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# Efficient Degradation of Lignocellulosic Plant Biomass, without Pretreatment, by the Thermophilic Anaerobe "*Anaerocellum thermophilum*" DSM 6725[▽]

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**Very few cultivated microorganisms can degrade lignocellulosic biomass without chemical pretreatment. We show here that "*Anaerocellum thermophilum*" DSM 6725, an anaerobic bacterium that grows optimally at 75°C, efficiently utilizes various types of untreated plant biomass, as well as crystalline cellulose and xylan. These include hardwoods such as poplar, low-lignin grasses such as napier and Bermuda grasses, and high-lignin grasses such as switchgrass. The organism did not utilize only the soluble fraction of the untreated biomass, since insoluble plant biomass (as well as cellulose and xylan) obtained after washing at 75°C for 18 h also served as a growth substrate. The predominant end products from all growth substrates were hydrogen, acetate, and lactate. Glucose and cellobiose (on crystalline cellulose) and xylose and xylobiose (on xylan) also accumulated in the growth media during growth on the defined substrates but not during growth on the plant biomass. *A. thermophilum* DSM 6725 grew well on first- and second-spent biomass derived from poplar and switchgrass, where spent biomass is defined as the insoluble growth substrate recovered after the organism has reached late stationary phase. No evidence was found for the direct attachment of *A. thermophilum* DSM 6725 to the plant biomass. This organism differs from the closely related strain *A. thermophilum* Z-1320 in its ability to grow on xylose and pectin. *Caldicellulosiruptor saccharolyticus* DSM 8903 (optimum growth temperature, 70°C), a close relative of *A. thermophilum* DSM 6725, grew well on switchgrass but not on poplar, indicating a significant difference in the biomass-degrading abilities of these two otherwise very similar organisms.**

Utilization of lignocellulosic biomass derived from renewable plant material to produce ethanol and other fuels is viewed as a major alternative to petroleum-based energy sources (19). The efficient conversion of plant biomass to fermentable sugars remains a formidable challenge, however, due to the recalcitrance of the insoluble starting materials (13, 21, 36). Thermal and chemical pretreatments must be used to solubilize and release the sugars, but such processes are costly and not very efficient (17, 28). Most pretreatments utilize acids, alkali, or organic solvents (39). Moreover, the plant feedstocks vary considerably in their compositions. The main components of plant biomass and the sources of the fermentable sugars, cellulose and hemicellulose, are combined with lignin, which can occupy 20% (wt/wt) or more of the plant cell wall. The development of technologies to efficiently degrade plant biomass therefore faces considerable obstacles. The discovery or engineering of new microorganisms with the ability to convert the components of lignocellulosic biomass into sugars is therefore of high priority.

Not many microorganisms are able to degrade pure crystalline cellulose, and the cellulose in plant biomass has a high order of crystallinity and is even less accessible to microbial or enzymatic attack (1, 12–14). Aerobic cellulolytic microorganisms usually secrete (hemi)cellulolytic enzymes containing carbohydrate-binding modules that serve to bind the catalytic domains to insoluble substrates. On the other hand, some anaerobic bacteria and fungi produce a large extracellular multienzyme complex called the cellulosome. This binds to and efficiently degrades cellulose and other polysaccharides, although it has a limited distribution in nature (3, 7). The rate at which microorganisms degrade cellulose increases dramatically with temperature (20), but the most thermophilic cellulosome-producing bacterium that has been characterized, *Clostridium thermocellum*, grows optimally near only 60°C (3, 9). A few anaerobic thermophiles are known that are able to grow on crystalline cellulose even though they lack cellulosomes, and in those cases the highest optimum growth temperature is 75°C (4, 32). Biomass conversion by thermophilic anaerobic microorganisms has many potential advantages over fermentation at lower temperatures. In particular, the organisms tend to have high rates of growth and metabolism, and the processes are less prone to contamination (30).

The gram-positive bacterium "*Anaerocellum thermophilum*" strain Z-1320 is among the most thermophilic of the cellulolytic anaerobes (32). It grows optimally at 75°C at neutral pH and utilizes both simple and complex polysaccharides, although it does not grow on xylose or pectin (32). The end

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products of fermentation are lactate, ethanol, acetate, $CO_2$, and hydrogen. Although *A. thermophilum* Z-1320 grows very rapidly on crystalline cellulose (4), surprisingly, it has been studied very little since its discovery (32). We report here on the physiology of a very closely related strain, *A. thermophilum* DSM 6725, the genome of which was recently sequenced (16). The ability of *A. thermophilum* DSM 6725 to grow on different types of defined and complex substrates was investigated with a focus on switchgrass and poplar. These high-lignin plants have been selected as models for biomass-to-biofuel conversion by the BioEnergy Science Center (funded by the U.S. Department of Energy; http://bioenergycenter.org/). We show that *A. thermophilum* DSM 6725 is able to grow efficiently on both types of plant substrate without a chemical pretreatment step.

## MATERIALS AND METHODS

**Microorganisms.** *Anaerocellum thermophilum* strain DSM 6725 was obtained from the DSMZ (http://www.dsmz.de/index.htm). *Caldicellulosiruptor saccharolyticus* DSM 8903 was a gift from Robert Kelly of North Carolina State University.

