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Biochemical conversion of sweet sorghum bagasse to succinic acid

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Succinic acid, an important intermediate in the manufacture of plastics and other commodity and specialty chemicals, is currently made primarily from petroleum. We attempted to biosynthesize succinic acid through microbial fermentation of cellulosic sugars derived from the bagasse of sweet sorghum, a renewable feedstock that can grow in a wide range of climates around the world. We investigated pretreating sweet sorghum bagasse (SSB) with concentrated phosphoric acid at mild conditions (40–85°C) at various residence times and biomass concentrations. We then subjected the pretreated SSB to enzymatic hydrolysis with a commercial cellulase to release glucose. The highest glucose yield was obtained when SSB was pretreated at 50°C for 43 min at 130 g/L biomass concentration on dry basis. Fermentation was carried out with *Actinobacillus succinogenes* 130Z, which readily converted 29.2 g/L of cellulosic glucose to 17.8 g/L of succinic acid in a 3.5-L bioreactor sparged with CO_2 at a rate of 0.5 vvm, thus reducing the carbon footprint of the process. Overall, we demonstrated, for the first time, the use of SSB for production of succinic acid using practices that lower energy use, future equipment cost, waste generation, and carbon footprint.

[**Key words:** Succinic acid; Sweet sorghum bagasse; Acid pretreatment; Phosphoric acid; Biochemical conversion]

Succinic acid, a four-carbon dicarboxylic acid used in a wide range of applications in the chemical, food, and pharmaceutical industries (1), is currently produced almost exclusively from oil. Sustainability concerns about the use of fossil sources and the energy intensity of high-temperature and high-pressure manufacturing processes have created interest in producing succinic acid via fermentation of sugars (2). However, use of sugars derived from food crops, like corn and sugarcane, raises significant societal concerns about sustainability, especially regarding food security and land use change.

To enhance the sustainability of fermentative succinic acid production, we investigated the use of sweet sorghum bagasse (SSB) as a renewable resource in a biochemical process that incorporates sustainability practices. Sweet sorghum is a promising source of sugar and fiber thanks to its ability to grow in a wider range of climates and soils and survive drought conditions compared to sugarcane, while its cellulosic fiber can be converted to ethanol (3,4). SSB is currently employed as animal feed, soil fertilizer, and solid fuel for power generation (5). However, it can be further valorized through chemical pretreatment, making it a potential source of cellulosic sugars and eventually of valuable bioproducts via enzymatic hydrolysis and fermentation (6,7). Although several lignocellulosic species, such as pinewood, corn stover, and sugarcane bagasse, have been used before for fermentative succinic acid production (8,9), SSB has not been investigated, prompting us to explore its potential as a renewable feedstock for succinic acid biosynthesis. Importantly, previous work on succinic acid production from sweet sorghum utilized the juice as feedstock, but no attempts have been reported on using its bagasse (10,11).

Chemical processing of biomass at large scale is mostly practiced with strong acids, primarily sulfuric. Although effective in conditioning biomass, such processes require high temperatures (160–240°C) and pressures (~10 atm) (12–14), thus necessitating high energy consumption and use of expensive materials of construction for the required equipment. Furthermore, the use of sulfuric acid results in the generation of significant amounts of gypsum, an environmental liability, during acid neutralization following pretreatment (14). Steering towards green chemistry practices, we examined SSB pretreatment under much milder conditions with concentrated phosphoric acid (15,16), a weak acid that is much less hazardous and corrosive compared to sulfuric acid (17). Importantly, neutralization of phosphoric acid at the end of the pretreatment process results in the formation of phosphate salts, which can serve as a fertilizer co-product, as opposed to gypsum waste generated by sulfuric acid. Moreover, the phosphates can serve as buffer and nutrient during microbial fermentation processes (18).

