BEHAVIOR AND SUPPORTIVE EVIDENCE OF A LARGE XYLANASE-CONTAINING MULTIENZYME COMPLEX OF *TEPIDIMICROBIUM XYLANILYTICUM* BT14

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Cellular behaviors and a xylanolytic-cellulolytic enzyme system of *Tepidimicrobium xylanilyticum* BT14 towards xylan-rich plant biomass degradation were characterized. During the exponential growth phase, the bacterial cells were bound tightly to the growth substrate where the degradation zones appeared mostly around the cells, indicating that the xylanolytic-cellulolytic enzyme system was linked to the cell surfaces. Interestingly, several cells appeared to secrete extracellular matrix to connect to their neighbors, and the matrix disappeared when cells passed to the stationary growth phase. Cationized-ferritin staining resulted in a dense assembly of bulbs, protuberance-like structures on the growing bacterial cell surfaces. The cell-associated proteins derived by sonication contained predominated xylanase with relatively low carboxymethyl-cellulase (CMCase) activities, suggesting that the xylanolytic-cellulolytic enzyme system occurred as a cell-associated enzyme. By means of gel-filtration chromatography, a high molecular mass protein with the estimated size of 2000 kDa was retrieved from the cell-associated enzymes, and it appeared as a single protein band on non-denaturing gel. However, more bands were obtained after the protein was boiled with sodium dodecyl sulfate and β-mercaptoethanol – which contained 4 xylanases and one CMCase – suggesting that these proteins were organized as a multienzyme complex (MEC) in natural form. Additionally, the predominated xylanolytic MEC preferred binding to cellulose rather than xylan.

Keywords: Cellulase; Multienzyme complex; Plant biomass; Polysaccharide binding; Tepidimicrobium xylanilyticum; Xylanase

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INTRODUCTION

Plant biomass, consisting of lignocellulosic materials, has been extensively evaluated as a potential raw material for bulk chemical production, such as ethanol, since it represents a rich source of fermentable sugars, is renewable, and is nonfood (Zhang 2008). The compositions of plant biomass (cell walls) can be roughly classified into two main types: polysaccharides and lignin, in which polysaccharides are target polymers for bioenergy and biorefinery processes (Ragauskas *et al.* 2006). The principle components of cell wall polysaccharides are cellulose and hemicelluloses. Cellulose is a polymer of

glucoses linked by β-1,4-glycosidic linkages, whereas hemicelluloses are heterogeneous polymers consisting of xylose, arabinose, mannose, galactose, and such acid sugars as glucuronic and uronic acids (Collins *et al.* 2005). For most terrestrial plants, a major component of hemicelluloses is xylan. The backbone of xylan is a β-1,4-linked xylose polymer, where some xylose units are decorated with such substituents as arabinose, glucuronic acid, as well as acetyl groups. In nature, hemicelluloses form complex-association with cellulose and lignin, thus resulting in limited accessibility and digestibility for glucan (Himmel *et al.* 2007).

Natural decomposition of plant biomass is mostly driven by microbial processes (Wilson 2008). Microorganisms are known to synthesize sets of enzymes to degrade plant cell wall materials, and their enzymatic strategies for effective degradation have been described, based entirely on hydrolysis of cellulose (Wilson 2008). For aerobic microorganisms, especially fungi and bacteria, they are believed to secrete individual cellulases – most possess carbohydrate-binding modules (CBMs) – to hydrolyze cellulose outside cell walls, as cellulose is a large molecular weight and insoluble material which cannot penetrate cell membranes. The resulting soluble sugars from cellulose hydrolysis are then transported across the cell membranes and further metabolized inside the cells. For anaerobic microorganisms, a cellulosomal mechanism has been employed to hydrolyze cellulose by several anaerobic bacteria, especially those from genus *Clostridium* (Bayer *et al.* 2004; Desvaux 2005; Doi and Kosugi 2004; Kakiuchi *et al.* 1998). Dockerin-bearing enzymes are integrated into a core protein, called scaffoldin, *via* dockerin-cohesin interaction to form a MEC, termed cellulosome. A few enzymes in cellulosomes contain CBMs; however, the scaffoldin contains CBM, which bring entire cellulosomal components organized on the scaffold to bind cellulose (Bayer *et al.* 2004). The advantages of cellulosome organization over free enzyme systems rely on enzyme proximity and targeting of enzymes to appropriate sites on the substrates, thereby leading to enhancement in synergy. Additionally, cellulosomes are located at outer surfaces of bacterial cells, thus mediating cells close to their nutrients (Schwarz 2001).

Since xylans often co-exist with cellulose in biomass, xylan hydrolysis is of interest, since the monomeric sugar components – most are pentose sugars, such as xylose and arabinose – can be also metabolized by natural or engineered pentose-utilizing microorganisms to yield valuable compounds (Deutschmann and Dekker 2012; Himmel *et al.* 2007). The current knowledge on bioconversion of xylan relies entirely on free-state enzymes produced by aerobic microorganisms. For example, complete hydrolysis of xylan requires at least two main types of enzymes: those that cleave the main backbones (*i.e.* β-1,4-endoxylanases [EC 3.2.1.8] and β-xylosidases [3.2.1.37]) and those that remove side-chain substituents (*i.e.* α-L-arabinofuranosidases [EC 3.2.1.55], α-L-glucuronidases [EC 3.2.1.1], and acetyl esterases [EC 3.1.1.6]) (Shallom and Shoham 2003). Debranching enzymes will remove substituents along pentosan (xylose) backbones; meanwhile β-1,4-endoxylanases and β-xylosidases will cleave β-linkages in the xylan backbone to release xylose and xylooligosaccharides (Biely *et al.* 1997). Several xylanases carry CBMs to facilitate polysaccharides binding; some single-domain xylanases have been reported to contain specific-xylan binding sites (or secondary binding sites) on their surface regions (Cuyvers *et al.* 2012).