**Growth substrates.** The following growth substrates with the indicated sources were used: D-(+)-cellobiose (catalog no. C7252), D-(+)-xylose (X1500), oat spelt xylan (X0627), and pectin (P9135; all from Sigma, St. Louis, MO); Avicel PH-101 (catalog no. 11365; Fluka, Switzerland); poplar and switchgrass (sieved −20/+80-mesh fraction; Brian Davison, Oak Ridge National Laboratory, Oak Ridge, TN); and Tifton 85 Bermuda grass and napier grass (sieved, −20/+80-mesh fraction; Joy Peterson, Department of Microbiology, University of Georgia). Samples of plant biomass were used as received without chemical or physical treatments and are referred to as untreated biomass (or without pretreatment).

**Growth medium.** *A. thermophilum* DSM 6725 and *C. saccharolyticus* DSM 8903 were grown in 516 medium (32) except that vitamin and trace mineral solutions were modified as follows. The mineral solution contained the following (per liter): $NH_4Cl$, 0.33 g; $KH_2PO_4$, 0.33 g; KCl, 0.33 g; $MgCl_2 \cdot 6H_2O$, 0.33 g; $CaCl_2 \cdot 2H_2O$, 0.33 g; yeast extract, 0.5 g; resazurin, 0.5 mg; vitamin solution, 5 ml; trace minerals solution, 1 ml. The vitamin solution contained the following (in mg/liter): biotin, 4; folic acid, 4; pyridoxine-HCl, 20; thiamine-HCl, 10; riboflavin, 10; nicotinic acid, 10; calcium pantothenate, 10; vitamin $B_{12}$, 0.2; *p*-aminobenzoic acid, 10; lipoic acid, 10. The trace mineral solution contained the following (in g/liter): $FeCl_3$, 2; $ZnCl_2$, 0.05; $MnCl_2 \cdot 4H_2O$, 0.05; $H_3BO_3$, 0.05; $CoCl_2 \cdot 6H_2O$, 0.05; $CuCl_2 \cdot 2H_2O$, 0.03; $NiCl_2 \cdot 6H_2O$, 0.05; $Na_4EDTA$ (tetrasodium salt), 0.5; $(NH_4)_2MoO_4$, 0.05; $AlK(SO_4)_2 \cdot 12H_2O$, 0.05. The medium was prepared anaerobically under a $N_2$–$CO_2$ (80:20) atmosphere, $NaHCO_3$ (1 g/liter) was added, and the mixture was reduced using (per liter) 0.5 g cysteine and 0.5 g $N_2S$. The final pH was 7.2. All soluble and insoluble biomass and defined substrates were used at a final concentration of 0.5% (wt/vol). Growth was at 75°C (*A. thermophilum*) or at 70°C (*C. saccharolyticus*) as static cultures in 100-ml serum bottles or with shaking (150 rpm) in 0.5- or 1.0-liter flasks. All media were filter sterilized using a 0.22-μm-pore-size sterile filter (Millipore Filter Corp., Bedford, MA). Insoluble substrates were added directly to sterilized culture bottles, followed by the addition of the filter-sterilized medium. The culture media containing the insoluble substrates without inoculation were used as negative controls.

**Growth on spent substrate.** The residual substrate was collected in late stationary phase. The residual substrate was separated from the cells by filtering it through glass filters (pore size, 40 to 60 μm), washed with distilled water to remove cells and media, and vacuum dried at 23°C for 18 h. This spent substrate was then used to grow new cell cultures. Unspent substrate was the unused and unwashed biomass (from the package) that was used in the first culture; first-spent substrate was that which remained after the first culture growth; and second-spent substrate was that which remained after growth of a culture on first-spent substrate.

**Conversion of insoluble substrate.** Conversion of insoluble substrate was calculated based on the amount of substrate remaining after cell growth had reached stationary phase (residual substrate). The residual substrate was determined by weight. So-called insoluble substrates derived from switchgrass, poplar, Avicel, and xylan were prepared by washing with water at 75°C (the growth temperature of *A. thermophilum* DSM 6725). Each substrate was suspended in distilled water (1 g/50 ml), stirred overnight at 75°C, and then washed twice with an equal volume of water at 75°C using a coarse glass filter (pore size, 40 to 60 μm). The substrate that remained on the filter was dried overnight at 50°C and was used for the growth experiments as insoluble substrate. The residual substrate was washed and dried similarly. The amount of insoluble substrate was measured after drying at 105°C overnight to a constant weight.

**Cell growth.** Cell density was monitored by cell count using a phase-contrast microscope with 40× magnification and expressed as cells per ml. To determine the extent to which *A. thermophilum* DSM 6725 adhered to insoluble substrate, the culture was shaken (150 rpm) for various time periods at 75°C in a closed 100-ml serum bottle with 50 ml of the mineral medium (pH 7.3) containing 0.5% (wt/vol) washed switchgrass in which the gas phase was replaced with $N_2$–$CO_2$ (80/20, vol/vol). The cultures were allowed to settle at room temperature for 15 min; then, 2 ml of the supernatant was withdrawn for the planktonic (free-floating) cell suspension. Both planktonic and substrate-bound cells were harvested by centrifuging the entire culture at 10,000 × *g* for 30 min. The centrifuged pellets were suspended in 50 ml of 50 mM Tris-HCl (pH 8.0), and the suspensions were sonicated on ice (six times for 30 s each time with 30-s intervals at 30 W). Cultures incubated under the same conditions without inoculation were used as the control for measuring cell protein concentration.

**Determination of structural carbohydrates and acid-soluble lignin.** Structural carbohydrates and acid-soluble lignin were determined using standard procedures at the National Renewable Resources Laboratory (http://www.nrel.gov/biomass/analytical_procedures.html). After the removal of water and ethanol extractives from switchgrass and poplar, the amount of lignin was estimated based on the absorption at 197 nm of the hydrolysate. Structural carbohydrates were determined by high-performance liquid chromatography (31).