Chemically pretreated biomass is amenable to enzymatic hydrolysis using cellulolytic enzymes, which depolymerize cellulose to fermentable glucose (19–21). A number of bacterial and yeast strains are known to ferment cellulosic glucose to succinic acid and other chemicals and biofuels (22–24). We elected to use *Actinobacillus succinogenes* 130Z, a promising succinic acid producer (25) with an interesting sustainability feature: whereas it anaerobically produces a mixture of organic acids and ethanol (25,26), when exposed to a CO_2-enriched environment its metabolism favors succinic acid formation, as glucose-derived glyceraldehyde (3-carbon molecule) is converted to succinic acid (4-

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carbon molecule) through CO_2 incorporation, as shown in Fig. 1. It has been reported that CO_2 enhances the activity of phosphoenol pyruvate (PEP) carboxykinase enzyme, which converts PEP to oxaloacetate and subsequently to succinate. In the absence of CO_2, PEP is instead converted to pyruvate and subsequently to formate, acetate, and ethanol, through the activity of pyruvate kinase enzyme (27). Thus, this bacterium can act as a CO_2 sink, while generating succinic acid with potentially high selectivity. Moreover, *A. succinogenes* can grow on a variety of carbon sources, including xylose, glycerol, sucrose, and cellobiose, producing high yields of succinic acid, up to near 1 g/g sugar (25,28,29).

In the present study, we investigated phosphoric acid pretreatment of SSB under mild conditions followed by enzymatic hydrolysis to glucose and then fermentation to succinic acid using *A. succinogenes* 130Z in a 3.5-L bioreactor. Our objective was to test our hypothesis that succinic acid can indeed be produced from mildly treated SSB, hence creating the foundation for future large-scale succinic acid production from such a renewable global resource.

MATERIALS AND METHODS

Pretreatment SSB was kindly provided by the Southern Region Research Center of the US Department of Agriculture in New Orleans, LA. It was dried at room temperature using a fan until there was no further change in dry weight. A food processor was then used to reduce the bagasse into small particles that passed through a 2 mm steel sieve. The bagasse composition was determined following the pertinent NREL protocol (30).

For chemical pretreatment with concentrated phosphoric acid, 50-mL glass centrifuge tubes with a round bottom were selected to serve as reactors. A shaking water bath able of operating in the range of 5–99°C was used for continuous heating and mixing during pretreatment. Using a partial factorial design (Table 1), we investigated the impact of three pretreatment independent variables, namely temperature, residence time, and biomass concentration, on SSB glucose release after subsequent enzymatic digestibility at several values of each variable: Temperature at 40°C, 50°C, 65°C, and 80°C, residence time at 30, 45, and 60 min, and biomass concentration (dry mass basis) at 100, 125, and 150 g/L. For data analysis we built an empirical model for the response variable, namely glucose released after the combined pretreatment and enzymatic hydrolysis, as a quadratic function of the 3 independent variables and their binary interactions. We then conducted statistical analysis using Minitab software (Minitab Inc., State College, PA, USA).

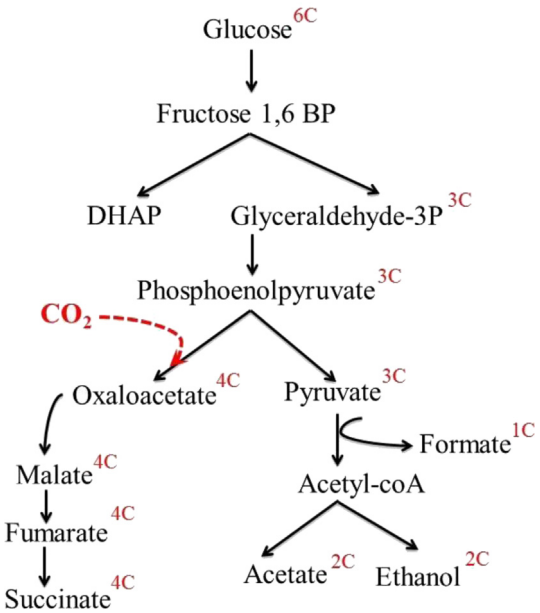

FIG. 1. Depiction of the pathways of glucose catabolism to succinic acid and other metabolites in *A. succinogenes* 130Z. Superscripts indicate the number of carbon atoms per molecule of the corresponding compound.

