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Bioconversion of acid-hydrolyzed poplar hemicellulose to acetic acid by *Clostridium thermoaceticum*

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SUMMARY

Clostridium thermoaceticum was used to ferment carbohydrate released from pretreated oat spelt xylan and hemicellulose isolated from hybrid poplar. Hydrolysis with dilute sulfuric acid (2.5% (v/v) for oat spelt xylan and 4.0% (v/v) for poplar hemicellulose) at 100 °C for 60 min was found to release the highest concentration of fermentable substrate. *C. thermoaceticum*, when grown in non-pH controlled batch culture at 55 °C under a headspace of 100% CO_2, typically produced 14 g l^{-1} acetic acid during a 48 h fermentation in medium containing 2% xylose. In fed-batch fermentations this organism was able to produce 42 g l^{-1} acetic acid after 116 h when the concentration of xylose was maintained at approximately 2% and the pH was controlled at 7.0.

INTRODUCTION

Considerable interest has focused on the development of fermentation processes utilizing carbohydrates derived from inexpensive lignocellulosic materials such as agricultural and forest residues [3,8,11,15,23]. Considering that cellulose and hemicellulose comprise the two most abundant renewable sources of carbon for fermentation to industrially useful chemicals, efficient utilization of both these constituents of lignocellulosic biomass is vital to the development of economically viable bioconversion processes [3,23].

The hemicellulose component comprises from 20–40% of plant dry weight and contains branched polysaccharides composed of various arrangements of pentoses such as xylose and arabinose, and hexoses such as mannose, glucose, and galactose [9]. The chemistry of the principal monomeric carbohydrates of these polymers is dependent upon their source with, for example, the hemicellulose of hardwoods consisting primarily of xylose [21].

The fact that hemicelluloses are considerably easier to hydrolyze to monomeric sugars than cellulose provides the possibility that the hemicellulosic fraction may be selectively hydrolyzed from the remaining plant biomass [10]. Enzymatic hydrolysis has been studied as a way to produce sugars from hemicellulosic materials because of the mild operating conditions required and the absence of toxic by-products such as furfurals [8,16,17]. Hemicelluloses are also readily depolymerized to their component sugars by dilute acid hydrolysis due to the low degree of polymerization and non-crystalline structure [3]. Dilute acid hydrolysis at moderate temperature results in the solubilization of hemicelluloses while leaving cellulose intact but with enhanced sensitivity to cellulase attack [8,10]. This pretreatment releases fermentable sugars from hemicelluloses while producing reduced amounts of degradation products that may be toxic to the fermenting organism [3].

Previous research has demonstrated that *Clostridium thermoaceticum* is potentially useful for the conversion of lignocellulosic biomass because of its ability to ferment the major sugars found in the hemicellulose fraction and cellulose [1,12]. This acetogenic thermophile is capable of converting glucose and xylose to acetic acid, as the only product, in near stoichiometric yields [1,6,12,13]. However, this organism does not possess the hydrolytic enzymes required to digest lignocellulosic polysaccharides and can therefore only utilize these substrates after they have been hydrolyzed to their component sugars [12].

Interest in the acetic acid fermentation of renewable lignocellulosic substrates has focused on the development of an inexpensive process to produce acetic acid for subsequent use in calcium-magnesium-acetate (CMA), an alternative to conventional road de-icing salt [13,14]. The utilization of the hemicellulose component of lignocellulosic materials has been considered crucial to the economics of biomass utilization strategies [11]. In this study, dilute sulfuric acid pretreatments were used to

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hydrolyze oat spelt xylan and the hemicellulose fraction isolated from hybrid poplar wood. The results of the fermentation of these pretreated substrates using *C. thermoaceticum* are reported here.