**Product analyses.** Acetate and lactate were measured using a high-performance liquid chromatography apparatus (model 2690 separations module; Waters) equipped with an Aminex HPX-87H column (300 mm by 7.8 mm; Bio-Rad, Hercules, CA) at 40°C with 5 mM $H_2SO_4$ as the mobile phase at a flow rate of 0.6 ml $min^{-1}$ with a refractive index detector (model 2410; Waters, Milford, MA). Ethanol was measured enzymatically using an ethanol kit (Megazyme, Wicklow, Ireland). Hydrogen was determined by a gas chromatograph (model GC-8A; Shimadzu, Kyoto, Japan) equipped with a thermal conductivity detector and a molecular sieve column (model 5A 80/100; Alltech, Deerfield, IL) with argon as the carrier gas. Reducing sugars were determined as described previously (22). Gas chromatography-mass spectrometry (GC-MS) was used to quantify the relative concentrations of targeted metabolites in the culture supernatants (μg sorbitol equivalents/ml). Sample preparation GC-MS operating conditions were as described elsewhere (37). Briefly, 100 μl of sorbitol (1 mg/ml aqueous solution) was added to each 2-ml sample as an internal standard, and samples were then dried in a helium stream. The internal standard was added to correct for differences in derivatization efficiency and changes in sample volume during heating. Dried exudates were dissolved in 500 μl of silylation-grade acetonitrile, followed by the addition of 500 μl *N*-methyl-*N*-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane (Pierce Chemical Co., Rockford, IL), and samples were then heated for 1 h at 70°C to generate trimethylsilyl derivatives. After 5 days, 2-μl aliquots were injected into a DSQII (Thermo Fisher Scientific, Waltham, MA) GC-MS, fitted with an Rtx-5MS (Crossbond 5% diphenyl–95% dimethyl polysiloxane) capillary column (film thickness, 30 m by 0.25 mm by 0.25 μm; Restek, Bellefonte, PA). The standard quadrupole GC-MS was operated in electron impact (70 eV) ionization mode, with six full-spectrum (70- to 650-Da) scans per second. Gas (helium) flow was set at 1.1 ml per minute with an injection port configured in the splitless mode. The injection port and detector temperatures were set to 220°C and 300°C, respectively. The initial oven temperature was held at 50°C for 2 min and was programmed to increase at 20°C per min to 325°C and held for another 11.25 min before cycling back to the initial conditions. The target metabolites were integrated using a key selected ion (and confirmed by three additional characteristic *m/z* fragments), rather than the total ion current, to minimize the quantification of interfering metabolites. Extracted peaks were quantified by area integration and the areas scaled to the total ion current using correction factors for each metabolite. The concentrations were normalized to the quantity of the internal standard (sorbitol) recovered.

## RESULTS

**Growth on defined carbohydrates.** *A. thermophilum* DSM 6725 grew well at 75°C with pectin, xylose, xylan, cellobiose, or crystalline cellulose as the carbon and energy sources. No significant growth of the organisms was observed in the standard medium in the absence of an added carbon source. As shown

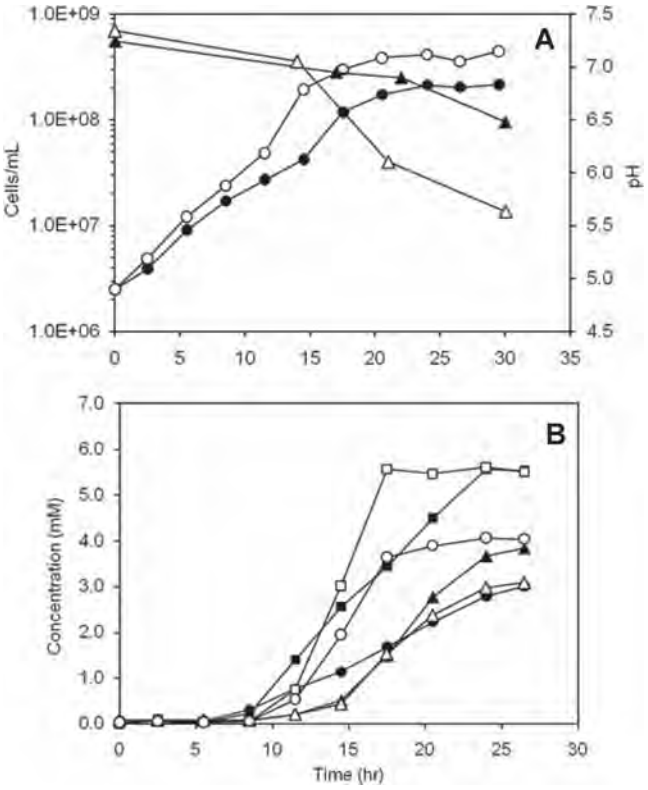


FIG. 1. Growth of *A. thermophilum* DSM 6725 on crystalline cellulose and xylan. Cell growth on unprocessed crystalline cellulose (solid symbols) and xylan (open symbols) was monitored by measuring cell density (circles) and pH (triangles) (A) and hydrogen (squares), lactate (triangles), and acetate (circles) (B).