TABLE 1. Set of values for the temperature, residence time, and biomass concentration selected for the pretreatment partial factorial design.

Temperature (°C)	Time (min)	Biomass concentration (g/L)
40	30	100
40	30	150
40	45	125
40	60	100
40	60	150
50	30	100
50	30	150
50	45	125
50	60	100
50	60	150
65	30	125
65	45	100
65	45	125
65	45	150
65	60	125
80	30	100
80	30	150
80	45	125
80	60	100
80	60	150

For each pretreatment run, 1 g of dry milled SSB was mixed with the appropriate amount of concentrated phosphoric acid (85% w/w, Fisher Chemical, Fair Lawn, NJ, USA) in a glass tube to achieve the desirable biomass concentration. The tubes were then incubated in the water bath for the appropriate number of minutes at the desired temperature. Afterwards, the pretreated SSB suspension was pH neutralized using a 10 g/L NaOH solution and the solids were separated from the liquid via vacuum filtration and oven dried overnight at 60°C.

Enzymatic activity measurement The enzymatic activity of the Cellic CTec2 cellulase batch, kindly provided by Novozymes (Franklinton, NC, USA), was determined following NREL's analytical procedure (31) using Whatman No.1 filter paper strips, acetic buffer (pH 5.0), and various dilutions of the enzyme batch. Enzymatic hydrolysis of the filter paper was initiated by placing all the tubes in a 50°C water bath for 60 min. Glucose released during enzymatic hydrolysis was measured using a Sigma glucose assay kit with an Infinite M200 PRO microplate reader (Tecan, Männedorf, Switzerland). Activity expressed in filter paper units (FPU) was calculated using the following equation:

$$\text{Filter paper unit} = \frac{\mu\text{mol glucose released}}{\text{Minute} \times \text{mL enzyme}} \quad (1)$$

Enzymatic hydrolysis of pretreated SSB Following common literature practices and the enzyme manufacturer's recommendations, hydrolysis of pretreated SSB was performed in a shaking water bath at selected biomass concentrations using acetic buffer (pH 5.0) at 50°C and 50 rpm over 72 h with 40 FPU/g pretreated SSB of Cellic CTec2 cellulase (32). The liquid phase was separated from the solids by centrifugation and the sugar content (glucose and xylose) in the liquid was measured using a YSI 2700 Select Biochemistry Analyzer (YSI Inc., Yellow Springs, OH, USA). These readings were validated by reanalyzing randomly selected samples with HPLC (Thermo Fisher Scientific, Waltham, MA, USA).

Microorganism inoculation and cultivation in a bioreactor The selected microorganism, *A. succinogenes* 130Z, was obtained from the American Type Culture Collection (Manassas, VA, USA). For inoculum preparation, *A. succinogenes* was grown overnight in trypticase soy broth medium in flasks equipped with stoppers to create an anaerobic environment in a shaker (New Brunswick Scientific Excella E24, New Brunswick Scientific, Edison, NJ, USA) at 37°C and 200 rpm. The fermentation medium consisted of NaCl at 1.00 g/L, $MgSO_4 \cdot 7H_2O$ at 0.1025 g/L, K_2HPO_4 at 6.40 g/L, $NaH_2PO_4 \cdot H_2O$ at 17.71 g/L, NaHCO3 at 10.00 g/L, and yeast extract at 10.00 g/L, taking into account previous studies (26,28,33). Fermentation was carried out in a 3.5-L New Brunswick BioFlo benchtop fermentation system equipped with temperature and pH monitoring and control systems (New Brunswick Scientific). Inoculum was added at 10% v/v to a final volume of 1 L of fermentation medium for an initial OD_{600} of approximately 0.1–0.2. During fermentation, the bioreactor temperature was maintained at 37°C using a heating pad and the pH was kept at 6.8–7.2 using a 2.5 M Na_2CO_3 solution. CO_2 was continuously sparged into the bioreactor through a 0.22 μm filter at a flow rate of 0.5 vvm.