MATERIALS AND METHODS

Culture and culture conditions

C. thermoaceticum ATCC 39073 was obtained from Dr. Harold Drake (The University of Mississippi, University, MS) and was cultivated at 55 °C under a headspace of 100% CO_2 in the medium described by Ljundahl [12]. This medium was made as three solutions which were sterilized separately and contained per liter: Solution A, 20 g of xylose in 100 ml of distilled water; Solution B, $NaHCO_3$, 16.8 g; K_2HPO_4, 7 g; KH_2PO_4, 5.5 g; and resazurin (1 ml of a 0.01% stock solution) in 100 ml of distilled water; and Solution C, yeast extract, 5.0 g; $(NH_4)_2SO_4$, 0.5 g; sodium thioglycolate, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; $CaCl_2 \cdot 2H_2O$, 0.025 g; $Fe(NH_4)_2(SO_4)_2$, 0.04 g; H_2SeO_4, 0.01 g; $NaMoO_4 \cdot 2H_2O$, 0.06 g; $Na_2WO_4 \cdot 2H_2O$, 0.01 g; $CoCl_2 \cdot 6H_2O$, 0.017 g; $NiCl_2 \cdot 6H_2O$, 0.005 g; H_3BO_3, 0.00015 g; $ZnCl_2$, 0.00015 g; $AlK(SO_4)_2 \cdot 12H_2O$, 0.00015 g; $CuCl_2 \cdot 2H_2O$, 0.00015 g; and EDTA, 0.005 g in 800 ml of distilled water. After sterilization, these solutions were combined and sparged with sterile CO_2.

Fermentation experiments were conducted in 10 ml Hungate tubes, 100 ml bottles sealed with butyl rubber stoppers, and in a 2.0 l fermentor (New Brunswick Scientific Co., Edison, NJ). The initial pH ranged between 7.2 and 7.4, except where indicated, and all fermentations were maintained at 55 °C with a headspace of 100% CO_2. The pH in the fermentor was automatically controlled at pH 7.0 by the addition of 10 N NaOH. An actively growing culture was used as inoculum at a concentration of 5% (v/v) in all experiments.

Hemicellulose pretreatment

The hemicellulose fraction was isolated from debarked *Populus tremuloides* wood, which had been ground in a Wiley mill to pass a 40 mesh screen and extracted with benzene-ethanol (2 : 1) for 24 h in a Soxhlet apparatus according to the method of Timell [21]. After air drying in a vacuum hood, 150 g of extracted wood were shaken with 900 ml of 24% (w/w) KOH for 3 h at room temperature. The solubilized fraction was filtered through sintered glass and precipitated by pouring into 2.5 l of ethanol : acetic acid (15 : 1). After centrifugation, the hemicellulose precipitate was washed successively with 70% and 90% ethanol, and dried under vacuum in the presence of NaOH. The final product was ground to a fine powder before use in acid hydrolysis experiments. Oat spelt xylan was obtained from Sigma (St. Louis, MO).

Suspensions of hemicellulose and oat spelt xylan were hydrolyzed over a range of sulfuric acid concentrations at 100 °C for 60 min with the ratio of liquid to solids equal to 20 : 1. The suspensions were then adjusted to pH 7.0 by the addition of solid $Ca(OH)_2$ and centrifuged to remove any insoluble materials. These neutralized hydrolysates were filter sterilized and concentrated as needed before use in fermentation experiments.

Methods of analyses

Growth of *C. thermoaceticum* was measured at time intervals by monitoring the increase in optical density (OD) at 660 nm using a Beckman DU-2 (Beckman Instruments, Fullerton, CA) spectrophotometer with a 1 cm lightpath [7]. Samples having an absorbancy greater than 0.5 were diluted 1 : 10 with distilled water to obtain more accurate measurements.

Pentose released from pretreated hemicellulose and oat spelt xylan was determined by the *p*-bromoaniline method using xylose as standard [2]. Reducing sugars were determined by the dinitrosalicyclic acid (DNS) method of Miller [18] based on xylose reducing equivalents. Total carbohydrate was measured by the phenol-sulfuric acid method [4].

Carbohydrates released by hydrolysis treatments were identified using a Perkin Elmer series 10 HPLC equipped with a BioRad HPX-42A column (BioRad, Richmond, CA) maintained at 85 °C with water as eluant and a flow rate of 0.5 ml/min. Detection was by UV absorbancy at 192 nm. The elution times of xylose, furfural, and hydroxymethylfurfural (Sigma, St. Louis, MO) were used to identify sample peaks.

