Development of a thermophilic coculture for corn fiber conversion to ethanol

Dhananjay Beri, William S. York, Lee R. Lynd, Maria J. Peña & Christopher D. Herring

The fiber in corn kernels, currently unutilized in the corn to ethanol process, represents an opportunity for introduction of cellulose conversion technology. We report here that *Clostridium thermocellum* can solubilize over 90% of the carbohydrate in autoclaved corn fiber, including its hemicellulose component glucuronoarabinoxylan (GAX). However, *Thermoanaerobacterium thermosaccharolyticum* or several other described hemicellulose-fermenting thermophilic bacteria can only partially utilize this GAX. We describe the isolation of a previously undescribed organism, *Herbinix* spp. strain LL1355, from a thermophilic microbiome that can consume 85% of the recalcitrant GAX. We sequence its genome, and based on structural analysis of the GAX, identify six enzymes that hydrolyze GAX linkages. Combinations of up to four enzymes are successfully expressed in *T. thermosaccharolyticum*. Supplementation with these enzymes allows *T. thermosaccharolyticum* to consume 78% of the GAX compared to 53% by the parent strain and increases ethanol yield from corn fiber by 24%.

1 Thayer School of Engineering, Dartmouth College, Hanover, NH 03755, USA. 2 Centre for Bioenergy Innovation, Oak Ridge National Laboratory, Oak Ridge, TN 37830, USA. 3 Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602, USA. 4 Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30602, USA. 5 Enchi Corporation, Lebanon, NH 03766, USA. 6 Department of Biological Sciences, Dartmouth College, Hanover, NH 03755, USA. ✉ email: lee.r.lynd@dartmouth.edu; mpena@ccrc.uga.edu
Conversion of cellulose feedstocks into biofuels is challenging due to their high recalcitrance, typically addressed with thermochemical pretreatment followed by large amounts of cellulase and hemicellulase enzymes to release soluble carbohydrates\(^1,2\). Recently, there has been interest in low-capital Generation 1.5 projects that produce ethanol from corn fiber at existing corn ethanol facilities\(^3,4\). Corn fiber makes up about 10% of the weight of corn kernels and consists of cellulose and hemicellulose from the aleurone and pericarp layers of the corn kernel. In current corn ethanol facilities, corn fiber ends up in the Distillers Dried Grains with Solubles (DDGS)\(^5\). If the fiber could be economically converted to ethanol, leveraging existing infrastructure, it would allow the facility to produce up to 13% more ethanol per bushel of corn while preserving and enriching the protein components as animal feed\(^5,6\). The cellulosic ethanol produced would generate additional revenue while serving as a transitional proving ground for advanced technology.

Cellulolytic bacteria and bacterial consortia have been identified that are mesophiles, moderate thermophiles and extreme thermophiles. *Clostridium thermocellum* is a moderate thermophile that has been extensively characterized and is one of the most effective organisms described to date at deconstructing cellulose biomass\(^7,8\). In particular, several recent studies have found *C. thermocellum* to be 2- to 4-fold more effective than commercial fungal cellulase at solubilizing both woody and herbaceous lignocellulosic feedstocks\(^9,10\). Its thermostable operating temperature may also be advantageous for industrial biomass conversion\(^11\). However, *C. thermocellum* is unable to ferment most of the hydrolyzable sugars present in hemicellulose, which represents a large portion of lignocellulosic biomass. As a potential coculture partner, *Thermoanaerobacterium saccharolyticum* can utilize xylan and other hemicellulosic carbohydrates. It has also been engineered to produce ethanol at >90% of theoretical yield and up to 70 g/L at 10 g/L solids. By comparison, commercial fungal cellulase (CTEC2) only solubilized 23%, while autoclaving alone released 10 ± 1% (Table 1). The poor performance with fungal cellulase could be due to inhibition of *T. reesei* cellulases in CTEC2 by phenols released by DCB hydrolysis\(^20\). Consistent with this possibility, better performance of fungal cellulase on wet-milled corn fiber (WMCF) has been shown, with 43 and 56% solubilization using 5 and 20 mg/g protein/solids, respectively\(^9\).

To ferment both cellulose and hemicellulose, a sequential coculture of *C. thermocellum* with *T. saccharolyticum* was performed (Table 1). *T. saccharolyticum* was originally isolated for its ability to utilize xylan, so we expected the utilization of the corn fiber hemicellulose to be very high. Surprisingly, only 38 ± 3% of the non-glucose sugars (measured by liquid Quantitative Saccharification (QS)) that were solubilized by *C. thermocellum* were found to be consumed by *T. saccharolyticum* at both 10 and 20 g/L solids loading. The remaining sugars were left over in the broth. Reinoculating this broth aseptically did not result in further growth. Comparing acid-hydrolyzed (liquid QS) to unhydrolyzed broth by HPLC indicated that only 10% of the carbohydrate was present as monosaccharides. Fermentations with a coculture of *C. thermocellum* and *T. thermosaccharolyticum* on corn fiber showed 45 ± 5% consumption of the non-glucose sugars.

### Results and Discussion

*C. thermocellum* efficiently digests corn fiber. Corn fiber from a wet-milling process as well as destarched corn bran (DCB) from a dry mill, were both digested readily by *C. thermocellum* without added enzymes or pretreatment aside from autoclaving. We measured 90% solubilization within 3 days and 95% within 5 days of fermentation at 10 g/L solids. By comparison, commercial fungal cellulase (CTEC2) only solubilized 23%, while autoclaving alone released 10 ± 1% (Table 1). The poor performance with fungal cellulase could be due to inhibition of *T. reesei* cellulases in CTEC2 by phenols released by DCB hydrolysis\(^20\). Consistent with this possibility, better performance of fungal cellulase on wet-milled corn fiber (WMCF) has been shown, with 43 and 56% solubilization using 5 and 20 mg/g protein/solids, respectively\(^9\).

To ferment both cellulose and hemicellulose, a sequential coculture of *C. thermocellum* with *T. saccharolyticum* was performed (Table 1). *T. saccharolyticum* was originally isolated for its ability to utilize xylan, so we expected the utilization of the corn fiber hemicellulose to be very high. Surprisingly, only 38 ± 3% of the non-glucose sugars (measured by liquid Quantitative Saccharification (QS)) that were solubilized by *C. thermocellum* were found to be consumed by *T. saccharolyticum* at both 10 and 20 g/L solids loading. The remaining sugars were left over in the broth. Reinoculating this broth aseptically did not result in further growth. Comparing acid-hydrolyzed (liquid QS) to unhydrolyzed broth by HPLC indicated that only 10% of the carbohydrate was present as monosaccharides. Fermentations with a coculture of *C. thermocellum* and *T. thermosaccharolyticum* on corn fiber showed 45 ± 5% consumption of the non-glucose sugars.