Experimental reproducibility and data analysis All experiments were performed in duplicate to confirm reproducibility and obtain the mean and standard deviation (SD) values, as determined using Microsoft Excel's built-in statistical tools for data analysis. As detailed earlier, the data collected from the combined pretreatment and enzymatic hydrolysis runs were fitted to a quadratic empirical model for glucose released using the regression program Minitab 17 to identify the optimal set of pretreatment conditions for SSB among those tested. All

fermentation samples were analyzed using Thermo Scientific UltiMate 3000 ultra high-performance liquid chromatography (HPLC) equipped with a refractive index detector. Briefly, the HPLC was operated using 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min at a column and detector temperature of 50°C. Prior to analysis, fermentation samples were collected and centrifuged at 10,000 rpm and 10°C for 10 min (Eppendorf Centrifuge 5430 R, Eppendorf, Hamburg, Germany). The supernatant was filtered using 0.22 μm syringe filter, diluted 10-fold, and analyzed via HPLC using a Bio-Rad Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA). The obtained chromatograms were analyzed with the HPLC software Chromeleon 7.2.6 Chromatography Data System (Thermo Scientific).

RESULTS AND DISCUSSION

Pretreatment The glucan and xylan content of fresh SSB was determined to be 34.8 ± 0.8 g and 23.6 ± 1.2 g per 100 g SSB (dry basis), respectively. The studied combinations of pretreatment temperature, residence time, and biomass concentration (partial factorial design) are summarized in Table 1. Release of glucose during pretreatment remained mostly below 5 g per 100 g of SSB (data not shown) indicating only minor biomass decomposition. We also tested for the presence of hydroxymethyl furfural (HMF) and furfural, which are thermal degradation products of glucans and xylans, respectively, and could be inhibitory to *A. succinogenes* during the subsequent fermentation. Only at the harshest pretreatment conditions of 80°C for 60 min we detected low levels of HMF at 1–2 g per 100 g SSB. Under all other conditions, no sugar degradation was detected, an indication that concentrated phosphoric acid at the milder temperatures (40–65°C) does not degrade the biomass constituents, unlike dilute sulfuric acid at commonly used temperatures of 180–200°C even for short residence times of 5–10 min (13,34,35).

Enzymatic hydrolysis of pretreated SSB All pretreated SSB samples were subjected to cellulase hydrolysis under identical conditions, as described earlier. The amounts and yields of glucose released upon hydrolysis by cellulase after pretreatment are depicted in Fig. 2. A glucose yield of 24–32 g per 100 g SSB was obtained after pretreatment at the 40°C conditions and enzymatic hydrolysis at 50°C, whereas the highest glucose yield of 30–35 g per 100 g SSB was observed when the material was pretreated at 50°C and subsequently hydrolyzed by the cellulase at the same temperature. As the pretreatment temperature increased to 65°C and 80°C, the glucose yield dropped significantly to 19–27 and 10–16 g per 100 g SSB, respectively. It appears that at 50°C the biomass was pretreated most effectively, as indicated by the higher overall glucose yield observed after enzymatic hydrolysis. This implies that biomass conditioning was relatively less efficient at 40°C and 60°C, whereas at 80°C biomass started to degrade, as evidenced by the detection of HMF, a glucose degradation product, as mentioned earlier.

Optimal pretreatment conditions Using the Minitab 17 regression and optimization algorithm, a quadratic empirical equation of the independent variables was fitted to the experimental glucose release data, demonstrating a satisfactory coefficient of determination (R^2) of 0.87:

$$EGR = 48.8 - 1.87\times T + 0.81\times t + 6.1\times S + 0.0092\times T^2 - 0.00118\times t^2 - 0.276\times S^2 - 0.00106\times T\times t + 0.0058\times T\times S + 0.0194\times t\times S \quad (2)$$

where EGR is the expected glucose release (g/100 g SSB) after enzymatic hydrolysis following pretreatment; T is the pretreatment temperature (°C); t is the pretreatment residence time (min); and S is the biomass concentration (g/100 mL on dry basis) during pretreatment. The model identified as optimal pretreatment conditions, among those tested, the combination of 50°C, 43 min residence time, and 130 g/L biomass concentration, projecting a glucose release of 35 ± 3.0 g per 100 g SSB. Moreover, among the 3 pretreatment variables, temperature was clearly the most significant factor influencing glucose release from SSB, which is in agreement with previous reports of phosphoric acid pretreatment of other biomass feedstocks (36,37).

