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Thermophilic Anaerobic Bacteria from Biocompost Able to Degrade Cellulose and Xylan

Cellulolytic and Xylanolytic Bacteria

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Abstract

Nine thermophilic cellulolytic clostridia and four other non-cellulolytic bacteria were isolated from self-heated biocompost via preliminary enrichment culture on microcrystalline cellulose. All cellulolytic isolates grew vigorously on cellulose with the formation of either ethanol and acetate or acetate and formate as principal fermentation products, as well as lactate and glycerol as minor products. In addition, two out of nine cellulolytic strains were able to utilize xylan and pretreated wood with roughly the same efficiency as cellulose. The major products of xylan fermentation were acetate and formate, with minor contribution of lactate and ethanol. Phylogenetic analyses of 16S rRNA and glycosyl hydrolase family 48 (GH48) gene sequences revealed that two xylan-utilizing isolates were related to *Clostridium clariflavum* strain and represent a distinct novel branch within the GH48 family. Both isolates possessed high cellulase and xylanase activity induced independently by either cellulose or xylan. Enzymatic activity decayed after growth cessation with more rapid disappearance of cellulase than xylanase activity. A mixture of xylan and cellulose was utilized simultaneously with a significant synergistic effect observed as a reduction of lag-phase in cellulose degradation.

Keywords Thermophilic clostridia, cellulose fermentation, biocompost, cellulase, xylanase.

Introduction

Plant biomass represents an abundant and valuable renewable natural resource that may be used for a wide range of purposes, including the production of fuels and chemicals in addition to food and feed (41). Microbial conversion of cellulosic biomass is a promising strategy for low-cost biomass processing (21). After cellulose, xylan is the most abundant polymer in plants (40). Because of this, microbial conversion of hemicellulose and hemicellulose sugars is a subject of active research (29) and xylanases are of interest for application in the pulp and paper (18) as well as other industries (4).

Several anaerobic thermophiles have been shown to utilize cellulose, including *Clostridium thermocellum*, *C. straminisolvens, C. stercorarium*, *Caldicellulosiruptor saccharolyticus* and *C. obsidiansis* (8, 12, 15, 23, 27, 44). *C. thermocellum* exhibits a high growth rate on crystalline cellulose (22), but does not utilize xylan, does not grow on xylose and other pentoses, and grows poorly on glucose (20). Extremely thermophilic cellulolytic *Caldicellulosiruptor saccharolyticus* can co-utilize glucose and xylose (42), and its close relative *Caldicellulosiruptor bescii* DSM 6725 has been found to degrade xylan and xylose by Yang et al (45), although the original report (36) about this organism described it to be unable to grow on xylose. *C. bescii* was recently shown to utilize cellulose and hemicellulose originating from lignocellulose (45) although with relatively low yield of fermentation products indicating substrate conversion degree below 20%. Several mesophilic *Clostridium* strains have been reported to utilize both cellulose and xylan, including *C. phytofermentas* and *C. cellulovorans* (17, 34, 43). However, *C. stercorarium* is the only thermophilic cellulolytic *Clostridium* species reported to utilize xylan, and cellulose degradation rate by *C. stercorarium* is modest as compared to *C. thermocellum* (1, 47).

In our preceding study (13), we obtained three cellulolytic consortia from the self-heated biocompost. They contained cumulatively 30 and 11 OTUs based respectively on the 16S rRNA and GH48 (glycosyl hydrolase family 48) gene clone-libraries survey. Here we report on the next step of our biocompost study that resulted in isolation, purification and characterization of nine thermophilic, cellulolytic bacteria. Most of the isolates turned out to be different from *C. thermocellum* and display similarly high cellulolytic activity. Two isolates are able to degrade cellulose, xylan and their mixture with the same efficiency and rates.

Materials and methods

Field site

Samples were collected from the Middlebury College compost facility in May 2008 at locations 40 to 50 cm below the surface and temperatures ranging from 52 and 72^{o}C across sampling sites. The original compost material consisted of three roughly equal parts: wood chips, horse manure and food waste.

Sampling and enrichment procedure

Strictly anaerobic conditions were maintained by flushing with pure N_2 throughout primary sampling and subsequent inoculation. Compost samples were obtained with a T-shaped steel corer with a 2 cm diameter at three distinct spots designated as CO-4, CO-5 and CO-6. Between 8 and 15 g of extracted material were transferred into serum bottles containing 100 ml of mineral medium, pH 7 and one gram of microcrystalline cellulose (Avicel PH105; FMC Corp., Philadelphia, Pa.) or filter paper (Whatman, #1). After inoculation, each bottle was immediately flushed with N_2. In addition, 'cellulosic traps' consisting of stainless steel tea strainers containing about 1 g of filter paper strips were placed in the same compost. Traps were placed at a depth of about 40 cm and left *in situ* to obtain growth of cellulolytic microorganisms. After one week of incubation, the traps were retrieved, delivered to the lab and partly decayed filter paper was used for inoculation of cellulose-mineral media.

Nutrient medium

The enrichment medium did not contain sulfates and nitrates to preclude development of sulfate reducing or denitrifying bacteria competing with fermenting cellulose-degrading microorganisms. PE medium for primary enrichments contained (g/l): KH_2PO_4, 2.08; K_2HPO_4,

2.22; $MgCl_2 \times 6H_2O$, 0.1; NH_4Cl, 0.4; $CaCl_2 \times 2H_2O$, 0.05. Upon arriving at the laboratory, primary enrichments were incubated at 55 ^oC and transferred to fresh medium every 4 to 6 days. For subsequent transfers, chemically-defined minimal medium M was used (g/l): Avicel, 3; KH_2PO_4, 1.04; K_2HPO_4, 1.11; $NaHCO_3$, 2.5; $MgCl_2 \times 6H_2O$, 0.2; NH_4Cl, 0.4; $CaCl_2 \times 2H_2O$, 0.05; $FeCl_2 \times 4H_2O$, 0.05; L-cysteine HCl, 0.5; resazurin 0.0025. SL-10 trace element, 1 ml/L (3) and vitamin, 4 ml/L, solutions were added as concentrated solutions. The vitamin solution contained (g/l): pyridoxamine dihydrochloride, 0.2; PABA, 0.1; D-biotin, 0.05; vitamin B12, 0.05; thiamine-HCl, 0.0125; folic acid, 0.5; Ca-pantothenate, 0.125; nicotinic acid, 0.125; pyridoxine-HCl, 0.025; thioctic acid, 0.125; riboflavin, 0.0125 (all purchased from Sigma-Aldrich). Vitamins were sterilized by filtration. Phosphates were autoclaved separately to avoid precipitation. Other minerals and L-cysteine were prepared as a 100× concentrated stock solution, flushed with N_2 and autoclaved. All sterile and reduced ingredients were combined in serum bottles inside an anaerobic glove box, sealed and crimped.