### Structural characterization of GAX oligosaccharides

To identify the corn fiber carbohydrates solubilized but not utilized by *C. thermocellum* in monoculture and in coculture with *T. saccharolyticum* or *T. thermosaccharolyticum*, the fermentation broths were analyzed by NMR spectroscopy and MALDI-TOF mass spectrometry. These analyses showed that the main carbohydrates present in the broth after fermentation with *C. thermocellum* were arabinose and xylose monosaccharides, and xyloglucan and GAX oligosaccharides. Analysis of the Coculture Broths showed that the monosaccharides were almost completely consumed by *T. saccharolyticum* and *T. thermosaccharolyticum* but most of the GAX oligosaccharides remained unchanged with a degree of polymerization (DP) ranging from 4 to 20 (Supplementary Figs. 1 and 2). Xyloglucan oligosaccharides remained in the TT-Coculture Broth, meaning *T. thermosaccharolyticum* was unable to hydrolyze them. However, these oligosaccharides were absent in the TS-Coculture Broth suggesting *T. saccharolyticum*’s ability to consume them (Supplementary Fig. 2). For this reason, the TS-Coculture Broth was used to partially purify the GAX

---

**Table 1 Solubilization of corn fiber by various biocatalysts.**

| Biocatalyst | Corn fiber type | Solids loading (g/L) | % carbohydrate solubilization |
|------------|----------------|----------------------|-------------------------------|
| None       | DCB            | 10                   | 10%a                          |
| CTEC2 (5 mg/g) | DCB          | 10                   | 20 ± 2%                      |
| CTEC2 (20 mg/g) | DCB          | 10                   | 23 ± 1%                      |
| *C. thermocellum* | DCB      | 10                   | 95 ± 2%                      |
| *C. thermocellum* | WMCF    | 20                   | 96 ± 3%                      |
| *T. saccharolyticum* | WMCF  | 10                   | 96 ± 3%                      |
| *C. thermocellum* + WMCF | WMCF | 20                   | 92 ± 2%                      |

All the incubations were carried out for 5 days. *Denotes n = 2. ‡Denotes n = 1. For all other values, n = 3. Source data are provided as a Source Data file.
oligosaccharides by size-exclusion chromatography and to characterize their structure. Four main types of side chains (1–4) were identified in the GAX oligosaccharides in the broth (Fig. 1). The side chain 1 was identified as the disaccharide β-D-Xylp-(1,2)-α-L-Araf-(1,3) and represented 5% of the total carbohydrate in the TS-Coculture Broth (Fig. 1, Supplementary Table 1). This disaccharide is a common side chain in GAX from grasses21,24. The side chain 2 consisted of the disaccharide 1 with an α-1-3-Galp residue attached at O-2 to the β-D-Xylp and represented 13% of the total carbohydrate in the broth (Fig. 1, Supplementary Table 1). This side chain belongs to the series of side chains containing the unusual 1-Galp residue that are abundant in corn fiber but have also been found in other cereal GAXs18,22,23. The side chain 3 represented 13% of the total carbohydrate and contained α-D-Xylp, another unusual sugar in plant cell wall xylans (Fig. 1, Supplementary Table 1). This disaccharide has been found previously in the pericarp of corn kernels19. The structure 4 was the most complex identified in the fermentation broth and represented 8% of the total carbohydrate (Fig. 1, Supplementary Table 2, Supplementary Fig. 3). This fragment contained two side chains, an α-1-3-GlcPA residue linked at O-2 to the backbone xylose at the non-reducing end and the following backbone xylose that was double substituted with a β-D-Xylp residue at O-3 and the side chain 3 at O-2. The presence in corn fiber GAX of backbone xyloses double substituted with xylose and arabinose residues was proposed previously, but the oligosaccharide was never isolated or its structure characterized16,24. Interestingly, the oligosaccharides containing structure 4 were the only ones found in the Coculture Broths to contain uronic acids. Oligosaccharides carrying a single GlcPA or 4-O-Me-GlcPA side chains were identified in the Monoculture Broth and when GAX was extracted from corn fiber with alkali and digested with a xylanase (Supplementary Fig. 1). Their absence in the Coculture Broths indicated that enzymes in T. saccharolyticum and T. thermosaccharolyticum could hydrolyze simple uronic acid side chains and only the proximity to a large side chain in 4 prevented its cleavage.

Bacterial utilization of GAX oligosaccharides. A survey of thermophilic organisms, including several described hemi-cellulose-utilizers, was conducted to find ones that were better than T. saccharolyticum and T. thermosaccharolyticum in utilizing the corn fiber GAX (Table 2). Bacteroides are gut bacteria that have been extensively studied for their ability to utilize complex glycans25 such as corn xylan16, and though it is not thermophilic, Bacteroides cellulosilyticus was included in this comparison. Only a few of the species could consume more than 60% of the total carbohydrate solubilized by C. thermocellum, consistent with the described recalcitrance of GAX (Table 2). Among these were B. cellulosilyticus, which utilized 85% of available carbohydrate, and Caldanaerobius polysaccharolyticus (AKA Thermoanaerobacter polysaccharolyticus), a thermophile with described ability to breakdown and consume complex xylan26–28, which utilized 83%. A mixed thermophilic (55 °C) consortium maintained on switchgrass29 was also tested and found to consume 99% of the carbohydrate in the Monoculture Broth. This led to a quest to isolate organisms from the consortium with ability to utilize the recalcitrant corn fiber GAX.

Isolation and sequencing of strain LL1355. In order to isolate an organism that is able to utilize the GAX oligosaccharides that T. saccharolyticum could not, the consortium was enriched and simplified on TS-Coculture Broth. Colonies were isolated on plates containing arabinose, cellobiose, and xylose. Sequencing of the 16S ribosomal RNA genes of 120 colonies revealed a variety of organisms, with the closest BLAST hit for the most abundant ones shown in Supplementary Table 3. The isolated colonies were grown on TS-Coculture Broth to check for GAX utilization in liquid culture, but only two could grow. The pure cultures of these organisms, with closest BLAST matches to Herbinix hemicellulosilytica and Ruminococcus champanellensis, were named LL1355 and LL1354. They consumed 75% and 50% of the carbohydrates in the TS-Coculture Broth, respectively. Coculture of these two organisms did not give increased utilization. Also, LL1355 consumed 85% of the total carbohydrates in the Monoculture Broth.

Their genomes were sequenced by JGI, and the ORFs were analyzed using the dbCAN tool30 to get the list of all Carbohydrate-Active (CAZy) enzymes (Supplementary Table 4). The 16S ribosomal RNA gene sequence for LL1355 shows only 96.4% identity with Herbinix hemicellulosilytica, suggesting that we have isolated a species designated Herbinix spp. LL1355. BLAST results from housekeeping genes from the LL1355 genome show Herbinix hemicellulosilytica as the top hit, confirming the proposed genus assignment.