in Fig. 1A, cells reached stationary phase on cellulose at 75°C within approximately 20 h with a cell density of $1.5 \times 10^8$ cells/ml and a decrease in pH from 7.1 to 6.4. The predominant product after this time was hydrogen, with smaller amounts of lactate. Ethanol was not detected (<100 μM). The ratio of $H_2$ to acetate produced after 21 h of growth on cellulose was 2.0, indicating that acetate was produced entirely as an end product of fermentation. In contrast, this ratio dropped to 1.4 after a similar growth period on xylan. This shift to more acetate formation is presumably because xylan contains acetyl substituents which are removed as part of the xylan degradation process. *A. thermophilum* DSM 6725 exhibited similar growth kinetics when cellobiose was used as the carbon source, although the end products differed, with more lactate produced than hydrogen (data not shown). Contrary to what was previously reported for *A. thermophilum* strain Z-1230 (32), strain DSM 6725 also grew well on xylose and pectin. As shown in Fig. 1A, growth on xylan was slightly better than on crystalline cellulose, with a higher cell density and a greater decrease in pH (to 5.5). The predominant end product was also hydrogen rather than lactate (Fig. 1B), and no ethanol was detected. Growth on xylose and pectin was similar to that observed on xylan (data not shown). When *A. thermophilum* DSM 6725 was grown on crystalline cellulose in the presence of acetate (50 mM) or lactate (50 mM) or under hydrogen (1 atm), there was little effect on the growth kinetics of the organism, although slightly lower cell densities were obtained (data not shown).

**Growth on untreated plant biomass.** Three grasses and one hardwood were selected as plant biomass substrates for growth. Tifton Bermuda grass and napier grass have relatively low lignin contents (3 to 4%), with cellulose and hemicellulose constituting 20 to 28% and 29 to 42% (wt/wt), respectively (15, 25). The high-lignin plants were switchgrass (acid-soluble lignin was measured at 17.8%, wt/wt) and the hardwood poplar (21.8% acid-soluble lignin). Chemical analyses of the biomass also indicated that, compared to poplar, switchgrass contains more xylose (19.2 versus 14.8%), arabinose (3.3 versus 0.4%), and galactose (1.8 versus 1.0%) but less glucose (31.0 versus 46.2%) and no mannose (which is found in poplar at 2.8%). Poplar and switchgrass also differed in relative amounts of water extractives (2.2 and 14.5%, respectively) and ethanol extractives (3.7 and 1.4%, respectively).

*A. thermophilum* DSM 6725 was able to grow on all four types of plant material when each was added to the standard growth medium without any pretreatment (the plant substrates were used as received and were added to filter-sterilized growth media). In closed static cultures (50 ml), growth on all plant materials was similar to that seen with the defined substrates, with cell densities reaching approximately $1.8 \times 10^8$ cells/ml within 20 h (data not shown). In closed stirred cultures (500 ml), *A. thermophilum* DSM 6725 grew on switchgrass and poplar, with cell densities after 21 h of $1.3 \times 10^8$ and $1.1 \times 10^8$ cells/ml, respectively (Fig. 2A). In all cases, stationary phase was reached after approximately 10 h of growth and the growth was accompanied by slight acidification of the media. As shown in Fig. 2B, hydrogen was the predominant end product. The ratios of hydrogen to acetate produced during growth on switchgrass and poplar after 21 h of growth were also less than that (2.0) found using cellulose. The values were 0.97 and 1.3, respectively, indicating that about half of the acetate that is produced originates from these highly acetylated plant materials. Chemical analyses of the residual switchgrass and poplar at periodic times throughout the growth phase up to 15 h revealed that the proportions of the constituents of the two types of biomass did not change significantly from those described above (data not shown).

**Growth on insoluble plant biomass.** Both switchgrass and poplar contain significant amounts of water-soluble material (14.5 and 2.2%, wt/wt, respectively), in addition to potential growth substrates such as protein. To determine if the ability of *A. thermophilum* DSM 6725 to grow on the untreated plant biomass, as well as on crystalline cellulose and xylan, was due to soluble rather than to insoluble substrates, a simple washing procedure was utilized. Both switchgrass and poplar, as well as crystalline cellulose and xylan, were incubated with unbuffered water at 75°C for 18 h to remove soluble material. The amounts of insoluble material remaining after this treatment were 72, 93, 84, and 34% (by weight) of the starting material, respectively. Thus, xylan had the majority of hot-water-extractable components (66%) and poplar the least (7%). These remaining materials were designated insoluble substrates. For the plant materials, chemical analyses revealed that the proportions of the constituents of the biomass did not change significantly after they had been incubated for 15 h at 75°C (in the absence of the organism; data not shown).