Isolation of pure cultures and total cell counts

Isolation of pure cultures of cellulose-utilizing bacteria was performed on agar-avicel and agar-cellobiose media after 10 consecutive transfers on M medium with the following changes: 15 g/l of agar were added, vitamins substituted with 2.0 g/l of yeast extract, and the concentration of Avicel increased to 20 g/l. Cellulolytic consortia were serially diluted into agar-avicel medium that was preliminarily melted and cooled to 55-60 ^oC and then plated under strictly anoxic conditions in anaerobic glove box. After solidifying, plates were incubated under anaerobic conditions at 55º C.

Cellulose-utilizing bacteria formed colonies with clearing zones. Colony forming units were counted after 4 – 7 days of incubation at 55º C. Single colonies were picked with a syringe needle and inoculated into cellulose- and cellobiose-mineral liquid M medium. Isolates first grown on cellobiose medium were transferred onto avicel medium to evaluate the ability to utilize cellulose.

Cultivation and growth measurement

Batch fermentation of various substrates was carried out in serum bottles with a total volume of 240 ml filled with 100 ml of defined M medium and 1.5-5.0 g/l of Avicel, xylan (from Birchwood, Sigma or Oats Spelts, Tokyo Chemical Industry CO., LTD, Japan), xylose, pretreated wood (obtained from Mascoma Corp. as a mixed steam-pretreated hardwood and then washed, grounded, and dried before addition to media) or mixture of Avicel and xylan. One volume percent of actively growing Avicel batch cultures were used as inocula. Two or three bottles of each culture were incubated at 55º C with agitation at 180 rpm. Two replicate 2-ml subsamples were taken periodically from each bottle and immediately centrifuged at 10,000 g. Supernatants were used to measure pH (pH-meter UB-10, Denver Instrument; Orion 410, Orion Research Inc.) and fermentation products by HPLC (19). Pellets were washed and analyzed for the total pellet C (TPC) and total pellet N (TPN) content with Shimadzu TOC-V Combustion Analyzer coupled with a Total Nitrogen module TNM-1 (26). A glycine solution (1 g/L) was used as a standard. The TPC and TPN data allowed calculation of cell mass and residual insoluble substrate as described earlier (26): the TPN was interpreted as microbial N, assuming that contribution of extracellular enzymes to the pellet N was negligible and that cellulose, xylan and pretreated wood were N-free. In preliminary tests, it was found that the majority of N-compounds interfering with cell-N determination (NH_4^+, amino acids, peptides and extracellular

proteins) were completely removed by pellet washing. The bacterial N-content was measured in cells of *Clostridium thermocellum* and isolate 4-2a grown on cellobiose, it was observed to vary in a relatively narrow range 0.27±0.02 g N/g C. The yield of tested strains was calculated within a period of intensive batch growth as a cell mass increment (based on TPN with indicated above N:C ratio) per unit of consumed insoluble substrate (respective decline in residual TPC of cellulose or xylan corrected for cell mass).

Enzyme assay

Cellulase activity was analyzed with MarkerGene™ Fluorescent Cellulase Assay Kit (Marker Gene Technologies, Inc.) with fluorescent substrate Resorufin Cellobioside (6). Fluorescence was recorded using a Gen5™ Microtiter Plate Reader (BioTeck Instruments, Inc.) with 530/25 nm excitation filter and 595/35 nm emission filter. Fluorescence reading was taken at 2-minute intervals for 60 minutes.

Xylanase activity analysis was performed with EnzChek® Ultra Xylanase Assay Kit (Molecular Probes™, Inc.) using fluorescent substrate, 6,8-difluoro-4-methylumbelliferyl [beta]-d-xylobioside (DiFMUX2) (9). Fluorescence was recorded with the same instrument using the 360/40 nm excitation filter and 460/40 nm emission filter. Fluorescence reading was taken at 2-minute intervals for 90 minutes. Both activities (cellulase and xylanase) were recorded in the original cultural liquid (sum of free and bound enzymes) and in the supernatant after centrifugation of suspension at 13,000 rpm (free enzymes). Enzymatic activity was calculated from the slope of linear segment of product accumulation curve or as initial rate of fluorescent product release when hydrolysis rate was progressively declining during measurement period. Control for self-decay of fluorescent substrate was set up according to manufacturer's

instructions. Three to six replicates of cultural liquid and spent medium was used to determine enzymatic activity at each time point to calculate the mean and standard deviation.

DNA extraction, PCR amplification and phylogenetic analysis

Genomic DNA was extracted from microbial biomass with the GenElute Genomic DNA Kit (Sigma) according to manufacturer's instructions. PCR amplification of the 16S rRNA gene and sequencing was done as described before (33). Amplification of GH48 genes was performed with GH48F and GH48R degenerate primers as previously described (13). For *C. thermocellum* and *C. straminisolvens*-type isolates, co-amplification of *celS* and *celY* genes was resolved by constructing clone libraries with amplicons using pGEM-T-easy vector (Promega, Madison, WI) as previously described (13). *C. thermocellum* ATCC 27405 was used as a positive control. Amplified PCR products were sequenced at Agencourt Bioscience Corporation (Beverly, MA). Nucleotide sequences were aligned with sequences from GenBank using BioEdit v.7.0.5 (11) or CLUSTALX (39). Phylogenetic trees were reconstructed using the ME-algorithm (28) via the MEGA4 program package (37). Screening for sequence similarity was carried out with BLAST (2).

Microscopy

Microscopic observation was performed after smear staining with DTAF [5-(4,6-dichlorotriazin-2-yl)amino-fluorescein] using a Leica DMLB microscope equipped with a Mercury short ARC photo optic lamp (33). A K3 filter (illumination path 470-490 nm, observation path 515 nm) was used to visualize DTAF-stained microbial cells.

