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Selection of Multienzyme Complex-Producing Bacteria Under Aerobic Cultivation

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Abstract The selection of multienzyme complex-producing bacteria under aerobic condition was conducted for improving the degradation of lignocellulosic substances. The criteria for selection were cellulase and xylanase enzyme production, the presence of cellulose-binding domains and/or xylan-binding domains in enzymes to bind to insoluble substances, the adhesion of bacterial cells to insoluble substances, and the production of multiple cellulases and xylanases in a form of a high molecular weight complex. Among the six *Bacillus* strains, isolated from various sources and deposited in our laboratory, *Paenibacillus curdlanolyticus* B-6 strain was the best producer of cellulase and xylanase enzymes, which have both cellulose-binding factors (CBFs) and xylan-binding factors (XBFs). Moreover, multiple carboxymethyl cellulases (CMCases) and xylanases were produced by the strain B-6. The zymograms analysis showed at least 9 types of xylanases and 6 types of CMCases associated in a protein band of xylanase and cellulase with high molecular weight. These cells also enabled to adhere to both avicel and insoluble xylan, which were analyzed by scanning electron microscopy. The results indicated that the strain B-6 produced the multienzyme complex, which may be cellulosome or xylanosome. Thus, *P. curdlanolyticus* B-6 was selected to study the role and interaction between the enzymes and their substrates and the cooperation of multiple enzymes to enhance the hydrolysis due to the complex structure for efficient cellulases and xylanases degradation of insoluble polysaccharides.

Key words: Cellulase/cellulose-binding factor, lignocellulosic substances, multienzyme complex, xylanase/xylan-binding factor

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Many cellulolytic microorganisms and their cellulase systems have been studied extensively for degradation of naturally abundant lignocelluloses to valuable products such as fermentable sugars, chemicals, and liquid fuel [6, 10, 14, 18, 34]. Efficient enzymatic degradation of insoluble polysaccharides requires a tight interaction between the enzymes and their substrates and the cooperation of multiple enzymes to enhance the hydrolysis due to the complex structure [21, 22]. Cellulases and xylanases that contained cellulose-binding domains (CBDs) and/or xylan-binding domains (XBDs) are associated into a high molecular weight multienzyme complex, cellulosome, and have been found in many anaerobic cellulolytic microorganisms [2–4, 20, 29] and multienzyme complexes. However, xylanosome was found in only one microorganism [16]. The most intensively studied multienzyme complex is the cellulosome produced by anaerobic thermophilic *Clostridium thermocellum* [21, 4]. The cellulosome in *C. thermocellum* comprises numerous subunits such as different types of glycosyl hydrolases, which are packed into polycellulosomal protuberance-like organelles on the cell surface and serve to promote their synergistic action [2, 25, 26, 36]. Thus, the arrangement of cell wall-degrading enzymes into a multienzyme complex has advantages over free enzyme systems. These cellulosomes are dedicated to hydrolyze lignocellulosic substances because of their ability to bind to insoluble cellulose and/or xylan via CBDs and XBDs, respectively [3]. However, it has been reported that aerobic bacteria produced different cellulolytic enzymes, which exhibited separately [9, 23]. Therefore, we searched for multienzyme complex-producing bacteria under aerobic cultivation to improve the degradation of lignocellulosic substances. This study is necessary to

expand the knowledge concerning the diversity of cellulosome systems in nature [5]. The selection criteria for cellulolytic and xylanolytic multienzyme complexes-producing bacteria were the production of cellulase and xylanase enzyme activities, the presence of CBD and/or XBD in enzymes to bind to insoluble substances, the adhesion of bacterial cells to insoluble substances, and the production of multiple cellulases and xylanases in a form of a high molecular weight complex. Six cellulolytic and xylanolytic *Bacillus* strains, isolated from different sources and deposited in our laboratory, were studied for the production of multienzyme complexes in aerobic condition [17, 28]. This paper describes the selection of the cellulolytic and xylanolytic multienzyme complex-producing bacterium *P. curdlanolyticus* B-6.