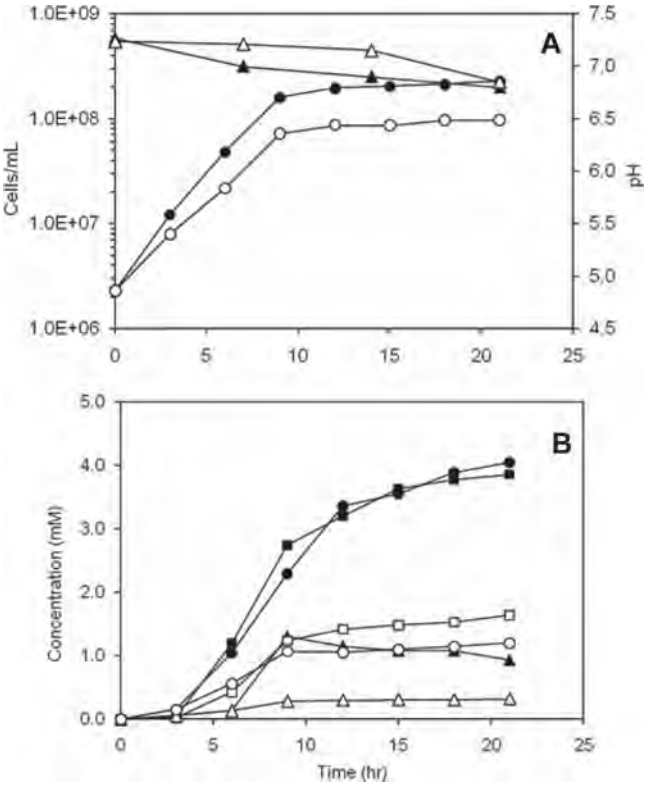


FIG. 2. Growth of *A. thermophilum* DSM 6725 on unprocessed switchgrass and poplar. Cell growth on unprocessed switchgrass (solid symbols) and poplar (open symbols) was monitored by measuring cell density (circles) and pH (triangles) (A) and hydrogen (squares), lactate (triangles), and acetate (circles) (B).

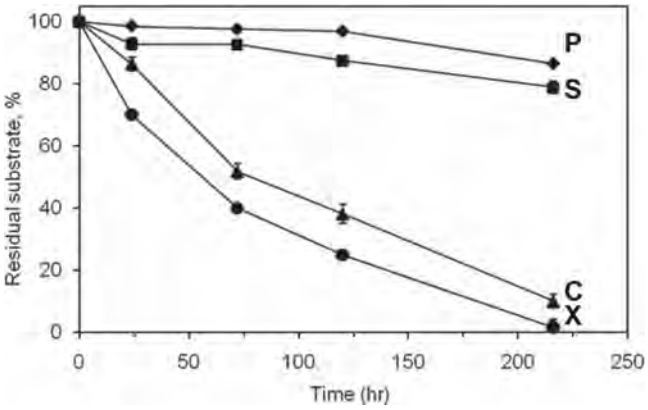


FIG. 3. Utilization of the insoluble forms of poplar, switchgrass, xylan, and crystalline cellulose by *A. thermophilum* DSM 6725. The amounts of substrate remaining after cell growth on the insoluble forms of poplar (P; diamonds), switchgrass (S; squares), xylan (X; circles), and crystalline cellulose (C; triangles) were determined by dry weight.

A. *thermophilum* DSM 6725 was able to utilize the insoluble material derived from poplar and switchgrass, as well as from crystalline cellulose and xylan, as sources of carbon and energy. The growth kinetics on each of the substrates were very similar to those observed on the unwashed (untreated) substrates, with cell densities of $\sim 2 \times 10^8$ cells/ml after 20 h or so (data not shown). To investigate the mechanism by which *A. thermophilum* DSM 6725 degraded the insoluble plant biomass, hot-water-washed insoluble switchgrass was used as the carbon and energy source, and the total amount of protein that was generated during the growth phase was determined for the planktonic cells and for all sedimented material, which included both planktonic cells and those adhered to the plant biomass. There were no significant differences between the two sets of measurements, indicating that a significant fraction of the cells is not complexed with the undegraded biomass (data not shown). Consequently, the cell densities determined in the experiments reported herein are an accurate estimate of cell growth on the insoluble plant biomass. Moreover, *A. thermophilum* DSM 6725 is predominantly in the planktonic state when it degrades biomass, and direct and permanent attachment to the insoluble substrate is apparently not necessary.

**Production of reducing sugars from insoluble substrates.** While the growth kinetics of *A. thermophilum* DSM 6725 on all four insoluble substrates were similar, there was an important difference in the responses to the insoluble plant material and the insoluble defined substrates upon prolonged incubation. As shown in Fig. 3, after almost 10 days, most of the xylan (98%) and cellulose (90%) had been solubilized by the organism, but the conversions of switchgrass and poplar were less extensive, with 26% and 15%, respectively, being utilized. Accordingly, chemical analysis of the growth media for reducing sugars revealed that insoluble crystalline cellulose was continuously degraded throughout the 10-day period, as shown by the continuous production of reducing sugars that approached 20 mM in concentration (after 10 days). In contrast, only low concentrations (<1 mM) of reducing sugars were produced from insoluble switchgrass and poplar, even after 10 days (data not shown). As shown in Table 1, metabolomic analyses revealed that after a 90-h incubation with *A. thermophilum* DSM 6725, high concentrations of glucose and cellobiose and, to a lesser extent, cellotriose were generated from crystalline cellulose, with comparable amounts of xylose and xylobiose and, to a lesser extent, xylotriose released from xylan. In contrast, only trace amounts of glucose were produced from poplar (Table 1). Trace amounts of cellobiose, galactose, xylose, and xylobiose were released from switchgrass, and in this case the amount of glucose produced was significant, reaching about 27% of that released on crystalline cellulose. Clearly, there is a difference in the mechanisms by which the organism metabolizes the two insoluble plant materials, and, more importantly, these also differ from those that are used to degrade the defined polysaccharide substrates.