There are a few reports demonstrating the presence of multiple xylanases as a protein aggregate/MEC—referred as xylanosomes—in some microorganisms, including *Butyrivibrio fibrisolvens* (Lin and Thomson 1991), *Streptomyces olivaceoviridis* (Jiang

et al. 2004), *Bacillus licheniformis* (van Dyk *et al.* 2010a), *Bacillus subtilis* (Jones *et al.* 2012), *Paenibacillus curdlanolyticus* (Pason *et al.* 2006). *Thermoanaerobacterium saccharolyticum* (Zeikus *et al.* 1991), *Thermoanaerobacterium thermosaccharolyticum* (Chimtong *et al.* 2011), and *Chaetomium* spp. (Ohtsuki *et al.* 2005). Integration of xylanases into a complex results in enhanced deconstruction of xylan present in complex lignocellulosic materials rather than free, unstructured xylanases (McClendon *et al.* 2012; Moraïs *et al.* 2011). While cellulosome is best-known as a discrete, cellulose-degrading machine, xylanosome is considered to be an analogue of cellulosomes since its vital role is suggested for xylan hydrolysis, owing to its predominated activity towards xylan rather than cellulose (Gonzalez-Vogel *et al.* 2011; Jiang *et al.* 2005; Sunna and Antranikian 1997). However, the complex formation of such xylanases is still unanswered since there have been no reports revealing the presence of scaffoldin subunits in those complexes. Additionally, besides *Thermoanaerobacterium* species, there has been no evidence to show that the xylanosome complexes are associated with cell walls (Moraïs *et al.* 2011).

Xylanases offer a broad range of applications, including the biobleaching of kraft pulp to prepare it for papermaking applications, the manufacture of food and animal feed, and production of xylooligosaccharides (Subramaniyan and Prema 2002). Importantly, xylanases can be deployed in combination with cellulases to achieve effective bioconversion of lignocellulosic materials to generate simple sugars for bioenergy (McClendon *et al.* 2012; Moraïs *et al.* 2011), which might improve overall economics of production processes (Zhang 2011). Previously, we isolated a thermophilic, anaerobic, predominantly xylanase-producing bacterium *Tepidimicrobium xylanilyticum* BT14, which grows vigorously on xylan-rich materials. The question that needs to be addressed is how does the bacterium thrive and overcome recalcitrance of natural plant biomass? In this work, we studied behaviors of this certain microorganism towards plant biomass degradation and partially characterized its xylanolytic-cellulolytic enzyme system, which appears as a large, cell-associated MEC.

EXPERIMENTAL

Chemicals

Carboxymethyl-cellulose (CMC) (medium viscosity, 400-800 cP), cellulose powder (Sigmacell® Cellulose, Type 20; particle size = 20 μm), and xylans from birch wood and oat spelts were purchased from Sigma Chemical Company (St. Louise, MO, USA). Crystalline cellulose, Avicel (Avicel® PH-101) was purchased from Fluka (Ireland). All other reagents were analytical grade.

Bacterial Strain and Culture

T. xylanilyticum BT14, isolated from a soil sample in Bangkuntien, Bangkok, Thailand, was deposited at the Mircen Culture Collection, Thailand Institute of Scientific and Technological Research (TISTR), Bangkok Thailand and Korean Collection of Type Cultures (KCTC) under accession numbers TISTR1899 and KCTC5875, respectively. Strain BT14 was cultured as previously described by Phitsuwan *et al.* (2010) with a modified composition of mineral salts (250 g of $MgCl_2 \cdot 6H_2O$, 37.5 g of $CaCl_2 \cdot 2H_2O$, and 0.3 g of $FeSO_4 \cdot 6H_2O$; per liter).

Observation of Cellular Growth on Corn Husk

Cells were cultured on several pieces of 2×3 cm corn husk strips, about 0.5 g dry basis in 100 mL of basal medium under growth conditions (Phitsuwan *et al.* 2010). Samples were taken at appropriate times for scanning electron micrograph (SEM) observation. The samples for SEM analysis were prepared according to the methods of Bozzola and Russell (1999), with minor modification.

In order to observe protuberance structures on cell surfaces, cationic ferritin was used as tracking aids. The samples were prepared as described by Lamed *et al.* (1987a).

Protein Preparation

One liter of corn husk-grown culture of *T. xylanilyticum* BT14 was harvested at the exponential growth phase by centrifugation at 10000 rpm, 4 °C for 10 min, yielding two fractions: the culture supernatant and the corn husk pellets (cells and proteins). The culture supernatant fraction was filtered using a 75 mm filter unit (Nalgene) and subsequently concentrated by ultrafiltration using a 30 kDa-cutoff membrane (Pellicon, Millipore). The protein preparation of this fraction was considered as the unbound protein fraction. The corn husk pellets were washed several times with 50 mM carbonate buffer (CBB) by centrifugation until there were no detectable proteins in the washing solutions. Then, a small volume of CBB was added to the pellets and resuspended prior to sonication. The suspension of corn husk pellets was sonicated on ice with 30% amplitude for 10 min (10 sec breaks for every 1 min) using the Sonics-Vibra cell sonicator (Switzerland). The sonicated sample (fluid) was collected by centrifugation at 10000 rpm, 4 °C for 10 min. The protein preparation was considered as the bound protein fraction. All protein preparations were kept at -20 °C until use. Corn husk alone was used as a control for protein preparations.