Screening and characterization of enzymes from LL1355. Rogowski et al.16 characterized enzymes from Bacteroides ovatus involved in the breakdown of different xylans, including corn fiber GAX. In order to determine whether the same enzymes
show activity on the GAX oligosaccharides released into solution by *C. thermocellum* fermentation, selected enzymes were tested for monosaccharide release on TS-Coculture Broth. Activity was observed for α-xylanase-BoXyl131 (BACOVA_03422) and α-1-galactosidase-BoGalp95A. The arabinofuranosidases enzymes also showed activity when combined with these two (Supplementary Table 5).

Based on the elucidated structures of GAX oligosaccharides present in TS-Coculture Broth, the following LL1355 glycoside hydrolases were hypothesized to be important for degrading the recalcitrant linkages in GAX oligosaccharides: (i) α-D-xylanidase—GH31; (ii) α-1-galactosidase—GH95; (iii) α-L-arabinofuranosidase—GH43 and GH51; (iv) β-D-xylosidase—GH10 and GH110. In addition, α-D-galactosidase and α-glucuronidase enzymes were also indicated as small amounts of these residues were detected in the GAX side chains. In total, 27 ORF sequences from LL1355 were cloned and tested for activity, first by colorimetric assays measuring hydrolysis of sugar-nitrophenol complexes, then by checking for structural analysis of the oligosaccharides after digestion not just carbohydrate, but some residual enzyme activity as well. The selected enzymes were purified using His-tag protein purification (Supplementary Fig. 4). The α-L-Galp_687 proved difficult to purify. Since it had similar but lower activity than α-L-Galp_687, it was not characterized further. Activity of the purified enzymes was determined using p-nitrophenyl glycosides (Supplementary Table 7). The specific activity of β-Xylp_1710 is one of the highest reported for β-xylanidases31–35 while the measured activities on pNP α-1-arabinofuranosidase were up to 100-fold lower than others31,36. There have been very few α-xylanidases described37. Most of them are active on xyloglucan oligosaccharides and/or on pNP α-D-xylopyranoside while only two have been shown to be active on corn xylan: BACOVA_03422 from *Bacteroides ovatus* and CjXyl31 from *Cellvibrio japonicus*38. The CjXyl31 has very low activity against corn xylan and prefers p-xylosides31 while only two have been isolated and analyzed before and after the enzymatic treatment (Fig. 3; Supplementary Fig. 7, Supplementary Tables 2 and 8). These analyses showed that α-Xylp_1211 was able to completely remove the α-D-Xylp residues from structure 4. However, β-Xylp_1710 was not active against the side chain 4 even after the α-D-Xylp residues were removed by α-Xylp_1211.

This result was not surprising given that the β-D-Xylp residue is surrounded by other sugar residues in this oligosaccharide. It is likely that the GlcA residue attached to the adjacent xylose limited the action of the β-xylanidase. Considering this, it is possible that side chain 3 is a product of *C. thermocellum* β-xylanidases, which could act against the double substituted xylose when it is not in the proximity of a GlcA residue.

Combinations of the selected enzymes were tested for monosaccharide release from TT-Coculture Broth (Supplementary Table 9), keeping in mind that the broth may have contained not just carbohydrate, but some residual enzyme activity as well. Structural analysis of the oligosaccharides after digestion confirmed that the targeted linkages had been hydrolyzed. We found a high degree of synergy between the enzymes, especially for the arabinofuranosidases, which have negligible effect on their own but considerable release of monosaccharides in conjunction with α-1-galactosidase and α-xylanidase. This can be understood structurally if the terminal glycosyl residue of the side chain needs to be removed before the other enzymes cleave the next residue. The five enzymes together deconstructed the oligosaccharides in the TT-Coculture Broth and released 42 ±3% of the carbohydrate as monosaccharides (Supplementary Table 9). For comparison, the CFE from both LL1355 and *B. cellulosolyticus* released 45% of the carbohydrate as monosaccharides. Interestingly, the supernatant for all the tested organisms showed negligible activity (0–5%) (Supplementary Table 9), suggesting that the proteins are predominantly intracellular.

### Table 3 Selected enzymes from LL1355.

| Enzyme activity | Locus tag                  | Enzyme name            | GH family |
|-----------------|----------------------------|------------------------|-----------|
| α-x-xylosidase   | Ga0256695_1211             | α-Xylp_1211            | 31        |
| α-1-galactosidase| Ga0256695_0687             | α-1-Galp_687           | 95        |
| α-1-galactosidase| Ga0256695_0697             | α-1-Galp_697           | 95        |
| β-D-xylosidase   | Ga0256695_1710             | β-Xylp_1710            | 120       |
| α-L-arabinofuranosidase | Ga0256695_0996 | α-Araf_996             | 43        |
| α-L-arabinofuranosidase | Ga0256695_1120 | α-Araf_1120            | 43        |
of the total carbohydrate while *T. thermosaccharolyticum* consumed only 53 ± 3% without enzyme addition. The most important enzyme for this purpose was α-Xyl_1211, which when added alone resulted in 68 ± 2% of carbohydrate being utilized. Although α-1-Galp_687 releases monosaccharides from *TT*-Coculture Broth, it did not greatly enhance carbohydrate utilization. This is due to the inability of *T. thermosaccharolyticum* to utilize the uncommon l-isomer of galactose that it releases. Addition of α-Xylp_1211 and α-1-Galp_687 along with any of the other three enzymes helped *T. thermosaccharolyticum* utilize almost the same amount of carbohydrate as addition of all enzymes (76% vs 78%, see Supplementary Table 10). This suggests that the α-Araf_1120, α-Araf_996, and β-Xylp_1710 had similar activity on the carbohydrates in Monoculture Broth.

The effect of the addition of LL1355 CFE is also shown in Supplementary Tables 9 and 10. It is interesting to note that the performance of the five enzymes identified above and the LL1355 CFE was similar in catalyzing monosaccharide release (42 ± 3% vs 45 ± 4%), but the efficacy for increased carbohydrate utilization by *T. thermosaccharolyticum* was higher with the addition of LL1355 CFE (89 ± 1% vs 78 ± 1%). This may indicate the presence of additional enzymes in LL1355 CFE that did not show activity in our screen. An example of such an enzyme would be an endo-acting hydrolase, which might not release monosaccharides, but would nonetheless be important for efficient deconstruction. The existence of such enzymes is also suggested by considering *C. polysaccharolyticus* for which the endoxylanase Xyn10A has been reported to be important for xylan utilization[26]. It showed a high utilization (83%) of the *TT*-Coculture broth (Table 2) but its CFE and supernatant showed low activity in terms of monosaccharide release, at 17% and 0%, respectively (Supplementary Table 9).

Taken together, results from both LL1355 and *C. polysaccharolyticus* suggest the potential to identify additional endo-acting hydrolases that may improve arabinoxylan utilization further.

