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A NOVEL MULTIENZYME COMPLEX FROM A NEWLY ISOLATED FACULTATIVE ANAEROBIC BACTERIUM, *PAENIBACILLUS* SP. TW1

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A multienzyme complex from newly isolated *Paenibacillus* sp. TW1 was purified from pellet-bound enzyme preparations by elution with 0.25% sucrose and 1.0% triethylamine (TEA), ultrafiltration and Sephacryl S-400 gel filtration chromatography. The purified multienzyme complex showed a single protein band on non-denaturing polyacrylamide gel electrophoresis (native-PAGE). The high molecular mass of the purified multienzyme complex was approximately 1,950 kDa. The complex consisted of xylanase and cellulase activities as the major and minor enzyme subunits, respectively. The complex appeared as at least 18 protein bands on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and as 15 xylanases and 6 cellulases on zymograms. The purified multienzyme complex contained xylanase, α-L-arabinofuranosidase, carboxymethyl cellulase (CMCase), avicelase and cellobiohydrolase. The complex could effectively hydrolyze corn hulls, corncobs and sugarcane bagasse. These results indicate that the multienzyme complex that is produced by this bacterium is a large, novel xylanolytic-cellulolytic enzyme complex.

Keywords: Enzyme purification – facultative anaerobic bacterium – multienzyme complex – *Paenibacillus* sp. TW1 – xylanolytic-cellulolytic enzymes

INTRODUCTION

The hydrolysis of plant cell wall polysaccharides into soluble sugars is potentially important for the production of value-added products such as fermentable sugars, chemicals and liquid fuel [7]. Cellulose and hemicellulose are the major components of plant cell walls. In nature, cellulose fibers are embedded in a matrix of other structural polysaccharides, primarily hemicelllulose and lignin, which together create a rigid structure strengthening the plant cell wall [10, 18, 26]. Therefore, the hydrolysis of plant cell walls requires efficient cellulolytic and xylanolytic enzyme systems for complete degradation. A cellulase system comprises three types of enzymes: endoglucanases, exoglucanases and β-glucosidases. The first type, endoglucanases (EC 3.2.1.4), randomly cleaves at internal amorphous regions of cellulose chains. The

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second type, exoglucanases, includes cellobiohydrolase (EC 3.2.1.91). The mode of action of exoglucanases is to release cellobiose, primarily from chain ends, and also to act on microcrystalline cellulose [30, 33]. The last type is β-glucosidases (EC 3.2.1.21), which cleaves cellobiose to glucose. A xylanolytic enzyme system comprises endo-1,4-β-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), α-L-arabinofuranosidase (EC 3.2.1.55), acetyl esterase (EC 3.1.1.6), and α-D-glucuronidase (EC 3.2.1.1). In general, the xylanases attack internal xylosidic linkages on the backbone, and the β-xylosidases release xylosyl residues by an endwise attack of xylooligosaccharides. α-L-Arabinofuranosidase and α-glucuronidase remove the arabinose and 4-*O*-methyl glucuronic acid substituents from the xylan backbone, respectively. Acetyl esterases hydrolyze the ester linkages between xylose units of xylan and acetic acid [6]. Many anaerobic bacteria have been reported to produce multiple cellulases and xylanases containing a cellulose-binding domain and/or a xylan-binding domain that are associated into a discrete, high molecular weight cellulolytic or xylanolytic enzyme complex, known as cellulosome [1, 3, 7, 8, 23] or xylanosome [13]. These enzyme complexes exhibit high activity against recalcitrant crystalline components of plant cell walls [1, 8, 23]. Multienzyme complex producer microorganisms are mainly found among anaerobic bacteria and fungi [2]. Cellulosomes have been found in several genera such as *Clostridium*, *Acetovibrio*, *Bacteroides*, *Butyrivibrio*, and *Ruminococcus* [8]. To our knowledge, few reports concern cellulolytic-xylanolytic multienzyme complex production by facultative anaerobic or aerobic bacteria under aerobic conditions. For example, the facultative anaerobic bacterium *Paenibacillus curdlanolyticus* B-6 has been reported to produce a multienzyme complex under aerobic conditions, [20] and subsequently, the aerobic bacterium *Bacillus licheniformis* SVD1 was reported to produce a multienzyme complex [9].

