EFFECT OF ACETATE ON FERMENTATION PRODUCTION OF BUTYRATE

ADAM JAROS,[*,**] ULRIKA ROVA[*,**] and KRIS A. BERGLUND[*,**]

[*]*Luleå University of Technology, SE-971 87 Luleå, Sweden*
[**]*Michigan State University, East Lansing MI 48824, USA*

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A carbon source for the fermentation production of butyrate is xylose extracted from ligno-cellulosic material by hot water extraction. Although this auto-hydrolysis of hemicellulose can provide a low-cost source of xylose, the process generates a high level of acetic acid that might inhibit subsequent fermentations. This study focuses on the effects of acetate on the production of butyrate from xylose by batch fermentations with a selected strain *Clostridium tyrobutyricum*.

At initial acetate concentrations of 17.6 g L^{-1} and 26.3 g L^{-1} in the media, *C. tyrobutyricum* cultures exhibited a lag phase (45 and 118 hours, respectively) in terms of sugar consumption, butyrate production and cell biomass growth, lowering the overall production rate. Butyrate fermentations performed with high concentrations of acetate in the media demonstrated a re-uptake of acetate into the butyrate production pathway and after the lag phase, all cultures adapted to the inhibitory acetate, which increased the final butyrate yields by 12.6% (32.6 g L^{-1} compared to 28.5 g L^{-1}).

Keywords: *Clostridium tyrobutyricum*, butyrate, xylose fermentation, hemicellulose utilization, acetate inhibition

INTRODUCTION

Economically feasible fermentation processes, involving low-cost renewable substrates, have potential applications in manufacturing butyrate as a food additive or as a bulk organic commodity for further refinement into biodegradable plastics.[1] In terms of a food product, butyrate is listed by the Food and Drug Administration (US) as a flavoring substance generally recognized as safe (GRAS). As a flavor additive, butyrate has a wide array of uses, from enhancing the sensorial quality of cattle feed to acting as a chemical feedstock in the production of flavor esters.

Butyrate is mainly produced industrially from petrochemical sources, but can also be made by fermentation of sugar or other starch sources by anaerobic bacteria. Most of these bacteria are obligate anaerobes, meaning that they require oxygen-free conditions for them to perform their metabolism, typical of the conditions in the gastrointestinal tracts of mammals from which many of these bacteria are isolated. Microorganisms from the genus *Clostridium* are the most studied in terms of butyrate fermentation and within this group, the Gram-positive anaerobic spore-former *Clostridium tyrobutyricum* can produce butyrate utilizing either glucose or xylose as a carbon source.[1,2]

Using xylose as a carbon source, yields of up to 0.4 g g^{-1} have been obtained with immobilized *C. tyrobutyricum* in a fibrous bed reactor.[3]

Hemicellulose is a valuable biomass resource and its extraction could be integrated into current industrial pulp and paper processes, yielding hemicellulosic sugars for fermentation, while retaining the cellulose fraction to be used in subsequent pulp production.[4] One of the main issues concerning an integration is that near-neutral pre-extraction of hemicellulose from hardwood releases not only xylose, but also acetic acid, a microbial growth inhibitor influencing energy generation and intracellular pH homeostasis. Depending on process constraints, a hydrolysate of hardwood extracted xylose, conceivable for fermentation, typically contains up to 70 g L^{-1} xylose and 40 g L^{-1} acetic acid.[4]

Butyric acid production by *C. tyrobutyricum* is an attractive potential industrial approach for ligno-cellulosic based fermentations, but for hemicellulose derived xylose to be competitive as a low-cost substrate, either the inhibiting acetic acid must be removed from the hydrolysate or the fermentation strain should be adapted to tolerate such high levels. Previous research has shown *Clostridia* to be tolerant to high levels of initial

acetate and butyrate, and that the presence of these organic acids results in increased product yields not only for butyrate fermentation, but butanol fermentation as well.[2,5] The addition of 8.9 g L^{-1} sodium acetate (6.518 g L^{-1} acetic acid equivalents) to a *Clostridium beijerinckii* BA101 fermentation increased the final butanol yield by 14%.[5] *Clostridium thermobutyricum* fermentations challenged with 30 to 360 mM acetate (1.8 to 21.6 g L^{-1}) exhibited a 2-to-4 fold increase in butyric acid formation.[6] Furthermore, the reinforcement of a cassava based medium with 30 mM ammonium acetate (1.8 g L^{-1} acetic acid equivalent) increased 2.6 fold *Clostridium acetobutylicum* strain EA 2018 butyric acid fermentation.[7] As *C. tyrobutyricum* is from the same genus as these bacteria and share analogous metabolic pathways, one can expect an increase in butyric acid yield when challenging *C. tyrobutyricum* with acetic acid.