Expression of enzymes in *T. thermosaccharolyticum*. Consolidated bioprocessing (CBP) entails production of required enzymes by the fermenting organism[11]. In order to enable CBP of corn fiber, we sought to express arabinoxylan degrading enzymes in *T. thermosaccharolyticum*, enhancing its ability to utilize corn fiber hemicellulose and thus improving product yield. The five selected enzymes in Table 3 were individually expressed in *T. thermosaccharolyticum* using a replicating plasmid construct shown in Fig. 4a. The activity of the cloned enzymes was confirmed in the CFEs of the resulting strains. The strain expressing α-Xylp_1211 could utilize 32 ± 2% of the carbohydrates in the *TT*-Coculture Broth (Table 4) compared with 8 ± 5% by the parent strain. Subsequently, four 2-gene operons were constructed, all containing α-Xylp_1211 plus another gene, then transformed into *T. thermosaccharolyticum* (Fig. 4b). All pairs of enzymes were determined to be active in CFEs.

Cloning of 4-gene operons was attempted with α-Xylp_1211, an α-1-galactosidase and two of the other three genes. Although α-1-Galp_687 was initially included, operons containing that gene could not be successfully cloned, despite multiple attempts. Replacing it with α-1-Galp_697 led to successful cloning. The three successfully expressed four-gene constructs are shown in Supplementary Table 11. Strains containing these constructs were transferred 8–10 times on *TT*-Coculture Broth to adapt them for better GAX utilization. The % carbohydrate utilization of all the developed strains is shown in Table 4. The best performing strain, expressing α-Xylp_1211 + β-Xylp_1710 + α-Araf_996 + α-1-Galp_697 (strain LL1703), was able to consume 49 ± 7% of the
carbohydrate in TT-Coculture Broth after 10 days of incubation. Strains expressing \( \alpha \)-Xylp_1211 + \( \alpha \)-L-Galp_697 and any of the \( \alpha \)-arabinofuranosidase enzymes utilized an average of 47 ± 1% of the carbohydrate in TT-Coculture Broth. For comparison, addition of LL1355 CFE resulted in a total utilization of 66 ± 1% on the same broth.

In Monoculture Broth, the LL1703 strain utilized 67 ± 2% of the carbohydrates after 10 days, compared with 50 ± 2% by the
parent strain. As shown in Fig. 5, LL1703 grew slower (lower OD600) than the parent strain, although both strains consumed the same amount of carbohydrates after 2 days. At this point in the growth curve, the parent strain stopped growing while LL1703 continued slowly consuming more carbohydrate even after 10–12 days, possibly due to enzyme release upon cells lysis. The difference in carbohydrate utilization between engineered strains expressing LL1355 enzymes and the parent strain is more distinct on TT-Coculture Broth, where the parent strain has no carbohydrate it can utilize, and lysis may happen earlier (Table 4).

The supernatant of LL1703 showed negligible activity in releasing monosaccharides from the TT-Coculture Broth (Supplementary Table 9). These results suggest that the heterologous enzymes were expressed intracellularly in LL1703 but extracellular expression of the selected enzymes is likely needed for optimal growth on GAX. It is notable that the selected enzymes from LL1355 did not have secretion signals and are likely expressed intracellularly. However, some CAZymes from LL1355 do have secretion signals, particularly endoxylanases. In line with these observations, the supernatant from a LL1355 culture did not have much activity in terms of monosaccharide release (Supplementary Table 9) but did increase the utilization of Monoculture broth carbohydrates by 63% (Supplementary Table 10). The LL1355 genome contains a large number of oligosaccharide transporters that presumably allow the intake of the small GAX oligosaccharides resulting from the action of secreted endoxylanases. Similar to some human-gut

### Table 4 Utilization of carbohydrates in TT or TS-Coculture Broths by various T. thermosaccharolyticum strains with heterologous expression of identified enzymes.

| Enzyme | % Carbohydrate utilization | Plasmid |
|--------|---------------------------|---------|
| pL1270 | 8 ± 5%                    | ✓       |
| pL1271 | 32 ± 2%                   | ✓✓      |
| pL1272 | 34 ± 3%                   | ✓✓      |
| pL1274 | 37 ± 2%                   | ✓✓      |
| pL1273 | 38 ± 2%                   | ✓✓      |
| pL1277 | 44 ± 6%                   | ✓✓      |
| pL1275 | 48 ± 6%                   | ✓✓      |
| pL1276 | 49 ± 7%                   |         |

Growth experiments were done on 2 different days with replicates n ≥ 4, with two different Coculture Broths from 20 g/L corn fiber fermentations: TT-Coculture Broth with 4.3 g/L or TS-Coculture Broth with 4.0 g/L total carbohydrates. Fresh media components were not added other than 5 g/L MOPS.

**Fig. 5** Growth of *T. thermosaccharolyticum* strain LL1703 on Monoculture Broth. The growth curves are representative of two replicates. The final % utilization (n = 6) was 65 ± 2% for the enzyme-expressing strain, 51 ± 1% for the parent strain, and 76 ± 2% for the strain supplemented with enzymes. Red circles—LL1548 (parent strain); black squares—LL1703 (LL1548 expressing four enzymes from LL1355); blue triangles—LL1548 with added LL1703 CFE. Source data are provided as a Source Data file.
Table 5 Fermentations on 40 g/L corn fiber using various biocatalysts.

| Biocatalyst | % Carbohydrate solubilization | % GAX utilization |
|-------------|-------------------------------|-------------------|
| C. thermocellum + T. thermosaccharolyticum | 33 | 33% |
| C. thermocellum + T. thermosaccharolyticum + LL1355 CFE | 46 | 64% |
| C. thermocellum + LL1355 | 63 | 90% |

Each reactor was run with two replicates. Source data are provided as a Source Data file.

Corn fiber coculture fermentations with added enzymes/CFE.

To study the solubilization of corn fiber fermentation with the addition of LL1355 or its CFE, pH-controlled bioreactor fermentations of 40 g/L fiber-enriched corn bran were conducted (Table 5). A coculture of C. thermocellum and T. thermosaccharolyticum solubilized 33% of the carbohydrate and consumed only 33% of the solubilized GAX. Addition of CFE from LL1355 increased GAX utilization to 64% and the solubilization to 46%, while coculture with LL1355 increased these values to 90% and 63%, respectively. These results indicate an association between increasing GAX utilization and increasing solubilization of the fiber. In another experiment, five purified enzymes identified from LL1355 were added at a total loading of 1 mg/g (protein/corn fiber) to C. thermocellum and T. thermosaccharolyticum broth fermentations on 8.8 g/L corn fiber. The ethanol titer increased to 2.4 ± 0.08 g/L compared with 1.93 ± 0.02 g/L for the control without enzyme addition (n = 3 for both).