This paper describes the isolation and characterization of a newly isolated *Paenibacillus* sp. that produces xylanolytic-cellulolytic enzymes as a complex form. This study also reports the purification and partial characterization of the multienzyme complex in the genus *Paenibacillus,* which was produced by the isolated strain under aerobic conditions.

MATERIALS AND METHODS

Bacterial strain and culture medium

Paenibacillus sp. TW1 is a facultative anaerobic bacterium that is isolated from a waste treatment reactor. The bacterium was grown in Berg's mineral salts medium, pH 7.0 [5] containing 0.2% $NaNO_3$, 0.05% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.002% $MnSO_4 \cdot H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$, 0.002% $CaCl_2 \cdot 2H_2O$, and 0.5% corn hull as the sole carbon source. The culture was incubated in a rotary shaker at 200 rpm and 37 °C under aerobic conditions.

Identification of microorganism and morphology

The morphological and taxonomic characteristics of the isolate were analyzed according to the methods in Bergey's Manual of Systematic Bacteriology [27]. The isolated strain was also identified by 16S rRNA gene analysis. The 16S rRNA gene was amplified by PCR with 8F primer (5'-AGAGTTTGATCCTGGCTCAG-3') and 1942r primer (5'-GGTTACCTTGTTACGACTT-3'). The PCR protocol was performed in accordance with a previous report [21]. The purified PCR product was ligated to a *p*GEM-T Easy vector cloning kit (Promega) and then transformed into a competent cell, *Escherichia coli* DH5α. The nucleotide sequence of the 16S rRNA gene fragment was determined by the T7 and SP6 primers. Gram- and endospore staining were performed as described by Singleton [25].

Scanning electron microscopy

Cells of strain TW1 grown on xylan were harvested at the late exponential growth phase. Afterward, the cell samples were filtered through a Nucleopore membrane filter, dehydrated by a series of graded ethanol solutions, and critical point dried with liquid CO_2. The samples were coated with gold and examined with a JEOL JEM-35 scanning electron microscopy.

Enzyme assays

All assays were conducted in triplicate. Xylanase activity was measured by determining the reducing sugar released from oat spelt xylan (Sigma Chemical Co., St. Louis, MO, USA). The reaction mixture consisted of 0.5 mL of 1% oat spelt xylan in 100 mM sodium phosphate buffer (pH 7.0) and 0.1 mL of enzyme. After incubation for 10 min at 50 °C, the increase in reducing sugar was determined by the Somogyi–Nelson method [19] using xylose as a standard. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmole of reducing sugars per minute under assay conditions. CMCase and avicelase activities were measured under the same conditions as described above using carboxymethylcellulose and Avicel as substrates, respectively. Cellobiohydrolase, β-xylosidase, β-glucosidase and α-L-arabinofuranosidase activities were determined in accordance with previous reports [20, 22]. Protein concentration was determined by the Lowry method [17] using bovine serum albumin as a standard.

Purification of multienzyme complex

All purification steps were performed at 4 °C. The isolated TW1 was grown in Berg's mineral salts medium (pH 7.0) containing 0.5% corn hull (40 mesh) as the sole car-

bon source. The culture was incubated in a rotary shaker at 200 rpm and 37 °C until the late exponential growth phase (2 days) and was harvested by centrifugation (8,000×*g* for 5 min) at 4 °C. To collect the pellet-bound enzymes, which are made up of the multienzyme complex, the pellets were washed four times with a large amount of phosphate buffer saline (PBS: 150 mM sodium chloride in 100 mM phosphate buffer, pH 7.0) and eluted with 0.25% sucrose. The pellets were then washed again several times with the same buffer until no protein was detected in the washing fractions, and then the pellets were eluted with 1.0% TEA. The eluate was dialyzed and concentrated by ultrafiltration with a 5-kDa molecular weight cut-off membrane (Tangential flow filtration; PALL, U.S.A). The concentrated eluate (10 mg protein) was subjected to a Sephacryl S-400 high-resolution column (0.7 × 50.0 cm), which was equilibrated with 50 mM phosphate buffer (pH 7.0) at 4 °C and was eluted with the same buffer at a flow rate of 10 mL h^{-1}. The elution of the standard proteins, blue dextran (2,000 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), and catalase (232 kDa) (Amersham Pharmacia Biotech), was conducted in the same manner.

