Cel9D, an Atypical 1,4-β-D-Glucan glucohydrolase: Characteristics, Catalytic Residues and Synergistic Interactions with Other Cellulases.

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Running Title: Cel9D in *Fibrobacter succinogenes*

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ABSTRACT

The increasing demands of renewable energy have led to the critical emphasis on novel enzymes to enhance cellulose biodegradation for biomass conversion. To identify new cellulases in the ruminal bacterium *Fibrobacter succinogenes* a cell extract of cellulose-grown cells was separated by ion-exchange chromatography, cellulases located by zymogram analysis, and identified by peptide-mass fingerprinting. An atypical family 9 glycoside hydrolase (GH), Cel9D, with less than 20% identity to typical GH9 cellulases was identified. Purified recombinant Cel9D enhanced the production of reducing sugar from acid swollen cellulose (ASC) and Avicel by 1.5 to 4-fold when separately mixed with each of 4 other glucanases although it had low activity on these substrates. Cel9D degraded ASC and cellodextrins with a degree of polymerization higher than 2 to glucose with no apparent endoglucanase activity and its activity was restricted to β-1→4 linked glucose residues. It catalyzed the hydrolysis of cellulose by an inverting mode of reaction, releasing glucose from the non-reducing end. Unlike many GH9 cellulases, calcium ions were not required for its function. Cel9D had increased k_{cat}/K_m values on cello-oligosaccharides with higher degree of polymerization. The k_{cat}/K_m value on cellohexaose was 2300 times higher than on cellobiose. This indicates that Cel9D is a 1,4-β-D-glucan glucohydrolase (EC 3.2.1.74) in GH9 family. Site-directed mutagenesis of Cel9D identified Asp166 and Glu612 as the candidate catalytic residues while Ser168, which is not present in typical GH9 cellulases has a crucial structural role. This enzyme has an important role in crystalline cellulose digestion by releasing glucose from accessible cellooligosaccharides.

INTRODUCTION

Plant material is now viewed as the bioenergy feed stock of the future. Cellulose which accounts for the bulk of plant materials is recalcitrant which necessitates a combination of the classes of cellulases for biodegradation. These include endoglucanases (EC3.2.1.4) that cut randomly at internal amorphous sites in the cellulose chain, exoglucanases (EC3.2.1.91 and EC3.2.1.74) that act processively on the reducing or non-reducing ends of cellulose chains releasing either cellobiose or glucose as major products, and β-glucosidases (EC3.2.1.21) that hydrolyze soluble cellodextrins and cellobiose to glucose (18).

Fibrobacter succinogenes is a highly cellulolytic bacterium that is closely related to the *Cytophaga-Flavobacterium-Bacteroides* group (8). It is commonly found in the rumen of ruminant animals and appears to be one of the most active rumen cellulose degraders (4). Recently, the genome was sequenced (21) and a number of the cellulases (24) and cellulose binding proteins (12) identified. We reported synergistic interaction among 5 cellulases of this organism (24). Although these enzymes had a degree of synergism up to 3.7 and the combination of Cel9B, Ce51A and Cel8B gave the highest activity, the extent of hydrolysis of cellulose was low. In addition, Cel10A, known as the exo-acting Cl-stimulated cellobiosidase, had limited synergistic interaction with other enzymes.

In the following study, a novel cellulase gene *cel9D*, was cloned and protein encoded was purified and characterized. An investigation of the synergistic effect of Cel9D with 4 other cellulases, Cel9B, Cel51A, Cel8B and Cel45C from *F. succinogenes* revealed that Cel9D acted synergistically with these glucanases. Our data also documents that this novel enzyme is the first glucan 1,4-β-D-glucohydrolase in glycoside hydrolase family 9 with enhanced activity on long chain cello-oligosaccharides.

MATERIALS AND METHODS

Bacterial strains and plasmid. *Fibrobacter succinogenes* S85 was used as the source of genomic DNA. It was maintained and cultured in chemically defined medium (CDM) with either glucose or microcrystalline cellulose (Avicel) PH105 (FMC Biopolymer) as the carbon source as previously described (30). The *Escherichia coli* strains DH10B (Invitrogen) was used as the host for genetic manipulation. *E. coli* BL21 (DE3) was the host for protein production. Cultures were grown at 37 ^{o}C or 18^{o}C in Luria-Bertani medium or Luria-Bertani medium supplied with 1M sorbitol and 2.5mM betaine glycine for enhanced solubility of the recombinant proteins (29). When required, media were supplemented with kanamycin at 34 µg/ml. Solid media contained 1.6% (w/v) of agar.

Identification of Cel9D. *F. succinogenes* S85 was grown in 300 ml CDM with 0.3% (w/v) Avicel cellulose PH105 as the sole carbon source at 37^{o}C for 24 h with reciprocating shaking at 150 rpm. After removal of residual cellulose by centrifugation at 300 rpm for 10 min, cells were harvested by centrifugation at 5000 x g and then resuspended in 10 ml of 30 times concentrated culture fluid prepared as described before (20). The cells were then broken by three passes through a French pressure cell at 1200 psi. The cell lysate was centrifuged at 12,000 x g for 30 min. The clarified supernatant containing 25 mg of protein was separated by a 2.5 x 10 cm column of DEAE-Sepharose CL-6B as described (20) except that imidazole buffer (20 mM) was used instead of potassium phosphate buffer. Approximately 40% of the activity that eluted in the application buffer before NaCl gradient was applied and these fractions were kept for further study. Four hundred micro liters of each fraction eluted by the NaCl gradient buffer were mixed with 100 µl 2.5% Avicel PH105 suspension and incubated at 37^{o}C for 20 h. Reducing sugar produced was determined by the PAHBAH methods (16).

