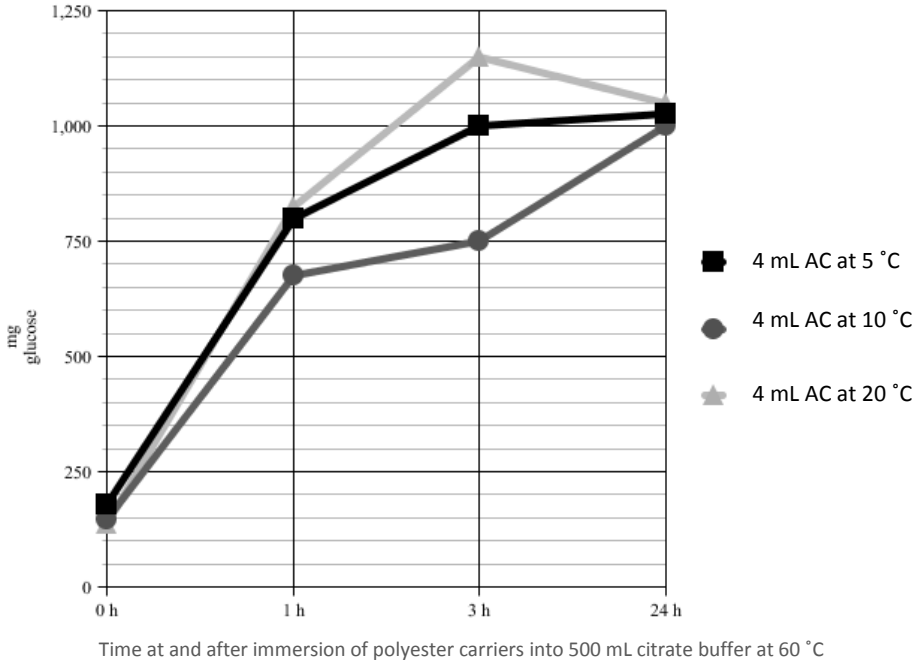

Fig. 2a. Time course of total glucose produced from 5 g of NaOH pretreated CS following 1 h exposure to 4 mL AC in 100 mL citrate buffer pH = 4.5 at 5 °C, 10 °C, and 20 °C. Porous polyester fabric was used as carriers.

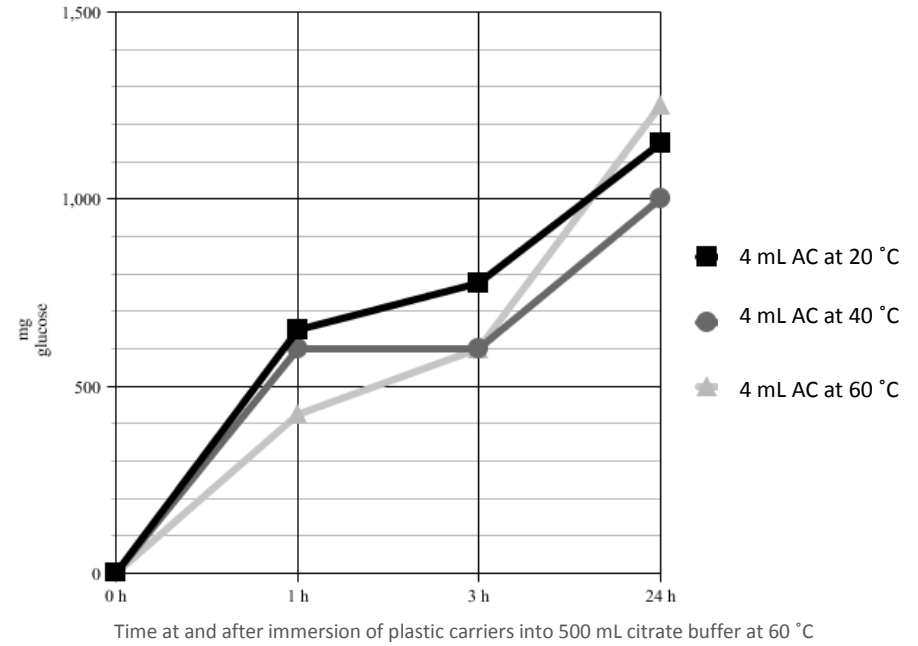

Fig. 2b. Time course of total glucose produced from 5 g of NaOH pretreated CS following 1 h exposure to 4 mL AC in 100 mL citrate buffer pH = 4.5 at 20 °C, 40 °C, and 60 °C. Hard plastic cylinders with slits were used as carriers.