Cellulose- and xylan-binding assays

The preparation of insoluble xylan was performed by the method of Irwin et al. [12]. The xylan-binding assay was conducted as described by Pason et al. [20]. The purified multienzyme complex (0.23 mg protein) was added to 50 mg of insoluble xylan in 1.0 mL of 100 mM phosphate buffer (pH 7.0) in 1.5-mL Eppendorf tubes. Samples were shaken at intervals for 30 min at 4 °C before centrifugation. The amount of activity remaining in the supernatant was determined by the xylanase assay method as described above. The activity lost from the supernatant was assumed to be the bound activity. The cellulose-binding assay used Avicel and was also conducted according to the above procedure.

pH and temperature optima and stability of the multienzyme complex

The optimum pH of the enzyme was determined using different buffers (100 mM) with a pH ranging from 4.0 to 9.0 (pH 4.0–6.0, citrate buffer; pH 6.0–7.0, phosphate buffer; pH 7.0–9.0, Tris-HCl buffer; pH 9.0–11.0, carbonate buffer), and the optimum temperature for enzyme activity was determined between 40 and 90 °C at pH 7.0. pH stability was conducted by incubating the enzyme at 50 °C for 1 h at a pH between 4.0 and 11.0 (25 mM of each buffer concentration). The thermostability was determined by incubating the enzymes at different temperatures ranging from 40 to 90 °C at pH 7.0 for 30 min. The residual xylanase activity was measured by the above described enzyme assay.

Preparation of soluble xylan

The preparation of soluble xylan was performed as described in a previous report [11]. Ten grams of commercial oat spelt xylan was suspended in 200 mL of distilled water, and the pH was adjusted to 10 using 1 M NaOH. Then, the mixture was stirred for 1 hour at room temperature and was centrifuged for 10 min at 10,000×*g*. After centrifugation, the supernatant was neutralized with 1 M acetic acid and then freeze dried.

Gel electrophoresis and zymogram analysis

SDS-PAGE was performed in a 10% polyacrylamide gel using the method of Laemmli [15], whereas native-PAGE was performed the same as the SDS-PAGE but without SDS, β-mercaptoethanol and heat. After electrophoresis, the proteins were stained with Coomassie brilliant blue R-250. Zymograms for cellulase and xylanase activities were performed on SDS-10% polyacrylamide gel containing 0.1% (w/v) carboxymethylcellulose and 0.1% (w/v) soluble oat spelt xylan, respectively. After the gels were incubated with 50 mM sodium phosphate buffer at 60 °C for 30 min, Congo red solution (0.1%, w/v) was used for 30 min at room temperature to stain residual polysaccharides. Destaining was performed with 1 M NaCl until clear bands occurred. Afterward, the gels were fixed with 5% acetic acid (v/v) [20, 21].

Hydrolysis of lignocellulosic substances

Insoluble lignocellulosic substances such as agricultural residues (corn hull, corncob, sugarcane bagasse, rice straw and rice husk) were ground (40-mesh) and washed several times in warm distilled water to remove remaining free sugars and dried at 50 °C. Each insoluble substance (1% dry weight) was incubated with multienzyme complex (5 units of xylanase activity and 0.84 units of CMCase activity) at 50 °C, using 100 mM phosphate buffer, pH 7.0. After 30 min, the hydrolysis products were taken, and the amount of reducing sugars released was determined by the Somogyi–Nelson method [19].

