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Fermentation of Xylose into Acetic Acid by *Clostridium thermoaceticum*

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Abstract

For optimum fermentation, fermenting xylose into acetic acid by *Clostridium thermoaceticum* (ATCC 49707) requires adaptation of the strain to xylose medium. Exposed to a mixture of glucose and xylose, it preferentially consumes xylose over glucose. The initial concentration of xylose in the medium affects the final concentration and the yield of acetic acid. Batch fermentation of 20 g/L of xylose with 5 g/L of yeast extract as the nitrogen source results in a maximum acetate concentration of 15.2 g/L and yield of 0.76 g of acid/g of xylose. Corn steep liquor (CLS) is a good substitute for yeast extract and results in similar fermentation profiles. The organism consumes fructose, xylose, and glucose from a mixture of sugars in batch fermentation. Arabinose, mannose, and galactose are consumed only slightly. This organism loses viability on fed-batch operation, even with supplementation of all the required nutrients. In fed-batch fermentation with CSL supplementation, D-xylulose (an intermediate in the xylose metabolic pathway) accumulates in large quantities.

Index Entries: Xylose; fermentation; *Clostridium thermoaceticum*; acetic acid.

Introduction

Acetic acid is an important feedstock for many chemicals including vinyl acetate polymer, cellulose acetate, terephthalic acid/dimethyl terephthalate, acetic acid esters, acetic anhydride, and calcium magnesium acetate. At present these products are made from petroleum-derived acetic acid *(1)*. Fermentation is potentially a cost-effective alternative for acetic acid production. Production of acetic acid via fermentation using renewable biomass feedstock has been studied extensively since the late 1970s *(2–4)*. The cellulose and hemicellulose in lignocellulosic biomass are the two most abundant renewable sources of carbon for fermentation to industrially

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useful chemicals. Efficient utilization of these constituents is vital to the development of economically viable bioconversion processes. Homoacetate anaerobic organisms such as *Clostridium thermoaceticum* convert glucose and xylose to acetic acid with a theoretical weight yield of 100% *(5–7)*.

Fermentation of glucose to acetic acid by the modified *C. thermoaceticum* strain (ATCC 49707) has been extensively studied *(8,9)*. However, its ability to ferment xylose into acetic acid is unknown. In this article, we report on the characteristics of acetic acid production by this organism using xylose as the carbon source.

Materials and Methods

Microorganism and Growth Media

A modified/mutant strain of *C. thermoaceticum* registered as ATCC 49707 *(10)* and renamed *Moorella thermoacetica* was used. The culture was grown in Difco Reinforced Clostridial medium at 59°C. It was maintained in the active state by transferring it alternately between this medium and medium containing 3% sodium acetate. To acclimatize this strain to using xylose as the principal sugar source, it was transferred to the fermentation medium described in the next section for 48 h for three generations alternating with growth in Clostridial broth. This adapted strain was stored at 4°C for future fermentation runs using xylose as the principal sugar source.

Fermentation Medium

The fermentation medium, similar to that used by Ljungdahl *(2)*, contained the following: 1.0 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, 0.25 g/L of cysteine·HCl·H_2O, 5 g/L of yeast extract or corn steep liquor (CSL) (variable), 7.5 g/L of KH_2PO_4, 4.4 g/L of K_2HPO_4, 0.415 g/L of NaOH, 5 g/L of $NaHCO_3$, and xylose (variable) with resazurin to detect trace amounts of oxygen.

The medium was prepared in five parts and sterilized separately at 121°C for 20 min:

1. Xylose.
2. Yeast extract or CSL.
3. Cysteine·HCl.
4. Mineral solution with resazurin.
5. Buffer (KH_2PO_4, K_2HPO_4, NaOH, $NaHCO_3$).