Polyester carriers were used for the Fig. 2a experiments, while hard plastic carriers were used for Fig. 2b results. The polyester material was porous at 0.5 mm. The hard plastic carriers were cylindrical with 1 mm slits around them. The 5 g of CS was loosely accommodated in the polyester fabric while it was closely packed into the plastic carriers. As shown in Fig. 2a, adsorption at 20 °C, as measured by subsequent glucose release during volume expansion, was better at 3 h than with adsorption done at the lower temperatures, although they appeared comparable at 24 h. The glucose yield for 20 °C with the polyester material was somewhat higher than that for the paper carriers shown in Fig. 1 after subtracting glucose produced from the paper carrier. As shown in Fig. 2b, the early time course of glucose production using the plastic carriers was suppressed compared with that shown in Fig. 1 and 2. This was likely due to the dense packing of CS into the plastic carriers that could have initially impeded hydrolysis. However, by 24 h glucose production had increased so that the yield for the 20 °C group in Fig. 2b was comparable to the 20 °C group at 3 h in Fig. 2a. The 60 °C group in Fig. 2b was not much better than the 20 °C group possibly due to release of glucose during enzyme treatment that was lost in the enzyme bath.

Considering the cost of heating to 60 °C compared to enzyme adsorption at 20°C, it appears that enzyme adsorption should be done at room temperature. As shown in Fig. 2a, the temperatures 5 °C and 10 °C for enzyme adsorption appeared inferior at 3 h. However, the lower temperatures might inhibit glucose production in the enzyme bath. As shown in Table 1 this appears to be the case.

Table 1. Amount of Glucose in Cellulase Bath after 1 h Exposure to 5 g Alkali Pretreated Corn Stover (CS) at Three Temperatures from Two Experimental Trials

	mg glucose		
	5°C	10°C	20°C
1st trial	34	--	90
2nd trial	28	34	92

(-- not done)

Given the small amount of glucose lost in the enzyme bath and the cost that would be incurred to achieve the lower temperatures, it appears that enzyme adsorption at room temperature (20 °C) is practical. The flexibility of the polyester carrier is an advantage over the rigid plastic carrier; however, except for the inhibiting effect of close packing found with the rigid plastic carrier, either material could possibly be used as a carrier.

Effect of Cellulase Loading on Cellulase Adsorption Using Fiberglass Carriers

The amount of cellulase in the bath available to adsorb onto the alkali-pretreated biomass is perhaps the most important factor in converting biomass to glucose, considering its cost. To determine a minimum effective load of cellulase, five AC loadings per 5 g alkali pretreated CS were tested using glucose yields as the measure of effectiveness. Fiberglass mesh was used as carrier material in these experiments. Enzyme adsorption was conducted at 20 °C for 1 h, after which the fiberglass carriers were removed, and

when liquid stopped draining they were immersed in 500 mL citrate buffer pH = 4.5 at 0 h. Results are shown in Figs. 3a and 3b.

