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Simultaneous Saccharification and Fermentation of Cellulosic Biomass to Acetic Acid

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Abstract

A strain of *Clostridium thermoaceticum* (ATCC 49707) was evaluated for its homoacetate potential. This thermophilic anaerobe best produces acetate from glucose at pH 6.0 and 59°C with a yield of 83% of theoretical. Enzyme hydrolysis of two substrates, a-cellulose and a pulp mill sludge, yielded 68% and 70% digestion, respectively. The optimum conditions for the simultaneous saccharification and fermentation (SSF) were substrate dependent: 55°C, pH 6.0 for α-cellulose, and 55°C, pH 5.5 for the pulp mill sludge. In the SSF with α-cellulose, the overall yield of acetate was strongly influenced by the enzyme loading. In a fed-batch operation of SSF with α-cellulose, an overall acetic acid yield of 60 wt% was obtained. Among the factors limiting the yields were incomplete digestion by the enzyme and the end-product inhibition. In the SSF of pulp mill sludge, inhibitors present in the sludge severely limited bacterial action. A large accumulation of glucose developed over the entire process, changing the intended SSF operation into a separate hydrolysis and fermentation operation. Despite a long lag phase of microbial growth, a terminal yield of 85% was obtained with this substrate.

Index Entries: Biomass; SSF; acetic acid; *Clostridium thermoaceticum*.

Introduction

With a US production of 2.2 billion kg/yr *(1)*, acetic acid is a widely used commodity chemical. Applications are ubiquitous, from paints to plastics, vinegar to vinyl, inks to insulation *(2)*. Growth in production has been moderate, averaging 2 to 3%/yr. However, calcium-magnesium-acetate could bolster acetate production immensely with a potential demand of multimillion tons per year. There are two large volume potential end uses for this chemical: noncorrosive road deicer and a coal combustion

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additive *(3)*. Acetic acid accounts for 80% of the mass of calcium-magnesium-acetate and 85–90% of its feedstock costs *(4)*.

Although almost exclusively produced from petrochemical derivatives *(5)* at present, the production of acetic acid through homoacetate fermentation is receiving considerable attention. The advantage of the fermentation route is that it relies on renewable resources rather than nonrenewable (petroleum) resources. Homoacetate organisms such as *Clostridium thermoaceticum* make full use of this advantage by completely converting both glucose and xylose to acid with a theoretical weight yield of 100% *(6–8)*.

There are two major cost factors in this biological process. One is the high downstream processing cost, which is mainly owing to the low acetate concentration that develops under the strong inhibition by acetic acid. A substantial amount of research has been undertaken to alleviate this particular problem. One line of research dealt with increasing bacterial tolerance of acetate *(9–13)*, and another investigation novel separation methods such as nanofiltration and solvent extraction *(14–16)*. The second major cost item is the feedstock. Traditionally, hydrolyzates of corn starch and corn-steep liquor have been used for glucose/nitrogen sources for this process *(1,17)*.

The purpose of this present study was to evaluate cellulosic biomass as an alternative feedstock for this process. Conversion of cellulosic biomass to acetic acid involves two different biological processes, the enzymatic hydrolysis of biomass and the fermentation of glucose to acetate. When these two processes were carried out separately, the mode of operation was referred to as separate hydrolysis and fermentation (SHF), and when they were carried out simultaneously it was called simultaneous saccharification and fermentation (SSF). The two individual elements in the SHF have been investigated extensively. However, SSF has not been tested. Our primary interest was therefore in the development of the SSF. To our knowledge, our investigation represents the first such attempt to understand this process. The SSF was first studied using α-cellulose, a standard substrate. The resulting data were then applied to the development of a process using a more practical feedstock, a pulp mill sludge. The scope of this study thus covers the basic factors surrounding the SSF process and the assessment of the overall performance.