Fermentation

All fermentation experiments were conducted in a New Brunswick Bioflo model C-30 bioreactor at 59°C with a working volume of 400 mL of the previously described fermentation medium. Anaerobic environment was achieved by sparging filtered CO_2 until the oxygen indicator resazurin changed from pink to colorless and then was maintained by supplying CO_2

in the headspace of the reactor. The pH was maintained at 6.7–6.9 with 8 *N* NaOH so that there would be no appreciable change in working liquid volume. Fermentation was initiated by transferring 7 mL of 24-h xylose adapted inoculum to the reactor medium. Xylose concentration and amount of CSL added were varied in batch and fed-batch experiments. The yield of acetic acid to xylose was based on consumed xylose.

Analytical Methods

The fermentation samples were analyzed for sugar and acetic acid by high-performance liquid chromatography (HPLC) (Water Associates) equipped with an RI detector. Bio-Rad's HPX-87H column was used at 65°C with 0.005 *M* H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min. For mixed sugars, the substrate profiles were analyzed using a Bio-Rad HPX-87-P column operated at 85°C with deionized water as the mobile phase and a flow rate of 0.55 mL/min.

The cell density of the fermentation medium was measured by a turbidimeter (Hach Model 2100N) *(11)*. The data on nephelometric turbidity units were calibrated with four different Formazin standards prior to use.

Results and Discussion

The original strain of *C. thermoaceticum* ATCC 49707 was maintained through growth in medium with glucose as the only carbon source. On transfer to a medium containing a mixture of glucose and xylose, it consumed xylose first before consuming glucose. However, xylose uptake was very slow. With subsequent transfers in xylose medium, the rate of utilization increased. This culture was stored at 4°C and used in all fermentation experiments.

Profiles in Xylose Fermentation

Figure 1 shows typical batch fermentation profiles of cell growth, xylose utilization, and acetic acid production through fermentation at pH 6.9 and 59°C by *M. thermoacetica* (ATCC 49707). A lag phase was observed for the first 20 h, after which an exponential growth phase occurred for about 60 h, as depicted in Fig. 2. Almost all the xylose consumption and acetic acid production occurred during the log phase, indicating a growth-associated acid production. Cell numbers decreased after the log phase of growth, indicating that there was an autolytic decay of cells.

Batch fermentation experiments were conducted over a range of initial xylose concentrations to find the optimal initial sugar concentration for acetic acid production. The data indicated that a concentration of 15 g/L resulted in a maximum yield of acetic acid at 0.84 (g of acetic acid/g of xylose consumed). The maximum concentration of product was 15.2 g/L, which occurred with a 20 g/L xylose concentration with a yield of 76%. With increases in xylose concentration, the amount of unconsumed xylose

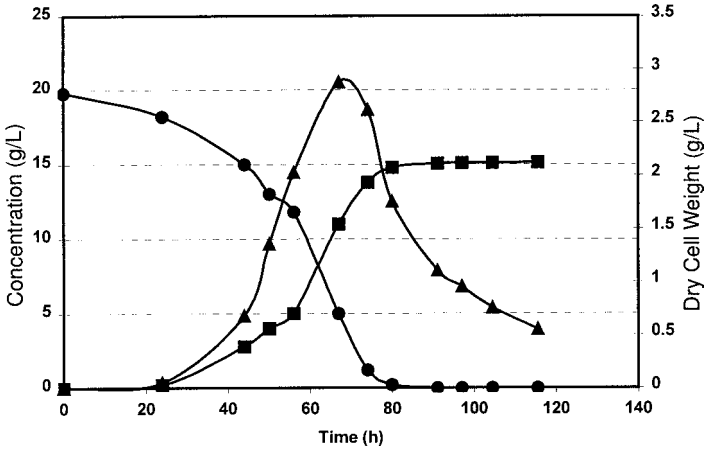

Fig. 1. Batch fermentation profile of *C. thermoaceticum*. Fermentation conditions: 59°C, pH 6.8. (—●—), xylose; (—■—), acetate; (—▲—), cell dry weight.