Fractions eluting in the buffer gradient exhibiting Avicelase activities were loaded to a 7.5% (w/v) polyacrylamide gel without SDS and subjected to electrophoresis at 100 V for 2 h. The gel was then laid on a plate containing 20 ml 0.1% (w/v) carboxymethyl cellulose

(CMC) solidified with 0.7% agarose and incubated at 37°C for 4 h. The gel was then removed and stained with colloidal Coomassie blue and the agar layer on the plate was stained by 0.1% Congo Red for 30 min and destained by 1M NaCl solution. The bands on the gel that corresponded to clear zones on the CMC plate were excised and subjected to MALDI-TOF mass spectrometry analysis and Cel9D was identified by matching the mass spectra of peptides to the *F. succinogenes* protein database as described previously (14).

Cloning, expression and purification of Cel9D and its mutants. Genomic DNA was prepared from *F. succinogenes* S85 using the cetyl trimethylammonium bromide (CTAB) method as described before (1). Intact *cel9D* gene was amplified from the genomic DNA using Cel9D_1 and Cel9D_2C primers (Table 1) and *Pwo* polymerase (Roche). The restriction endonuclease sites NdeI and XhoI in the 5'-primer and 3' primer, respectively, allowed for cloning of the gene into the expression vector pET30a (Novagen). The coding sequence of Cel9D was fused in frame with a downstream sequence of the vector encoding six histidine residues. The mutants of Cel9D, D166A, E612A, S168A and ΔS168 were generated by the PCR overlap extension methods (27) using the primers listed (Table 1) to replace amino acid residues as indicated and to remove the Ser168, respectively. The wild-type and mutant genes were sequenced to ensure the correct nucleotides changes and no PCR-induced errors. Cel9D and its mutants were produced in *E. coli* BL21 (DE3) and purified by immobilized metal affinity chromatography. Wild-type Cel9D was further purified by molecular sieve chromatography as described (14). Cel8B, Cel9B and Cel51A were produced and purified as described previously (24).

Enzyme assays. Glycoside hydrolase activity was assayed with various substrates as described (24). The standard reaction period for Cel9D was 16 h on Avicel PH105 and 2 h for all other substrates in 0.05 M sodium phosphate buffer pH 6.5. The reducing sugar produced was measured. Substrates used were either Avicel PH105 (0.5%, w/v) or phosphoric acid

swollen cellulose (ASC; 0.25%, w/v). The degree of synergism (DoS) was calculated as follows: (observed activity of a mixture of enzymes)/(additive activities of the individual enzymes making up the mixture).

To determine the kinetics parameters for hydrolysis of cello-oligosaccharides, 0.1 µg purified Cel9D enzyme was mixed with different concentrations of cellooligosaccharide substrates in 0.05 mM phosphate buffer, pH 6.5. Samples were taken at given intervals, appropriately diluted in 0.15M sodium hydroxide to stop the reaction and hydrolysis products analyzed by HPLC.

Analysis of degradation products. Cello-oligosaccharides and their hydrolysis products as well as products from synthetic substrates *p*-Nitrophenol glucopyranoside (pNPG), *p*-Nitrophenol β-D-cellobioside (pNPC) and acid swollen cellulose were analyzed by high-performance anion-exchange chromatography coupled with a Waters model 464 pulsed amperimetric detector system with a base resistant reference electrode as described before (14).

^{1}H NMR Spectroscopy. ^{1}H NMR spectra were generated on a Bruker Avance-400 NMR spectrometer in 5-mm diameter samples tubes. Samples (0.6 ml) were analyzed at 27 ^{o}C in 50 mM potassium phosphate buffer, pH 6.5 containing 20 mM pNPC or 30 mM cellotetraose as the substrate. Chemicals and substrates were dissolved in D_2O. Cel9D enzyme was transferred to buffer containing D_2O by ultrafiltration using a PM-10 (Amicon) membrane. Spectra were collected in 32K data points using a spectral width of 5 KHz and a relaxation delay of 2 s. The spectra were referenced against an external standard of trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP; sodium salt) at 27^{o}C. One hundred microgram of purified Cel9D was added to each reaction.

Nucleotide sequence accession numbers. The nucleotide sequences of the gene *cel9D* from *F. succinogenes* S85 have been deposited in the GenBank database under accession number ########.

RESULTS

Identification and sequence analysis of a novel family 9 enzyme from the genome of *F. succinogenes*. When the avicelases present in the whole cell extract from cellulose-grown *F. succinogenes* were separated on DEAE-Sepharose, a sharp single peak of activity was eluted in the buffer gradient at 0.15~0.25 M NaCl, followed by several minor peaks at 0.35 M and 0.45 M NaCl, respectively (Fig S1). The fractions (#15, #16, #17) corresponding to the major peak were subjected to native PAGE and zymogram analysis (Fig S2). The three fractions yielded similar profiles with activity zones at about 62, 75 and 80 kDa, respectively. Proteins in the corresponding bands of fraction #16 on the native PAGE were extracted and analyzed by peptide mass fingerprinting. No proteins involved in cellulose degradation were identified from the two smaller bands, while in the 80 kDa band a cellulase with an amino acid sequence corresponding to FSU2558 known as *cel9D*, was identified in the genome of *F. succinogenes* S85. MALDI-TOF MS analysis of tryptic peptides of the 80 kDa bands accounted for 22% of the Cel9D protein and the peptide sequences were dispersed throughout the entire protein (Fig. S3).

The gene *cel9D* encoded a protein that had an N-terminal signal peptide, and a putative cleavage site located between Ala23 and Glu24. The mature protein contained 697 amino acid residues with a theoretical mass of 77.0 kDa and a theoretical isoelectric point of 5.27. Following the signal peptide, there was a 100 amino acid region that had low similarity to an immunoglobulin-like module, followed by a putative family 9 catalytic domain and a 69-amino-acid basic C-terminal domain with a theoretical pI of 10.5. The protein sequence had highest sequence similarity to several putative endoglucanases or hypothetical proteins

mainly from the genus of *Vibrio*, *Photobacterium* and *Cytophaga,* but had low similarity to nearly all endo- or exo- glucanases ('Classical GH9s'). Phylogenetic analysis showed that the group of GHs in which Cel9D resides is a unique branch of the GH9 family (Fig. S4). Multiple sequence alignment (Fig. 1) showed that the putative catalytic residues of Cel9D (D166 and D170) were separated by three other amino acids; while in all other characterized cellulases, they were separated by two. The extra residue (S168) seems to be conserved in the Cel9D group (Data not shown). E612 was identified as a putative catalytic base, however, the surrounding amino acid residues, which is conserved in classical GH9s, were not aligned in the Cel9D group.