Acetic acid production in sampled fermentation broth was measured by a model 2400 Varian gas chromatograph (Varian Inc., Sunnyvale, CA) using a flame ionization detector and equipped with a stainless steel column (1.82 m by 0.31 cm) packed with Porapak Q (Supelco Inc., Bellefonte, PA). The column was maintained at 210 °C, the injector at 200 °C, and the detector at 235 °C.

RESULTS AND DISCUSSION

Fermentation experiments were run over a range of initial xylose concentrations to determine the substrate level for optimal acetic acid production in batch fermentation. The data in Table 1 indicate that yields of acetic acid of greater than 71% can be obtained from 72 h fermentations in which the initial xylose concentration was 3.3% or less. A maximum product concentration of 14.1 g l^{-1} was realized when the initial xylose level was 2%.

In experiments in which the xylose concentration was greater than 3.3%, carbohydrate utilization and the acetic

acid yield were significantly lower (Table 1). These results are similar to those previously reported for glucose tolerance, which indicate that, while growth rates were not affected over an initial range of glucose concentrations from 1–10%, product yields sharply declined at the higher substrate concentrations [13]. As with xylose, 2% glucose has been reported to provide for maximum conversion to acetic acid in yields from 75 to 85% [13,20,22]. The less than theoretical yield of 100% conversion has been reported elsewhere in the literature and may be attributable to loss of carbohydrate to cell mass and thermal degradation over the course of the fermentation [20].

Fig. 1 shows a typical time course fermentation of xylose at an initial concentration of 2% and without pH adjustment. The culture grew exponentially for the first 24 h reaching a maximum specific growth rate of 0.14 h^{-1}. At this point, cell density reached a peak of 2.2 optical density units, corresponding to 1.4 g l^{-1} cell dry weight and approximately 10 g l^{-1} acetic acid had been produced. Acetic acid production continued until 48 h at which time over 90% of the xylose had been consumed and the pH had decreased from an initial value of 7.3 to a final pH of 5.0. During this 48 h time course, acetic acid concentrations of greater than 14 g l^{-1} were obtained.

The fermentation profile in Fig. 1 is closely related to those found for growth and acetic acid production using glucose as substrate [13]. In these studies, cell density typically increased for the first 30 h and acetic acid formation continued until 48 h at which time the glucose was exhausted, resulting in product concentrations of 14 to 16 g l^{-1} in batch fermentations [20,22]. Acetic acid production is known to be both growth and non-growth associated when either glucose or xylose is used as substrate [20].

In fermentation processes for chemical production, the accumulation of end products often inhibits cell growth and product formation. Studies to determine the effect of product inhibition on glucose fermentation [19,20,22] indicate that in batch cultures, acetic acid levels above 10 g l^{-1} cause a reduction in specific growth rate and product formation. Table 2 shows the effect of acetic acid concentration on the ability of *C. thermoaceticum* to ferment xylose. These data indicate that acetic acid concentrations of greater than 10 g l^{-1} significantly affect product formation. The addition of acetic acid to initial concentrations of 10 to 20 g l^{-1} results in lowered xylose utilization and acetic acid production.