RESULTS

Identification and morphology of the microorganism

Strain TW1 was facultative anaerobic, Gram-positive, spore-forming and rod-shaped. The 16S rRNA gene analysis of strain TW1 was performed by PCR with an 8F primer and a 1942r primer. The amplified sequence (1,513 bp) showed high similarity to the 16S rRNA gene fragment of *P. cellulosilyticus* (97.6%), *P. curdlanolyticus*

(97.4%) and *P. kobensis* (97.1%). The nucleotide sequence data of the 16S rRNA gene of strain TW1 was deposited in the GenBank database under accession number FJ532373. The isolate could grow in Berg's medium containing corn hull as a sole carbon source and produce xylanolytic-cellulolytic enzymes under aerobic conditions. The cells grew at pH and temperature optima of 7.0 and 37 °C, respectively (data not shown). Strain TW1 was deposited at the MIRCEN Culture Collection, Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, Thailand under accession number TISTR1914.

Adhesion of cells to xylan

To determine the adhesion of xylan-grown cells to xylan, the cells were harvested at the late exponential growth phase and were analyzed by scanning electron microscopy. Figure 1 shows that the surfaces of cells grown on xylan were adhered to this substrate.

Purification of multienzyme complex

The multienzyme complex of *Paenibacillus* sp. strain TW1 was purified from pellets by 0.25% sucrose and 1.0% TEA elution followed by ultrafiltration and gel filtration chromatography. Strain TW1 was grown in a medium containing 0.5% corn hull as the sole carbon source, and the culture was harvested at the late exponential growth phase. Product analogue and a mild denaturant were used to elute the multienzyme complex from pellets, as previously reported [28]. The multienzyme complex was

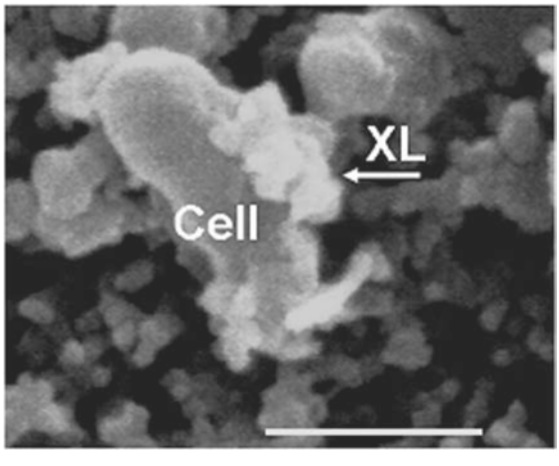

Fig. 1. Scanning electron microscopy of a *Paenibacillus* sp. TW1 cell harvested at the late exponential growth phase. Adhesion of the cell to xylan (XL) is indicated by the arrow. The scale bar in the micrograph is 1 μm

Table 1
Specific activity of the purified multienzyme complex from *Paenibacillus* sp. TW1

Enzyme	Specific activity of multienzyme complex (U/mg protein)
Xylanolytic enzymes	
Xylanase	21.45
α-L-Arabinofuranosidase	1.42
β-Xylosidase	n.d.
Cellulolytic enzymes	
CMCase	3.60
Avicelase	0.94
Cellobiohydrolase	0.42
β-Glucosidase	n.d.

n.d. – not detected under the assay conditions.

eluted with 0.25% sucrose to eliminate weak binding proteins from the pellets and then eluted with 1.0% TEA. The mild denaturant, TEA, may deform the protein by promoting unfolding, especially of the carbohydrate-binding region of the complex [28]. The eluted pellet-binding proteins were dialyzed and concentrated as described in the Materials and Methods. The concentrated pellet-binding protein was then subjected into a Sephacryl S-400 column. A major protein peak (fractions 20 to 26) was eluted closely with blue dextran. The molecular mass of the protein was estimated to