To test the accuracy of the model prediction, we pretreated SSB at the conditions identified by the model as optimal and then performed enzymatic hydrolysis under conditions identical to those used earlier. Glucose release was monitored over 96 h and samples were taken every 24 h (data not shown). Glucose release was essentially complete within 24 h and the total glucose generated was 32.75 g per 100 g SSB, which is in good agreement with

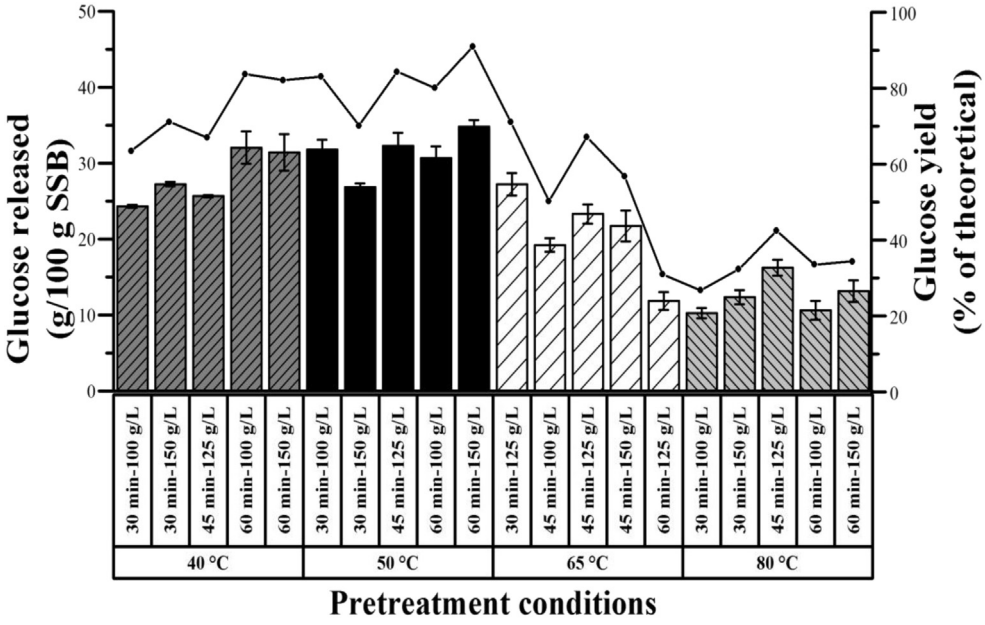

FIG. 2. Mass of glucose released per 100 g of pretreated sweet sorghum bagasse (SSB) (bars) and the corresponding glucose yield (as % of initial SSB glucose content, dots and line above bars) at the studied pretreatment conditions of temperature, residence time, and biomass concentration (dry basis) following subsequent enzymatic hydrolysis.

the model prediction of 35 ± 3.0 g per 100 g SSB. Moreover, since the average glucan content was 34.8 g per 100 g SSB, which corresponded to 38.7 g of glucose per 100 g SSB, the released glucose represented 85% of the total glucose available in untreated SSB, indicating that glucose release was highly efficient under the tested conditions.

Fermentation with pure glucose Before attempting to ferment SSB-derived glucose, we first assessed the ability of *A. succinogenes* 130Z to produce succinic acid from pure glucose in the presence of CO_2, as reported previously (25) and metabolically depicted in Fig. 1. The fermentation was conducted in the 3.5-L BioFlo fermentor starting with 25 g/L pure glucose as carbon source, as described earlier, and sparging CO_2 continuously at a volumetric rate of 0.5 vvm. As shown in Fig. 3, succinic acid formation began after an initial 4-h lag phase, and all glucose was consumed within 16 h resulting in the production of 14.1 g/L of succinic acid. As predicted from the metabolism of *A. succinogenes* (Fig. 1), acetic acid emerged as a byproduct during the fermentation, but in very limited amounts (less than 1 g/L). Thus, the vast majority of consumed glucose was successfully directed towards succinic acid biosynthesis by *A. succinogenes* in the CO_2-rich environment of the bioreactor.