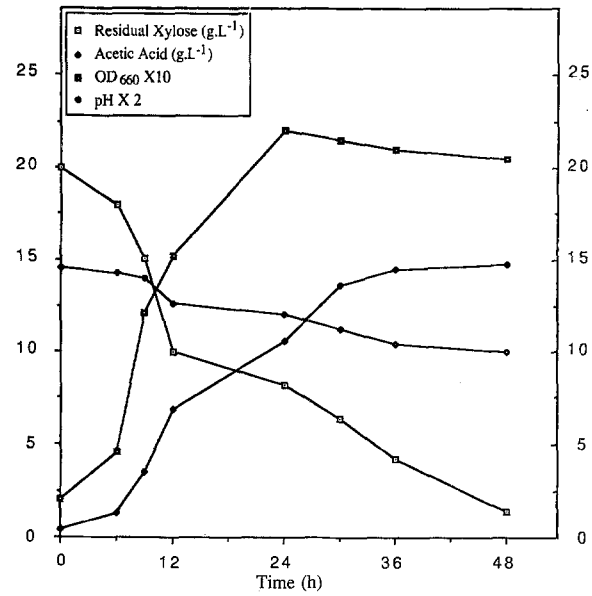

Fig. 1. The time course of growth, xylose utilization, and acetic acid production by *C. thermoaceticum* in non-pH controlled fermentation. Acetic acid produced (◆), residual xylose (□), OD_{660} × 10 (■), and pH × 2 (◇).

TABLE 1

Effect of xylose concentration on acetic acid production

% Initial xylose (w/v)	% Xylose consumed	Acetic acid produced (g l^{-1})[a]	Yield (g acetate/ g xylose)
0.0	–	0.3	–
0.1	95	0.73	0.77
0.5	94	3.4	0.72
1.0	93	6.6	0.71
1.5	93	9.9	0.71
2.0	93	14.1	0.76
2.4	68	13.2	0.81
3.3	44	11.6	0.80
5.3	35	11.1	0.60

[a] Represents acetic acid produced after 72 h fermentation.

TABLE 2

Effect of acetic acid concentration on xylose utilization

% Initial acetic acid (v/v)	% Xylose consumed[a]	Acetic acid produced (g l^{-1})[b]	Yield (g acetate/ g xylose)
0.0	92	14.3	0.78
0.5	89	14.0	0.79
1.0	85	13.5	0.79
1.5	80	12.3	0.77
2.0	78	11.0	0.70
5.0	38	5.3	0.70
10.0	23	2.8	0.61

[a] Initial xylose concentration was 2% (w/v).
[b] Represents acetic acid produced after 72 h fermentation.

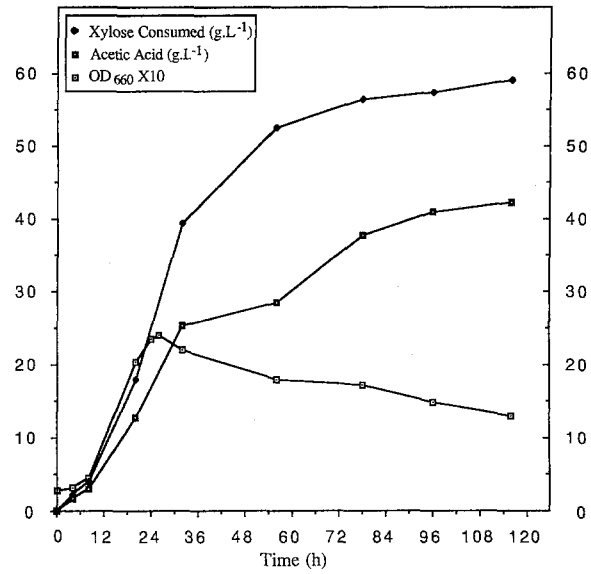

Fig. 2. Profile of a fed-batch fermentation with *C. thermoaceticum* in which pH was controlled at pH 7.0. Xylose concentration was maintained between 10 and 20 g l^{-1}. Acetic acid produced (■), xylose consumed (◆), OD_{660} × 10 (□).

Fig. 2 shows the results from a fed-batch fermentation controlled at pH 7 using 10 N NaOH for acetic acid neutralization and maintaining the xylose concentration between 10 and 20 g l^{-1}. A peak cell density of 2.43 OD units was obtained at 30 h followed by a gradual decline until 116 h at which time no additional xylose was consumed. Acetic acid production correlated closely to xylose consumption and reached a final concentration of 42 g l^{-1}, a yield of 71%. It has been reported that in fed-batch fermentations where glucose was substrate and was maintained at concentrations between 5 and 15 g l^{-1} as much as 56 g l^{-1} acetic acid was produced in yields ranging from 67 to 85% [22]. As in the non-pH controlled fermentation shown in Fig. 1, acetic acid production is related to cell growth for the first 24 h and continues thereafter when cell mass no longer increases.