The difference between how *A. thermophilum* DSM 6725 degrades defined polysaccharides and plant biomass was also evident from a kinetic analysis of end products. As shown in Fig. 4, the production of hydrogen and lactate closely followed cell growth, reaching a maximum in less than 20 h and showing only a slow increase even over 20 days. Note that both phenomena are seemingly independent of glucose production, which continues for about 10 days (Table 1), at which time only 10% of the cellulose remains (Fig. 3). In contrast, even though the growth kinetics on poplar and switchgrass are similar to those observed on cellulose, with a cell density of $>10^8$ cells/ml

TABLE 1. Production of simple sugars by *A. thermophilum* DSM 6725

| Growth substrate | Concn (mM)[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Glucose | Cellobiose | Cellotriose | Galactose | Xylose | Xylobiose | Xylotriose |
| Poplar | 0.06 | ND | ND | ND | ND | ND | ND |
| Switchgrass | 2.62 | 0.22 | ND | 1.05 | 0.40 | 0.31 | ND |
| Cellulose | 9.69 | 4.89 | 0.19 | 1.48 | 0.09 | 0.05 | ND |
| Xylan | 0.04 | ND | ND | ND | 9.26 | 4.00 | 0.09 |

[a] After 90 h of growth on insoluble forms of poplar, switchgrass, crystalline cellulose, and xylan, the concentrations were determined by GC-MS as described in Materials and Methods. ND, not detected.

reached within 20 h, hydrogen and lactate are produced continuously over the 20-day period. Moreover, there is a dramatic difference in the ratio of reduced products. Cellulose degradation results in the formation predominantly of lactate (the hydrogen/lactate ratio is 0.55 after 10 days), while hydrogen is the main product during growth on both switchgrass and poplar (the hydrogen/lactate ratio is 9.0 after 10 days).

**Growth on spent insoluble substrates.** The results shown in Fig. 3 prompted the following questions. Why does *A. thermophilum* DSM 6725 cease to grow significantly after 20 h or so on the insoluble plant substrates? Is it the recalcitrance of the material that remains? To address this issue, the insoluble material that remained after *A. thermophilum* DSM 6725 had reached stationary-phase growth on insoluble switchgrass and poplar was recovered and washed, and this so-called spent insoluble biomass was used as a carbon and energy source for a new *A. thermophilum* DSM 6725 culture. As shown in Fig. 5, the organism grew as well on spent switchgrass as, and even better on spent poplar than, it did on the unspent insoluble materials, with similar growth rates and cell densities. In addition, the amounts of the predominantly reduced products (hydrogen and lactate) were also virtually identical (data not shown). Moreover, the insoluble material that was left after the growth of the second culture was recovered and washed, and this second-spent insoluble biomass was used for a third fresh culture of *A. thermophilum* DSM 6725 (in all cases the initial concentration of the growth substrate was 5.0 g/liter). As shown in Fig. 5, the third culture supported similarly rapid growth and a cell density that approached the results obtained with the first and second cultures. The end products were once more comparable to those measured with the other cultures. Apparently, the mechanisms by which *A. thermophilum* DSM 6725 degrades and utilizes unspent, first-spent, and second-spent insoluble biomass from poplar and switchgrass are virtually identical. Compared to the original unspent insoluble biomass used in the first culture, the amounts of switchgrass and poplar that were solubilized after the third culture were 65.2% and 36.6%, respectively.

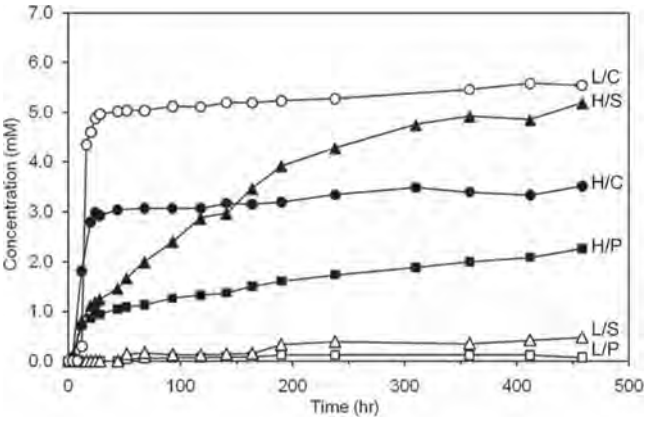


FIG. 4. End product analyses after prolonged growth of *A. thermophilum* DSM 6725 on the insoluble forms of poplar, switchgrass, and crystalline cellulose. Hydrogen (solid symbols) and lactate (open symbols) in cultures grown on the insoluble forms of poplar (squares), switchgrass (triangles), and crystalline cellulose (circles) were measured. H, hydrogen; L, lactate; P, poplar; S, switchgrass; C, crystalline cellulose.