Statistical calculations and data analysis

Descriptive statistics of primary data, including mean, confidence interval and standard deviation were performed with MS Excel. We used 2-5 replicates for all analytical measurements (HPLC and TOCN) and relative error did not exceed 5-10%. The batch growth experiments were done at least twice with two or three replicate bottles.

Phylogenetic trees were assembled using a bootstrap test with 1000 replicates to evaluate robustness. Sequences generated within this study have been deposited in GenBank under accession numbers FJ808599-FJ808612, GQ265352-GQ265362 and HM171683-HM171687. Strain 4-2a was deposited with ATCC under deposition number PTA10114.

Results

Isolation of pure cultures

Enrichment cultures after 5-10 consecutive transfers exhibited reproducible cellulose fermentation comparable to pure cultures (e.g. of *Clostridium thermocellum*) as indicated by fermentation products, extent and time required for cellulose utilization. These mixed cultures are referred to below as 'consortia' which are distinct from 'primary enrichment' having non-stabilized variable composition. In total, we obtained four anaerobic thermophilic cellulolytic consortia: three (CO-4, CO-5 and CO-6) were derived directly from three respective biocompost samples as described earlier (13) and one named CT was obtained from a cellulose trap. Plating of the serially diluted consortia cultures on cellulose agar revealed bacterial colonies with extensive clearing zones indicating cellulose solubilization by extracellular enzymes (Supplemental Material, Fig. 1).

Cell numbers for consortia were estimated by plate count and by direct microscopy. Both methods produced converging results. For example, at the end of active growth in liquid batch

culture when cellulose was used nearly completely, the consortia CO-4, CO-5 and CT contained about 2.5×10^8 CFU/ml, while CO-6 had one order of magnitude higher density, 2.5×10^9 CFU/ml. Direct microscopic counts in the same liquid cultures had 1×10^9 and 3×10^9 cell/ml respectively In total, nine cellulolytic and four non-cellulolytic bacteria were isolated from four tested consortia. Strains CT1 and CT2 were isolated from the CT-consortium, strains 4-1, 4-2a came from CO-4, strain 5-8 was isolated from CO-5 and the rest of strains (6-17a, 6-24, 6-26, 6-29) were isolated from CO-6. Non-cellulolytic strains 6-12, 6-16, 6-30 and 6-31 were obtained from CO-6.

Phylogenetic analysis of isolates. Phylogenetic analysis of cellulolytic and non-cellulolytic isolates based on 16S rRNA gene sequences (Fig 1) revealed cellulolytic bacteria belonging to three particular groups, all of them belonging to the genus *Clostridium*: i) closely related to *C. straminisolvens* (15*)*; ii) related to *C. thermocellum*; and iii) related to *C. clariflavum* recently isolated from methanogenic sludge (30, 32).

16S rRNA gene sequences similarity of strains 4-1 and 4-2a was 100% as related to each other and 99.7 % as related to *C. clariflavum*. In spite of the 16S rRNA gene sequences identity, these two strains showed phenotypic differences in cell morphology and fermentation pattern (see below), therefore we consider them as distinct although closely related organisms diverged significantly from other members of the *Clostridium* genus. Sequence similarity was only 96% compared to *C. thermocellum* and *C. straminisolvens*. Cellulolytic strains 6-17a, 5-8, 6-24, 6-26 and 6-29 formed a tight cluster (99-100% similarity) with *C. straminisolvens,* while isolates CT1 and CT2 clustered with *C. thermocellum* (99-100% similarity). Non-cellulolytic isolate 6-12 was related to *Clostridium thermosuccinogenes* and *Clostridium* sp. FG4 (16), strain 6-30 was similar to recently described *Lutispora thermophila* (31), while strains 6-16 and 6-31 turned out to be

related to previously uncultured microorganisms from glucose or cellulose degrading consortia of methanogenic reactors (5, 38).

Glycosyl Hydrolase Family 48 (GH48)

Phylogenetic analysis was also carried out with respect to exocellulases of glycosyl hydrolase family 48, a major enzyme of interest within cellulolytic microorganisms. PCR amplification of this region yielded an amplicon of variable length, between 1143 and 1167 nucleotides in length. Consistent with observations from 16S rRNA gene analyses, cellulolytic isolates formed three groups (Fig 2): i) isolates 6-17a, 5-8, 6-24, 6-26 and 6-29 were most closely related to the glycosyl hydrolase family 48 gene sequences in *C. straminisolvens celS*- and *celY*-like genes*;* ii) isolates CT1 and CT2 related to *C. thermocellum*, formed two separate clusters of sequences closely related to *celS* and *celY* genes of *C. thermocellum* type strain ATCC 27405 and strain DSM 1313; iii) strains 4-1 and 4-2a, related to *C. clariflavum*, formed a distinct cluster of identical nucleotide sequences with no known sequences closely related to them. The closest matches to *Clostridium clariflavum*-like sequences were *C. thermocellum celY* (74.1% similarity nucleotide, 87% deduced amino acid sequence similarity) and *C. straminisolvens* (73.4% similarity nucleotide, 87% deduced amino acid sequence similarity). No GH48 genes were detected in non-cellulolytic isolates.

Novel isolates related to *C. thermocellum* and *C. straminisolvens* were determined to have both free (*celY*-like) and cellulosome-bound (*celS*-like) exoglucanases from the glycosyl hydrolases family 48 as seen in reference strains (13). GH48 genes in *C. straminisolvens* and *C. clariflavum* related isolates displayed very similar grouping as observed in 16S rRNA gene analyses, suggesting a very strict conservation of this particular family of glycosyl hydrolases within

cellulolytic clostridia. GH48 genes retrieved from isolates 4-1 and 4-2a represent novel exoglucanases.

Cell morphology

The bacteria were found to be straight and curved rods of different length, long filamentous cells, and cells with terminal spores. All strains related to *C. straminisolvens* were represented by curved rods 5-15 × 0.1-0.3 µm or filamentous cells 10-40 × 0.1-0.3 µm attached to cellulose particles. We observed that isolates having almost identical 16S rRNA sequences displayed significant morphological diversity, even under uniform growth conditions. *C. thermocellum* related strains were represented by slightly curved rods 5-15 × 0.2-0.5 µm. The strains 4-2a and 4-1 were different from each other: strain 4-2a was presented by long curved rods 2-10 × 0.1-0.2 µm while 4-1 strain formed short and mostly straight rods 2-4 × 0.1-0.3 µm. Both strains changed their morphology as dependent on carbon substrate; the cellulose-grown cells were 2-3 times longer as compared with cells grown on xylan. The phylogenetic identity of strains 4-1 and 4-2a grown on different substrates was confirmed by 16S rRNA gene analyses. All cultures grown on Avicel produced a bright yellow or light cream pigment.