Production and purification of recombinant Cel9D. *cel9D* gene was cloned and protein Cel9D was produced in *E. coli* BL21 (DE3). Most of the recombinant Cel9D enzyme was associated with the membrane fraction perhaps in inclusion bodies. However, a small amount present in the non-sedimentable fraction was purified by immobilized metal affinity chromatography followed by molecular sieve chromatography. About 1 mg of pure protein was obtained from 1 liter of culture. The recombinant protein had a mass of 76 kDa as determined by SDS-PAGE (Fig. S5), which is in good agreement with the theoretical mass of 77 kDa based on the primary sequence.

Characterization of Cel9D. As with most other glycoside hydrolases identified in *F. succinogenes* S85, Cel9D had a pH optimum of 6.5 and retained 80% activity between pH 6.0 and 8.0. The temperature for maximum activity was 37^{o}C. Detailed substrate specificity assays (Table 2) on various types of polysaccharides showed that Cel9D had activity on all polymers with a β-1,4-linkage, but not on laminarin which has a β-1,3 linkage. By measuring the release of p-nitrophenol, no activity was detected on *p*-nitrophenyl (pNP)-α-L-arabinofuranoside, pNP-α-D-glucoside, pNP-β-D-cellobioside, pNP-β-D-fucoside, pNP-β-D-galactoside, pNP-N-acetyl-β-D-glucosaminide, pNP-β-D-glucoside, pNP-β-D-glucuronoside,

pNP-β-D-lactoside, or pNP-β-D-maltoside. Cel9D had 27% lower activity on medium viscosity (Average M*w* ~250,000) CMC compared to low viscosity CMC (Average M*w* ~90,000) (Table 2). This property of Cel9D was quite different from the Cel9B enzyme, which had high activity on both medium and low viscosity CMC (24). The hydrolytic activity of Cel9D on Avicel cellulose, ASC and CMC was tested and compared to those of Cel8B, Cel9B, and Cel51A from *F. succinogenes* (Table 3). It had less activity on CMC compared to other enzymes.

Although Cel9D did not release p-nitrophenol from any of the arylglycosides tested, it did efficiently cleave pNPC to pNPG and glucose, as indicated by HPLC analysis (Fig. S6A). This documented that Cel9D acts on the non-reducing end. Cel9D did not degrade sophorose (β1-2), laminaribiose (β1-3) and gentiobiose (β1-6) as assayed by HPLC analysis.

Hydrolytic activities on either CMC or cellopentaose of purified Cel9D was not enhanced by 1mM $CaCl_2$ or 1mM $MgCl_2$. EDTA (10 mM) did not inhibit the activities.

Synergism of Cel9D with other cellulases from *F. succinogenes*. To identify possible synergistic interactions among different glycoside hydrolase families of cellulases synthesized by *F. succinogenes*, acid swollen cellulose and Avicel cellulose hydrolysis by different combinations of cellulases was assessed. Reducing sugars released by different combinations of enzymes are presented in Fig. 2 and Table 4. For all the binary mixtures, the greatest degree of synergism was obtained from the reaction containing 1.2 nmol of Cel9D and 1.2 nmol of Cel51 on Avicel (Fig. 2,f), in which the actual activity was four times of the sum of individual enzymes activities (Table 4). The highest degree of synergism on the ASC was from the combination of Cel9D and Cel8B, with a synergism value of 3.4 (Fig. 2,p). The combinations of the two family 9 enzymes, Cel9D and Cel9B, gave a moderate degree of synergism of 1.5, which indicates there are differences between the two cellulases within the same family. Cellulose degradation by mixtures of up to 4 cellulases was also investigated

(Table 4). The enzyme mixtures containing Cel9D generally yielded a higher degree of synergism as well as a higher overall level of reducing sugar production. By incubation of the enzyme mixture containing all four enzymes for 60 h, an amount of reducing sugar released corresponded to hydrolysis of 2.8% of initial Avicel substrate present.

Hydrolysis products of Cel9D. Glucose was the only product released from acid swollen cellulose by Cel9D as detected by HPLC analysis (Fig. S6B). This indicated that Cel9D has β-1,4-glucosidase activity. Cellohexaose hydrolysis initially yielded cellopentaose and glucose (Fig. 3). No cellotetraose was detected (t = 0), until a larger amount of cellopentaose accumulated (t = 0.5). Similarly, no cellotriose was detected (t = 0, 0.5, 1), until a larger amount of cellotetraose accumulated (t = 2) and no cellobiose was detected (t = 0, 0.5, 1, 2, 4), until there was a larger amount of cellotriose accumulated (t = 8). This clearly showed that Cel9D catalyzed an exo-type of hydrolysis reaction. Cellobiose was degraded very slowly such that less than 5% of the cellobiose accumulated 120 min was digested to glucose after 3000 min (Fig. 3). In contrast, for the same amount of cellooligosaccharides with three or more glucose residues, complete hydrolysis was achieved within 2 h. Detailed relationship between the kinetic parameters (K_m, k_{cat}, and k_{cat}/K_m) and the cello-oligosaccharide substrates of Cel9D are shown in Table 5. The K_m decreased with increasing chain length of substrate. The k_{cat} for cellotetraose, cellopentaose and cellohexaose were similar while the k_{cat} for cellotriose and cellobiose hydrolysis were substantially smaller. Catalytic efficiency factors k_{cat}/K_m increased steadily with increasing DP. The k_{cat}/K_m for cellobiose was 2300 fold lower than for cellohexaose. The observation that cellooligosaccharides with a longer DP were degraded faster than those with a shorter chain length documented a preference for longer cellooligosaccharides.

After mixing Cel9D with up to 100 mM glucose and/or cellobiose and incubating at 37°C for 24 h, no longer cello-oligosaccharides were produced, indicating it does not catalyze the reverse transglycosylation reaction.