Protein concentration was determined by the method of Bradford (Bradford 1976), using the Bio-Rad protein assay kit (Bio-Rad).

Enzyme Assays

The enzyme assays were measured with a modified method of Moraïs *et al.* (2011). In brief, xylanase and CMCase activities were determined by the release of reducing sugars from the xylan and CMC substrates using the dinitrosalicylic (DNS) method. One hundred and twenty-five microliters of protein samples was mixed with 125 µl of 1% (w/v) xylan or CMC suspended in 50 mM citrate buffer, pH 6.5 and incubated at 60˚C for 30 min, or indicated otherwise. One unit of enzyme activity was defined as the amount of enzyme that produced 1 µmol of reducing sugars in 1 min under assayed conditions.

Chromatography

Gel-filtration chromatography was performed at room temperature using a HiLoad 16/60 Superdex 200 prep grade column (GE Healthcare, Piscataway, NJ) equipped with a fast protein liquid chromatography system (Akta Explorer; Amersham Biosciences, Sweden). The gel-filtration column was equilibrated with 25 mM Tris/HCl containing 175 mM NaCl and 2.7 mM KCl. The flow rate was 1 mL/min and the fraction volume of 1.7 mL was collected. Protein measurement by OD_{280} was used to monitor protein in column effluent. Fractions of interest were pooled and desalted by ultrafiltration using Amicon Ultra-4 3K (Millipore).

Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 10% polyacrylamide gel. Cellulase and xylanase zymograms were studied by using 0.1% (w/v) CMC or soluble birchwood xylan copolymerized with 10% polyacrylamide together with 0.1% (w/v) SDS for denaturing conditions (Phitsuwan *et al.* 2010). After electrophoresis, gels were washed two times with 2% (v/v) Triton X-100 for 20 min and two times with citrate buffer (50 mM, pH 6.5). Subsequently, gels were incubated with the same buffer at 60˚C for 10 min and were stained for residual polysaccharides with 0.5% (w/v) Congo red solution for 30 min at room temperature with mild shaking and destained with 1.0 M NaCl. The gels were fixed with 5% (v/v) acetic acid after clear bands occurred if needed.

Polysaccharide-Binding Assay

A polysaccharide-binding assay was performed using a modified method of Morais *et al.* (2011). Briefly, 200 μg of protein samples were mixed with 20 mg of Avicel or insoluble oat spelt xylan (in 10 mM CBB, pH 9.0); the mixture was brought to a final volume of 200 μL in a microcentrifuge tube. The sample was stirred gently at 4 °C for 1 h, and the tube was centrifuged at 4 °C, 13000 rpm for 2 min. The supernatant was retrieved and was considered as an unbound protein fraction, whereas the pellet containing cellulose/xylan-bound protein was washed three times by resuspension in 200 μL of the same buffer and centrifugation at 13000 rpm for 1 min. The washed pellet was resuspended in 60 μL of SDS-sample buffer and boiled for 10 min in order to desorb bound proteins. The unbound fraction was precipitated by 4-volume of acetone at -20 °C for 1 h, resuspened in 60 μL of SDS-sample buffer and boiled for 10 min. The unbound and bound protein fractions were analyzed by SDS-PAGE using 10% gels.

Quantitative analysis of polysaccharide binding (% binding) was calculated as described by Pason *et al.* (2006).

RESULTS AND DISCUSSION

In our previous work we isolated predominantly the xylanolytic bacterium *T. xylanilyticum* BT14, which demonstrated robust growth on natural plant materials (Phitsuwan *et al.* 2010). Besides the presence of carbohydrate active enzymes pertinent with the growth substrate, the bacterium exhibited binding ability *in vitro* to insoluble polysaccharides including xylan, microcrystalline cellulose (Avicel), and corn husk, a representative of complex polysaccharides. This raised the question whether adherence of cells to growth substrates might be essentially involved in nutrient utilization (degradation) for this certain microorganism. In order to prove our hypothesis, cellular behaviors of strain BT14 during growth on corn husk were investigated *in situ* using SEM imaging. The corn husk pellets were taken from the culture medium of strain BT14 according to time and washed several times before visualization (Fig. 1). After a 1-day incubation (lag phase), very few bacterial cells were observed on corn husks, and the corn husks remained intact (Fig. 1A). On day 3 (early-exponential growth phase), an increasing number of cells were present and greatly distributed over parenchyma tissues between the veins of the corn husks. The corn husk degradation could be seen where the degradation zone mainly appeared around the individual cells, suggesting that xylanolytic-cellulolytic enzyme activities might be associated with the bacterial cell

surfaces (Fig. 1B). On day 5 (exponential growth phase), the deconstruction of the corn husk structure appeared as a great number of cells loaded on the surfaces of corn husk (Fig. 1C). Most parts of corn husk were severely degraded on day 9 (stationary growth phase) (Fig. 1D).

Additionally, a more complicated event appeared during microbial degradation. As observed on day 5, each cell seemed to produce an exo-cellular matrix that connected it to its neighbors, likely forming a biofilm that covered degrading zones (Fig. 1C). On day 9, the matrix seemed to be destroyed; meanwhile, some of the bacteria underwent a stationary growth phase, as evident by the longer, thinner morphology with the presence of endospores (Fig. 1D). Some cellulolytic microorganisms, such as *Clostridium cellulolyticum* and *Cellulomonas flavigena*, are known to produce biofilm (*i.e.* exopolysaccharides) in order to collect nutrients (Desvaux 2005; Kenyon *et al.* 2005). The films might be formed as a net that prevent the leak of hydrolysis products to environments, thus reducing in food competition with other microorganisms. Later, the films disappeared by hydrolytic actions when cells were going through stationary phase of growth (Desvaux 2005).