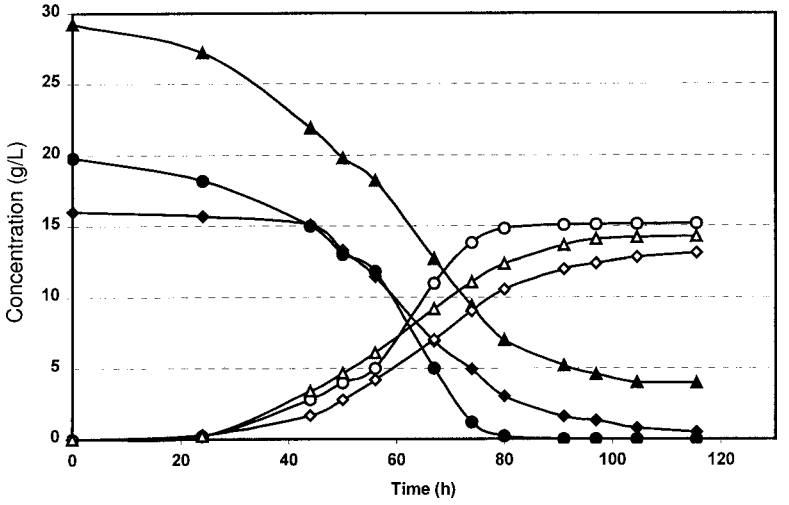

Fig. 2. Effect of initial xylose loading on the acetic acid yield. Fermentation conditions: 59°C, pH 6.8. (—◇—), acetic acid (1.5% xylose); (—○—), acetic acid (2% xylose); (—△—), acetic acid (3% xylose); (—◆—), 1.5% xylose; (—●—), 2% xylose; (—▲—), 3% xylose.

in the medium increased, which decreased the yield. The effects of initial xylose are summarized in Table 1 and Fig. 2. Subsequent fermentation experiments were conducted using an initial xylose concentration of 15–20 g/L.

Effect of CSL as Nitrogen Source

One obstacle to successfully commercializing this bioconversion process is the high cost of nutrients, such as yeast extract, required by *C. thermo-*

Table 1
Batch Fermentation of Xylose into Acetic Acid by *C. thermoaceticum* ATCC 49707 at 59°C, pH 6.8

Initial loading (g/L)	Xylose consumed (%)	Acetic acid		Nutrient		Fermentation time (h)
		Productivity (g/[L·h])	Yield (g acid/g xylose)	Yeast extract (g/L)	CSL (g/L)	
16	95	12.78	0.84	5	—	100
20	100	15.13	0.76	5	—	100
29.23	84.3	14.16	0.574	5	—	100
19.4	90	10	0.557	—	10	100
19.4	97.5	14.94	0.79	—	20	100
19.4	100	16.57	0.85	—	25	100

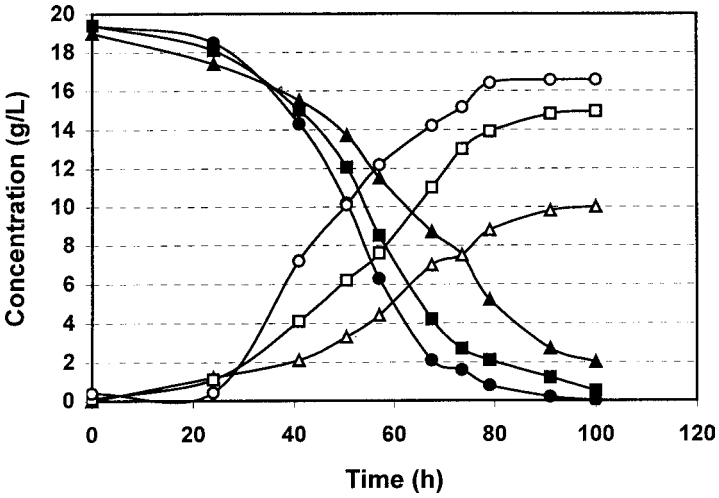

Fig. 3. Effect of concentration of CSL on xylose utilization. Fermentation conditions: 59°C, pH 6.8. (—△—), acetic acid (10 g/L CSL); (—□—), acetic acid (20 g/L CSL); (—○—), acetic acid (25 g/L CSL); (—▲—), 10 g/L CSL; (—■—), 20 g/L CSL; (—●—), 25 g/L CSL.

aceticum. CSL *(12)* has been identified as an inexpensive nitrogen-rich nutrient source *(4,13)*.