Fermentation of cellulose

Table 1 shows major fermentation products found in all tested strains on the second day of batch culture grown on 3 g/L cellulose. All strains produced acetate, ethanol and lactate. With the exception of strain CT2, all isolates produced formate. Ethanol concentration at the end of fermentation varied from 0.3 to 10.5 mM. The most active strains 6-29 and 6-24 (*C. straminisolvens* group) produced 9.2 – 10.5 mM of ethanol, which accounted 22-30% of the theoretical maximal yield (assuming 2:1 stoichiometry between ethanol and glucose) with the

acetate-to-ethanol ratio being close to 1:1. In other strains, acetate was the predominant fermentation product. In the *C. clariflavum* group, acetate:ethanol ratios reached values between 10:1 (strain 4-2a) and 22:1 (strain 4-1); these strains had formate (not ethanol) as a second major fermentation product. There was a very clear relationship between phylogenetic position and fermentation profile. Only members of the *C. clariflavum* group generated significant amounts of formate, while all isolates related to *C. straminisolvens* and *C. thermocellum* fermented Avicel mostly to acetate and ethanol. Lactate accumulated at the late stage of fermentation as the pH dropped.

Fermentation and enzymatic activity of strain 4-2a grown on various carbon substrates

The most intriguing members of our collection were strains 4-1 and 4-2a related to *C. clariflavum.* Isolated from the CO-4 consortium developed on cellulose, both strains were able to utilize wide spectrum of plant polymers including xylan, xylose and pretreated wood (Table 2). Xylan supported very intensive growth of these bacteria. Growth on pretreated wood remained quite vigorous although less intensive. Xylose supported anaerobic growth of both strains but its fermentation was extremely slow and incomplete, after 10 days of incubation at 55^{o} C about 50% of xylose remained unutilized. Major fermentation products of xylan were acetate and formate, pretreated wood was transformed mainly into acetate. Ethanol concentrations varied from 0.8 to 0.9 mM. The acetate: ethanol ratio ranged from 10:1 to 16:1, very similar to the ratio observed in cellulose fermentations. Major fermentation products of xylose were acetate and lactate, with no ethanol detected.

Dynamics of Avicel and xylan fermentation was observed for strain 4-2a in batch culture with 5 g/L of separately added polymeric substrates (Fig. 3). Although inocula were obtained on

medium with cellulose, degradation of xylan started immediately while fermentation of Avicel was delayed by 11-15 hours. Beyond the lag-phase, both polymeric substrates were consumed within 20 hours indicating exceptionally high degradation rate comparable to the reported cellulolytic activity of *C. thermocellum* (22). Growth efficiency turned out to be roughly the same on cellulose and xylan with cell yield 0.14 g C biomass per g C of each substrate. After utilization of more than 50% of added C-substrate, the solubilization process slowed down accompanied by acidification of cultural liquid (pH drop from 8 to 6 units, not shown). Metabolic acidity was the most probable reason for microbial growth cessation before complete utilization of xylan and cellulose. The major fermentation products (>5 mM) from Avicel were acetate, formate, and lactate; the minor products (<2 mM) were ethanol and glycerol. Two main intermediates of Avicel depolymerization were cellobiose and glucose. All listed products formed two distinct clusters: i) acetate, formate, glycerol and ethanol closely followed dynamics of cell mass and ii) lactate, cellobiose and glucose lagged behind growth. With xylan, product dynamics was very similar. Xylose, the main depolymerization intermediate of xylan, was accumulated up to 3.5 mM during stationary phase and eventually degraded after prolonged culture starvation (data not shown).

In the second experiment, strain 4-2a was grown on i) 1.5 g/L of Avicel, ii) 1.5 g/L of xylan and iii) mixture of 1.5 g/L Avicel and 1.5 g/L xylan to see a possible competition or synergy in conversion of two substrates. Apart from cell mass, fermentation products and pH we also monitored enzymatic activity of cellulase and xylanase (Fig 4). There was a high degree of synchrony between cell growth (Fig 4A), accumulation of fermentation products (Fig 4B) and the level of cellulase (Fig 4C) and xylanase (Fig 4D) activity. It becomes evident from these data that synthesis of cellulases and xylanases was induced by both polymeric substrates. Cellulase

activity correlated with cell mass and although it was the highest in the culture with Avicel, its level remained high enough in culture grown on xylan and the xylan-cellulose mixture. In a similar way, xylanase activity was expressed to the same degree in the medium with xylan or cellulose alone, the difference being mainly explained (r^2 0.86) by the variation in bacterial cell mass concentration. Again (like in the first experiment) xylan started to be degraded without lag-phase contrary to about one day delay in the Avicel-containing medium. In the culture grown on mixture of two substrates (dotted lines in all panels of Fig 4), growth was even more intensive and instant than on xylan. It was accompanied by faster acidification (Fig 4E) with pH drop to the tolerance limit of pH 5.4 with concomitant abrupt decline in enzymatic activity and slowing down of the fermentation. The indicated acidification prevented complete utilization of the cellulose-xylan mixture. Nevertheless, we have clear indication (Fig 4F) that presence of xylan stimulated degradation of cellulose: cellobiose (unique intermediate deriving from cellulose) was released as early as after 8 hours of bacterial growth on mixture of two substrates while with cellulose alone, it was detected after 50 hours. In the xylan- and xylan-cellulose media (black and grey diamonds first day of incubation, Fig 4F) initial rate of xylose release was the same; however, the positive effect of cellulose became clear on the 2nd day being expressed as a stimulation of xylose uptake.

The majority of enzymatic activity in the strain 4-2a was found to be associated with particulate matter, no more than 15% of enzymatic activity being detected in a supernatant. The only exception was the cellulase activity in xylan-grown culture where up to 75% of enzymes were detected in a supernatant. It indicates that strain 4-2a produces free extracellular enzymes which are easily bound to insoluble substrates. Xylan forms smaller sediment because of partial thermal degradation and solubilization during autoclaving, therefore it binds less enzymes and mainly

xylanase leaving the most of cellulase free. Both enzymatic activities (cellulase and xylanase) quickly declined after growth cessation indicating abrupt inactivation of extracellular enzymes.