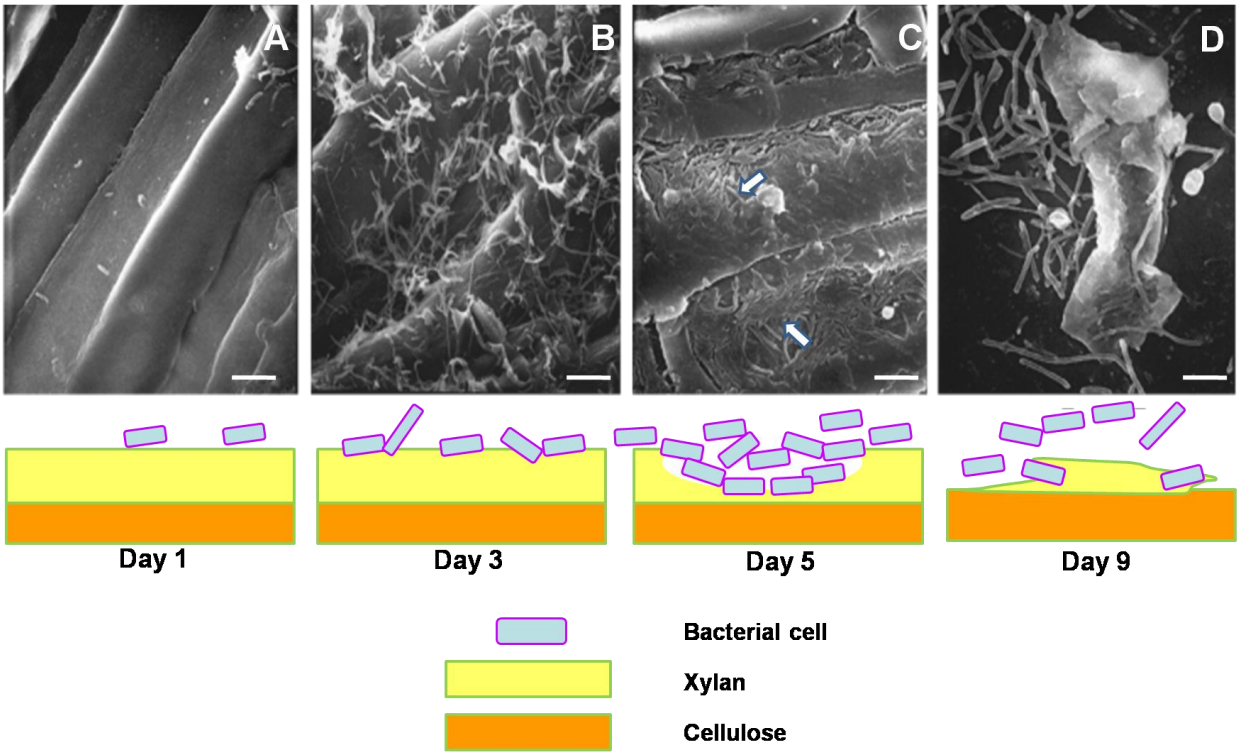

Fig. 1. Behavioral phenomena and simplified diagrams of *T. xylanilyticum* BT14 towards plant cell wall degradation according to time. Cells were anaerobically grown at pH 9.0 and 60 °C using corn husk as a substrate. Very few bacterial cells adhered to the growth substrate after 1-day incubation (A) and the numbers of cells increased rapidly from days 3 to 5 (B-C). Noticeably, several cells likely produced extracellular matrix that connected to their neighbors (C) during the exponential growth phase, and they appeared singly and desorbed from corn husk (D) while going to the stationary growth phase. Arrows indicate extracellular matrix formation. Bar, 300 μm.

Although biofilm formation is an advanced process developed by microorganisms in order to harvest food, hydrolytic actions might play a vital role as a primary degradation strategy to overcome the recalcitrance of plant biomass. The microorganisms might evolve and form special structural organization, synthesizing an array of enzymes suitable for available growth substrate. As degradation machinery of strain BT14 is unknown, it is of our primary interest to further investigate the xylanolytic-cellulolytic enzyme system produced by this certain microorganism.

Cationized ferritin staining is generally used to study cell-associated multienzyme complexes, termed cellulosomes, of several microorganisms (Kenyon *et al.* 2005; Lamed *et al.* 1987b; Liang *et al.* 2009). Based on that, positive charges of Fe-containing proteins could interact with negative charges of proteins on the bacterial cell surfaces, resulting in rough, bulging, and protuberant structures when observed under SEM (Lamed *et al.* 1987a). As can be seen previously, the degradation zones of corn husks mostly occurred around the bacterial cell surfaces; thus, this technique was employed to provide preliminary evidence that some proteins were associated with the cell surfaces. Apparently, dense, protuberance-like structures were observed on the cell surfaces of strain BT14 growing on corn husk strips (Fig. 2A) – and few bulges appeared on the surfaces of some free cells collected at the stationary growth phase (Fig. 2B). Although cationized ferritin staining confirmed the presence of protein on the cell surfaces, one might say that what was seen might not involve in hydrolytic action. Therefore, the protein fraction was prepared from the corn husk-bound cells by sonication and ultrafiltration using a 30 kDa-cutoff to retrieve small proteins, and evaluated the potential hydrolyzing capability.