Recent studies have shown inhibition of C. thermocellum by hemicellulosic hydrolysis products, which establishes the need for a coculture partner proficient in hemicellulose utilization to assist in lignocellulosic solubilization13,14. Engineering LL1355 for high ethanol yield could make it a useful coculture partner for C. thermocellum grown on corn fiber. We have shown improvement in solubilization with this coculture as compared with C. thermocellum alone or along with T. thermosaccharolyticum. Two other organisms, Bacteroides cellosolititsicus and Caldanaerobius polysaccharolyticus, showed high GAX utilization similar to LL1355, but B. cellosolititsicus is a mesophile while C. polysaccharolyticus grows poorly at 55 °C. Neither is a suitable coculture partner for C. thermocellum. However, enzymes from C. polysaccharolyticus in addition to endo-acting enzymes from LL1355 may warrant further study. The gap we observed between performance of heterologous enzyme expression and exogenous enzyme supplementation suggests further improvements (e.g., oligosaccharide transport or enzyme secretion) that may be required for full transfer of carbohydrate utilization from one organism to the other.

This study has demonstrated that C. thermocellum is much better than fungal cellulase at solubilizing the carbohydrate in corn fiber, achieving solubilization yield of >90%. This is substantially higher than its yields (<70%) on other lignocellulosic feedstocks such as corn stover or switchgrass7,10. We have also shown that various thermophiles described to be efficient xylanolytic organisms struggle to deconstruct and utilize corn GAX. To overcome this limitation, we exploited natural microbial diversity to isolate an organism Herbinix spp. strain LL1355 and used it as a source of enzymes for deconstruction of corn GAX. We have also demonstrated how a detailed structural characterization of the recalcitrant linkages in corn GAX helped in identifying enzymes for their processing, which could complement specific activities missing in our original host. Addition of just two enzymes, α-Xylp_1211 and α-1-Galp_687, increased utilization of complex corn GAX by T. thermosaccharolyticum from 53 ± 3% to 76 ± 1%. Addition of five enzymes resulted in a 24% improvement in ethanol titer from corn fiber by the thermophic coculture of C. thermocellum and T. thermosaccharolyticum.

Methods

Fermentation conditions. Fermentations were carried out with C. thermocellum strain M157715, the M1442 strain of T. saccharolyticum16 and LL1548, a high ethanol yielding derivative of T. thermosaccharolyticum strain LL1244, also known as ATCC 31960 and HG-8.

Wet-milled corn fiber was obtained from a Midwestern wet-milling facility and dry milled corn bran was obtained from Grain Millers, Inc. (Marion, IN). Distarched corn bran was prepared by treating 130 g of milled (0.5 mm) corn bran with 75 mL of a-amylase (Sigma-Aldrich A7595) and 25 mL of Sprizyme (Novozymes A/S, Bagsvaerd, Denmark) at 60 °C for 30 h followed by 5–6 water washes to get rid of all the solubilized starch. The bran was then dried in a 50 °C oven followed by milling to 0.2 mm.

Growth media were prepared as 10× concentrates and either passed through a 0.22 µm syringe filter (Corning Inc., Corning, NY) to be inoculated into autoclaved, nitrogen-flushed serum bottles. The media components were obtained from Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA). The composition of the 1X CC6 medium used for bioreactors was 5 g/L yeast extract, 5 g/L trisodium citrate·2H₂O, 0.5 g/L KH₂PO₄, 0.5 g/L KH₂PO₄, 2 g/L MgSO₄·7H₂O, 0.1 g/L CaCl₂, 0.1 g/L FeSO₄·7H₂O, 0.5 g/L L-cysteine and 1 g/L urea (for fermentations with C. thermocellum and T. saccharolyticum) or 1 g/L (NH₄)₂SO₄ (for fermentations with T. thermosaccharolyticum). For bioreactors, a 50 mL (3%) inoculum of C. thermocellum was grown in a nitrogen-flushed 125 mL serum bottle with 10 g/L avicel PH-105 (FMC Corp., Philadelphia, PA) and CC6 medium supplemented with 5 g/L MOPS, pH set to 7.1 ± 0.1. T. saccharolyticum inoculum was prepared in the modified DSMZ 122 media17 adjusted to pH 6.5, and with 5 g/L cellobiose. T. thermosaccharolyticum inoculum was prepared in CTFUD17 with 5 g/L cellobiose, pH 6.7. For both these partner organisms, 50 mL inoculum was used in bioreactors.

Bioreactor fermentations were carried out in 3 L (1–2 L working volume) Biostat A-plus bioreactors (Sartorius Stedim, Bohemia, NY) with the temperature maintained at 55 °C using a resistive heat blanket and stirred at 200 rpm25. The pH was controlled at 6.95 with a Mettler-Toledo pH probe (Columbus, OH) by the addition of KOH and temperature was recorded. pH and temperature were recorded every 5 min. Fermentations were carried out in a 30 °C water bath. The media was then added into the reactor and pH was adjusted to 6.95 before inoculating. In case of sequential cocultures with T. saccharolyticum, fermentation was allowed to proceed with C. thermocellum for ~24 h before adjusting the pH to 6.25 with slow dropwise addition of 1 N HCl and then adding a 5% inoculum of T. saccharolyticum. Fermentations were carried out for 5 days unless otherwise noted.

Culture broths. The broth resulting from fermentation was centrifuged to remove solids, then prefiltred through glass fiber filters without binder (MilliporeSigma, Burlington, MA). It was then filtered through a 0.45 µm Nylon Net Filter (HNPW04700, MilliporeSigma, Burlington, MA) and finally filtered through a 0.22 µm bottle-top filter (Corning Inc., Corning, NY) into a sterile bottle. The broth from a C. thermocellum fermentation is called ‘Monoculture Broth’, whereas the broths from a C. thermocellum plus T. saccharolyticum or T. thermosaccharolyticum fermentation are called ‘TS-Coculture Broth’ or ‘TT-Coculture Broth’, respectively.

Carbohydrate composition analysis. The dry residual solids from a fermentation were analyzed using complete acid hydrolysis and HPLC (Quantitative Sacchar- idine from a C. thermocellum (DH5a) strain. The residual solids were dried in a 55 °C oven till constant weight, which was noted. Approximately 0.1 g of the dried solids was then measured into a 50 mL round-bottom glass centrifuge tube (Kimble-chase 45212-50). 1.5 mL of conc. H₂SO₄ (for strong-acid hydrolysis) was added to the tube and a glass rod was put in for stirring. The tube was then incubated in a 30 °C water bath for 1 h with manual breaking of solid lumps at 15 min intervals. After the incubation, 42 mL of DI H₂O was added immediately. The tubes were then capped tightly and autoclaved for 1 h (weak-acid hydrolysis) in a liquid cycle. This...
treatment converted the carbohydrates in the residual solids into monomer sugars that were then measured by HPLC as described below. Measured amounts of pure monosaccharides—glucose, arabinose, and xylose, were also taken through the same treatment and their final amount measured to normalize for degradation of carbohydrate. The composition of wet-mill corn fiber, expressed as monomer sugar equivalents, was 24% glucose, 35% xylose + galactose, and 13% arabinose. For the destarched corn bran (DCB), the composition was 22% glucose, 38.5% xylose + galactose, and 5.9% arabinose. For the fiber, the composition was 17.5% glucose, 23% xylose + galactose, and 12% arabinose. The total soluble carbohydrate in the liquid broth was analyzed by a similar acid hydrolysis method we refer to as liquid QS, which requires only the weak-acid hydrolysis step. Broth carbohydrate in the liquid broth was analyzed by a similar acid hydrolysis method.