Close correlation between cellulase and xylanase activities could be explained by either their co-expression in the same operon(s) or that both polymers are degraded by a single enzyme with wide substrate specificity (10). To test the second option, we plotted the pooled data on xylanase and cellulase activities versus each other (Fig 5). Three domains of data points were formed: i) in cellulose-grown culture, the pellet-associated activities of xylanase and cellulase were closely correlated (r^2=0.987); ii) in xylan and xylan + cellulose medium the relationship between two enzyme activities was non-linear and iii) in supernatants of all cultures the activity of free xylanases and cellulases displayed a weak correlation (r^2=0.671) and with much shallower slope of respective regression line as compared with the first group. Behind wide variation in the xylanase/cellulase ratio seems to be a slower inactivation of xylanase as compared with cellulase activity in the stationary phase of batch culture (Fig 4C and D). The revealed difference is sufficient to decline the hypothesis that the same hydrolytic enzymes catalyze a breakdown of both polymers.

Discussion

In this paper, we describe isolation, characterization and primary testing of anaerobic thermophilic bacteria from mixed cellulolytic culture.

The isolation of single colony of anaerobic microorganisms is an intricate procedure, so we paid special attention to methodology in an attempt to find optimal plating conditions for cellulolytic thermophiles. Our experience supports the following major conclusions:

1. Serial dilution of bacterial suspension before plating should be done with full-strength reduced medium. Using buffer or water for serial dilution resulted in lowered plating efficiency.
2. Consortia or enriched cultures should be growing actively before plating. Substrate-depleted cultures resulted in lower cell counts and poor reproducibility of plating.
3. Plating and single colony isolation should be done several times to maximize the probability of isolating pure cultures of cellulolytic organisms. There is a high likelihood that non-cellulolytic colonies happen to be present within a clearing zone produced by cellulose-degrader. We found 2-3 times repetitive plating to be effective in eliminating non-cellulolytic contaminants.

In natural microbial communities, plate counts typically result in 100-1000 times lower microbial abundance as compared to microscopic counts and this discrepancy stems from broad metabolic diversity of indigenous populations and the presence of 'viable-non-culturable' cells (35). Lack of such discrepancy in our consortia indicates narrow metabolic diversity and absence of stressed organisms, which most probably have been lost in the course of consecutive transfers. Low diversity was also detected in 16S rRNA gene and glycosyl hydrolase family 48 sequences of these consortia (13). Limited diversity can be also explained by high cultivation temperature in combination with cellulose recalcitrance: only limited numbers of species are able to develop under such conditions. Similar observation on restricted diversity and lack of significant difference between plated and counted microbial abundances was found in extreme natural communities, like polar sea ice (14).

Contrary to previous studies (25), we have found wider phylogenetic and metabolic diversity of clostridia, probably due to methodological improvements, such as the use of reduced media for

serial dilution, avoiding starvation and oxygen stress and use of pour-plating techniques instead of rolling tubes.

The isolated strains formed three distinct groups. The first group of moderately thermophilic bacteria contained four isolates that were not able to utilize cellulose. They were related to different taxonomic groups including non-cellulolytic clostridia (*C. thermosuccinogenes*), recently described anaerobic bacterium *Lutispora thermophila* (31) and previously uncultured microorganisms from cellulose-degrading methanogenic reactors (5, 38). All of these isolates were able to grow as pure cultures on cellobiose medium with L-cysteine as reducing agent and they sustained ~10 transfers within cellulolytic consortia on defined cellulose-mineral medium. There are two tentative explanations of their stable coexistence with cellulolytic microorganisms in consortia: i) they have higher affinity to depolymerization products (cellobiose and glucose) than cellulolytic bacteria and therefore successfully compete with them for common carbon substrates, or ii) they are able to utilize non-cellulosic organic compounds in nutrient media (L-cysteine, vitamins) as a carbon and energy source and therefore do not compete or stimulate cellulolytic bacteria. Concentration of vitamins was too low to sustain stable growth, and therefore L-cysteine was the most probable candidate for the non-cellulosic carbon source. It is known that *C. thermosuccinogenes* is able to use glucose, xylose, and cellobiose (7). On the other hand, *L. thermophila* did not utilize any of detected Avicel fermentation products or intermediates but was able to degrade cysteine (31). Thus, it is likely that strain 6-12 related to *C. thermosuccinogenes* survived in consortia by utilizing cellobiose and glucose, while strain 6-30 related to *L. thermophila* had been enriched on L-cysteine.

The second and the most abundant group of isolates were related to *C. straminisolvens* and *C. thermocellum*. They are moderately thermophilic bacteria specializing on utilization of cellulose

only. Finally, two out of nine obtained cellulolytic isolates identified as members of *C. clariflavum* group were able to degrade cellulose, xylan and xylose, representing the most versatile group. Cultivation of strain 4-2a on individual polymers and their mixtures led us eventually to a tentative explanation of this versatility: cellulose, even without xylan, provokes synthesis of xylanase while xylan induces biosynthesis of cellulase. Taking an ecological viewpoint, such a tight coupling in expression of hydrolytic activity does not look wasteful because under natural conditions, cellulose is never supplied separately from hemicellulose or lignin. Therefore, bacteria related to *C. clariflavum* represent a specialized group of microorganisms adapted to degrade *the entire plant litter* under hot and anoxic conditions. Xylan, as the most soluble component of lignocellulose, probably acts as a signaling factor initiating cell growth on lignocellulose. Such assumption agrees with the fact that xylan stimulates degradation of cellulose by reducing lag phase to a minimum. Under natural conditions these bacteria probably stay dormant most of the time and can be triggered to 'wake up' by the combined effect of chemical signal from xylan and the rise of temperature above 50 ^{o}C. A warming-up should be at least a short-term event occurring locally at decomposition hotspots due to biogenic heat production by other soil microorganisms. In a biocompost pile, the process of self-heating is scaled-up both in time and in space giving a chance to thermophilic bacteria to approach high cell density.

C. thermocellum, one of the most successful cellulose degraders known combines traits of cellulase and xylanase activities (24, 46). However contrary to strain 4-2a and *C. stercorarium* (1, 46), it does not utilize xylan and xylose (24). Synthesis of xylan degrading enzymes without ability to assimilate all of the released xylose is another subject worthy of discussion and further studies.

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Tables

Table 1. Fermentation products formed by various isolates from cellulose (3 g/l of Avicel).