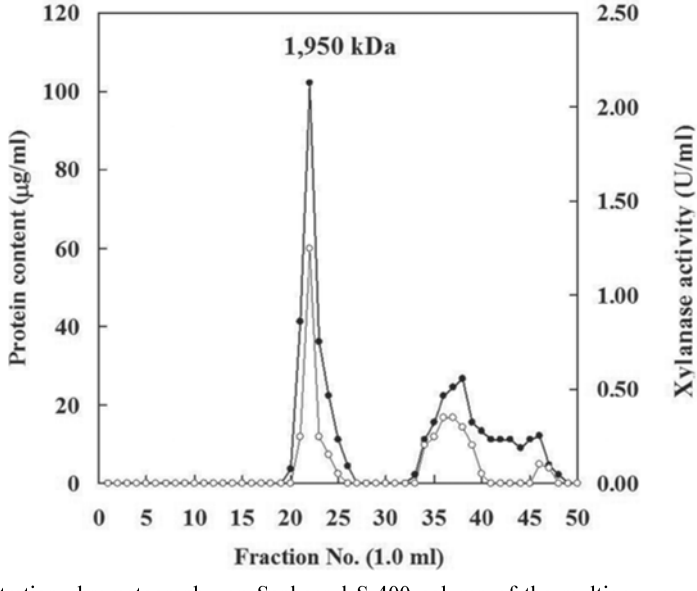

Fig. 2. Gel filtration chromatography on Sephacryl S-400 column of the multienzyme complex from *Paenibacillus* sp. TW1. Closed and open circles indicate protein content and xylanase activity, respectively

be 1,950 kDa (Fig. 2). Other small protein peaks appeared at fractions 33 to 48. Thus, we supposed that the 1,950 kDa protein is a major multienzyme complex of strain TW1 and used this protein for further characterization. The purified multienzyme complex of strain TW1 consisted of xylanolytic-cellulolytic enzymes, as shown in Table 1. Xylanase was a major enzyme in the multienzyme complex of strain TW1, whereas the CMCase activity was lower than the xylanase activity. Xylanase and CMCase were 21.45 and 3.60 U/mg protein, respectively.

Cellulose- and xylan-binding abilities

The purified multienzyme complex was found to be able to bind to both insoluble xylan and Avicel at 69% and 41%, respectively. The result indicated that carbohydrate-binding modules are possibly present with the complex enzyme.

Characterization of purified multienzyme complex

Characterization of the purified multienzyme complex is shown in Table 2. The optimum pH and the stability of the pH of the purified multienzyme complex were 7.0 and 6.0–9.0, respectively. The optimum and stability temperature of the purified multienzyme complex were 60 °C and up to 50 °C, respectively.

Native-PAGE, SDS-PAGE and zymograms of protein components

The protein composition of the purified multienzyme complex was analyzed by native-PAGE, SDS-PAGE and zymograms. The results are shown in Fig. 3. The purified multienzyme complex was subjected to native-PAGE, which showed only one protein band (N1) on top of the gel (Fig. 3A). When subjected to SDS-PAGE, the purified multienzyme complex showed at least 18 proteins with molecular masses in

Table 2
Characterization of the purified multienzyme complex from *Paenibacillus* sp. TW1

Properties	Purified multiezyme complex
Molecular weight (kDa) by gel filtration chromatography	1,950
pH optimum	7.0
pH stability	6.0–9.0
Temperature optimum	60 °C
Temperature stability	up to 50 °C
Enzyme subunits Xylanase CMCase	 15 6
The main hydrolysis products (corn hull as a substrate)	Oligosaccharides