As *Clostridia* are prone to tolerance adaptation regarding toxic fermentation products, *C. tyrobutyricum* is a candidate for selective enrichment towards acetate tolerance. The present work focuses on the impact of high levels of acetate/acetic acid on *C. tyrobutyricum* fermentation growth kinetics and product yields in order to use xylose extracted from hemicellulose as a fermentative substrate without requiring the removal of the acetate from the feedstock.

EXPERIMENTAL

Bacterial strain, media and growth

A lyophilized stock culture of *C. tyrobutyricum* (ATCC 25755) was re-hydrated under sterile anaerobic conditions in Reinforced Clostridial Media (RCM; Difco) broth. Stock cultures were maintained at -70 °C in CRYOBANK™ vials. To prepare cultures for glucose fermentations, RCM containing 5 g L^{-1} glucose was used, while for the xylose batches a medium was prepared with the following composition per liter: 10 g peptone, 10 g beef extract, 3 g yeast extract, 5 g sodium chloride, 0.5 g L cysteine, 3 g sodium acetate anhydrous, 0.5 g agar and 900 mL distilled water. The medium was sterilized at 121 °C for 20 min; thereafter 10 mL of a separately sterilized xylose solution (50 g L^{-1}) were aseptically added to bring the xylose concentration to 5 g L^{-1}. The inoculum for each batch fermentation in these studies was prepared by anaerobically inoculating 50 mL Screw Cap Corning tubes containing 35 mL sterile glucose or xylose based RCM with 5 mL of the stock culture. The inoculated tubes were cultivated under anaerobic conditions at 36 °C, 80 rpm, until log phase, approximately when the optical density at 600 nm had reached a value of 2. The reason behind two types of reinforced inoculate media is to avoid any substrate based growth lag. *C. tyrobutyricum* inocula were pre-conditioned to the correct sugar substrate in the inoculation media prior the batch fermentation.

Fermentations

One liter batch fermentations were conducted in New Brunswick Bioflo 310 reactors of 2.5 L working volume at 36 °C. For each batch, 950 mL minimal media of the following composition was used: 6 g L^{-1} yeast extract, 5 ppm $FeSO_4 \cdot 7H_2O$, and 200 mL xylose or glucose at 300 g L^{-1} sterilized separately. To examine the effect of acetate on butyrate production by *C. tyrobutyricum* various amounts of sodium acetate (4.4, 8.8, 17.6 or 26.3 g L^{-1}) were added to the media before sterilization. Fermentations without acetate are referred to as controls. Anaerobiosis was reached by sparging the vessel with nitrogen prior to inoculation. The batches were inoculated with 50 mL log phase *C. tyrobutyricum* cultures. Agitation was kept at 250 rpm and the nitrogen sparging was maintained until logarithmic growth was observed.

In order to maintain the *C. tyrobutyricum* cultures in the acidogenic phase, pH 6.0 was sustained by automatic addition of 5 M NaOH throughout the fermentation. Samples (10 mL) were withdrawn aseptically at regular intervals for analytical measurements. The batch fermentations were conducted until all sugar had been consumed.

Analytical methods

Organic acids and residual sugar were analyzed by HPLC (LC-20AT dual pump and 10A RI detector, Shimadzu) equipped with an ion exchange column (Aminex HPX-87H, 9 um, 7.8 mm x 300 mm, Bio-Rad) and a cation-H guard column (Micro-guard, 30 mm x 4.6 mm) using 50 mM sulfuric acid as the mobile phase. The flow rate of the mobile phase was maintained at 1 mL min^{-1} during analysis with 20 L of sample injected into the system with an auto-injector (SIL-20AHT, Shimadzu) with the column and guard maintained at 65 °C in a column oven (CT0-20A, Shimadzu). To remove cellular debris, the samples were centrifuged at 10000 rpm for 5 min in a micro-centrifuge prior to the analyses. Data for each sample was acquired and analyzed with Shimadzu EZ Start 7.4 SP1 chromatography software, using standards with known concentrations of glucose, xylose, butyrate, acetate and lactate.