Fermentation of SSB hydrolysate Next, we conducted fermentation runs under identical conditions, but using as carbon source the glucose present in the SSB hydrolysate (instead of pure glucose) prepared with enzymatic hydrolysis of SSB pretreated at the previously identified optimal conditions. During the first 4 h of the fermentation, the pH remained close to 7.0 as *A. Succinogenes* again went through a lag phase of culture adaptation. Afterwards, the bacteria started utilizing glucose (Fig. 4) and the pH of the culture dropped (not shown) due to the production of organic acids, primarily succinic acid. During SSB hydrolysate fermentation, which reached completion within 20 h, formic and acetic acid were also detected as byproducts in the fermentation broth (6.4 g/L and 4.0 g/L). Still, succinic acid remained by far the main metabolic product with its final concentration reaching 17.8 g/L, which corresponds to a yield of 0.61 g/g glucose (based on 29.2 g of glucose consumed) and 0.24 g/g SSB (based on 29.2 g glucose in 75.45 g of SSB). Importantly, the succinic acid yield from SSB hydrolysate was nearly identical to the yield from pure glucose, which was 0.56 g/g (based on 14.1 g/L of succinic acid produced from 25 g/L of glucose consumed) at a similar CO_2 sparging rate of 0.5 vvm, implying that pretreated and enzymatically hydrolyzed SSB can serve as an effective carbon source in succinic acid fermentation. Similar yields have been reported in previous work, but from biomass feedstocks other than SSB (38,39). In general, succinic acid yields of 0.43 up to 1.02 g/g have been reported from various types of renewable feedstocks, including lignocellulosic biomass, using several strains of *A. succinogenes*, as detailed elsewhere (25). Still, the yield can be further improved, since the maximum theoretical yield is 1.30 g/g in the presence of CO_2 and additional reducing agents (40), possibly through means such as genetic engineering to direct more carbon flux towards succinate at the expense of the competing organic acids.

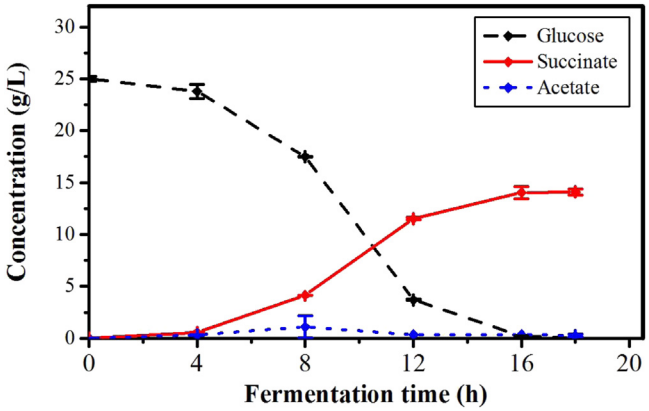

FIG. 3. Time profile of pure glucose fermentation and succinic acid formation by *A. succinogenes* 130Z in a bioreactor under anaerobic conditions with continuous CO_2 sparging.

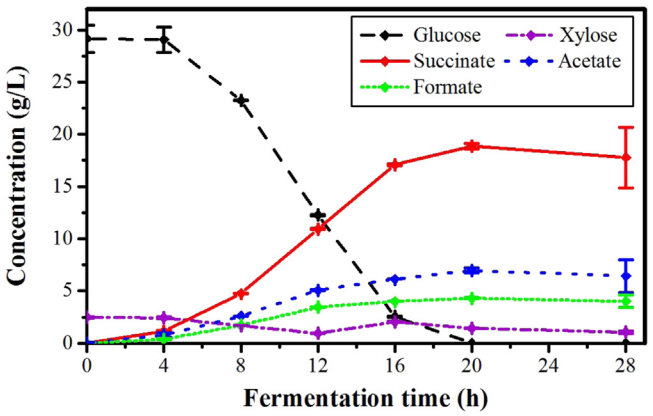

FIG. 4. Time profile of sweet sorghum bagasse (SSB) hydrolysate fermentation by *A. succinogenes* 130Z in a bioreactor under anaerobic conditions with continuous CO_2 sparging. SSB was pretreated at the identified optimal conditions and subjected to enzymatic hydrolysis prior to fermentation.