Materials and Methods

Enzymes and Feedstocks

Five commercially available cellulase enzymes were examined for their pH and temperature optima (Table 1) for the applicability in the SSF. Four different cellulosic substrates were analyzed for the composition (Table 2): α-cellulose (Sigma, St. Louis, MO), pulp mill sludge I (primary sludge for a kraft mill, Mead-Beit, Columbus, GA), pulp mill sludge II, and newsprint wastepaper. The α-cellulose and the sludge I were chosen for further investigation because of high glucan content. For the digestibility test, a 100-mL net reaction volume was chosen, with biomass consistent

Table 1
Enzymes Analyzed for Digestibility over a Broad pH Range

Enzyme	Optimum pH	Optimum temperature (°C)
ROCKSOFT NCE-L600[a]	6.5	55–57
ROCKSOFT SUPERACE[a]	4.8	55
ROCKSOFT CP POWDER[a]	4.8	55
IOGEN[b]	4.8	50
CELLUPRACT AL-100[c]	5.0	57

[a]Supplied by Dyadic, Jupiter FL.
[b]Supplied by National Renewable Energy Laboratory, Golden, CO.
[c]Supplied by Biopract GmbH, Berlin, Germany.

Table 2
Composition of Lignocellulosic Feedstock

Substrate	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ash (%)
α-Cellulose	79	21	0	0
Pulp mill sludge I	59	9	20	6
Pulp mill sludge II	28	3	30	24
Wastepaper (newsprint)	16	2	22	31

with 1 wt% cellulose, 0.5 mL of enzyme, and the remainder 0.1 *N* buffer solution. Citrate buffer was used for pH 5.0 to 6.0, and phosphate buffer for pH 6.0 to 7.0.

Fermentation

The microorganism employed was *C. thermoaceticum* (recently renamed to *Mooriella thermoacetica*), ATCC 49707. Inoculum cultures were grown at 59°C for 48 h in undefined Difco Clostridial medium (Difco, Detroit, MI). The fermentation medium contained: 5 g/L of yeast extract, 1 g/L of $(NH_4)_2SO_4$, 0.25 g/L of $MgSO_4 \cdot 7H_2O$, 0.04 g/L of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.00024 g/L of $NiCl_2 \cdot 6H_2O$, 0.00029 g/L of $ZnSO_4 \cdot 7H_2O$, 0.000017 g/L of Na_2SeO_3, and 0.25 g/L of cysteine·HCl·H_2O. In addition, 0.1 *N* phosphate/citrate buffers were utilized, and the oxygen indicator resazurin was added in trace amounts. Two weight percent of glucose or the equivalent of biomass was added.

A New Brunswick, Bioflo model-C30 was used as the bioreactor. It was operated with temperature and agitation control and with a 400-mL working liquid volume. An oxygen-free environment was maintained by initially sparging 0.3-μm of filtered CO_2 until the resazurin indicator changed from red to colorless and then constantly supplying CO_2 in the headspace of the bioreactor. The pH was controlled with 8 *N* NaOH so that there would not be an appreciable change in the working liquid volume. Samples were boiled to eliminate any bacterial activity and then stored at

4°C for further analysis. A 12- to 24-h period with no NaOH addition was taken as the fermentation end point.

Analytical Methods

Samples taken from the fermentation and hydrolysis experiments were analyzed for sugars and acetic acid by high-performance liquid chromatography equipped with an RI detector. A Bio-Rad-HPX-87H column (Bio-Rad, Hercules, CA) with 0.005 *M* H_2SO_4 mobile phase was used at 65°C at a flow rate of 0.55 mL/min.

Results and Discussion

Batch Enzyme Hydrolysis

Of the five enzymes listed in Table 1, ROCKSOFT SUPERACE (RS) performed best with respect to operable pH range, temperature proximity compatible with the *C. thermoaceticum* optimum of pH 6.0, and temperature of 59°C (Fig. 1). Under optimum conditions, 68% of α-cellulose and 70% of the mill waste was digestible using RS enzyme. Although IOGEN enzyme outperformed RS slightly in reactivity, its temperature optimum (55°C) concurred better with that of *C. thermoaceticum* (59°C). At pH 5.5, digestibilities of the pulp mill sludge I and α-cellulose were 61 and 60%, respectively, using RS enzyme. Increasing the pH to 6.0, the digestibilities reduced to 49% for α-cellulose and 36% for the sludge.