Fungal cellulase incubation. 0.5 g of destarched corn bran (0.2 mm) in 40 mL Milli-Q water was autoclaved for 40 min (liquid cycle) in a 125 mL serum bottle. CTEC2 (Novozymes) cellulase enzyme solution was prepared by 10x dilution in water and sterilized by filtering through a 0.22 µm syringe filter. The protein concentration was then measured by Bradford assay (Pierce™ Coomassie Plus Bradford Assay Kit—Thermo Fisher Scientific). The enzyme along with 5 mL of 10x concentrated sodium acetate buffer (pH 5), 75 µL penicillin G (2 µg/L), and Milli-Q purified water was added to bring the total volume up to 50 mL. Final concentrations were as follows: sodium acetate: 50 mM; penicillin G: 5 ppm; CTEC2: 5 or 20 mg protein/g of corn bran. The incubation was done at 35 °C for 120 h at 200 rpm in a 150 mL screw cap conical flask. The sterilization was carried out by weighing residual solids and measuring their sugar composition by QS. It was confirmed by measuring the sugar amount in the released soluble carbohydrate by liquid QS.

Growth of various organisms on Monoculture Broth. The growth conditions for various organisms tested on the Monoculture broth is given in Supplementary Table 1. A microbial consortium was obtained from Xiaoyu Liang in our lab who was maintaining a semi-continuous anaerobic digester at 55 °C on switchgrass at 30 g/L solids loading and 20 day residence time.

Isolation of organisms. To simplify the consortium obtained from Xiaoyu Liang, it was transferred 10 times on TS-Coculture Broth using a 10% inoculum. To simplify the consortium further, a dilution-to-extinction series was made, with the inoculum serially diluted 1/10 until the last few dilutions did not show any growth. These simplified consortia were then plated using the same media (CC6) as was used for the coculture and 5 g/L each of xylose, arabinose, and cellulose. The colonies were grown at 37 °C and sent for 16S rDNA gene sequencing and results were obtained for about 120 colonies. Most colonies were streaked out to ensure purity. The pure cultures of a Ruminococcus-like and Herbaspirillum-like isolate (likeness determined by 16S ribosomal RNA gene sequence matches) were added to the lab’s strain collection as LL1354 and LL1355, respectively. Their genomic DNA was sequenced by the Joint Genome Institute. The ORF sequences for both strains were analyzed with dBCAN, which is a web server and database for automated Carbohydrate-active enzyme annotation.

Analysis of corn fiber xylan-oligosaccharides. Wet-milled fiber was washed sequentially with aqueous 80% (v/v) EtOH and absolute EtOH. The residue was incubated for 1 h at room temperature with 1 M KOH (final concentration 1% w/v) sodium borohydride. The 1 M KOH extract, which contains mainly xylose, was adjusted to pH 5 with glacial acetic acid, dialyzed against deionized water, and lyophilized. The xylen-enriched material was dissolved on 50 mM sodium phosphate buffer, pH 7.0 and incubated for 16 h at 37 °C with CelI7S, a recombinant xylanase from C. thermocellum. The oligosaccharides enzymatically generated were analyzed by MALDI-TOF MS and NMR.

MALDI-TOF mass spectrometry. Positive ion MALDI-TOF mass spectra were recorded using an Applied Biosystems Voyager-DE biospectrometry workstation, and the data collection software used was Bruker Daltonics FlexControl version 3.0 (Bruker Corporation, https://www.bruker.com). Oligosaccharides samples (5 µL of a 1 mg/mL solution) were incubated with 1 µL of a suspension of Dowex-50 cation exchanger resin for 1 h. After centrifugation, 1 µL of the supernatant was mixed with an equal volume of matrix solution (20 mg/mL 2.5-dihydroxybenzoic acid in aqueous 50% MeOH) and dried on MALDI target plate. Typically, spectra from 200 laser shots were summed to generate a mass spectrum.

NMR spectroscopy. NMR spectra were recorded with a Varian Inova NMR spectrometer with a Vnmr version 4.2 Revision A software (Agilent Technologies) operating at 600 MHz using a 5 mm cold probe and a sample temperature of 25 °C. Lyophilized oligosaccharides were dissolved in D2O (0.3 mL, 99.9%; Cambridge Isotope Laboratories, Tewksbury, MA, USA) and placed in a 3 mm NMR tube. All two-dimensional spectra (gCOSY, TOCSY, NOESY, HSQCQAD, and gHMBCQAD) were recorded using standard Varian pulse programs. The 1D and (1H–1H) homonuclear 2D experiments were acquired using Presat for water suppression. The TOCSY and NOESY mixing times were 80 and 200 ms, respectively, and 256 FIDs consisting of 1024 points were recorded. For high resolution gCOSY spectra, 800 FIDs were collected.

Protein expression. Protein expression with pEXP-5-NT plasmid was performed using slight modifications to the manufacturer’s protocol. The strains were grown in a 30 mL volume in baffled Luria-Bertani (LB) broth (1× LB; Biolabs, Madrid) or Terrific Broth, modified (Sigma-Aldrich) with 50 µg/mL carbenicillin added. The pellet was resuspended in 2 mL KH2PO4 buffer (67 mM, pH 7.2) and 1X BugBuster reagent (MilliporeSigma). The cells were lysed with Ready-Lyse Lysozyme and DNase I (New England Biolabs) was added to reduce viscosity. Finally, sonication was performed using a microtip probe and 14 cycles of 10 s at 50% amplitude with 20 s intervals. The lysate was then centrifuged to remove cell debris, incubated at 55 °C for 30 min to precipitate host cell proteins, then centrifuged again to remove these proteins. The supernatant was filtered through a 0.22 µm microspin syringe filter to obtain the cell-free extract (CFE).

Protein purification. Protein purification was performed using Econo-Column® Chromatography Columns, 2.5 × 10 cm (Bio-Rad, Hercules, CA) packed with Ni- Sepharose High Performance histidine-tagged protein purification resin (GE Lifeiences, Pittsburgh, PA). Details are included in the Supplementary Method 2.

Nitrophenyl assays. The following para-nitrophenyl compounds were used according to the expected activity of the enzyme: a-oxo-pyranoside,
with 10 g/L MOPS. The study are available from the corresponding author upon request. The genomic DNA for
Supplementary Information

Bottle fermentations with 8.8 g/L corn of all were incubated in a 55 °C shaker at 200 rpm for 5 days. An equimolar enzyme mix

Monosaccharide release tests. Crude enzyme CFE or purified enzymes were added to 7T-Coculture Broth in 1:9 ratio with the final enzyme concentration being 0.15 mg/mL. Incubation was done at 55 °C for 24 h. After incubation, the monosaccharides released by the added enzymes were determined by HPLC as above. The % monosaccharide release was calculated by comparing to total carbohydrate content measured by liquid Q8.