It should be noted that since the hydrolysate carries over cellulase enzyme into the fermentor from the hydrolysis process, it is possible that the enzymatic activity on cellulose may continue during the fermentation process. However, the fermentation was operated at 37°C and pH 6.8–7.2, conditions that are quite far from the optimal conditions of the employed cellulase, as reported by the manufacturer (50°C and pH 5.0). It is therefore unlikely that significant additional amounts of glucose were released during the short time of the fermentation (20 h). Notably, it has been reported that *A. succinogenes* 130Z can catabolize both glucose and xylose (28). However, in our fermentation, the low-level xylose (less than 3 g/L) that was carried over from the enzymatic hydrolysis step was not consumed by the bacteria. It should be noted that xylose is known to co-elute with other sugars, such as fructose and galactose, on the HPLC chromatogram. Hence, it is possible that the residual xylose content may include co-eluting sugars, but still all sugars other than glucose were at very low levels.

Overall, our data demonstrate that sweet sorghum bagasse pretreated with concentrated phosphoric acid under mild conditions can indeed be efficiently converted to succinic acid after enzymatic hydrolysis by a commercial cellulase and fermentation by *A. succinogenes*. Hence, production of biobased succinic acid from sweet sorghum, a crop that can be cultivated in a broader range of locations and climates than sugarcane or corn around the world, looks promising. Moreover, the successful use of mild pretreatment conditions indicates that it is feasible to apply green chemistry practices (low energy consumption and low or no waste generation) to improve the sustainability of biobased succinic acid, compared to succinic acid produced from crop sugars or petroleum.

As outlined earlier, CO_2 purging during fermentation pushed the microbial metabolism of *A. succinogenes* towards the formation of the 4-carbon succinic acid rather than the 3-carbon lactic acid, the 2-carbon acetic acid and ethanol or the 1-carbon formic acid (Fig. 1). From a sustainability standpoint, this means that the biobased succinic acid production approach described here can also act as a sink of CO_2, a potent greenhouse gas, thus reducing the carbon footprint of the proposed process. Nevertheless, a life-cycle

analysis will be needed to more accurately quantify the overall carbon emissions associated with the proposed succinic acid production process from sweet sorghum bagasse. The significant xylan content of SSB (23.6 ± 1.2 g per 100 g SSB) represents a potentially additional carbon source that warrants future investigation for fermentative conversion of xylose to succinic acid or other value-added metabolites by the same or another microorganism.

In conclusion, this is the first report on succinic acid production from the bagasse of sweet sorghum. During the course of this work we demonstrated that cellulosic glucose can be efficiently released from sweet sorghum bagasse after mild phosphoric acid pretreatment and subsequent enzymatic hydrolysis. Analysis of glucose release data helped us identify the optimal pretreatment conditions among those tested and confirmed that temperature was the most influential factor for pretreatment effectiveness. Overall, we showed that low temperature pretreatment with concentrated phosphoric acid followed by enzymatic hydrolysis and anaerobic fermentation by CO_2-sequestering *A. succinogenes* 130Z is a process with a promising sustainability profile for producing biobased succinic acid from sweet sorghum bagasse. Future investigation will focus on rendering the process more commercially appealing by enhancing productivity through fed-batch or continuous mode of operation and by employing genetic engineering on *A. succinogenes* to bring the current 0.61 g/g succinate yield closer to the theoretical yield of 1.30 g/g.

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G.P.P. and I.D. conceived and designed the experiments. E.L. and L.C.B. conducted the experiments under the supervision of I.D. and E.L. and E.M.A. analysed the data. All authors participated in the preparation of the manuscript. E.M.A., G.P.P., and I.D. edited and reviewed the manuscript.

The authors declare that there are no competing financial interests.

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