Anomeric Configuration of Hydrolysis Products. Proton-NMR was used to investigate the anomeric configuration of oligosaccharides released during hydrolysis of p-nitrophenyl-cellobioside by Cel9D (Fig. S7). And the results indicate that Cel9D catalyzes the hydrolysis of the β-(1,4)-linkage with inversion of configuration.

Site-directed mutagenesis of Cel9D. To investigate whether D166 and E612 were the catalytic residues, the two amino acid residues were replaced with Alanine, respectively. The mutant proteins (D166A and E612A) were produced and purified by immobilized metal ion affinity chromatography (IMAC).. No activity was detected on either 1% (w/v) CMC after 4 h of incubation or 5 mM cellopentaose after 24 h of incubation determined by either reducing sugar analysis or by HPLC. Thus, D166 and E612 are good candidates for catalytic residues.

To investigate whether the serine residue at position 168 had a role in catalysis, two Cel9D mutants were made. ΔS168, which was constructed with the Ser168 removed had no activity on CMC or cellopentaose when assayed under the same conditions with Cel9D as the parallel positive control. Although when this serine residue is replaced by an alanine residue (S168A), the mutants retained about 40% percent of activity compared to the wild type.

DISCUSSION

Cel9D is a glucan 1,4-β-glucohydrolase. The present experiments showed that Cel9D hydrolyzed cellooligosaccharides from the non-reducing end releasing glucose units. There is similarity between β-glucosidase (EC 3.2.1.21) and glucan 1,4-β-glucohydrolase (EC 3.2.1.74) as both cleave the non-reducing terminal glycosyl residues from cello-oligosaccharide substrates. Cel9D hydrolyzed cello-oligosaccharides with a degree of

polymerization (DP) greater than two, or pNPC at much higher rates than cellobiose or pNPG, showing that the rate of hydrolysis increases with the DP of the cello-oligosaccharides. Cel9D released glucose from the non-reducing end of the substrate and its activity was restricted to β-1,4-linkages. Cel9D also had activity on soluble and insoluble β-glucans including CMC, ASC, and Avicel. This capability is distinct from β-glucosidases (EC 3.2.1.21), which have a decreased rate of hydrolysis with increasing DP (11, 25). Thus, Cel9D is classified as a glucan 1,4-β-glucohydrolase (34).

Since HPLC showed that the enzyme removed glucose residues from the non-reducing ends of cello-oligosaccharides, the subsites for binding substrate were designated as -1, +1, +2, +3, …, where "-1" subsite binds to the non-reducing terminal glucose and hydrolysis occurs between -1 and +1. Subsite affinities of Cel9D for glucose residues in cello-oligosaccharides were estimated (Table S1). Highest affinity was seen at the +2 subsite, which was 15.8 kJ/mol. Due to the difference in the subsite affinities between -1 (5.7 kJ/mol) and +2 (15.8 kJ/mol), cellobiose and pNPG would prefer to stay across the site +1 and +2, or +2 and +3 other than the "productive" sites -1 and +1, which explains the low rate of hydrolysis of cellobiose and pNPG.

Glucan 1,4-β-D-glucohydrolase is a type of exoglucanase that releases glucose from β-1→4 linked glucan. This type of enzymes has been identified in many organisms, including bacteria (15, 34), fungi (33) and plant (10, 22). Previously, all glucan 1,4-β-glucohydrolases and most β-glucosidases were classified as glycoside hydrolase family 1 or as family 3 ((9), http://www.cazy.org/fam/GH1.html and http://www.cazy.org/fam/GH3.html), which catalyze not only hydrolysis with retention of anomeric configuration but also catalyzed the reverse transglycosylation reaction (17). Our data showed that Cel9D could not produce long chain cello-oligosaccharide from glucose or cellobiose, indicating it does not catalyze the reverse transglycosylation reaction. Thus Cel9D is a unique inverting glucan 1,4-β-glucohydrolase.

Cel9D exhibits less than 20% identity to classical GH9s while it shows about 35% similarity to a series of putative endoglucanases mainly from the genus *Vibrio*. Among its many relatives, Vch_BglA (Vch0615) from *Vibrio cholerae* was previously characterized and was subsequently classified as a β-glucosidase (EC 3.2.1.21) (http://www.cazy.org/fam/GH9.html) that releases p-nitrophenol from pNPG and hydrolyzed cellobiose efficiently (23). Similar to Cel9D, Vch_BglA degraded cello-oligosaccharides and produces glucose as the major product. Like Cel9D, Vch_BglA did not cleave β-1,2-, β-1,3- and β-1,6- glucosidic linkages. Unfortunately, Park et al. (23) did not test the activity of the enzyme on cellulosic polymers. A key difference between the two enzymes is that Vch_BglA does not contain a signal peptide at the N-terminus, which may indicate that it is intracellular. Cel9D has a signal peptide, and presumably is exported across the cytoplasmic membrane. The differences in the localization suggested a difference in their physiological functions.

Why does Cel9D have a different mode of action compared with other family 9 glycoside hydrolases? Most members in the glycoside hydrolase family 9 are endoglucanases or exoglucanases that hydrolyze soluble or insoluble cellulosic substrate and produce cellobiose or cellotetraose as the major product (32, 36) which are further degraded by β-glucosidase or cellobiase. Although Cel9D belongs to GH family 9, the major hydrolytic product was glucose. It also has very distinct sequence features compared to other family 9 cellulases (classical GH9 enzymes) (Fig. 1) and phylogenetic analysis (Fig. S4). Most remarkably, the two catalytic residues (D166 and D170 in Cel9D), which are thought to deprotonate the water molecule that carries out the nucleophilic attack on the C1 carbon of the substrate (28) were separated by 3 amino acid residues instead of 2. The extra amino acid residue, S168, which is conserved in the Cel9D group, is not in the classical family 9 enzymes (Fig. 1). Removal of Ser168 by site-directed mutagenesis indeed completely abolished the enzyme activity, while S168A retained about 40% of activity. These data