Cell biomass determination

Cell growth was monitored during fermentation by measuring the optical density at 600 nm. The biomass from 40 mL cell suspension, removed in triplicate, was dried in a 80 °C drier for 48 hours and and the dry cell weight (DCW, g L^{-1}) was determined. The optical densities were then converted to dry cell weight using the following equation: $DCW = 0.38 (OD_{600})$.

RESULTS AND DISCUSSION

Acetate increases final butyrate yield, but results in an extended lag phase.

Although *Clostridia* prefer glucose as a sugar source, other metabolic pathways enable the utilization of alternate sugar sources, such as xylose. Once glucose is either completely consumed or lacking in the fermentation media, these alternate pathways are activated.[8] Repression of xylose metabolism by the presence of glucose is a common trait of these strains and causes a significant lag phase in substrate uptake during diauxic fermentations between glucose depletion and the xylose being consumed. Previous studies conclude this xylose consumption lag is caused by the requirement of the new metabolic system to be activated by the *Clostridia* in addition to an alternate xylose transport system.[9] *C. tyrobutyricum* activation of the xylose metabolism systems requires 48 hours to overcome. [10]

By avoiding the growth lag of *C. tyrobutyricum* caused by altered metabolic pathways for the carbon source, a direct study of the growth lag generated by inhibitory acetate was done using xylose as sugar source. *C. tyrobutyricum* cultures inoculated into media with 8.8 g L^{-1} initial acetate or less began log phase growth and thus sugar consumption at roughly the same time (Fig. 1). Cultures grown in 17.6 g L^{-1} acetate required 45 hours to adapt to the acetate environment before the consumption of xylose started. The lag phase for the 26.3 g L^{-1} initial acetate batch was even longer, stretching over 118 hours as the culture adapted to the acid. For all of the trials performed in this study, once the lag phase had passed, the xylose consumption occurred at nearly similar rates (Fig. 1). Other than the inhibitory acetate, the conditions were ideal for the *C. tyrobutyricum* fermentation as the control, 4.4 g L^{-1} and 8.8 g L^{-1} acetate initial concentration batches all entered log phase within the first 24 hours. The extended lag phase for the 26.3 g L^{-1} initial acetate concentration is one of the main drawbacks of utilizing a xylose feed derived from hardwood hemicellulose, as the acetate level, similar to those found in the hydrolyzate, is so inhibitorial to cellular growth that the extended lag phase lowers productivity. Although it took over 100 hours to enter the logarithmic growth phase, the 26.3 g L^{-1} initial acetate concentration batch did eventually reach the same biomass levels as the control and lower initial acetate concentrations batches, as well as surpassed them in final butyrate yield (Figs. 2 and 3). When xylose was used as carbon source, the 26.3 g L^{-1} initial acetate concentration batch resulted in 32.6 g L^{-1} butyric acid, compared to the control batches – 28.5 g L^{-1} butyric acid, which means a 12.6% increase. Given previous reports of the tolerance adaptability of *C. tyrobutyricum*, it appears that, given a long exposure time, even at 26.3 g L^{-1} acetate, the culture adapts and then carries out a butyric acid fermentation fully utilizing the xylose present. The glucose fermentations with additional acetate did not show any increase in yield and were also less sensitive according to the response in lag time.

Slower cell-mass generation and sugar uptake result in reduced overall productivity as fermentations with higher initial acetate concentrations exhibited periods of limited xylose consumption (Figs. 1 and 3). Control batches on glucose and xylose generated butyrate at a productivity of 0.24 and 0.23 g L^{-1} h^{-1} respectively, while the presence of 26.3 g L^{-1} acetate in the media resulted in a productivity decrease to 0.12 and 0.14 g L^{-1} h^{-1} (Table 1). The controls or fermentations with lower initial acetate concentration (i.e., 8.8 g L^{-1} or less) required 100 hours for consumption of nearly all sugar, and most of the butyrate was produced within this time (Figs. 1 and 2). However, the fermentation with initial 26.3 g L^{-1} acetate took over 100 hours to begin consuming xylose or producing butyric acid at detectable levels and more than 200 hours were required before all xylose was converted. As stated above, inoculation cultures were pre-conditioned with the appropriate sugar source prior to inoculation, so adjustment to a new sugar source was not the cause of the lag.