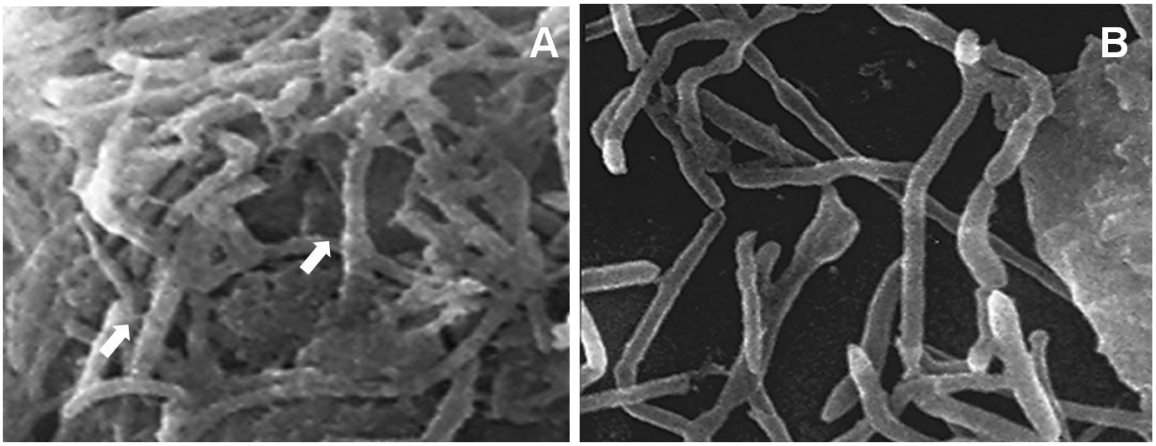

Fig. 2. Cationized-ferritin staining showing protuberance-like structures on the cell surfaces of *T. xylanilyticum* BT14 growing on corn husks (A) and free cells (B). Samples were collected at the exponential and stationary growth phases for photography for A and B, respectively. Arrows indicate protuberance-like structures.

The protein preparation from the pellet-bound enzymes exhibited strong xylanase activity (0.72 U/mL), with relatively low CMCase activity (0.04 U/mL). This result indicates that protein preparation from the cell-pellets possessed enzymatic ability; thus, the protein preparation was considered the cell-associated enzyme. In order to determine the size and to retain its natural form, the cell-associated enzyme was further characterized by gel-filtration chromatography using HiLoad Superdex 200, in which the highest size exclusion limited at 6×10^5 for globular proteins and 1×10^5 for dextrans.

Protein elution was directly measured by OD280 and followed by Bradford's assay to confirm protein concentration. Both protein measurements showed similar profiles, but the peak F1 likely showed exaggerated protein concentration by OD280, which might be due to interference of DNA contamination (not shown). The protein elution containing xylanase activity distributed over the entire column, where four major xylanase-containing peaks were observed (F1-F4) (Fig. 3). CMCase activity was also tracked; however, the activity could not be seen due to its relatively low activity under assayed conditions. The peak F1 was eluted at the position of void volume, whereas peaks F2 and F3 were positioned at the region where the sizes were larger than ferritin (M_r= 440 kDa) and IgG (158 kDa), respectively, followed by very low molecular weight xylanases. However, the size distributions from peaks F2 to F4 were not well-separated, possibly due to the similarity in molecular mass. The size of the F1 protein was determined by analytical column and its size was estimated to be 2000 kDa (not shown).

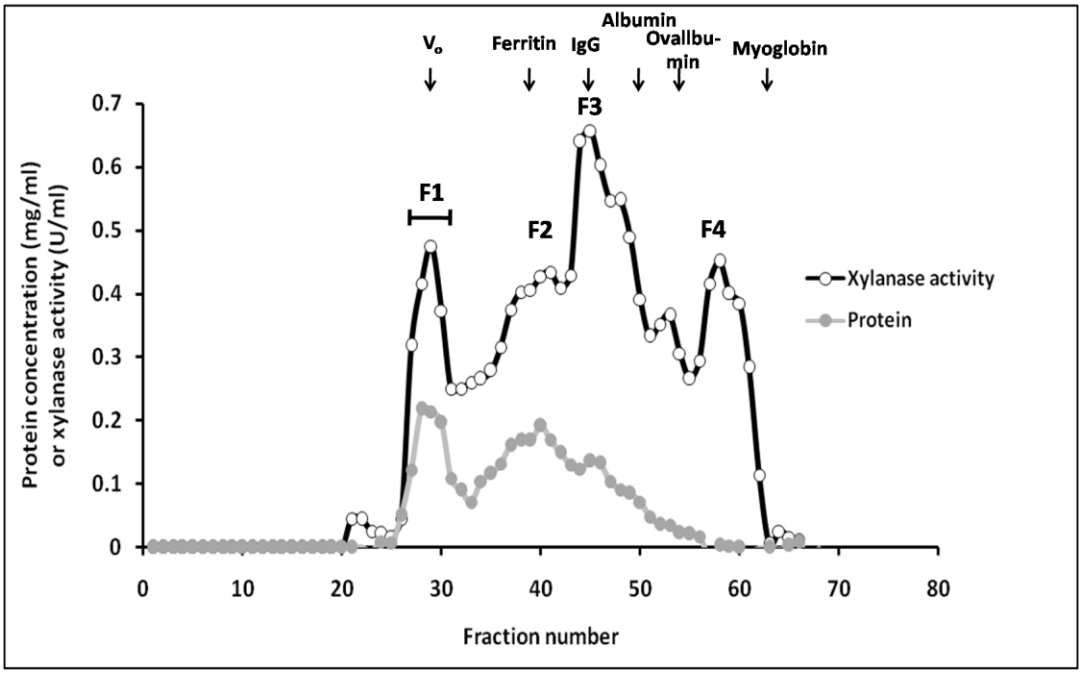

Fig. 3. Hiload Superdex 200 chromatogram of ultrafiltrated cell-associated enzymes derived from corn husk-bound cells