Average and standard deviation calculated out of two replicas.

Isolate	Closest known relative	Lactate	Formate	Acetate	Ethanol	Acetate/Ethanol ratio
		mM				
4-1	*Clostridium clariflavum*	0.2±0.04	2.74±0.07	7.78±0.55	0.35±0.03	22.2
4-2a	*Clostridium clariflavum*	0.97±0.02	3.53±0.09	9.2±0.61	0. 9±0.04	10.3
6-24	*Clostridium straminisolvens*	0.31±0.03	0.37±0.05	8.58±1.65	9.21±0.91	0.9
6-26	*Clostridium straminisolvens*	0.06±0.001	0.13±0.02	4.87±0.99	3.82±1.96	1.3
6-29	*Clostridium straminisolvens*	0.41±0.03	0.26±0.08	8.19±1.59	10.49±0.99	0.8
6-17a	*Clostridium straminisolvens*	0.4±0.006	0.31±0.002	9.57±1.86	6.79±0.5	1.4
5-8	*Clostridium straminisolvens*	0.03±0.01	0.12±0.02	2.75±0.31	1.22±0.09	2.2
CT1	*Clostridium thermocellum*	0.08±0.01	0.38±0.03	8.64±1.0	4.17±0.3	2.1
CT2	*Clostridium thermocellum*	0.26±0.04	0.0	3.77±0.41	1.43±0.07	2.6

Table 2 Fermentation products formed by isolates 4-1 and 4-2a from xylan, pretreated wood and xylose (3 g/l). Average and standard deviation calculated out of two replicas.

Growth substrate	Strain	Lactate	Formate	Acetate	Ethanol	Acetate/Ethanol
		mM				ratio
Xylan	4-1	0.32±0.03	3.63±0.09	12.83±0.43	0.79±0.13	16.3
Xylose		0.21±0.19	0	2.63±0.76	0	-
Pretreated wood		0.88±0.88	0.57±0.17	10.28±2.1	0.87±0.3	11.8
Xylan	4-2a	0.43±0.03	3.05±0.13	12.18±0.47	0.88±0.15	13.7
Xylose		0.41±0.06	0	2.85±0.25	0	-
Pretreated wood		0.44±0.43	0.22±0.06	9.4±1.47	0.91±0.26	10.3

Figures legend

Fig. 1 Phylogenetic tree of anaerobic thermophilic cellulolytic (◊), cellulolytic/xylanolytic (♦) and non-cellulolytic (○) isolates from biocompost based on 16s rRNA gene sequence comparisons.

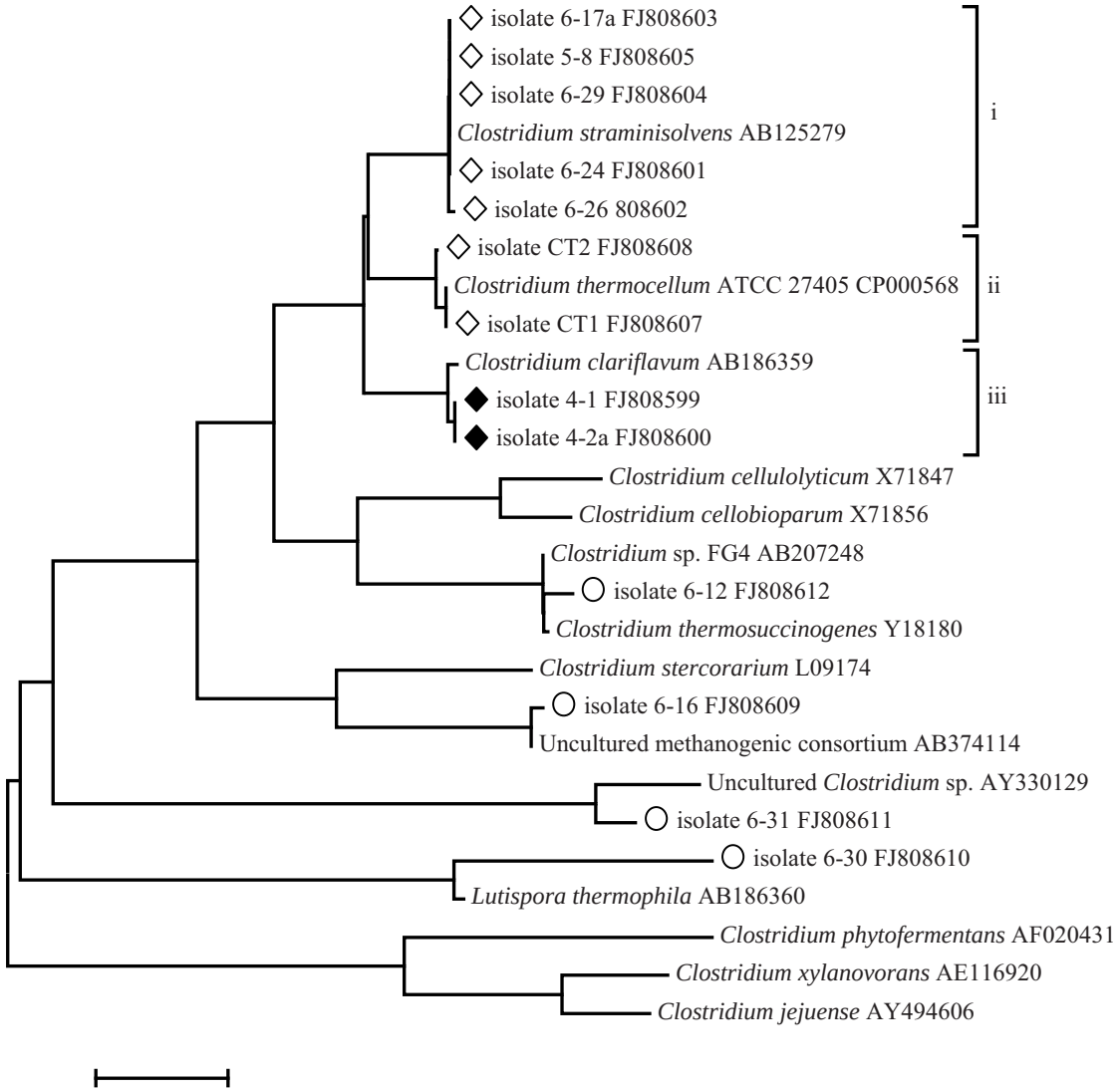
Fig. 2 Phylogenetic tree of thermophilic anaerobic cellulolytic (◊) and cellulolytic and xylanolytic (♦) isolates from biocompost based on GH48 gene sequence comparisons.