Unfortunately, the lengthy lag phase presents an issue for the large-scale feedstock potential of hemicellulose derived xylose, as such long periods of cellular non-growth leaves fermentation tanks open to acetate tolerant contaminating micro-organisms. The issue of lowered equipment turnover due to the low 0.14 L^{-1} h^{-1} overall production rate increases the economic burden on plant design. Also, operating tanks must be held at anaerobic conditions until the culture enters log phase and starts generating CO_2 requiring a much longer period of nitrogen sparging due to inhibitory acetate.

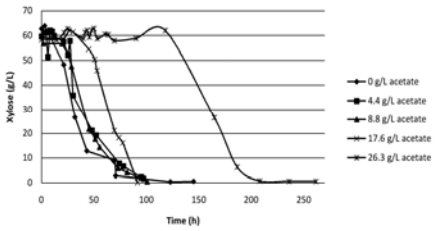

Figure 1: Effect of acetate inhibition on xylose consumption in *C. tyrobutyricum* batch fermentation system (fermentations performed as 1 liter batches in 2.5 liter working volume vessels under anaerobic conditions; temperature was maintained at 36 °C, agitation at 250 rpm, and pH at 6.0 with 5M NaOH; the media contained 60 g L^{-1} xylose, 6 g L^{-1} yeast extract and 5 ppm $FeSO_4 \cdot 7H_2O$; 10 mL samples were withdrawn from the fermentation and analyzed by HPLC)

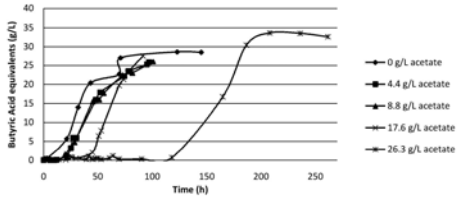

Figure 2: Effect of acetate inhibition on butyrate production in *C. tyrobutyricum* batch fermentation system (fermentations performed as 1 liter batches in 2.5 liter working volume vessels under anaerobic conditions; temperature was maintained at 36 °C, agitation at 250 rpm, and pH at 6.0 with 5M NaOH; the media contained 60 g L^{-1} xylose, 6 g L^{-1} yeast extract and 5 ppm $FeSO_4 \cdot 7H_2O$; 10 mL samples were withdrawn from the fermentation and analyzed by HPLC)

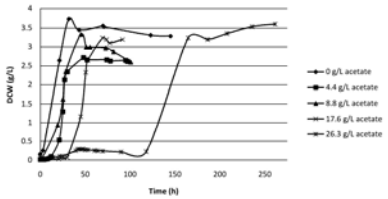

Figure 3: Effect of acetate inhibition on cell biomass production in *C. tyrobutyricum* batch fermentation system (fermentations performed as 1 liter batches in 2.5 liter working volume vessels under anaerobic conditions; temperature was maintained at 36 °C, agitation at 250 rpm, and pH at 6.0 with 5M NaOH; the media contained 60 g L^{-1} xylose, 6 g L^{-1} yeast extract and 5 ppm $FeSO_4 \cdot 7H_2O$; 10 mL samples were withdrawn from the fermentation and analyzed for OD by spectrophotometer at a wavelength of 600 nm)

Acetate re-utilization

Of note is the increased final butyrate concentration at high acetate concentrations with xylose as compared to glucose as a feedstock, 32.6 g L^{-1} and 22.3 g L^{-1} butyric acid, respectively (Table 1). In fact, all of the xylose feedstock batches performed better in terms of butyric acid production than the comparable glucose batches (Table 1). Glucose is preferred over xylose as a carbon source for *C. tyrobutyricum*, yet our findings demonstrate that there are no adverse affects on acid product selectivity or final butyric acid concentration with a xylose feedstock batch fermentation. In contrast, *C. tyrobutyricum* immobilized onto a fibrous bed bioreactor exhibit higher productivity on glucose feeds, compared to xylose, as the lower energy efficiency of metabolizing xylose lowered butyric acid yields.[3] To the best of our knowledge, this is the first report challenging *C. tyrobutyricum* with such high concentrations of initial acetic acid (17.6 and 26.3 g L^{-1}) in batch fermentations. Research has been performed on similar *C. tyrobutyricum* fermentations in a glucose fed batch style with the dilution rate of D = 0.1 h^{-1} and an acetate input of 10 g L^{-1} leading to the utilization of acetate and conversion into butyric acid.[11] As discussed below, providing *C. tyrobutyricum* with a high initial acetic acid equivalent not only challenges the fermentation, but has been reported to increase butyric acid yields as re-utilization of acetate for conversion to the 4 carbon product might be energy favorable for the microorganism.[12,13]