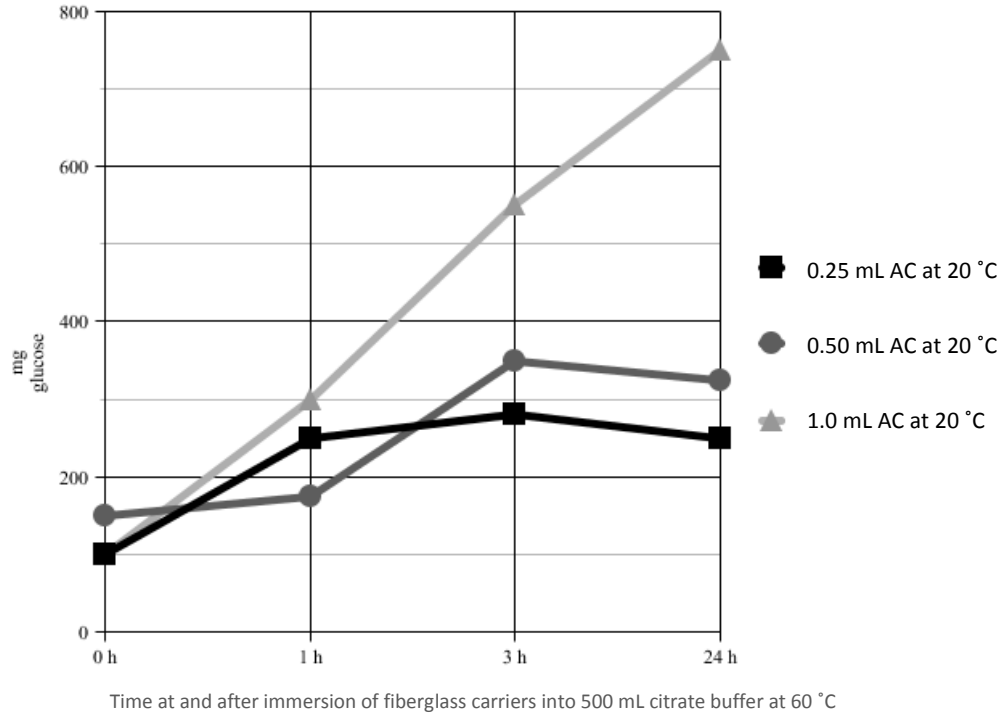

Fig. 3a. Time course of total glucose produced from 5 g of NaOH pretreated CS following 1 h exposure at 20 °C to 0.25 mL, 0.5 mL, and 1.0 mL of AC in 100 mL citrate buffer pH = 4.5. Fiberglass carriers were used.

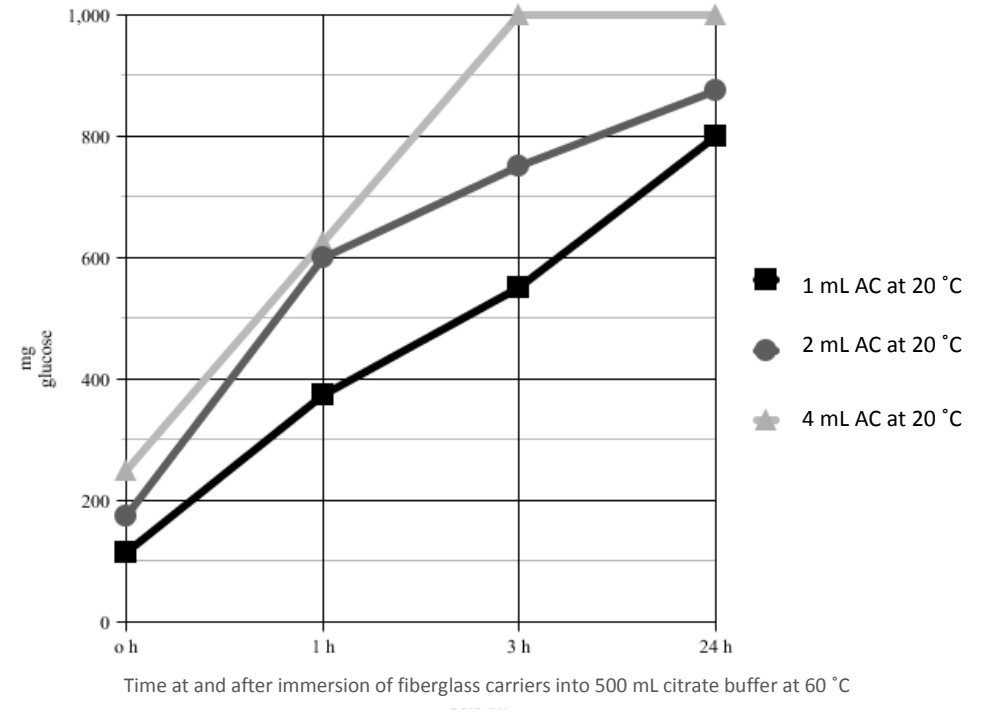

Fig. 3b. Time course of total glucose produced from 5 g of NaOH pretreated CS following 1 h exposure at 20 °C to 1.0 mL, 2.0 mL, and 4.0 mL of AC in 100 mL citrate buffer pH = 4.5. Fiberglass carriers were used.