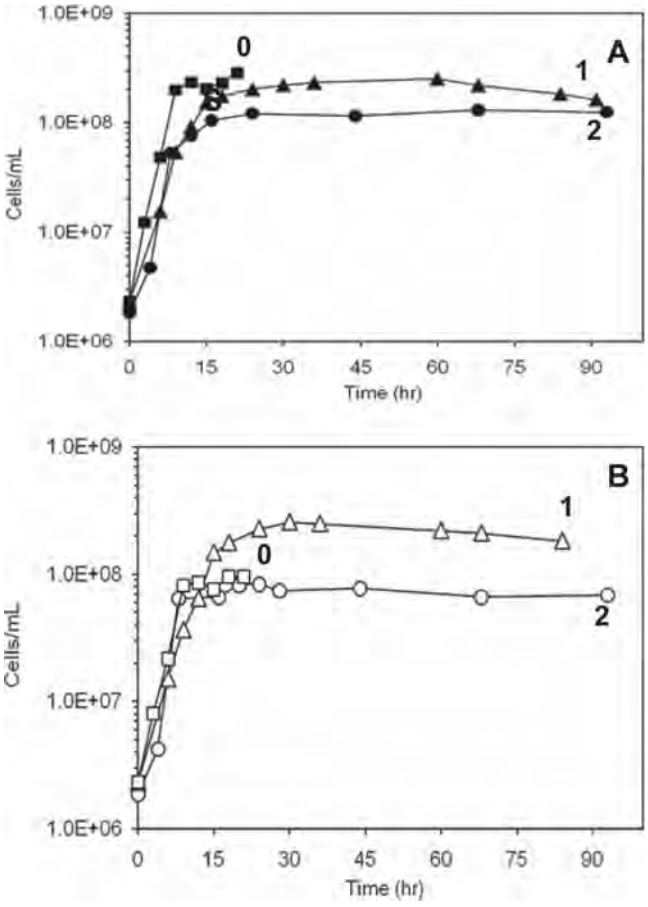


FIG. 5. Growth of *A. thermophilum* DSM 6725 on unspent, first-spent, and second-spent insoluble switchgrass (A) and insoluble poplar (B). Cells were grown on unspent (0; squares), first-spent (1; triangles), and second-spent (2; circles) switchgrass or poplar.

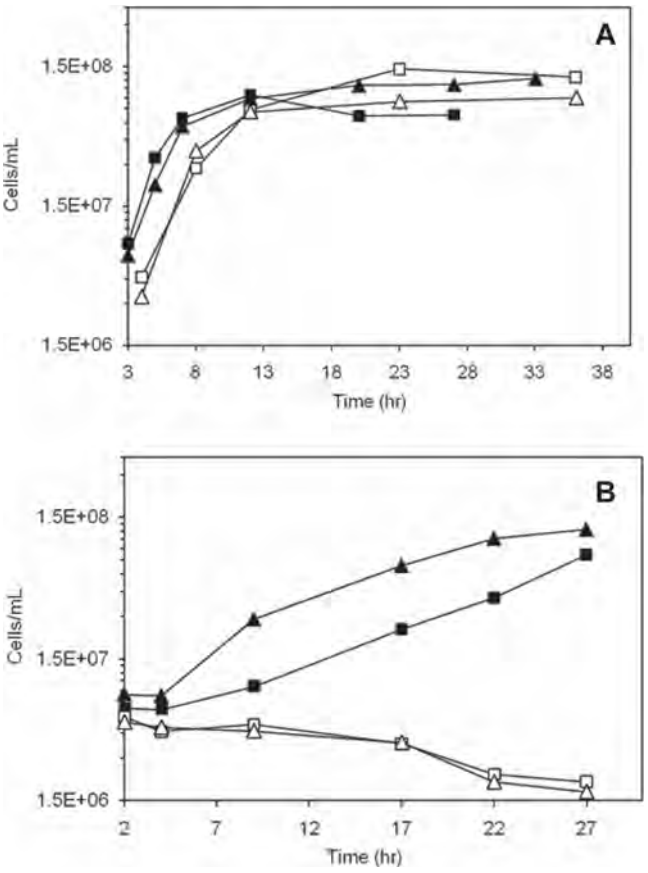


FIG. 6. Growth of *A. thermophilum* DSM 6725 and *C. saccharolyticus* DSM 8903 on insoluble and soluble fractions of switchgrass (A) and poplar (B). *A. thermophilum* (solid symbols) and *C. saccharolyticus* (open symbols) were grown on the insoluble (triangles) and soluble (squares) fractions of switchgrass or poplar.

**Growth of *Caldicellulosiruptor saccharolyticus* DSM 8903.** *C. saccharolyticus* is a close relative of *A. thermophilum* DSM 6725 and is known to degrade crystalline cellulose (26). As shown in Fig. 6, it was able to grow at 70°C on insoluble switchgrass in a manner similar to that of *A. thermophilum* strain 6725. The two organisms also exhibited comparable growth kinetics on so-called soluble switchgrass, which is the material released after the hot water wash of the plant material. Interestingly, however, while *A. thermophilum* DSM 6725 also grew on soluble poplar, as well as on the insoluble poplar material, *C. saccharolyticus* DSM 8903 did not. No significant growth of *C. saccharolyticus* DSM 8903 was detected on either the soluble or insoluble poplar, suggesting a significant difference in the biomass-degrading abilities of the two organisms.

## DISCUSSION

*A. thermophilum* DSM 6725 grew very well on crystalline cellulose and xylan, the two main components of plant biomass. The ability of *A. thermophilum* strain DSM 6725 to grow on xylose and pectin, and also the lack of detectable ethanol as a fermentation product, is in contrast to what was previously reported with *A. thermophilum* strain Z-1320 (32), showing that the two strains are not identical. This conclusion was also reached from a comparison of the limited gene sequence data available for *A. thermophilum* strain Z-1320 with the relevant genes in the complete genome sequence of *A. thermophilum* strain DSM 6725 (16). Specifically, their 16S rRNA sequences have 14 mismatches, insertions, or deletions and the nucleotide sequences of their CelA genes have 23 mismatches (16, 27). In our hands, *A. thermophilum* DSM 6725 behaved as a stable, pure strain, and in all cultures examined by phase-contrast and scanning electron microscopy, we observed only one type of rod-shaped cell (data not shown). Similarly, all experiments with strain DSM 6725 were reproducible, and two cultures obtained from the DSMZ culture collection more than 2 years apart showed identical properties, including the ability to grow on xylan, xylose, and pectin. In addition, the genome sequence of *A. thermophilum* DSM 6725 was readily assembled (16), consistent with its having a pure culture as the DNA source. *A. thermophilum* DSM 6725 (16) and *A. thermophilum* Z-1320 (26, 32, 41) are therefore closely related but are not identical strains.