In the fermentations with the xylose feed, there is a correlation between the initial acetate concentration and the butyrate production with the highest butyrate yield of 0.9 (mol butyrate mol^{-1} xylose) occurring with the highest initial acetate concentration, 26.3 g L^{-1} (Table 1 and Fig. 2). This result indicates that the acetate end-

product pathway in the organism is being inhibited by the acetate and less of the carbon is entering this metabolic pathway. This result is a beneficial aspect of using xylose derived from a hemicellulose substrate streams, since the high concentration of acetate found in these streams will push the fermentation towards butyrate and away from generating more acetate.

The experiments with 17.6 and 26.3 g L^{-1} initial acetate concentrations and xylose as the carbon source show reduced levels of acetate at the end of the fermentation (14.8 and 22.0 g L^{-1} respectively), suggesting re-utilization of acetate. The utilization of acetate is also evident in the 26.3 g L^{-1} acetate batches of both feedstocks, which have lower sugar consumption rates than the batches with lower acetate concentrations, as acetate is being used as an additional carbon source. The control batches demonstrated sugar consumption rates of 1.09 and 1.12 g L^{-1} h^{-1} for glucose and xylose feedstocks, respectively, while the 26.3 g L^{-1} acetate batch rates were of 0.72 and 0.86 g L^{-1} h^{-1} (Table 1).

Table 1
Effect of acetate inhibition on butyrate, acetate and biomass production in *C. tyrobutyricum* cultures

Sugar	Acetate (g L^{-1})	Lag time[2] (h)	Sugar consump.[3] (g L^{-1} h^{-1})	Butyrate yield[4] (mol/mol)	Final concentration (g L^{-1})			Overall prod.[6] (g L^{-1} h^{-1})
					Butyrate	Acetate	Biomass[5]	
Glu[1]	0	0	1.09	0.72	23.6	5.3	3.3	0.24
Glu	4.4	0	0.97	0.80	23.0	11.3	2.4	0.32
Glu	8.8	0	0.77	0.80	22.0	11.0	3.1	0.30
Glu	17.6	26	1.68	0.85	24.4	21.4	3.2	0.34
Glu	26.3	118	0.72	0.76	22.3	27.7	3.0	0.12
Xyl[1]	0	0	1.12	0.62	28.5	2.0	3.3	0.23
Xyl	4.4	24	1.71	0.56	24.8	8.6	2.6	0.33
Xyl	8.8	20	1.07	0.60	26.0	11.6	2.6	0.26
Xyl	17.6	45	1.10	0.73	32.3	14.8	3.2	0.28
Xyl	26.3	118	0.86	0.90	32.6	22.0	3.6	0.14

[1]Glucose and xylose respectively; [2]Calculated as time until sugar consumption started; [3]Calculated for the linear sugar consumption phase; [4]Yield was calculated as mol butyrate per mol glucose or xylose consumed during fermentation; [5]Calculated as DCW g L^{-1}; [6]Overall productivity calculated from the start of the fermentation until the sugar source was completed

Re-utilization of acetate is a known phenomenon for *Clostridium* strains being used for acetone-butanol-ethanol (ABE) fermentations, where free acetate present in the media exchanges with the acetyl-CoA pool obtained from glycolysis and results in higher butyrate production.[14] *C. tyrobutyricum* utilizes the Embden-Meyerhof-Parnas metabolic pathway after glycolysis to generate ATP with butyrate, acetate and lactate as end-products.[6] The main metabolic pathway generates butyrate, but the presence of acetate or lactate in the media induces an even higher use of this pathway and the production of butyrate.[15] The hypothetical evolutionary function of this mechanism is in uncoupling the cellular membrane pH gradient from undissociated acids in the environment.[11] The butyrate pathway generates less ATP than the one producing acetate, but this energy sacrifice is made by the cell to maintain a functional pH gradient.[11] An important factor determining acid product selectivity is that during log phase growth the cell requires more ATP than during stationary phase, leading to the necessity of acetate production during log phase but mainly butyrate production during stationary phase.[3] Nevertheless, the acetate uptake pathway provides another positive aspect of xylose substrate stream derived from hemicellulose as some of the inhibitory acetate is converted into the butyrate product.