In Fig. 3a the 1.0 mL loading was superior to the lower amounts. In Fig. 3b the 4.0 mL loading was superior to 1.0 mL and 2.0 mL. However, 2.0 mL appears most practical, considering the amount of enzyme used and the recycling of residual solids to obtain more glucose as will be shown later.

Effect of Biomass Loading on Cellulase Adsorption Using Fiberglass Carriers

In addition to the enzyme load, the biomass load is another critical factor in determining a practical yet economical Alkcell process. To compare with the 5 g CS loading used in Fig. 3b, two identical experiments were conducted using 10 g and 15 g of CS. Results are shown in Fig. 4a and 4b.

At the 10 g and 15 g CS loadings, there was a more rapid hydrolysis, as shown in both figures at 1 h compared with Fig. 3b for 5 g CS. However, the yield of glucose at 3 h and 24 h for the two higher amounts was not appreciably different from that obtained from the 5 g CS loading.

It appears that with more available substrate, the enzyme hydrolysis proceeds more rapidly but the yield is the same. Use of 5 g or 10 g CS per 2 mL AC appears optimal since the additional amount of biomass in the 15 g loading appeared unaffected. This can be seen in Table 2.

Table 2. % Residual Solids (RS) from 1 and 4 mL AC Load Processing of Corn Stover (CS) and from 2nd Processing of the 1st RS from the 15 g CS

Starting CS	10 g	10 g	15 g	15 g
CS AC load	1 mL	4 mL	1 mL	4 mL
% 1st RS of starting CS	22 %	11 %	50 %	41 %
RS AC load	--	--	2 mL	2 mL
% 2nd RS from 1st RS	--	--	53 %	37 %
% 2nd RS from starting CS	--	--	32 %	15 %
Glucose from 2nd RS at 3 h	--	--	700 mg	710 mg

(-- = not measured)

With 15 g of CS, nearly half of the starting CS remained as residual solids (1st RS) compared with less than a quarter of the starting material that remained from the 10 g loading. With the 15 g of CS, a second Alkcell processing again reduced the starting amount by around a half (2nd RS). This was about a fifth of the starting amount of CS. The glucose yield from the second processing showed that repeated cycling of residual solids rather than their disposal is useful. There appears to be no advantage of the extra amount of starting material in a 15 g CS loading compared with 10 g CS.