indicated that Ser168 is a structurally, but not catalytically important residue. Secondly, the catalytic residue, a glutamic acid (13) and surrounding amino acid residues, which are conserved in all the other 'classical' family 9 cellulases (Glu418 in Cth_CelD and Glu424 in Tfu_Cel9A), were not easy to be identified based on computer generated sequence alignment. E612 seems to be conserved within the Cel9D group and may be the catalytic residue comparing with the classical ones. Substitution of E612 of Cel9D with an alanine residue caused the enzyme to lose activity on both CMC and cellopentaose, and therefore E612 probably is a catalytic residue. A third difference of Cel9D from other GH9 enzymes is at position 559 to 562, where the classical enzymes have four conserved residues PHHR and in Cel9D, these residues were absent (Fig. 1). The four residues are situated in the cleavage groove of family 9 enzymes (13), and a substitution of the first histidine residue caused a 75% loss in activity of CelD from *Clostridium thermocellum* (31). The combination of these differences may result in a conversion of a classical endoglucanase or cellobiohydrolase to an exo-β-glucosidase like Cel9D, although further studies are needed to explore this possibility.

Many studies have reported that divalent metal ions, such as calcium and magnesium, enhanced the activity of GH9 cellulases (2, 24). However, this was not observed for Cel9D. This was corroborated by the fact that the calcium binding residues, which were identified by structural analyses of CelD (3), CbhA (28) from *C. thermocellum*, and E4 from *T. fusca* (26), were not identified in the Cel9D (Fig. 1).

Despite the observation that Cel9D has many differences in the arrangement of catalytically important residues (D166, D170 and E612) and different hydrolytic products, the proton NMR results (Fig. S7) clearly showed that Cel9D catalyzes hydrolysis of the β-1,4 linkage by an inverting mechanism, which is the same as other GH9 members (5).

Family 9 cellulases are classified into four different themes based on their domain organization (6). Theme A enzymes have only a family 9 catalytic domain. In addition to the

catalytic domain, theme B enzymes have a family 3 CBM at the C-terminus. Theme C and D members have immunoglobulin like domains at their N-termini and theme D enzymes have an additional family 4 CBM. Enzymes in themes B and D often have high activity towards crystalline cellulose and enzymes in themes A and C are usually potent endoglucanases.

A phylogenic tree based on the amino acid sequences from selected family 9 cellulases was constructed (Fig. S4). Theme A and theme B members were well separated in the phylogenic tree, indicating in addition to the conserved domain arrangement, amino acid sequences were conserved within the themes. Theme C and D enzymes were well separated from Theme A and B enzymes, but the two themes themselves could not be fully separated phylogenetically. Theme D is more like a subgroup of Theme C. Theme C and D enzymes are closer to members from theme A than from theme B. The Cel9D and Vch_BglA reside in a distinct branch separated from all other family 9 themes in the tree (Fig. S5).

During the course of evolution, a common ancestor of the members from the Cel9D branch and those in themes C and D gained the n-terminal Ig-like domain, and after that, it began to diverge. One branch contained the classical family 9 glycoside hydrolases, which are mainly endoglucanases and cellobiohydrolases, including themes C and D. The other branch includes the Cel9D in the present study as well as the Vch_BglA, which are exo-β-glucosidases. Interestingly, although Cel9D and Vch_BglA have similar modes of action, Cel9D has an N-terminal signal peptide while Vch_BglA does not, indicating their different localization and physiological roles.

Mode of synergistic interaction of Cel9D. The Cel51A of *F. succinogenes*, formerly CelF (19), contains three cellulose binding modules. In the present study, the Cel51A and Cel9D were more synergistic than other pairs of proteins (Fig 2). In the multiple enzyme mixtures, those containing both Cel51A and Cel9D gave the highest degree of cellulose degradation (Table 4). Thus, besides having the major role in cellulose binding, Cel51A may

also be a central role enzyme for cellulose hydrolysis. Previous studies showed that the major hydrolytic products of Cel51A were cellotetraose and cellotriose, which are the preferred substrates for Cel9D. Cel8A degraded acid swollen cellulose and the major product were cellodextrins with a DP ranging from 2 to 5 (24). So the synergistic effect between Cel8B and Cel9D is also expected. Cel9D has a much lower activity on cellobiose, which explains why Cel9D had less synergistic effect with Cel9B, since the latter enzyme was shown to degrade cellulose mainly to cellobiose and glucose (20). Since Cel9D cannot hydrolyze cellobiose, it can be presumed that β-glucosidases (EC3.2.1.21) would exert a synergistic effect with Cel9D. Indeed, cellobiase activity has been detected in *F. succinogenes* (7) and two GH3 β-glucosidases were identified in the genome of *F. succinogenes* (21).

A putative protein that has 37% sequence identity to Cel9D was identified from the genome of a phylogenetically related bacterium *Cytophaga hutchinsonii* (35). Cellulase systems of *F. succinogenes* and *C. hutchinsonii* are quite similar and distinct from other cellulolytic bacteria in that both bacteria lack genes encoding proteins with similarity to members from GH6 or GH48; most cellulases in both bacteria are cell associated but lack a carbohydrate binding module and no cellulosome structures were identified (21, 35). The Cel9D enzyme may be related to their unique but yet to be elucidated cellulose degradation system.

In conclusion, Cel9D is a novel family 9 glycoside hydrolase that has an important role in crystalline cellulose degradation by releasing of glucose from accessible cello-oligosaccharides during cellulose degradation by other cellulases. Further structural studies of Cel9D will provide greater insight into the function of this unique GH9 enzyme.