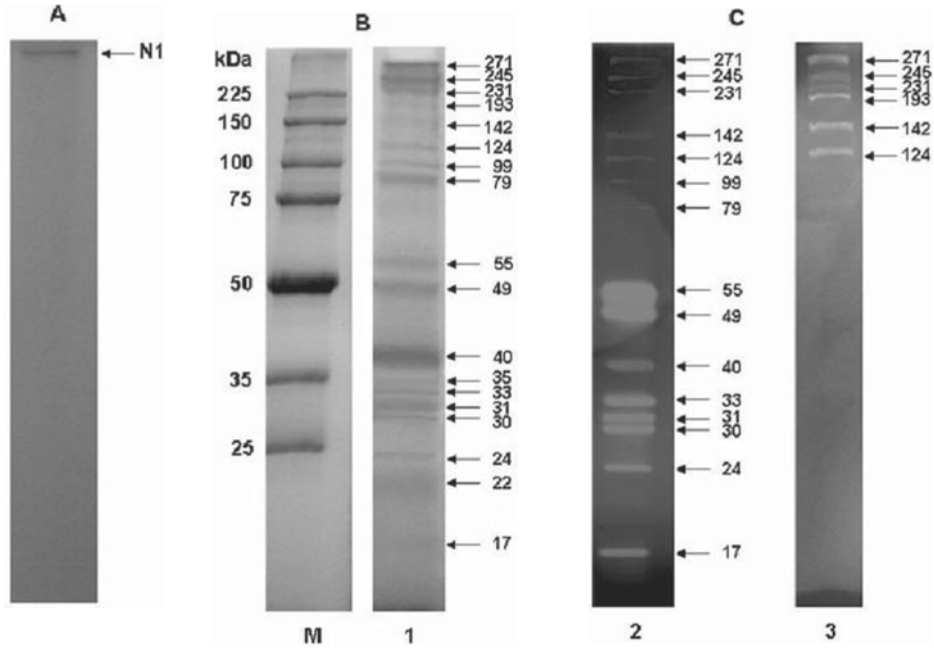

Fig. 3. Native-PAGE (A), SDS-PAGE (B) and zymograms (C) of the purified multienzyme complex from *Paenibacillus* sp. TW1. All samples contained approximately 70 μg of protein. Lane M, molecular weight markers; lane 1, proteins; lane 2, xylanase zymogram; lane 3, CMCase zymogram

the range of 271 to 17 kDa (Fig. 3B, lane 1), of which 15 proteins had xylanase activity (Fig. 3C, lane 2), and 6 proteins had CMCase activity (Fig. 3C, lane 3). Among them, five proteins (271, 245, 231, 142 and 124 kDa) showed both xylanase and cellulase activities.

Hydrolysis of lignocellulosic substrates

The reducing sugars released from non-pretreated corn hull, corncobs, sugarcane bagasse, rice straw and rice husk were 158, 118, 60, 17 and 10 mg/liter, respectively. The main hydrolysis product of corn hull by the purified multienzyme complex was oligosaccharides (data not shown).

DISCUSSION

In recent years, multienzyme complexes have been identified and described in many anaerobic cellulolytic bacteria and fungi [2, 7, 23]. Microorganisms primarily produce multienzyme complexes under anaerobic conditions. The bacterium adhesion to

cellulose is accomplished by means of a discrete, multifunctional, multicomponent cell surface protein complex, known as a cellulosome, which is exquisitely designed for efficient binding and hydrolysis of the substrates [16]. Cellulosomes are best known in the *Clostridium* genus such as *Clostridium thermocellum*, *C. cellulovorans* and *C. cellulolyticum* [2, 4, 8]. Subsequently, *Streptomyces olivaceoviridis* E-86 is reported to produce an ultra-large xylanolytic complex (xylanosome) with molecular mass of approximately 1,200 kDa [13]. In this study, we isolated a mesophilic, facultative anaerobic bacterium strain, TW1, from a waste treatment reactor. Strain TW1 produced a xylanolytic-cellulolytic multienzyme complex under aerobic conditions. The 16S rRNA gene analysis revealed that strain TW1 resembles *P. cellulosilyticus*, *P. curdlanolyticus* and *P. kobensis* (97.6–97.1% similarity). Therefore, the newly isolated strain TW1 was permitted into genus *Paenibacillus* and was tentatively identified as *Paenibacillus* sp. strain TW1. During cultivation, it was found that TW1 cells had the ability to bind to insoluble substrates. The xylanolytic-cellulolytic enzyme system of the *Paenibacillus* sp. strain TW1 was found to be associated with the cells. In addition, *P. curdlanolyticus* B-6 has been reported to produce a multienzyme complex containing these properties [20]. This association is facilitated by the multienzyme complex, which binds to both substrate and cell surface. This close association presumably facilitates degradation of the substrate and ready assimilation of the sugars, as they are produced by complex enzymes [14, 29].