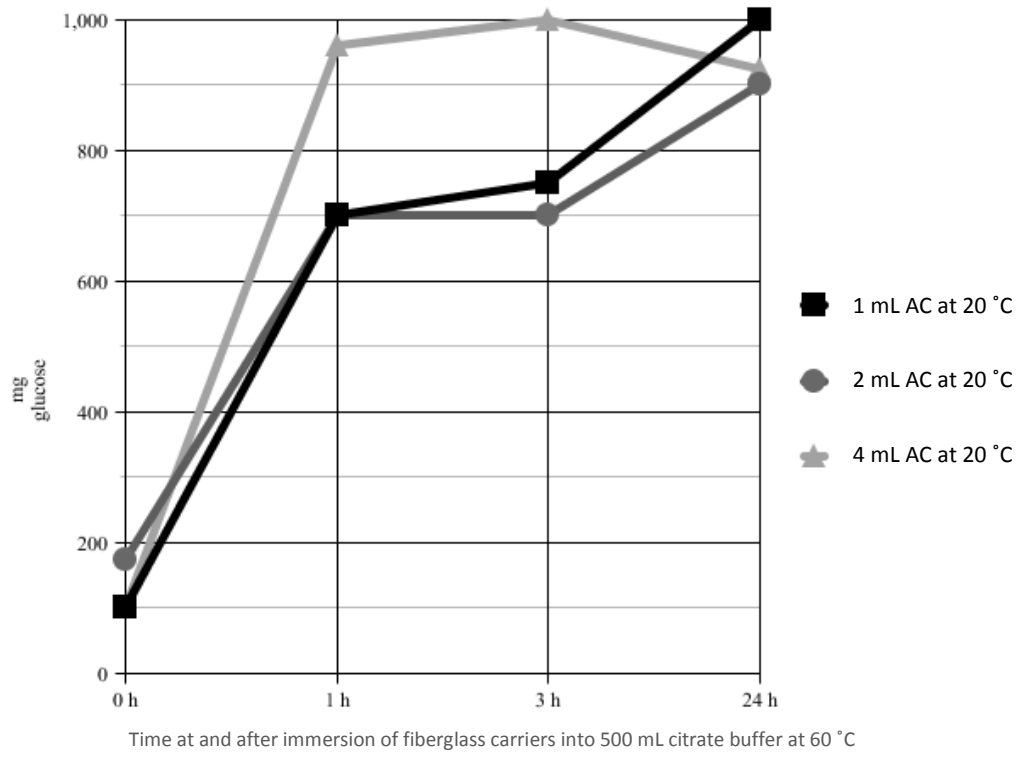

Fig. 4a. Time course of total glucose produced from 10 g of NaOH pretreated CS following 1 h exposure at 20 °C to 1.0 mL, 2.0 mL, and 4.0 mL of AC in 100 mL citrate buffer pH = 4.5. Fiberglass carriers were used.

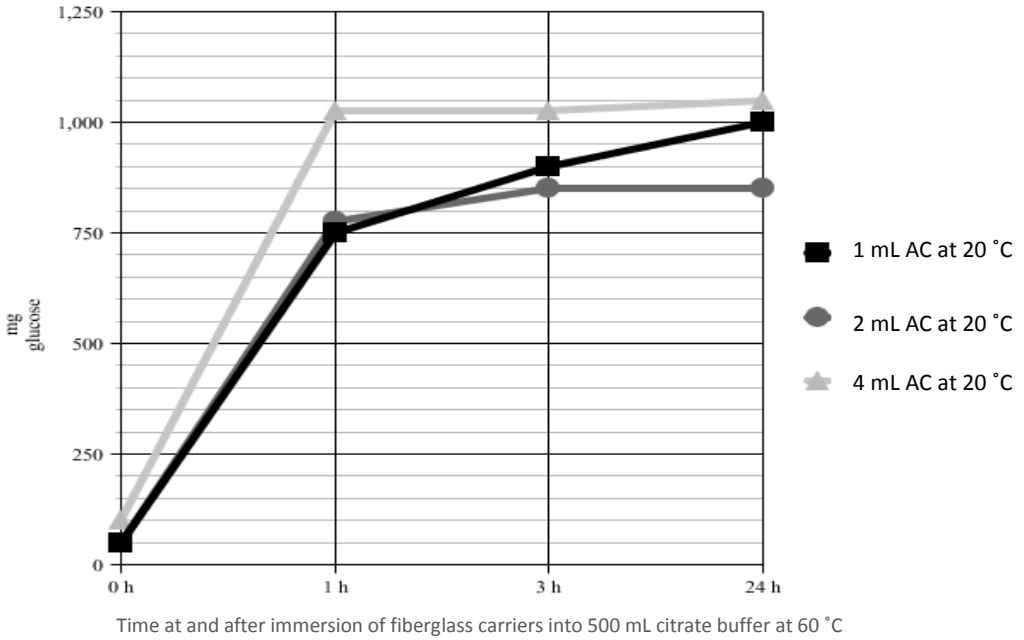

Fig. 4b. Time course of total glucose produced from 15 g of NaOH pretreated CS following 1 h exposure at 20 °C to 1.0 mL, 2.0 mL, and 4.0 mL of AC in 100 mL citrate buffer pH = 4.5. Fiberglass carriers were used.

Overall, 5 to10 g alkali pretreated CS per 100 mL citrate buffer containing 2 mL AC appears to be optimal and practical. Also, practical is the use of fiberglass mesh as carrier material. This material is readily available, handles easily, is resilient, and does not affect enzyme hydrolysis. Also, there was no need for antimicrobial treatment, most probably due to the short overall processing time of ten hours as well as the operating temperatures.