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FIGURE LEGENDS

FIG. 1. Alignment of Cel9D and Vch_0615 (AAF93781) with six classical family 9 glycoside hydrolases for which structures have been determined. The alignment was carried out using the ClustalX program and edited by eye. Putative catalytic sites of Cel9D are indicated by filled arrow heads. Regions of Cel9D that showed significant differences to classical GH9s are framed by dotted lines. Amino acid residues involved in Ca^{2+} binding in Tfu_Cel9A, Cth_CbhA, and Cth_CelD were boxed with solid lines. Tfu_Cel9A, *T. fusca* Cel9A, pdb:3TF4 (Theme B); Ccl_Cel9G, *C. cellulolyticum* Cel9G, pdb:1G87 (Theme B); Nta_Cel, termite, *Nasutitermes takasagoensis* endoglucanase, pdb:1KSD (Theme A); Ccl_Cel9M, *C. cellulolyticum* Cel9M, pdb:1IA7 (Theme A) ; Cth_CbhA, *C. thermocellum* CbhA, pdb:1RQ5 (Theme D); Cth_CelD, *C. thermocellum* CelD, pdb:1CLC (Theme C).

FIG. 2. Hydrolytic activity (line & points, right y-axis) and degree of synergism (grey bar, left y-axis) of various binary combinations of Cel9B, Cel51A, Cel8B, Cel9D and Cel45C at different molar ratios for the hydrolysis of either Avicel (a~j) or amorphous cellulose (k~t). The two enzymes used are indicated in each subfigure and proportions of enzymes in each reaction are shown at the bottom. Note: 100 stands for 0.98 nmol for Cel9B, 1.01 nmol for Cel51A, 1.18 nmol for Cel8B and 0.69 nmol for Cel9D per ml of reaction, respectively. [a] Degree of synergism is defined as observed cellulase activity by the combination of cellulases divided by the additive activities of the individual cellulases acting alone.

FIG. 3. Time course of hydrolysis of cellohexaose by Cel9D monitored by HPLC. Cellohexaose and their hydrolysis products were analyzed by high-performance anion-exchange chromatography coupled with a Waters model 464 pulsed amperimetric detector using Waters 625 LC system with a base resistant reference electrode.

TABLES AND FIGURES

TABLE 1. Oligonucleotide primers used in this study

Primers	Sequences[a]	Location	Note
Cel9D_1	5'-GTCCTCCATATGCGTATCTATAAGCTTTC-3'	1-20	Cloning, *Nde* I
Cel9D_2C	5'-ATATGCCTCGAGCTTCACAACAAACTTCTT-3'	2143-2160	Cloning, *Xho* I
D166A_1	5'-CGGTTGGTATG**C**TGCGAGCGGCG-3'	487-509	Mutagenesis
D166A_2C	5'-CGCCGCTCGCA**G**CATACCAACCG-3'	487-509 reverse	Mutagenesis
S168A_1	5'-GGTTGGTATGATGCG**GC**CGGCGATGTCAGTAAG-3'	487-519	Mutagenesis
S168A_2C	5'-CTTACTGACATCGCCG**GC**CGCATCATACCAACC-3'	487-519 reverse	Mutagenesis
ΔS168_1	5'-GGTTGGTATGATGCG~GGCGATGTCAGTAAG-3'	487-519	Mutagenesis
ΔS168_2C	5'-CTTACTGACATCGCC~CGCATCATACCAACC-3'	487-519 reverse	Mutagenesis
E612A_1	5'-GCGCTGGGATG**C**ACAGTGGCTGC-3'	1824-1846	Mutagenesis
E612A_2C	5'-GCAGCCACTGT**G**CATCCCAGCGC-3'	1824-1846 reverse	Mutagenesis

[a] Underline, restriction sites for cloning. Bold and tilde denote the substituted nucleotides for amino acid replacement and deletion, respectively.

TABLE 2 Substrate specificity of Cel9D

	Activity [a] (µmol product /min/µmol protein)	% [b]
CMC (Medium Viscosity)	0.275±0.050	100.0%
Acid swollen cellulose	0.250±0.063	90.4%
Barley β-glucan	0.102±0.031	37.2%
Lichenin	0.095±0.040	34.6%
CMC (Low Viscosity)	0.075±0.028	27.1%
Oat spelt xylan	0.075±0.035	27.1%
Sigmacel 100	0.056±0.022	20.2%
Avicel PH105	0.038±0.014	13.8%
Mannan	0.019±0.006	6.9%
Hydroxyethyl cellulose	0.019±0.009	6.9%
Laminarin	<0.001	0
Acyl glycosides[c]	<0.001	0
Disaccharides[d]	0	0

[a] Glycoside hydrolase activities were determined by reducing sugar (Glucose equivalents) produced from polysaccharides.

[b] Relative activity

[c] Includes: *p*-nitrophenyl (pNP)-α-L-arabinofuranoside, pNP-α-D-glucoside, pNP-β-D-cellobioside, pNP-β-D-fucoside, pNP-β-D-galactoside, pNP-N-acetyl-β-D-glucosaminide, pNP-β-D-glucoside, pNP-β-D-glucuronoside, pNP-β-D-lactoside, pNP-β-D-maltoside at 5 mM in 0.05 M sodium phosphate buffer pH 6.5 for 2 h at 37°C. Released p-Nitrophenol measured as described by Kam et al. (11)

[d] Includes: sophorose (β1-2), laminaribiose (β1-3) and gentiobiose (β1-6), products detected by HPLC.

1

TABLE 3. Catalytic properties of the cellulases from *F. succinogenes* S85

Protein	MW(kDa) /pI	Family	Specific activity (μmol Glc/min/μmol protein)		
			CMC	Amorphous cellulose	Avicel
Cel9D	79.4/5.38	9	0.275±0.050	0.250±0.063	0.038±0.003
Cel8B	81.4/5.55	8	6.38±1.25	1.56±0.29	0.056±0.014
Cel9B	67.3/6.06	9	1680±151	3.94±0.18	0.069±0.014
Cel45C	37.7/4.90	45	4.84±1.89	0.027±0.015	0.009±0.003
Cel51A	119/7.81	51	1300±35.9	2.93±0.12	0.067±0.016