Glucose Fermentations

The literature is abundant with information on the conversion of glucose to acetic acid by *C. thermoaceticum (9–13,16)*. Our main concern was to determine the pH effect on this strain—whether there was enough latitude to be compatible with the enzymatic reaction. A series of tests revealed that the microbial performance was unimpaired within the pH range of 6.9–6.0. A slight increase in yield was seen as the pH was lowered from 6.9 to 6.0. At the same time, the lag time increased with a decrease in pH. A further decrease in pH to 5.0 had a detrimental effect on the acetate tolerance, lowering it to 12 g/L of acetate. Tolerance is defined as the level of total acetate (undissociated and salt form) in the media at which the fermentation ceases.

A strong inhibition effect on this organism by acetate is well documented. Han and Cheryan *(16)* reported that the tolerance for this organism at pH 5.5 is only 18 g/L of acetate. This trend would extend further to lower pH because the undissociated acid is more inhibitory to growth and fermentation than the salt form.

Simultaneous Saccharification and Fermentation

On the basis of the data from repeated batch hydrolysis and glucose fermentation runs, our best estimate of the optimum conditions for the SSF

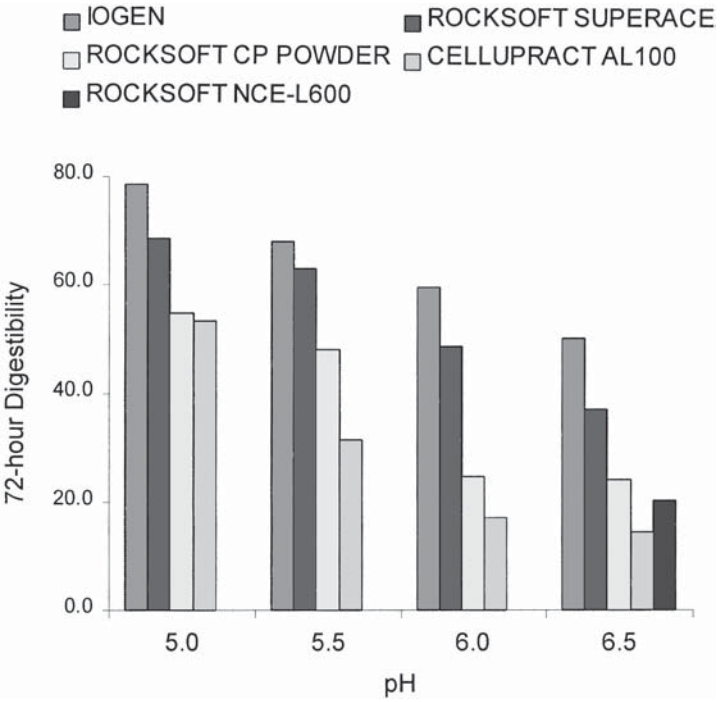

Fig. 1. Effect of pH on the enzymatic digestibility of various feedstocks. One weight percent initial glucan in media.

was determined to be 55°C and pH 5.5 for the pulp mill sludge, and 55°C and pH 6.0 for α-cellulose. This was done in consideration of the yield and productivity of the two biological processes. The discrepancy in pH optimum between the two substrates was unclear at this time. Somehow α-cellulose became less digestible at higher pH. For the conversion of pulp mill sludge, the reconciliation of the wider difference in the optimal pH of bacterial fermentation and that of enzyme hydrolysis led to a greater compromise in yield.