Lin and Thompson (1991) isolated a MEC with a molecular weight greater than 669 kDa from *Butyrivibrio fibrosolvens*, whereas Kim and Kim (1993) purified two MECs from *Bacillus circulans* with approximate molecular weights of 669 and 443 kDa. Pason *et al.* (2006) isolated two (MECs) from *Paenibacillus curdlanolyticus*, whose sizes were about 1450 and 400 kDa, respectively. Additionally, the sizes of discrete, multienzyme components, cellulosomes of several microorganisms were reported in a range of 650 kDa to 2500 kDa (Doi *et al.* 2003). The molecular weights of the F1 protein agreed in ranges with the sizes of MECs reported in the literature. Although size cannot entirely indicate the existence of MECs, it suggests a primarily-characteristic feature of MECs. Therefore, peak F1 was selected. The largest molecular weight protein and appropriate fractions of F1 protein (Fraction no. 26-30) were pooled, ultrafiltrated using

Amicon, and used for subsequent studies in order to provide supportive evidence of MEC formation.

To further characterize the complex organizations, the F1 protein was subjected to 6% non-denaturing PAGE, showing one single band protein at the top of the gel (Fig. 4A). Similar results were found in the works of Jiang *et al.* (2004) and Chimtong *et al.* (2011), in which the MEC appeared at the top of non-denaturing gel, possibly due to its high molecular mass. Denaturing conditions were established to learn more about protein interactions and compositions. The F1 protein was incubated with 1% SDS and 1.7% β-mercaptoethanol at room temperature for 1 h before being subjected to 10% SDS-PAGE. Under these mild-denaturing conditions, a few bands of proteins were dissociated from the F1 protein; however, most proteins remained at the top of the separating gel (Fig. 4B; lane 1), indicating the limited penetration of proteins into the gel, presumably owing to its intact large size or incomplete fragmentation. For totally-denaturing conditions, the F1 protein was boiled for 10 min under the same conditions, resulting in multiple, smaller protein fragments on SDS-PAGE (Fig. 4B; lane 2). This suggests very tight association of each protein component of the F1 protein – and possibly that the F1 protein might be formed as a discrete complex rather than a simple protein aggregation.

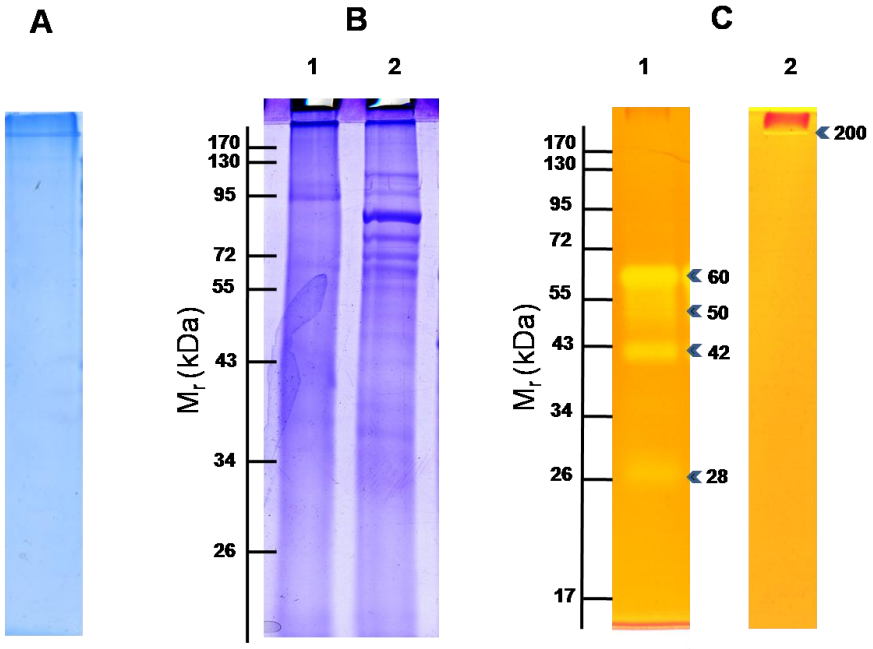

Fig. 4. Protein patterns and zymogram analyses. The F1 protein was run under non-denaturing conditions using 6% gels (A) and under mild- (B; lane 1) and strong-denaturing (B; lane 2) conditions using 10% gels. Xylanolytic and cellulolytic enzyme activities of protein components were revealed by zymograms containing soluble birch wood xylan (C; lane 1) and CMC (C; lane 2) using 10% gels.

Zymogram analyses were performed in order to reveal xylanolytic and cellulolytic enzyme activities corresponded by subunit compositions of the F1 protein. The complete-denatured protein sample was run on soluble birch wood or CMC-containing SDS-PAGE (10% gel) and was subsequently soaked with 2% Triton X-100 in order to remove SDS and to regenerate protein folds. At least four proteins with estimated molecular weights of

60, 50, 42, and 28 kDa exhibited xylanase activity, and one with a size of at least 200 kDa showed relatively low CMCase activity (Fig. 4C; lane 2).