Determination of the activity of purified enzymes. The 7S-Coculture Broth was fractionated by size-exclusion chromatography (SEC) using a Superdex-75 HR10-30 column eluted at 0.5 mL/min with water and refractive index detector. Under these conditions acidic oligosaccharides eluted from the matrix before neutral oligosaccharides of the same size. The fractions containing oligosaccharides were collected manually and lyophilized. The acidic oligosaccharides were further purified from the neutral oligosaccharides using a LC-18 column (Supelco; Sigma-Aldrich, USA) following the manufacturer’s instructions. The purified oligo-

References

1. Himmel, M. E. et al. Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 315, 804–807 (2007).
2. Lynd, L. R. et al. Cellulosic ethanol: status and innovation. Curr. Opin. Biotechnol. 45, 202–211 (2017).
3. Li, X., Chen, S., Huang, H. & Jin, M. In-situ corn fiber conversion improves ethanol yield in corn dry-mill process. Ind. Crops Prod. 113, 217–224 (2018).
4. Kurambhati, C. et al. Ethanol production from corn fiber separated after liquefaction in the dry grind process. Energies 11, 2921 (2018).
5. Grohmann, K. & Bothast, R. J. Saccharification of corn fiber by combined treatment with dilute sulfuric acid and enzymes. Process Biochem. 32, 405–415 (1997).
6. Golli, M., Kohlmann, K., Ladisch, M. R., Hespell, R. & Bothast, R. J. Assessment of ethanol production options for corn products. Bioresour. Technol. 58, 253–264 (1996).
7. Paye, J. M. D. et al. Biological lignocellulose solubilization: comparative evaluation of biocatalysts and enhancement via cotreatment. Biochim. Biophys. Acta 9, 8 (2016).
8. Xu, Q. et al. Dramatic performance of Clostridium thermocellum explained by its wide range of cellulase modalities. Sci. Adv. 2, e1501254 (2016).
9. Lynd, L. R. et al. Advances in consolidated bioprocessing using Clostridium thermocellum and Thermoanaerobacter saccharolyticus. Indus. Biotechnol. Microorg. 10, 365–394 (2016).
10. Holwerda, E. K. et al. Multiple levers for overcoming the recalcitrance of lignocellulosic biomass. Bioresour. Technol. 12, 15 (2019).
11. Lynd, L. R., Weimer, P. J., van Zyl, W. H. & Pretorius, I. S. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol. Mol. Biol. Rev. 66, 506–577 (2002).
12. Herring, C. D. et al. Strain and bioprocess improvement of a thermophilic anaerobe for the production of ethanol from wood. Bioresour. Biotechnol. 9, 125 (2016).
13. Shaw, A. J., Hoggett, D. A. & Lynd, L. R. Natural competence in Thermoanaerobacter and Thermoanaerobacterium species. Appl. Environ. Microbiol. 76, 4713–4719 (2010).
14. Bhandiwad, A., Guseva, A. & Lynd, L. Metabolic engineering of Thermoanaerobacterium thermosaccharolyticum for increased n-butilan production. Adv. Microbiol. 2013, 46–51 (2013).
15. Peña, M. J. et al. Structural diversity of xylans in the cell walls of monocots. Planta 244, 589–606 (2016).
16. Rogowski, A. et al. Glycan complexity dictates microbial resource allocation in the large intestine. Nat. Commun. 6, 7481 (2015).
17. Åger, J., Viks-Nielsen, A. & Meyer, A. S. Enzymatic xylose release from pretreated corn bran arabinoxylan: differential effects of deacetylation and defurfurylation on insoluble and soluble substrate fractions. J. Agric. Food Chem. 58, 6141–6148 (2010).
18. Allerdings, E., Ralph, J., Steinhardt, H. & Bunzel, M. Isolation and structural identification of complex furanocarboxylic acid side-chains from maize bran. Phytochemistry 67, 1276–1286 (2006).
19. Montgomery, R., Smith, F. & Sivartava, H. C. Structure of corn hemicelluloses. Part IV. Partial hydrolysis and identification of 3-O-α-D-Xylopyranosyl-L-arabinose and 4-O-β-D-Galactopyranosyl-β-D-xylose 1,2. J. Am. Chem. Soc. 79, 698–700 (1957).
20. Kim, D., Orrego, D., Ximenes, E. A. & Ladisch, M. R. Cellulose conversion of corn pericarp without pretreatment. Bioresour. Technol. 245, 511–517 (2017).
21. Ebingerová, A. Structural diversity and application potential of hemicelluloses. Macromol. Symp. 232, 1–12 (2005).
22. Appeldoorn, M. M., Kabel, M. A., Van Eijlen, D., Gruppen, H. & Schols, H. A. Characterization of oligomeric xylan structures from corn fiber resistant to pretreatment and simultaneous saccharification and fermentation. J. Agric. Food Chem. 58, 11294–11301 (2010).
23. Schendel, R. R., Meyer, R. R. & Bunzel, M. Quantitative profiling of feruloylated arabinoxylan side-chains from graminaceous cell walls. Front. Plant Sci. 6, 1249 (2015).
24. Saulnier, L., Marot, C., Chanlaud, E. & Thibault, J.-F. Cell wall polysaccharide interaction in maize bran. Carbohydr. Polym. 28, 279–287 (1995).
25. Martens, E. C., Koropatkin, N. M., Smith, T. J. & Gordon, J. I. Complex glycan catabolism by the human gut microbiota: the Bacteroidetes Sus-like paradigm. J. Biol. Chem. 284, 24673–24677 (2009).
26. Han, Y. et al. Biochemical and structural insights into xylan utilization by the thermophilic bacterium Caldanaerobius polysaccharolyticus. J. Biol. Chem. 287, 34946–34960 (2012).

27. Stroot, P. G., Mackie, R. L., White, B. A., Mackie, K. R. & Cann, I. K. Characterization of two novel saccharolytic, anaerobic thermophiles, Thermoanaerobacterium polysaccharolyticum sp. nov. and Thermoanaerobacterium zaee sp. nov., and emendation of the genus Thermoanaerobacterium. Int. J. Syst. Evol. Microbiol. 51, 293–302 (2001).

28. Lee, Y.-J., Mackie, R. L., Cann, I. K. O. & Wiegel, J. Description of Caldanaerobius fijienis gen. nov., sp. nov., an inulin-degrading, ethanol-producing, thermophilic bacterium from a Fijian hot spring sediment, and reclassification of Thermoanaerobacterium polysaccharolyticum and Thermoanaerobacterium zaee as Caldanaerobius polysaccharolyticus comb. nov. and Caldanaerobius zaee comb. nov. Int. J. Syst. Evol. Microbiol. 58, 666–670 (2008).

29. Liang, X. et al. Development and characterization of stable anaerobic thermophilic methanogenic microorganisms fermenting switchgrass at decreasing residence times. Biotechnol. Biofuels 11, 243 (2018).