While the xylose fed 17.6 and 26.3 g L^{-1} initial acetic acid concentration batches demonstrated acetate uptake, this phenomenon was not seen in glucose fed batches at the same initial acetic acid concentrations (Table 1). Glucose fed 17.6 and 26.3 g L^{-1} initial acetic acid batches generated a

net gain of acetic acid, of 3.8 g L^{-1} and 1.4 g L^{-1}, respectively. An interpretation of these results stems from the lower energy gains of xylose metabolism compared to that of glucose. Cells consuming xylose rather than glucose thus have less free energy to devote to maintaining the pH gradient across the cell wall and in turn must activate the acetate uptake mechanism to partially nullify the external acetic acid. It is possible that our glucose metabolizing batches with high initial acetic acid had enough energy throughout the fermentation to carry out maintenance of the pH gradient and thus continued generating acetic acid. Future work would test this theory by exposing glucose metabolizing cultures to even higher initial acetic acid concentrations than those presented in this study and noting acetate uptake.

Coinciding as to the acetate uptake results, acetic acid had less of an effect on extending lag phase in growth on glucose fed cultures, compared to *C. tyrobutyricum* consuming xylose (Table 1). Only at the 26.3 g L^{-1} initial acetic acid concentration did the glucose batch match the xylose batch in sustaining a 118 h long lag phase. It required 17.6 g L^{-1} acetic acid before the glucose consuming *C. tyrobutyricum* even demonstrated a lag phase, while some lag phase was noticed in xylose fed cultures with only 4.4 g L^{-1} acetic acid present. Such a marked difference in response to acetic acid inhibition likely emanates from the lowered energetic value of metabolizing xylose. Xylose consumption leads to less free energy for cellular maintenance than glucose. In the case of lag phase, the lower free energy from xylose causes an extension of the lag as the cells take longer to adapt to the high acetate environment. Further studies regarding the issue of lag phase both with glucose and xylose consuming *C. tyrobutyricum* will be conducted in order to determine if an adaptation technique can overcome the lag phase altogether.

CONCLUSIONS

Xylose derived from hardwood by near-neutral pre-extraction results in a feedstock stream that contains up to 40 g L^{-1} acetic acid.[4] The work presented here evaluates the challenges to design a butyric acid fermentation based on a xylose stream with similar inhibitory levels of acetic acid. Xylose is an excellent alternative to glucose as a feedstock for batch fermentation as a higher final butyric acid concentration was obtained compared to the glucose batches.

High initial acetic acid concentrations proved inhibitory to the *C. tyrobutyricum* and generated an extended growth lag phase before the culture could begin fermentation. Despite lowered production rates caused by the lag, the acetic acid challenged cultures all eventually adapted to the acid environment and fully consumed the xylose substrate. A benefit of the presence of acetate in the media was the acetate re-utilization mechanism, which induced higher final butyric acid concentrations. The re-utilization of acetate from the media back into the acidogenic pathway increased product yield up to 45%. Increased product yield is of economic importance in an acetic acid containing feedstock as *C. tyrobutyricum* will ferment carbon from the acid as well as the xylose, increasing the butyric acid yields in batch production.

The adaptive ability of *C. tyrobutyricum* makes hemicellulose derived xylose a feasible and attractive substrate for fermentations, as acetate present in the hydrolyzate can be used as a carbon source, thus saving the requirements of detoxification. Future work will focus on maintaining pre-adapted cultures to overcome the production inhibiting lag phase. In addition, examining the xylose metabolism at a molecular level will reveal insight into metabolic pathways affected by the acetic acid, which might illuminate a potential solution to overcome the inhibition. A further exploration of fermentation kinetics on acetic acid tolerant pre-adapted strains will provide a better understanding of hemicellulose derived xylose as a production feedstock.

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