The numbers of enzymatic components likely corresponded with the hydrolyzing capability of the complex, in which the activity of xylanase was greater than CMCase. The MECs with predominant xylanase activity were reported to contain 11, 5, 12, 4, and 5 xylanases for *Butyrivibrio fibrosolvens* (Lin and Thomson 1991), *B. subtilis* (Jones *et al.* 2012), *P. curdlanolyticus* (Pason *et al.* 2006), *S. olivaceoviridis* (Jiang *et al.* 2006), and *Th. thermosaccharolyticum* (Chimtong *et al.* 2011), respectively. As visualized by the zymogram, two polypeptides with estimated sizes of 60 and 42 kDa appeared to account for the major xylanase activity. Kosugi *et al.* (2001) found that the MEC of *C. cellulovorans* contained two polypeptides that showed great xylanase activity, one of which belonged to glycosyl hydrolase family 11. Family 10 and 11 hydrolases, consisting solely of xylanases, typically provide the major xylanase activity in several microorganisms, such as *C. thermocellum*, *C. stercorarium*, and *C. acetobutylicum*, and are known to preferably hydrolyze unsubstituted long-chain xylooligosaccharides (Collins *et al.* 2005; Kosugi *et al.* 2001). Since birch wood xylan (Sigma), which contains more than 90% xylose, was used as the substrate, it is possible that those two polypeptides with active xylanase activity might be lodged into family 10 or 11 glycoside hydrolase. However, protein identification using N-terminal sequencing and MALDI-TOF plus mass-spectrometry is needed to confirm the identities of those polypeptides.

The presence of one CMCase might imply significant degradation of hemi-celluloses, as xylans are likely associated with cellulose at amorphous regions (Collins *et al.* 2005). The co-occurrence of at least one cellulase and three or more xylanases might suggest a saprophytic lifestyle of bacteria (Medie *et al.* 2012). However, it is worth noting that the molecular weight of the cellulase-containing polypeptide (*ca.* 200 kDa) is relatively high, as compared to those reported in the literature (Wilson 2004). The occurrence of its large sizes might be due to modular structural organizations or that it appears as a part of other structural components of the complexes. One study showed that cellulase was an integral part of scaffoldin (CipV) of *Acetivibrio cellulolyticus* cellulosome, resulting in *ca.* 199 kDa in molecular mass (Ding *et al.* 1999). The rest of the polypeptides of the F1 protein complex might be xylanases or CMCases, both of which might be sensitive to boiling in the presence of SDS (Morag *et al.* 1990). Several MECs comprised other enzymes, such as exoglucanases (cellobiohydrolases), mannanases, and pectinases, or structural component, such as scaffoldins, which are typically found in cellulosomes (Doi and Kosugi 2004). Thus, additional protein components of the F1 complex in the present work can be further investigated.

Xylanases are commonly contained in the cellulosomes produced by anaerobic bacteria, which is considered the most efficient enzymatic system for cellulosic biomass degradation. However, there was a report showing that the cellulosomal xylanase components were likely released into environments instead of tightly binding inside the complex (Kosugi *et al.* 2001). It is possible that (1) cellulosome-producing micro-organisms might secrete high amounts of dockerin-bearing cellulases rather than xylanases—such that cellulases might compete for cohesin modules, thus preventing incorporation of xylanases into the complex or (2) the interaction between xylanase-bearing dockerins and cohesins might be relatively weak (Kosugi *et al.* 2001). In contrast, the MEC found in the present study comprised mostly xylanase polypeptides inside the complex. This might suggest an abundance of xylanase subunits secreted by

the bacterium and strong interaction of xylanase components inside the MEC. These atypical characteristics likely differ from cellulosomal integrity.

The binding ability and the specificity of enzymes to adhere insoluble substrates are usually due to carbohydrate-binding modules (CBMs). The binding ability of the F1 protein complex to insoluble polysaccharides corresponded to about 90% for Avicel and about 30% for xylan. Additionally, all subunits of the F1 protein were found in an Avicel-bound fraction (Fig. 5A; lane 2). This result likely supported the existence of the F1 protein as a MEC, in which CBMs might bring the entire subunits inside the complex to adsorb on cellulose. Despite binding to xylan, the majority of all components remained in an unbound fraction (Fig. 5B; lane 1), suggesting the role of the CBMs as cellulose targeting for this MEC.

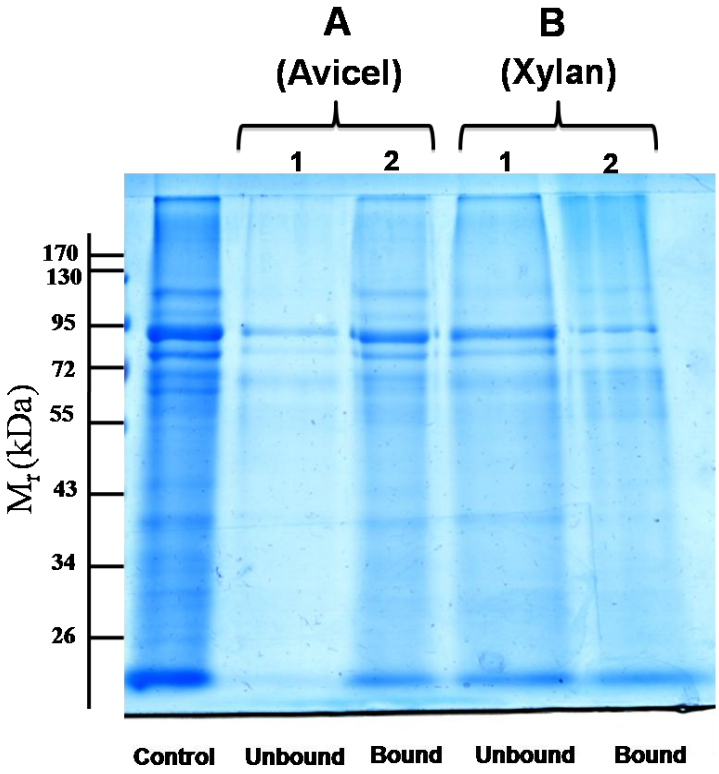

Fig. 5. Polysaccharide-binding ability of a xylanolytic MEC of *T. xylanilyticum* BT14. The F1 complex was incubated with Avicel (A) or insoluble xylan (B) at 4 ˚C for 1 h. After binding, the samples were further fractionated to Avicel-bound (A; lane 1) and unbound (A; lane 2) proteins or xylan-bound (B; lane 1) and unbound (B; lane 2) proteins (See Experimental). The pattern of the F1 complex at a total protein of 200 µg was shown in the lane Control. All samples were run on the SDS-PAGE containing 10% gel.