Figure 2 depicts batch SSF data based on α-cellulose with two different enzyme loadings. There was a significant amount of glucose accumulation in the early phase of the SSF owing to slow microbial growth and reactivity. We noted that the product formation pattern of this organism was predominantly growth associated *(1)*. Most of the fermentation process occurred over the span of 40–80 h. It was also observed that the lag phase of bacterial growth was excessively long and led to glucose accumulation, so that the SSF was not carried out in a normal pattern in which the process occurs under glucose-limited conditions. Instead, the overall process pattern was close to that of an SHF. The overall yield of acetate varied with the enzyme load. The apparent yields for the two runs were 68 and 33%, for 50 and 25 International filter paper units (IFPU)/g of glucan respectively. It is unclear whether the terminal yield was attained with 25 IFPU/g of glucan

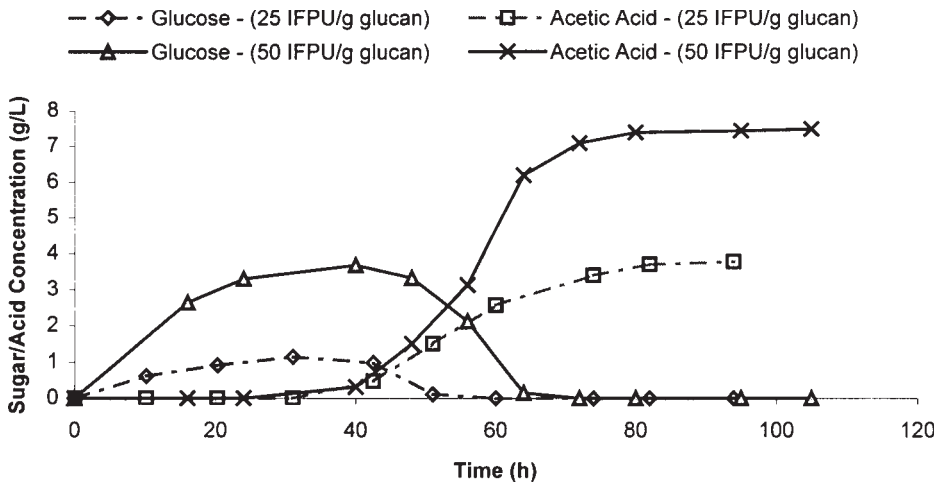

Fig. 2. SSF with α-cellulose. One weight percent initial glucan.

during this time span. An enzyme adapted to neutral pH conditions would allow for lower enzyme loadings, as well as increased acetate tolerance at higher operating pH. Enzyme systems with these qualities have been produced on a bench scale, with considerable activity at neutral pH and thermophilic temperature *(18).*

To accomplish a true SSF, a fed-batch operation was attempted. In this run, 1 g of α-cellulose was added every 9 h along with supplementation of the enzyme so that the overall enzyme input was maintained at a level of 50 IFPU/g of glucan. There were also two additional supplementations of minerals and yeast extract at 120- and 175-h points. Figure 3 shows the progression of the fed-batch SSFThat the SSF is operated under a stable condition for 250 h without any sign of system deterioration is significant, especially since this is the first attempt at the SSF on this organism. Under this mode of operation, the initial lag phase was followed by rapid glucose depletion. At the point of glucose depletion, the hydrolysis became the rate-limiting step. Over time, acetate inhibition lowered the activity of both the enzyme and the bacteria (hence a slight curvature in the line). The two sharp drops in the acetate curve at 120 and 175 h are the result of the dilution caused by minerals and yeast extract supplementation. Also, it was seen that the acetate production accelerated at these two points. This could be attributed to the effect of the addition of yeast extract. As the acetate level increases, the inhibition effect on bacteria also increases. Eventually it reaches a point where the microbial uptake of glucose becomes the rate-limiting step. This is indicated by accumulation of glucose at 180 h. The total acetate reached ~30 g/L at the end of the run. The overall yield in the fed-batch operation was 60%, somewhat lower than the batch yield. The limitation of the yield has to do with the acetate inhibition. The inhibition affects both the microorganism and the enzyme. The presence of