We also show here that *A. thermophilum* DSM 6725 is able to efficiently utilize untreated forms of both low-lignin (napier and Bermuda) and high-lignin (switchgrass) grasses and a hardwood (poplar) as carbon and energy sources, with cell densities of $>10^8$ cells/ml obtained in 20 h. Significant growth of an anaerobic thermophile such as *A. thermophilum* DSM 6725 on untreated poplar was unexpected, given that this hardwood contains a large amount of lignin and highly crystalline cellulose and it would be expected to be even more recalcitrant to microbial conversion than switchgrass. For example, softwood species contain cellulose of 52 to 62% crystallinity (1, 24) and the value for switchgrass is 55% (12), which compares with a value of 65% for poplar (38). This higher value is close to the range (66 to 75%) for the form of cellulose (Avicel) (18) used as a model substrate in the growth studies reported here. *A. thermophilum* DSM 6725 degraded more than 90% of this crystalline cellulose over a 10-day period (Fig. 3). The organism is comparable to the well-studied *Clostridium thermocellum* in its cellulose-degrading ability but has the advantage of a higher optimum growth temperature (75°C rather than 60°C) and the ability to hydrolyze xylan and consume xylose, an end product of xylan hydrolysis, which *C. thermocellum* lacks (11, 34). Like *C. thermocellum* (34, 40), *A. thermophilum* DSM 6725 generated high concentrations of glucose and cellobiose from cellulose, and similarly, xylan was converted mainly to xylobiose and xylose. These products are typical for cellulose and xylan hydrolysis by many other microorganisms, although the ratios may differ (2, 6, 40).

The concern that the ability of *A. thermophilum* DSM 6725 to grow on untreated or unprocessed plant biomass was due at least in part to its utilization of the more readily accessible, water-extractable components was found to be unwarranted by the demonstration that the organism grows just as well on what we term insoluble biomass, which is that remaining after an 18-h wash with water at 75°C (Fig. 4). Similarly, the recalcitrance of the biomass remaining at the end of the growth phase is not the reason why the organism ceases to grow, as the so-called first-spent and second-spent biomass substrates were as efficiently utilized as the unspent material (Fig. 5). The overall conversion of switchgrass (65%) and poplar (36%) after the third culture is an excellent starting point for cell

immobilization studies or the use of recycled bioreactors that might ultimately lead to almost complete solubilization of the plant material (10, 23, 35). What is not clear, however, is the fate of lignin, the other major component of plant cell walls. Lignin constitutes approximately 20% of both switchgrass and poplar biomass, and at present no anaerobic organism is known that can degrade lignin. Presumably, in the case of switchgrass, the 35% of the initial biomass that remains after the third culture is enriched with lignin and contains more recalcitrant cellulose and other components embedded into a lignin network than does the unspent switchgrass, although further analyses will be required to substantiate this.

Analysis of the end products formed upon growth on different substrates showed that on crystalline cellulose, xylan, switchgrass, and poplar, hydrogen was the predominant product, compared to lactate over the first 20 h or so. However, as shown in Fig. 4, continued incubation led to more lactate than hydrogen from cellulose, but then little of either product was produced after 30 h, even though accumulation of glucose continued (Table 1). In contrast, upon prolonged incubation on poplar and switchgrass, hydrogen remained the predominant product and production continued for up to 20 days. Changes in the ratio of hydrogen to lactate during the later stages of growth can originate from inhibition of hydrogenase by $H_2$ or by regulation of other enzymes involved in pyruvate conversion to lactate (8, 33). Hydrogen is clearly the predominant product when *A. thermophilum* DSM 6725 grows on plant biomass. Thus, for practical applications, the bacterium has the potential to be a hydrogen rather than ethanol producer.

*C. saccharolyticus* DSM 8903 could also grow on switchgrass (both soluble and insoluble fractions) but differed from *A. thermophilum* DSM 6725 in its response to poplar. *A. thermophilum* DSM 6725 grew on this substrate as well as on its water-extractive (soluble poplar) and extractive-free (insoluble poplar) fractions, while *C. saccharolyticus* DSM 8903 did not grow on either of these fractions. This may be because the insoluble fraction of poplar is too recalcitrant for this bacterium; because poplar has a higher lignin content, a higher relative amount of cellulose, and a higher crystallinity than switchgrass; and/or because mannan is present in poplar but not in switchgrass. A comparison of the genome sequences of the two organisms might indicate genes unique to *A. thermophilum* DSM 6725 that allow this bacterium to grow on untreated hardwood. Alternatively, it is known that the water-extractable part of hardwoods such as poplar, which contains alkaloids, tannins, sesquiterpenes, and lignans, can be toxic to microorganisms (5, 17, 28, 29). Chemical pretreatment of biomass, which is considered at present to be a necessary step in any applied biomass-to-biofuel conversion process, can lead to the release of additional potential inhibitors, such as furfural, metal ions, and various lignin degradation products (17, 28). The design of less severe pretreatment steps, or even avoidance of the pretreatment step altogether, is therefore of great importance. Presumably, a microorganism such as *A. thermophilum* DSM 6725 that utilizes untreated plant biomass has a great advantage.

In summary, *A. thermophilum* DSM 6725 has the ability to grow on plant biomass with a high lignin content and high crystallinity of cellulose; it is insensitive to inhibitors present in poplar biomass; its cells remain vital and produce hydrogen, which is an alternative biofuel to ethanol, for prolonged periods (20 days); it is able to hydrolyze highly crystalline cellulose almost completely with glucose and cellobiose as major products; and it grows on spent biomass efficiently. These unique properties might be of utility in any applied biomass conversion process.

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