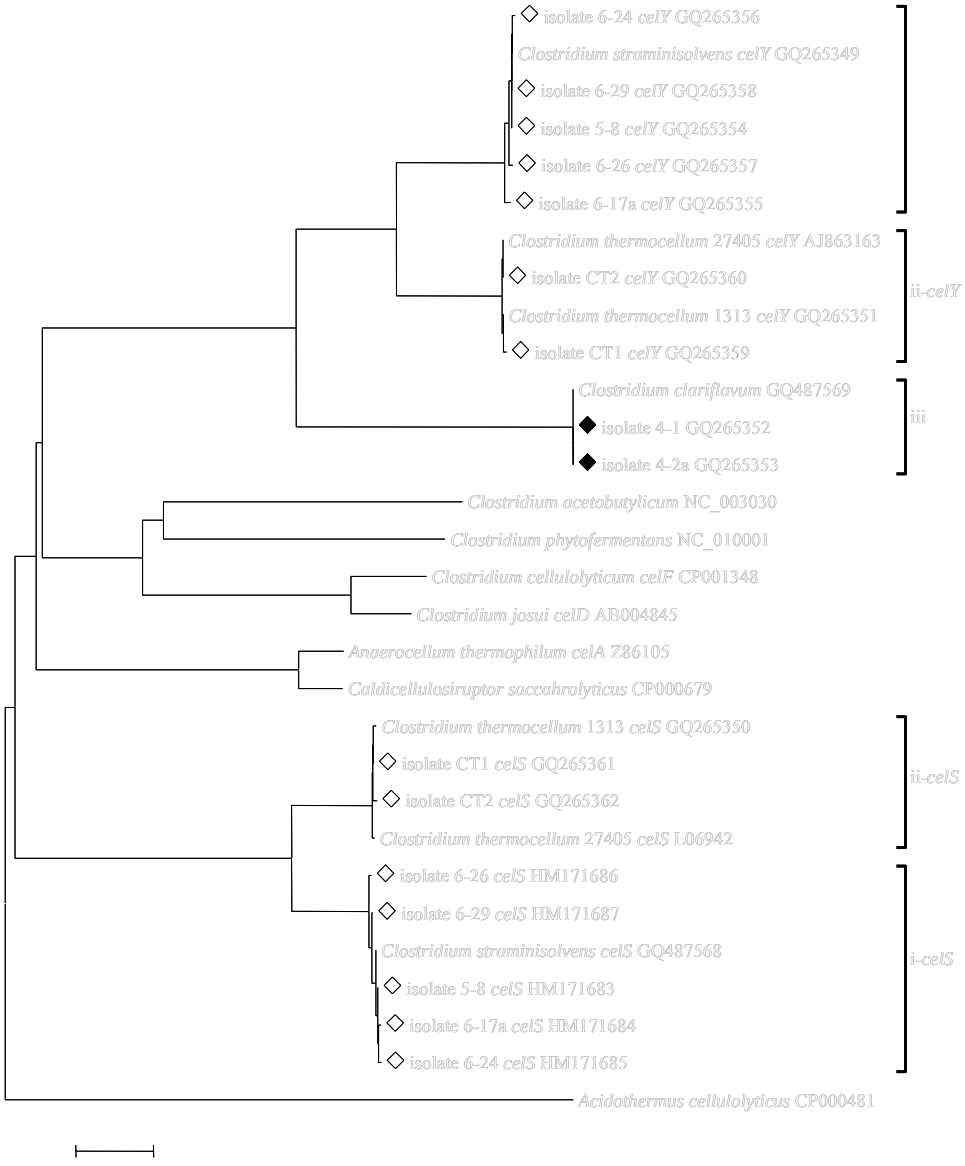
Fig. 3 Fermentation of cellulose (left column) and xylan (right column) by isolate 4-2a. Top panels: total pellet carbon (TPC = C-substrate + C-cell) and total pellet nitrogen (TPN, measure of cell mass concentration). Bottom panels: fermentation products and depolymerization intermediates.

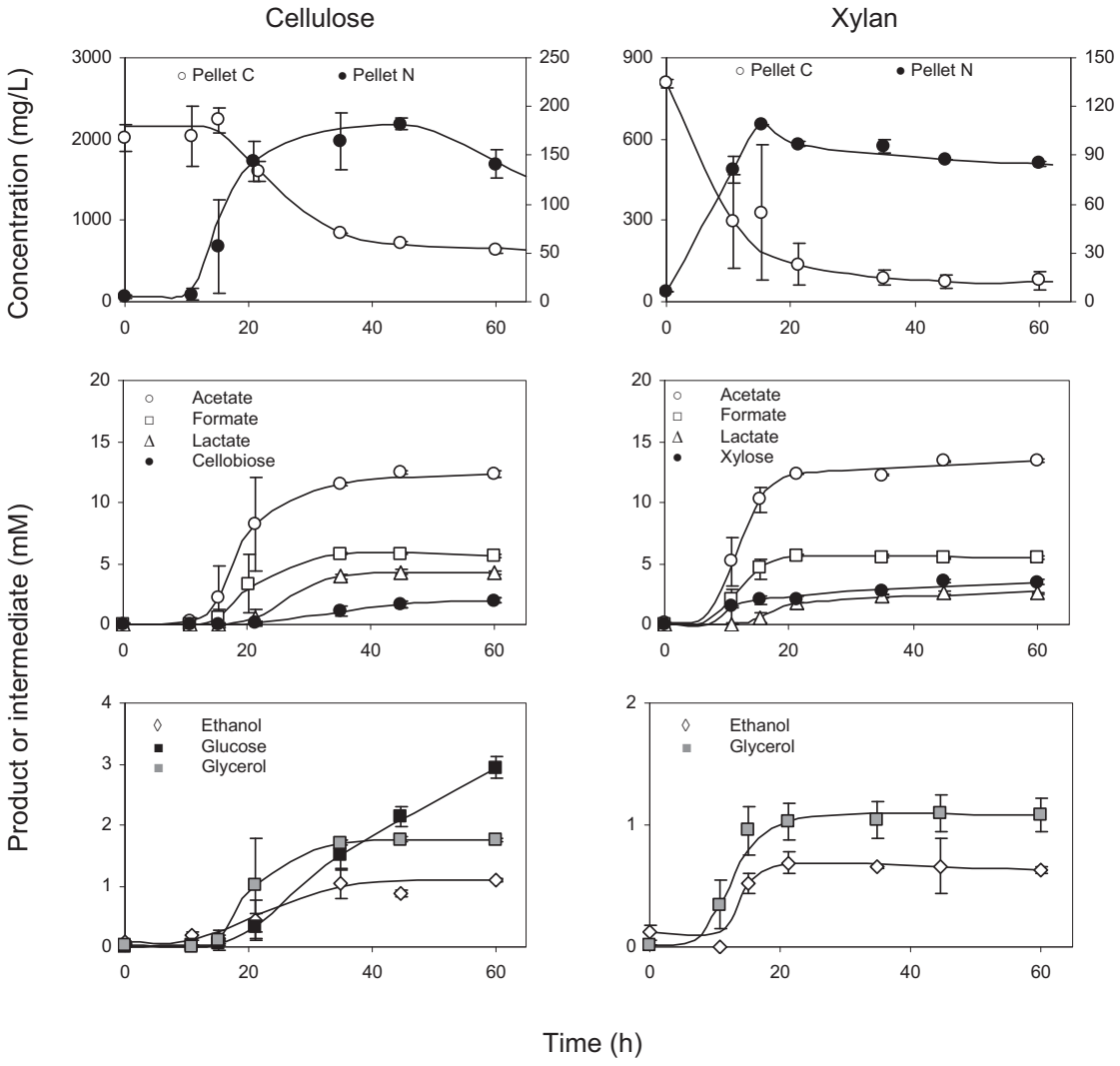
Fig. 4. Growth dynamics of isolate 4-2a on medium with cellulose (open symbols, continuous lines), xylan (grey-filled symbols, continuous lines) or mixture of cellulose + xylan (black-filled symbols, dotted lines).