30. Yin, Y. et al. dbCAN: a web resource for automated carbohydrate-active enzyme annotation. Nucleic Acids Res. 40, W445–W451 (2012).

31. de Camargo, B. R., Classens, N. J., Quirino, R. F., Noronha, E. F. & Kengen, S. W. M. Heterologous expression and characterization of a putative glycoside hydrolase family 43 arabinofuranosidase from Clostridium thermocellum B8. Enzym. Microb. Technol. 109, 74–83 (2018).

32. Ye, Y., Li, X. & Zhao, J. Production and characteristics of a novel xylanase- and α-L-arabinofuranoside-β-xylosidase from Enterobacter sp. identified from soil and forest soil bacteria. Microb. Res. 169, 213–220 (2014).

33. Shao, X. J. et al. Mutant selection and phenotypic and genetic characterization of ethanol-tolerant strains of Clostridium thermocellum. Appl. Microbiol. Biotechnol. 92, 641–652 (2011).

34. Cintra, L. C. et al. Characterization of a recombinant xylose tolerant β-xylosidase from Humicola grisea var. thermoidea and its use in sugarcane bagasse hydrolysis. Int. J. Biol. Macromol. 105, 262–271 (2017).

35. Yang, X. et al. A new GH43 α-arabinofuranosidase from Humicola insolens Y and its biochemical characterization and synergistic action with a xylanase on xylan degradation. Appl. Biochem. Biotechnol. 175, 1960–1970 (2015).

36. Larssrink, J. et al. Structural and enzymatic characterization of a glycoside hydrolase family 31 α-xylosidase from Cellvibrio japonicus involved in xylolucan saccharification. Biochem. J. 436, 567–580 (2011).

37. Krogh, K. B. R. M. et al. Polypeptides having a xylosidase activity and polynucleotides encoding same. US patent 9,603,378 (2017).

38. Okuyama, M., Morii, H., Chiba, S. & Kimura, A. Overexpression and characterization of two unknown proteins, Ycl and YihG, originated from Escherichia coli. Protein Expr. Purif. 37, 170–179 (2004).

39. Moracci, M. et al. Identiﬁcation and molecular characterization of the first Thermobacterium thermophilum α-L-fucosidase from an archaeon, J. Biol. Chem. 275, 22082–22089 (2000).

40. Katayama, T. et al. Molecular cloning and characterization of Bifidobacterium bifidum 1,2-alpha-L-fucosidase (AFCa), a novel inverting glycosidase (glycoside hydrolase family 95). J. Bacteriol. 186, 4885–4893 (2004).

41. Krogh, K.B.R.M. et al. Polypeptides having a- and /galactosidase activity and polynucleotides encoding same. US patent application no. 14/899,489 (2016).

42. Froese, A., Schellenberg, J. & Sparling, R. Enhanced depolymerization and utilization of raw lignocellulosic material by co-cultures of Ruminiclostridium thermocellum with hemicellulose-utilizing partners. Can. J. Microbiol. 65, 296–307 (2019).

43. Verbeke, T. J., Garcia, G. M. & Elkins, J. G. The effect of switchgrass loadings on feedstock solubilization and biofuel production by Clostridium thermocellum. Biotechnol. Biofuels 10, 233 (2017).

44. Argyros, D. A. et al. High ethanol titers from cellulosic by using metabolically engineered thermophilic, anaerobic microbes. Appl. Environ. Microbiol. 77, 8288–8294 (2011).

45. Lee, J., Venditti, R. A., Janneel, H. & Kenealy, W. R. Detoxification of woody hydrolysates with activated carbon for bioconversion to ethanol by the thermophilic anaerobic bacterium Thermoanaerobacterium saccharolyticum. Biomass–Bioenergy. 35, 626–636 (2011).

46. Olson, D. G. & Lynd, L. R. Transformation of Clostridium thermocellum by electroporation. Methods Enzym. 510, 317–330 (2012).

47. Ellis, L. D. et al. Closing the carbon balance for fermentation by Clostridium thermocellum (ATCC 27405). Bioresour. Technol. 103, 293–299 (2012).

48. Holwerda, E. K., Hirst, K. D. & Lynd, L. R. A defined growth medium with very low background carbon for culturing Clostridium thermocellum. J. Ind. Microbiol. Biotechnol. 39, 943–947 (2012).

49. Holwerda, E. K., Ellis, L. D. & Lynd, L. R. Development and evaluation of methods to infer biosynthesis and substrate consumption in cultures of cellulolytic microorganisms. Biotechnol. Bioeng. 110, 2380–2388 (2013).

50. Holwerda, E. K. et al. The exometabolome of Clostridium thermocellum reveals overflow metabolism at high cellulose loading. Biotechnol. Biofuels 7, 155 (2014).

51. Urbanowicz, R. B. et al. 4-O-methylation of glucuronic acid in Arabidopsis glucuronoxylan is catalyzed by a domain of unknown function family 579 protein. Proc. Natl. Acad. Sci. USA 109, 14253–14258 (2012).

52. Correa, M. A. S. et al. Crystal structure of a cellulolous family 3 carbohydrate esterase from Clostridium thermocellum provides insights into the mechanism of substrate recognition. J. Mol. Biol. 379, 64–72 (2008).

53. Protocol for Expression Using T7 Express (C2566). https://www.neb.com/ protocols/0001/01/01/protocol-for-expression-using-t7-express-c2566.

54. Peña, M. J. et al. Arabidopsis irregular xylan and irregular xylan: implications for the complexity of glucuronoxylan biosynthesis. Plant Cell 19, 549–563 (2007).

Acknowledgements

The authors would like to thank Xiaoyu Liang for providing the sample from her anaerobic digestor from which LL1335 was isolated, Daniel G. Olson who performed LL1335 genome analysis, and Eric Martens, who provided B. cellulosilyticus. This study was supported by the Bioenergy Science Center and Center for Bioenergy Innovation, which are US Department of Energy Bioenergy Research Centers supported by the Office of Biological and Environmental Research in the DOE Office of Science and by Enchi Corporation. Genome sequencing was performed by the Department of Energy Joint Genome Institute, a DOE Office of Science User Facility, which is supported by the Office of Science of the US Department of Energy under contract number DE-AC02-05CH11231.

Author contributions

D.B. performed the experimental work involving fermentations, microbiology and molecular biology. C.D.H. and L.R.L. supervised the biotechnology work. M.I.P. and W.S.Y. performed the structural analysis of carbohydrates and the activity characterization of the purified enzymes. All authors contributed to writing and editing of the paper.

Competing interests

L.R.L. and C.D.H. receive funding from Enchi Corporation. L.R.L. has personal financial interest in Enchi Corporation, which seeks to commercialize technology related to C. thermocellum and other similar bacteria. Other authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-15704-z.

Correspondence and requests for materials should be addressed to L.R.L. or M.J.P.

Peer review information Nature Communications thanks Isaac Cann, Richard Sparling and the other anonymous reviewer(s) for their contribution to the peer review of this work.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2020