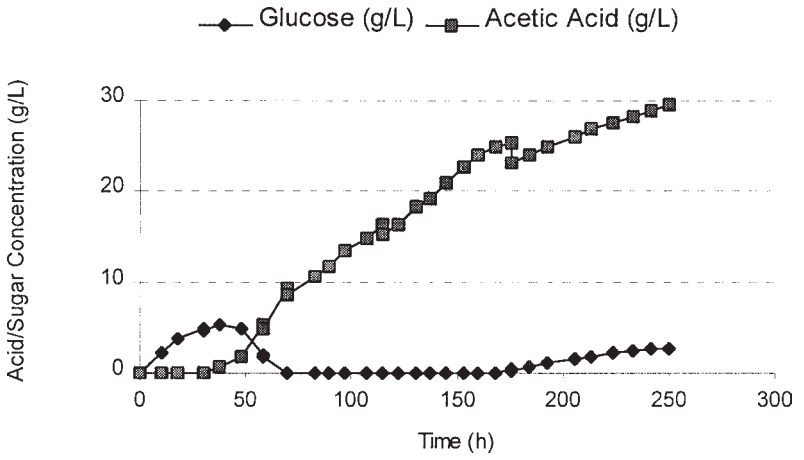

Fig. 3. Fed-batch SSF with α-cellulose. With each addition of biomass, 50 IFPU/g of glucan maintained.

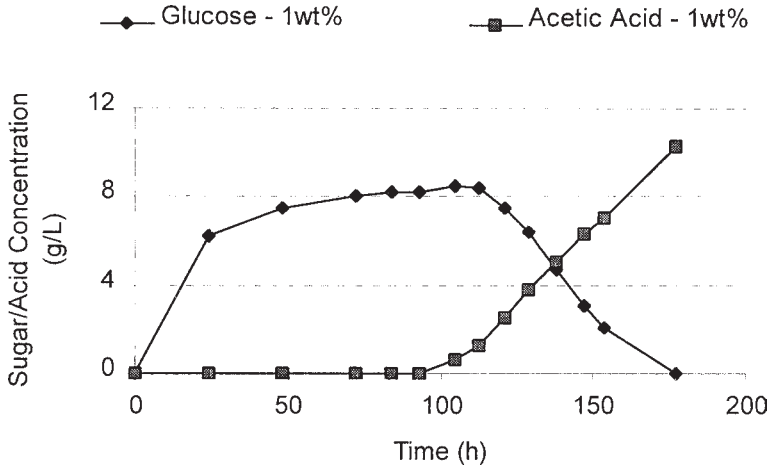

Fig. 4. SSF operation of paper mill sludge (pH 5.5, 55°C).

unconverted α-cellulose at the end of the run was observed, a sign of incomplete digestion. Inhibition of enzyme activity by acetate is therefore believed to be a significant factor limiting the yield. We have yet to determine which of the two inhibition factors is more important.

Follow-up experiments were conducted using the pulp mill sludge as the feedstock. Figure 4 presents the results from one of these experiments. A large amount of glucose accumulation was seen over the entire process. It appeared that the toxins released from the sludge were severely limiting glucose uptake. This led to a situation in which the microbial glucose uptake (0.13 g/[L·h]) was much lower than the enzyme hydrolysis rate (0.17 g/[L·h]). The intended SSF process was therefore operated under the mode of SHF. Despite the delay in microbial action, overall yield observed in this run was remarkably high at 85%. The spent solid residue retained no glucan

at the end of the run, indicating a complete digestion. Work is currently in progress in our laboratory to verify why the digestibility improved in the SSF over that of enzyme hydrolysis.

Conclusion

We have proven that a fed-batch SSF can be operated under a stable condition for production of acetic acid from α-cellulose. The overall yield of acetate in the SSF using α-cellulose was 60%. A total acetate concentration of 30 g/L was attainable in the SSF broth. Acetic acid inhibits not only the fermentation but also the enzymatic hydrolysis. Yields was higher (67%) in the batch SSF of α-cellulose in which acetate inhibition on enzyme was not as extensive. In the SSF using a paper mill sludge, bacterial growth was significantly inhibited by the toxins released from the sludge. The delay in microbial action changed the SSF into an SHF. Despite the long lag phase of microbial growth, the terminal yield obtainable from the paper mill sludge was substantially higher (85%). The toxins from the sludge do not appear to inhibit the enzymatic reaction in the SSF of the sludge.

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