Unlike hemicellulolytic MECs reported in the literature, the F1 protein displayed great binding towards cellulose rather than xylan, although xylanase was the predominant activity. Cai *et al.* (2010) found that several xylanases of *Cellulosilyticum ruminicola* possessed cellulose-binding CBMs rather than xylan-binding ones. The unusual finding of xylanases containing cellulose-binding CBMs might suggest the importance for natural substrate targeting inside a complex polysaccharide mixture in lignocellulosic mater-

ials (Cai *et al*. 2010). Possibly, cellulose-binding CBMs harbored by the xylanolytic MEC might bind to readily-accessible cellulose inside cellulosic biomass; meanwhile xylanases might work in a synergistic manner to degrade neighboring xylan components (Moraïs *et al.* 2011).

Among xylanolytic MEC reported to date (Table 1), the MEC of *T. xylanilyticum* BT14 characteristically differs from those in terms of polysaccharide-binding ability, cell-surface association, size, and numbers of enzymatic subunits. All known xylanolytic MECs, except that from strain BT14, prefer binding to xylan over cellulose. The MECs produced by aerobic microorganisms appear not to be associated with cell walls, whereas those from anaerobes; *T. xylanilyticum* and *Th. thermosaccharolyticum*, are cell-associated enzymes, presumably providing an economic way to obtain enzyme secretions (Bayer *et al.* 2008). The sizes of MECs vary among microorganisms, possibly due to number limitation of enzyme subunits (Jones *et al.* 2012).

Table 1. Comparisons of Characteristics of Xylanolytic MECs Produced by Several Microorganisms

Microorganism	O_2-demand	Enzyme fraction	MW. (kDa)	No. of enzyme subunits*		Binding ability‡		Reference
				Xylanase	CMCase	Xylan	Avicel	
Bacteria								
T. xylanilyticum BT14	Anaerobe	Cell-associated	2000	4	1	+	+++	This study
Th. thermo-sacchrolyticum NOI-1	Anaerobe	Cell-associated	1200	5	0	+++	++	Chimtong *et al.* (2011)
P. curdlanolyticus B-6	Facultative anaerobe	Culture medium	1450	12	9	+++	++	Pason *et al.* (2006)
Bu. fibrisolvens H17c	Anaerobe	Culture medium	669	11	3	ND	ND	Lin and Thompson (1991)
B. subtilis SJ01	Aerobe	Culture medium	371	5	ND†	+	+	Jones *et al.* (2012)
B. licheniformis SDV1	Aerobe	Culture medium	2000	7	2	+++	+	van Dyk *et al.* (2010b)
S. oliveceoviridis E-86	Aerobe	Culture medium	1200	4	ND	+++	+	Jiang *et al.* (2004)
Fungi								
Chaetomium sp. MS-017	Aerobe	Culture medium	468	5	4	ND	ND	Ohtsuki *et al.* (2005)

* Based on zymography
† ND, not determined
‡ Based on the difference between initial protein and remaining protein after binding; +++, good; ++,fair; +, poor

Structurally, xylans are recognized as being less recalcitrant than cellulose, as they contain side-chain substituents that primarily make their structure amorphous. The "free-state" xylanase enzymes show great hydrolysis efficiency towards commercial

xylans; however, the effectiveness of hydrolyzing performance towards real-world substrates has not been clearly shown. In this study, the robust xylan-utilizing bacterium *T. xylanilyticum* BT14 secreted cell-associated xylanolytic MEC to degrade xylan-rich materials apart from its cell-free enzymes (Phitsuwan *et al.* 2010). Thus, it is possible that there is a need for the microorganism to form a xylanolytic MEC, which might synergistically act with free xylanases, to degrade natural-occurring xylan-containing plant biomass. For further study, the relationships between noncomplexed and complexed xylanase systems produced by this microorganism are being studied.

CONCLUSIONS

1. Adherence of cells to substrates is essential for *T. xylanilyticum* BT14 to utilize its growth nutrient – where degradation strategy and biofilm formation appear as co-biochemical events during growth on the substrate.
2. Xylanolytic-cellulolytic enzyme activities were found to associate with the cell surfaces, as evident by the degradation zone and cationized-ferritin staining.
3. The cell-associated enzyme appeared as a large MEC of *ca*. 2000 kDa in molecular mass containing mainly xylanase activities – 4 xylanases and one CMCase, as evident by gel-filtration chromatography, gel electrophoresis, and zymogram analyses.
4. The newly discovered cell-associated, xylanolytic MEC preferred binding to cellulose rather than xylan. This feature is similar to the cellulsomal characteristic, but differs from previous reported characters of xylanolytic MECs or xylanosomes.

ACKNOWLEDGMENTS

Paripok Phitsuwan was supported by a grant of the Royal Golden Jubilee Ph.D. program of the Thai Research Fund. The authors are grateful for the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission for financial support.

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Article submitted: August 31, 2012; Peer review completed: October 4, 2012; Revised version received: October 22, 2012; Accepted: October 24, 2012; Published: October 29, 2012.