CSL, a byproduct of wet milling of corn, is a rich source of amino acids, minerals, and vitamins. It also contains other nitrogen compounds useful for microbial growth *(14)*. CSL has been used as medium for industrial production of penicillin *(15)*. Using this nutrient source, instead of yeast extract, can reduce the fermentation cost significantly *(16)*. Several experiments were performed to estimate the amount of CSL that would be required to obtain a yield of acetic acid comparable with that obtained using yeast extract. The results in Fig. 3 show that the fermentation profile with CSL is similar to that with yeast extract. When the concentration of CSL was 25 g/L and initial xylose loading was 20 g/L, the final concentration and yield of acetic acid were 16.57 g/L and 0.84 (g of acetic acid/g of xylose consumed), respectively. The profiles with varying CSL also are presented in Table 1.

Consumption of Mixture of Sugars

In a medium containing a mixture of glucose, xylose, galactose, fructose, arabinose, and mannose, *C. thermoaceticum* consumes fructose first and then xylose, as shown in Fig. 4. However, the rate of fructose consumption was faster than the rate of xylose consumption. Glucose was the third sugar to be utilized as the carbon source. When xylose, fructose, and glucose were completely consumed, the organism appeared to utilize arabinose, mannose, and galactose in that order, but at an extremely slow rate.

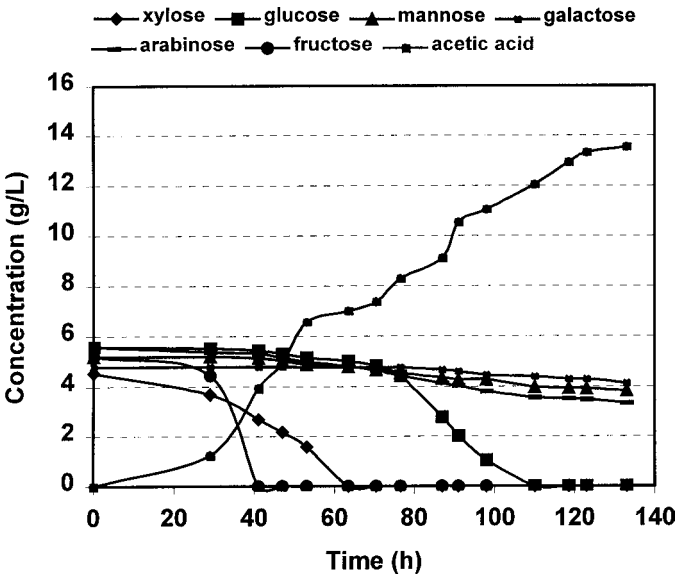

Fig. 4. Batch fermentation of a mixture of sugars into acetic acid. Fermentation conditions: 57°C, pH 6.8.

Fed-Batch Operation

Considering that the yield is higher at low sugar and high nitrogen source, a fed-batch mode of operation was perceived as a way to enhance yield by maintaining the optimal conditions in the reactor. Therefore, three sets of experiments were performed in which the xylose concentration was maintained at 15–20 g/L. The results are given in Table 2 and Fig. 5.

In experiment 1, adding 8 g of xylose led to slightly increased cell viability after the initial log phase. After 126 h of fermentation, however, there was no further uptake of xylose. In experiment 2, the same trend was observed despite adding 8 g of CSL to the 400 mL of medium. It is suspected that cell death could have resulted owing to lack of mineral supplementation. In experiment 3, mineral solution and cysteine-HCl were therefore added in addition to 8 g of CSL and 6 g of xylose. This strategy worked only up to 95 h of fermentation, a slight improvement over the previous set of experiments, in which growth ceased after 80 h. The organism produced acetic acid at the same rate as in the initial log phase of growth. However, further addition of sugar or nutrients did not increase the growth rate, sugar consumption, or acid yield. The organism could not be revitalized after the initial 80 to 90-h period. Accumulation of an intermediate product was also detected in experiment 3. This substance was identified to be D-xylulose using a pure standard in HPLC. It is an intermediate in the xylose metabolism of most bacterial systems formed by the action of xylose isomerase on xylose.