The molecular mass of the purified multienzyme complex of *Paenibacillus* sp. strain TW1 was estimated by gel filtration chromatography. The molecular mass of the purified multienzyme complex of strain TW1 was 1,950 kDa, whereas the cellulolytic-xylanolytic enzyme complex produced by *P. curdlanolyticus* B-6 revealed only 1,450 and 1,600 kDa when grown on xylan [20] and Avicel [32], respectively. SDS-PAGE analysis indicated that the purified multienzyme complex of strain TW1 consisted of at least 18 proteins, from which 15 proteins showed xylanase activity, and 6 proteins showed CMCase activity. The largest enzyme subunit of the multienzyme complex from strain TW1 was 271 kDa and showed xylanase and CMCase activities, whereas the large enzyme subunits of *P. curdlanolyticus* B-6 were 224 and 216 kDa for xylanase and CMCase, respectively, when grown on corn hull as the sole carbon source [31]. Recently, the aerobic bacterium *B. licheniformis* SVD1 was reported to produce a cellulolytic and hemicellulolytic multienzyme complex. The purified multienzyme complex of *B. licheniformis* SVD1 showed a molecular mass of 2,000 kDa by gel filtration chromatography [9]. Although the size of the multienzyme complex of *Paenibacillus* sp. strain TW1 is similar to that of *B. licheniformis* SVD1, the patterns of non-enzymatic and enzyme subunits are quite different. The multienzyme complex of strain TW1 was composed of more enzyme subunits than *B. licheniformis* SVD1 (8 bands on SDS-PAGE with 2 xylanases and 2 CMCases) [9]. These results strongly indicate that the multienzyme complex of the isolated strain TW1 is different from those of *P. curdlanolyticus* B-6 and *B. licheniformis* SVD1.

The pH and temperature profiles of the purified multienzyme complex of strain TW1 are similar to those of multienzyme complexes from several microorganisms [2, 4, 7, 9, 13, 20, 23]. The purified multienzyme complex from *Paenibacillus* sp. strain

TW1 efficiently hydrolyzed lignocellulosic substances. It is likely that the multienzyme complex from strain TW1 would degrade both cellulose and hemicelluloses in plant cell walls. The enzyme subunits of the multienzyme complex are held together into a complex, which serves to promote their synergistic actions. In addition, the binding ability of the multienzyme complex of strain TW1 to cellulose and xylan suggests the presence of cellulose and xylan-binding factors, probably to facilitate cellulose and xylan hydrolysis by bringing a biocatalyst in close proximity to the substrates and prolonged association with its recalcitrant substrate [21]. The multienzyme complex of strain TW1 showed high activity toward agricultural residues. This complex was different from other multienzyme complexes, such as the multienzyme complex produced by *S. olivaceovidis* E-86 that was highly specific towards oat-spelt xylan, and showed low activity toward corncob powder and carboxymethylcellulose [13], whereas a cellulosome of *C. thermocellum* showed high specificity toward crystalline cellulose [2, 3, 23]. These findings suggest that these multienzyme complexes have a different specificity to hydrolyze substrates.

Strain TW1 produced a xylanolytic-cellulolytic multienzyme complex with high molecular weight. The purified multienzyme complex consisted of cellulose- and xylan-binding regions and efficiently degraded lignocellulosic substrates without pretreatment. In conclusion, these unique properties of the purified multienzyme complex from newly isolated *Paenibacillus* sp. TW1 under aerobic conditions make this enzymatic complex attractive for several industrial applications. As far as it can be ascertained, this is the largest multienzyme complex in genus *Paenibacillus* reported to date. Moreover, studies to elucidate the functional features of the enzyme and gene involved with lignocellulose degradation of the isolated strain are in progress.

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