In experiments designed to generate fermentable carbohydrate, hemicellulose, isolated in the laboratory from poplar wood, and commercially obtained oat spelt xylan were hydrolyzed using dilute sulfuric acid pretreatments. These data are in Table 3. These pretreatments were selected to prevent the loss of fermentable substrate due to the formation of inhibitory products such as furfural and hydroxymethylfurfural (HMF) which may be toxic to the fermenting organism [8]. HPLC and chemical analysis by the *p*-bromoaniline method indicated that xylose was the principle reducing sugar released by these treatments and that furfural and HMF were not produced in detectable amounts.

The ability of *C. thermoaceticum* to ferment these pretreated substrates was tested and Table 4 shows fermentation data from experiments in which the hydrolyzed substrate was adjusted to an initial concentration of 2% carbohydrate, which is shown in Table 1 to be optimal for growth and acetic acid formation. After 72 h these fermentations were analyzed for carbohydrate consumption and acetic acid concentration.

Poplar hemicellulose and oat spelt xylan treated with 4.0% and 2.5% (v/v) H_2SO_4, respectively, were found to provide the optimal substrates for fermentation. *C. thermoaceticum* consumed 89% and 94% of the available carbohydrate in these experiments (Table 4). Optimal

TABLE 3

Pretreatment of oat spelt xylan and poplar hemicellulose

% Sulfuric acid (v/v)[a]	Oat spelt xylan		Poplar hemicellulose	
	Pentose released (g l^{-1})[b]	Reducing sugar (g l^{-1})[c]	Pentose released (g l^{-1})[b]	Reducing sugar (g l^{-1})[c]
0.0	1.0	1.2	0.7	0.8
1.0	16.5	17.0	3.0	3.2
1.5	28.3	29.7	6.0	6.4
2.0	34.8	36.5	8.9	9.3
2.5	42.8	44.9	12.6	13.1
3.0	41.0	43.1	13.0	13.5
4.0	–	–	14.0	14.7

[a] Hydrolysis treatments were carried out at 100 °C for 60 min at sulfuric acid concentrations indicated with a liquid to solids ratio of 20:1.

[b] Pentose concentration was determined by the *p*-bromoaniline method.

[c] Reducing sugars were determined using the DNS procedure.

TABLE 4

Fermentation of pretreated oat spelt xylan and poplar hemicellulose

Pretreatment % Sulfuric acid[a]	Oat spelt xylan		Poplar hemicellulose	
	% Pentose consumed[b]	Acetic acid produced ($g\,l^{-1}$)	% Pentose consumed[b]	Acetic acid produced ($g\,l^{-1}$)
0.0	–	–	–	–
1.0	–	–	–	–
1.5	89	12.1	92	11.9
2.0	92	13.4	91	11.8
2.5	94	14.4	87	11.3
3.0	90	11.2	87	11.3
4.0	–	–	89	11.5

[a] Hydrolysis treatments were carried out at 100 °C for 60 min under the conditions indicated with a liquid to solids ratio of 20:1.
[b] Initial pentose concentration was adjusted to 2%. Fermentations were incubated for 72 h.

acetic acid yields were also realized under these same pretreatment conditions. During 72 h fermentations, acetic acid concentrations reached 14.4 g l^{-1} from oat spelt xylan, and 11.5 g l^{-1} from poplar hemicellulose.

C. thermoaceticum is the current organism of choice for the development of an acetic acid fermentation process based on the utilization of lignocellulosic biomass because it can ferment both glucose and xylose [13,14]. The results of this study show that xylose, as well as acid-hydrolyzed hemicellulose and xylan, can serve as substrates for acetic acid production by *C. thermoaceticum*. This work, combined with previous studies for acetic acid production by *C. thermoaceticum* may ultimately be useful in improving the economic viability for the large scale production of CMA through the bioconversion of lignocellulosic materials.

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