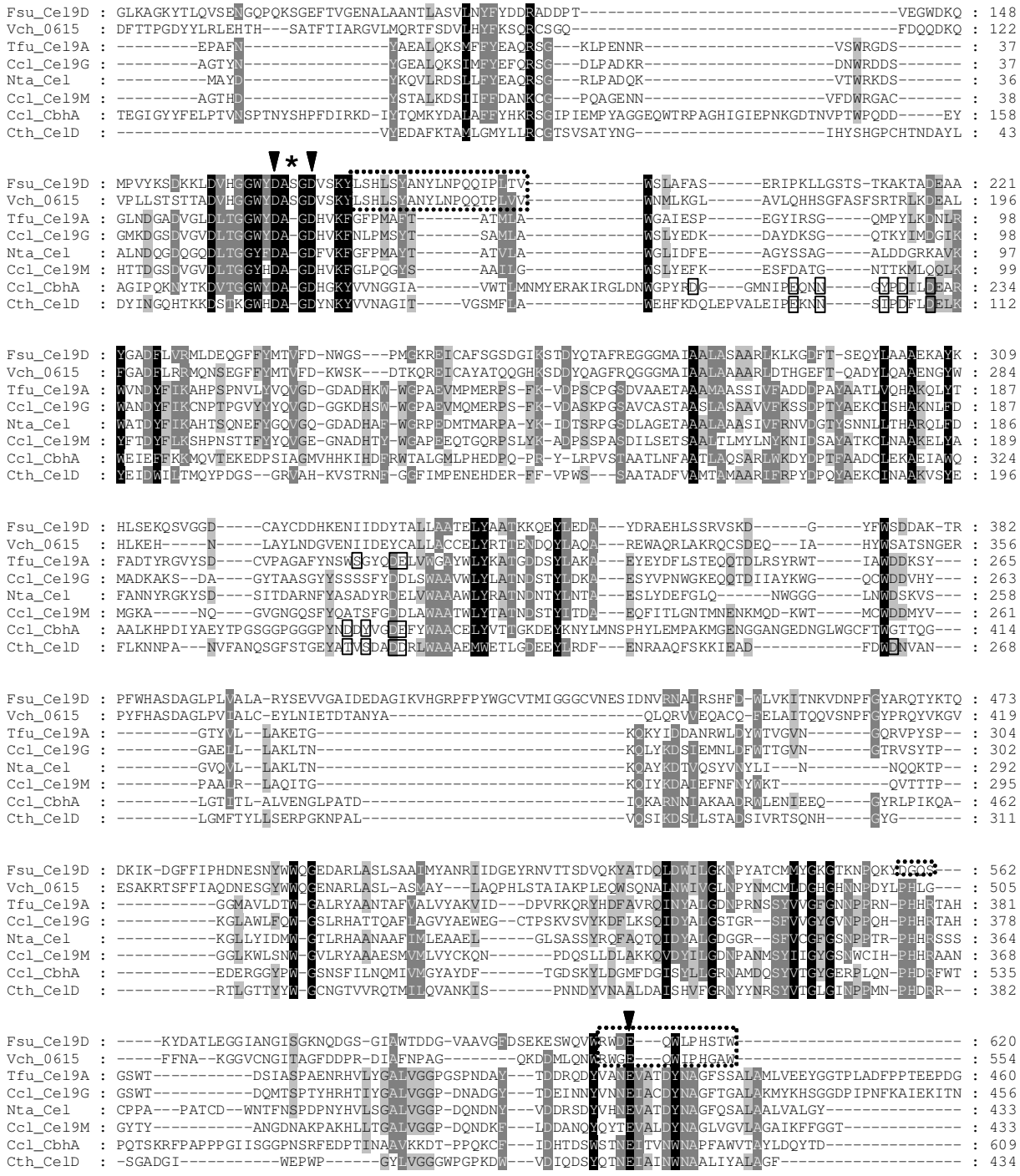
2

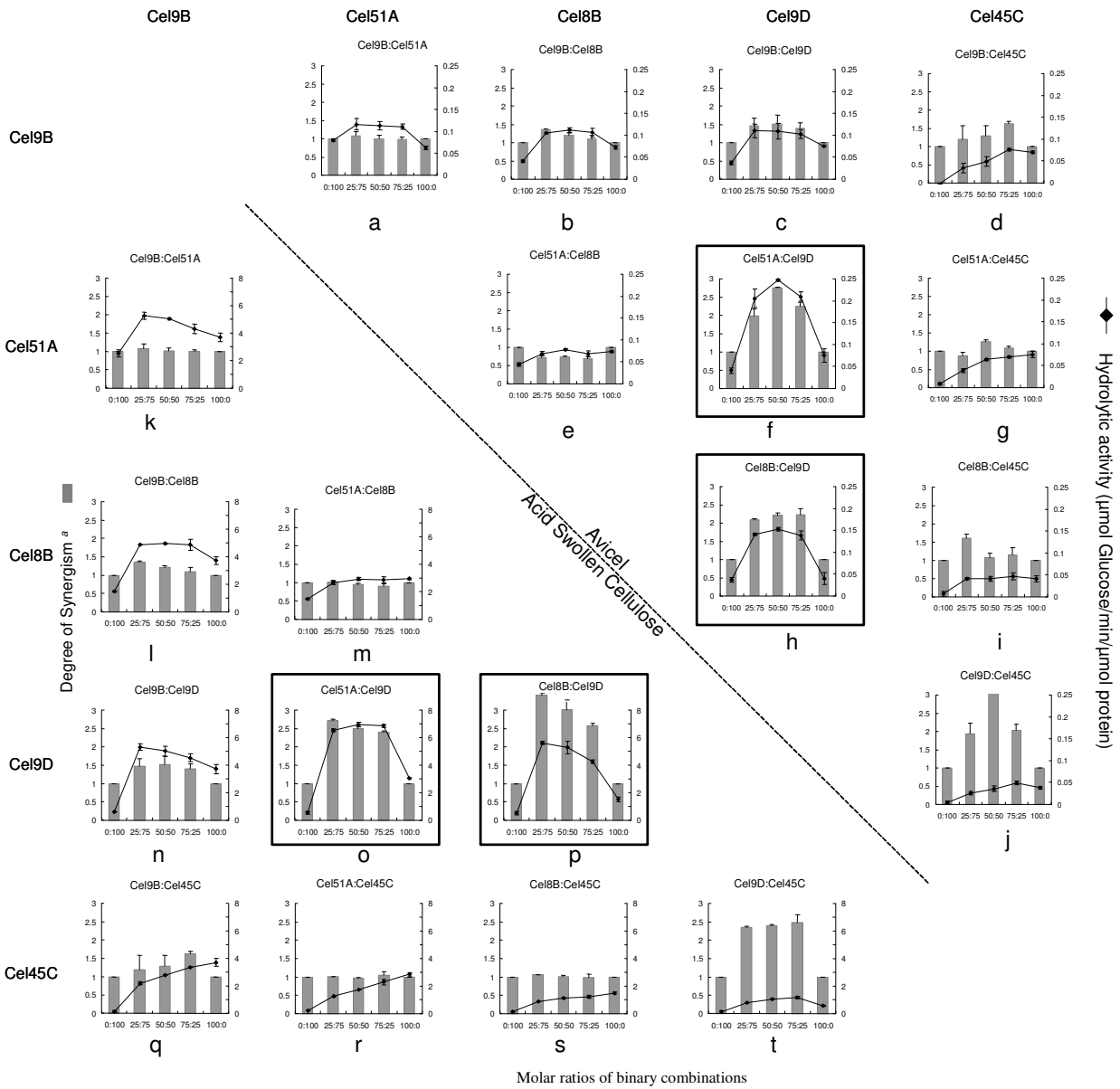
TABLE 4. Synergistic effect of various combination of Cel9D with Cel9B, Cel51A and Cel8B on the hydrolysis of Avicel cellulose. Reaction mixtures (100 µL) were incubated at 37 ^{o}C for 16 h.