Table 2
Fed-Batch Fermentation of Xylose into Acetic Acid by *C. thermoaceticum* ATCC 49707 at 59°C, pH 6.8

Volume		Xylose			Acetic acid			
Initial (mL)	Final (mL)	Initial (g/L)	Total (g/L)	Consumed (%)	Productivity (g/[L·h])	Yield (g/g xylose)	Time (h)	Additional nutrients
400	405	18.9	15.56	57	19.6	0.7	120	Xylose
400	420	18	15.2	52	17.48	0.7	120	Xylose + CSL
400	460	19.7	29.88	74	17.66	0.36	120	Xylose + CSL + minerals

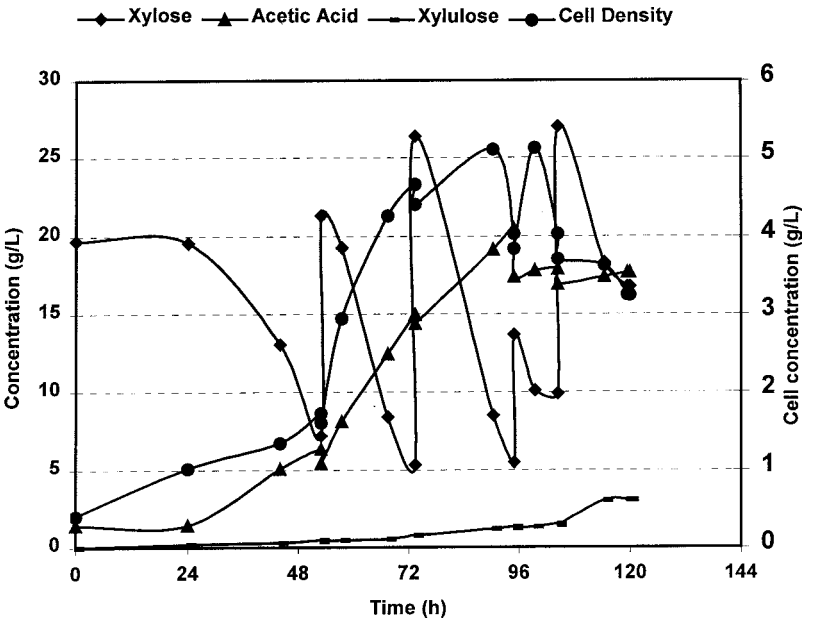

Fig. 5. Fermentation profile under the fed-batch mode of operation. Fermentation conditions: 59°C, pH 6.8. Experiment 3: total addition of 32 g of xylose, 16 g of CSL, and trace salts.

Conclusion

C. thermoaceticum needs to be acclimatized to a xylose environment to obtain high yields of acetic acid. It preferentially consumes xylose over glucose when grown in a medium containing a mixture of glucose and xylose. To maintain viability for xylose fermentation, it is necessary to grow the organism in xylose and glucose medium alternately. In a 20 g/L xylose medium containing 5 g/L of yeast extract, fermentation to acetic acid occurs within 80 h, resulting in a final acetate concentration of 15.2 g/L and yield of 0.76. CSL is efficiently used by this strain as its nitrogen source. With an initial CSL loading of 25 and 20 g/L of xylose, 0.86 yield and 6.6 g/L of final acetic acid concentration are attained. Replacing yeast extract with CSL can significantly reduce production cost. The organism consumes arabinose, mannose, and galactose only when each of these is present with xylose in the medium. In a batch fermentation of a mixture of sugars, the extent of consumption of mannose, arabinose, and galactose is <20% in 130 h. Fed-batch operation did not result in increased yield of acetic acid, because the organism lost viability after a certain period and was not revived by adding extra nutrients or trace elements. This proves to be a major drawback for acetate production from this strain using xylose as the carbon source. Accumulation of D-xylulose is detected in fed-batch fermentation of xylose with CSL as the nitrogen source.

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