Top: cell biomass concentration (A) and sum of fermentation products (B), see Fig 3 for individual products. *Middle*: cellulase (C) and xylanase (D) activity. *Bottom*: pH (E) and intermediate degradation products (F).

Fig.5 Relationship between cellulase and xylanase activities. The pooled xylanase activity plotted versus respective cellulase activity. The shaded areas indicate three domains with different relationship between two enzymatic activities that allows rejection of hypothesis that degradation of xylan and cellulose is catalyzed by the same enzyme.







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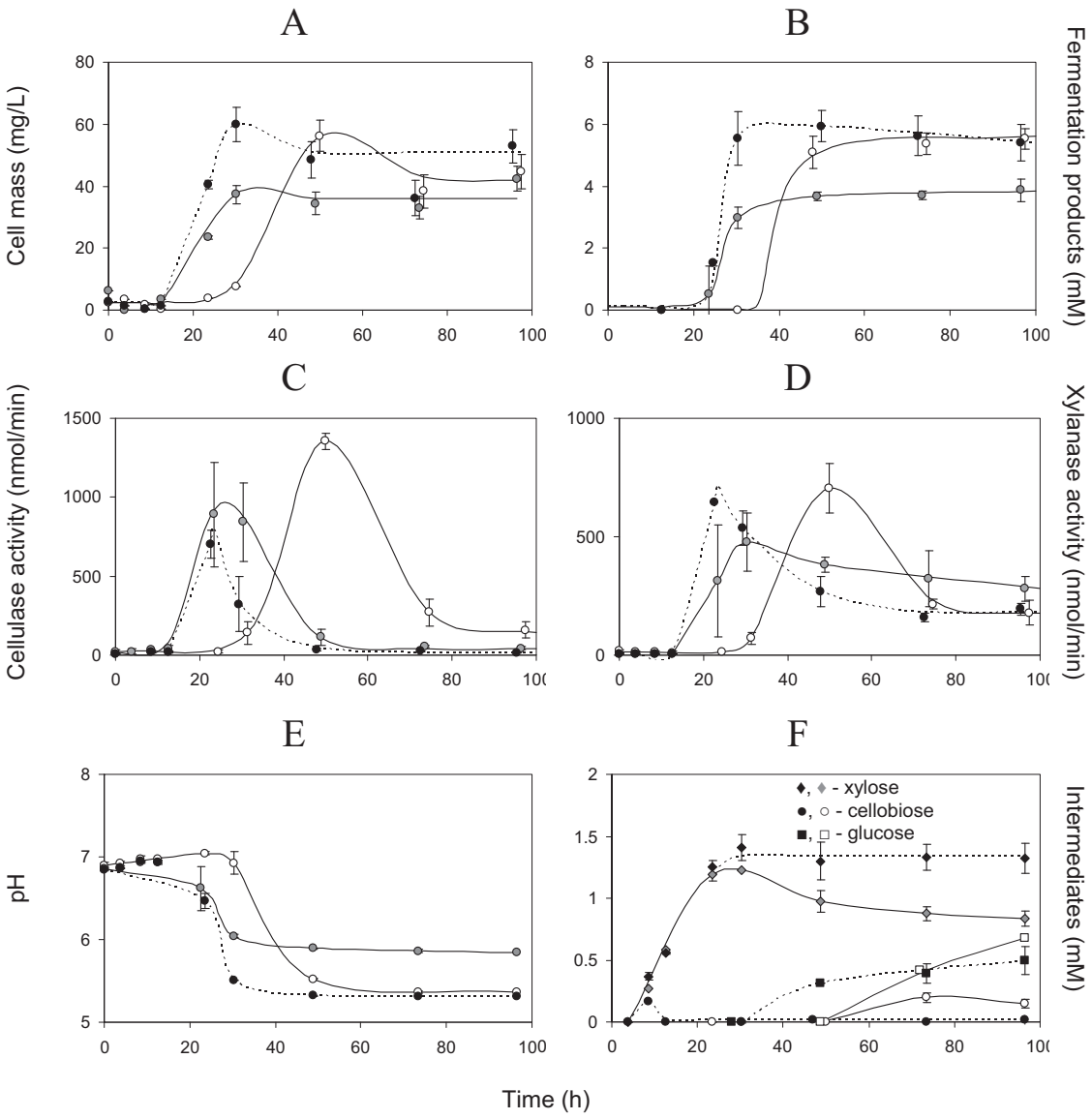

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