Cellulase mixture	Mol. Concn (nmol/ml) of each enzymes	Reducing sugar produced (µg)		Synergy degree	% Conversion
		Observed	Theoretical		
Cel9D	1.2	0.35±0.04	-	1	0.07
Cel9B	1.8	2.18±0.36	-	1	0.44
Cel51A	1.2	1.24±0.18	-	1	0.25
Cel8B	1.2	1.26±0.37	-	1	0.25
Cel9D + Cel9B	1.2 + 1.8	3.00±0.25	2.53	1.19	0.60
Cel9D+ Cel51A	1.2 + 1.2	6.36±1.24	1.59	**4.01**	**1.27**
Cel9D + Cel8B	1.2 + 1.2	3.71±0.24	1.60	2.31	0.74
Cel9B + Cel51A	1.8 + 1.2	3.61±0.57	3.43	1.06	0.72
Cel9B+ Cel8B	1.8 + 1.2	3.27±0.18	3.44	0.95	0.65
Cel51A + Cel8B	1.2 + 1.2	1.93±0.57	2.50	0.77	0.39
Cel9D+ Cel9B + Cel51A	1.2 + 1.8 + 1.2	6.30±0.39	3.77	**1.67**	**1.26**
Cel9D + Cel9B + Cel8B	1.2 + 1.8 + 1.2	5.34±0.23	3.79	**1.41**	**1.07**
Cel9D+ Cel51A + Cel8B	1.2 +1.2 + 1.2	6.62±0.31	2.84	**2.33**	**1.32**
Cel51A + Cel8B + Cel9B	1.2 + 1.2 + 1.8	4.27±0.13	4.68	0.91	0.85
Cel9D+ Cel9B + Cel51A + Cel8B	1.2 + 1.8 + 1.2 +1.2	7.20±1.28	5.03	**1.43**	**1.44**

TABLE 5. Kinetic parameters (Mean±SD) K_m, k_{cat}, and k_{cat}/K_m of Cel9D during the hydrolysis of cello-oligosaccharides of DP 2-6.

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($10^{-3}s^{-1}M^{-1}$)
Cellobiose	28.0±6.3	0.25±0.03	0.0097±0.003
Cellotriose	1.52±0.27	6.21±0.45	4.27±1.06
Cellotetraose	1.21±0.11	18.6±0.66	15.5±1.97
Cellopentaose	0.90±0.10	16.4±0.65	18.5±2.74
Cellohexaose	0.73±0.09	16.3±0.69	22.8±3.76




Cel9B
Cel51A
Cel8B
Cel9D
Cel45C
Cel9B:Cel51A
Cel9B:Cel8B
Cel9B:Cel9D
Cel9B:Cel45C
Cel51A:Cel8B
Cel51A:Cel9D
Cel51A:Cel45C
Cel8B:Cel9D
Cel8B:Cel45C
Cel9D:Cel45C
Cel9B:Cel51A
Cel9B:Cel8B
Cel51A:Cel8B
Cel9B:Cel9D
Cel51A:Cel9D
Cel8B:Cel9D
Cel9B:Cel45C
Cel51A:Cel45C
Cel8B:Cel45C
Cel9D:Cel45C
0:100 25:75 50:50 75:25 100:0
a
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s
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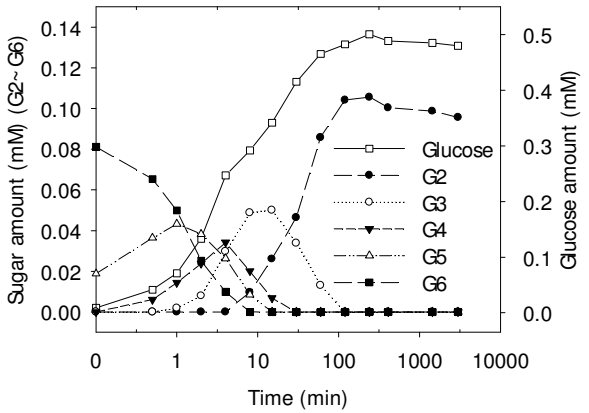
Avicel
Acid Swollen Cellulose
Degree of Synergism a
Hydrolytic activity (µmol Glucose/min/µmol protein)
Molar ratios of binary combinations


Sugar amount (mM) (G2~G6)
Glucose amount (mM)
Time (min)
Glucose
G2
G3
G4
G5
G6
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.0
0.1
0.2
0.3
0.4
0.5
0
1
10
100
1000
10000