CONCLUSIONS

1. The Alkcell process for converting biomass to glucose can be operated continuously by using biomass carriers that move through the various stages of the process. The dwell time in the different solutions has been determined based on this study and previous work (Savarese 2013a,b).
2. One continuous Alkcell process cycle takes ten hours and can be scaled for larger quantities of biomass. The materials and methods used are easily accessible, easy to use, and require operating conditions that can be achieved near farms.
3. In addition to a source of water, the process requires heat that can be provided by portable gas fuel. Movement of the carriers requires a simple mechanical system. The resulting dilute glucose solution can be concentrated with membranes or heat. The concentrated glucose solution can then be transported to any existing grain fermenting facility and is ready to use.

REFERENCES CITED

Blanch, H. W., Simmons, B. A., and Klein-Marcuschamer, D. (2011). "Biomass deconstruction to sugars," *Biotech. J.* 6(9), 1086-1102.

Brechbill, S. C., Tyner, W. E., and Heleji, K. E. (2011). "The economics of biomass collection and transportation and its supply to Indiana cellulosic and electric utility facilities," *Bioenerg. Res*. 4, 141-152.

Brethauer, S., and Wyman, C. E. (2010). "Review: Continuous hydrolysis and fermentation for cellulosic ethanol production," *Bioresource Technol*. 101, 4862-4874.

Haque, M., and Epplin, F. M. (2012). "Cost to produce switchgrass and cost to produce ethanol from switchgrass for several levels of biorefinery investment cost and biomass to ethanol conversion rates." *Biomass Bioenergy.* 46, 517-530.

Kadam, K. L., Chin, C. Y., and Brown, L. W. (2009). "Continuous biomass fractionation process for producing ethanol and low-molecular-weight lignin," *Environ. Prog. Sustainable Energy.* 28, 89-99.

Klein-Marcuschamer, D., Oleskowicz-Popiel, P., and Simmons, B. A. (2012). "The challenge of enzyme cost in the production of lignocellulosic biofuels," *Biotechnol. Bioeng.* 109(4), 1083-1087.

Kumar, R., and Wyman, C. E. (2009). "Access of cellulase to cellulose and lignin for poplar solids produced by leading pretreatment technologies," *Biotechnol. Prog.* 25(3), 807-819.

Larson, J. A., Yu, T.-H., English, B. C., and Mooney, D. F. (2010). "Cost evaluation of alternative switchgrass producing, harvesting, storing, and transporting systems and their logistics in the Southeastern USA," *Agr. Finance Rev.* 70(2), 184-200.

Lu, Y., Yang, B., Gregg, D., Saddler, J. N., and Mansfield, S. D. (2002). "Cellulase adsorption and an evaluation of enzyme recycle during hydrolysis of steam-exploded softwood residues," *App. Biochem. Biotech.* 98-100, 641-654.

Lynd, L. R., Laser, M. S., Bransby, D., Dale, B. E., Davison, B., Hamilton, R., Himmel, M., Keller, M., McMillan J. D., Sheehan, J., and Wyman, C. E. (2008). "How biotech can transform biofuels," *Nature Biotech.* 26(2), 169-172.

Sathitsuksanoh, N., George, A., and Zhang, Y.-H. P. (2013). "New lignocellulose pretreatments using cellulose solvents: A review," *J. Chem. Technol. Biotechnol.* 88(2), 169-180.

Savarese, J. J. (2013a). "Local biomass processing is practical for facilitating fermentation to bioethanol," *J. Tech. Innov. Renew. Energy*. 2(3), 239-245.

Savarese, J. J. (2013b). "Optimizing alkali-cellulase processing of biomass to glucose," *BioResources* 8(4), 5005-5013.

Yu, T. E., Fu, J. S., Larson, J. A., English, B. C., De La Torre Ugarte, D., Wilson, B., Yun, G., Gao, Y., and Calcagno, J. (2012). "The transportation emission impact of the biomass feedstock traffic of a potential commercial-scale biorefinery in east Tennessee," In: Transportation Research Board 91st Annual Meeting (No. 12-4035).

Article submitted: September 20, 2013; Peer review completed: October 9, 2013; Revised version received and accepted: October 9, 2013; Published: October 15, 2013.