Materials and Methods

Microorganisms

Six *Bacillus* strains, isolated from different sources and deposited in our laboratory, were used in this study. *Bacillus* sp. strain S-1 and *Bacillus* sp. strain B-6 were facultative and isolated from an anaerobic digester. *Bacillus* sp. strain H-4 was aerobic and isolated from a wastewater treatment plant of the paper industry in Khon Kean Province. *Bacillus* sp. strain X-11, *Bacillus* sp. strain X-24, and *Bacillus* sp. strain X-26 were alkaliphilic aerobic and isolated from a wastewater treatment plant of the pulp and paper industry in Ayutthaya Province, Thailand.

Production of Enzymes

The bacteria were grown on Berg's mineral salts medium [7] containing 0.5% microcrystalline cellulose (avicel) or xylan. For alkaliphilic bacteria, the pHs of the media were adjusted to pH 10.5 with 1% Na_2CO_3 after sterilization. The cultures were incubated in a rotary incubator at 200 rpm and 37°C and harvested at the late-stationary phase. After centrifugation at 12,000 $\times g$ for 10 min at 4°C, the culture supernatants were used as crude enzymes.

Adherence Test of Cells to Insoluble Substrates

An adherence test of cells to insoluble substrates was performed by a modified method of Bayer *et al.* [1]. Each type of microorganism was grown on microcrystalline cellulose (avicel) and insoluble xylan and harvested at the exponential phase by centrifugation. The cells were washed three times with phosphate-buffered saline (PBS) containing 0.15 M sodium chloride in 10 mM potassium phosphate (pH 7.0) by centrifugation. The washed cells suspension (about 5 mg of cells in 1 ml of PBS) was brought to a total volume of 3 ml with 1 ml of 20% (w/v in PBS) microcrystalline cellulose or insoluble xylan and 1 ml of PBS. The suspension was then vortexed for 40 sec and cellulose containing the adhered cell was allowed to settle at room temperature for 60 min. The turbidity (absorbance at 400 nm) of the suspension was measured and compared with that of an identical cell suspension wherein PBS was substituted for the cellulose suspension.

Scanning Electron Microscopy (SEM)

SEM was used for study of the cell surface [2]. The cells were collected by filtration through a membrane filter (0.45 micron pore size) and dehydrated by a series of graded ethanol solutions and critical-point dried with a critical point dryer (780 Samdri). The preparation was coating with gold and examined with a JEOL JSM-5410LV scanning electron microscope.

Carboxymethyl Cellulase (CMCase) and Xylanase Assays

The assay mixture consisted of 0.1 ml of enzyme solution and 0.5 ml of 1% carboxymethyl cellulose (CMC) or oat spelt xylan in 0.1 M Tris-HCl buffer, pH 7.0. After incubation at 50°C for 10 min, the increase in reducing sugars was determined by the Somogyi-Nelson method [33] using glucose or xylose as a standard. One unit of enzyme activity was defined as that amount of enzyme that liberated 1 μmole of reducing sugars in 1 min under the above conditions.

Protein Determination

Protein concentration was determined according to the Lowry method [24] using bovine serum albumin as a standard.

Cellulose- and Xylan-Binding Assays

In order to test for the cellulose-binding ability, binding of cellulase on avicel was performed by a modified procedure of Bayer *et al.* [4]. A sample of cell-free culture supernatant (1 ml) was mixed with an excess of avicel (50 mg) and the suspension incubated with shaking for 1 h at 4°C. After the suspension was centrifuged, the supernatant was removed and examined for CMCase activity. The amount of activity adsorbed was calculated by subtracting the unabsorbed activity from the value of the initial activity of the sample.

Insoluble xylan was prepared according to the method of Ghangas *et al.* [12]. The xylan-binding assay was performed according to the above procedure using insoluble xylan.

Zymograms of Nondenaturing Polyacrylamide Gel Electrophoresis (PAGE) and Sodium Dodecyl Sulfate-PAGE (SDS-PAGE)

Zymograms of nondenaturing (native) PAGE and SDS-PAGE for xylanase and CMCase activities were prepared on 10% polyacrylamide gels without or with SDS containing 0.1% soluble xylan or 0.1% CMC, as described previously [30].

Table 1. Production of enzymes in *Bacillus* spp.

Bacterial strain (*Bacillus* sp.)	Grown on avicel			Grown on xylan		
	Protein (μg/ml)	Specific activity (U/mg protein)		Protein (μg/ml)	Specific activity (U/mg protein)	
		CMCase	Xylanase		CMCase	Xylanase
Strain S-1	590	0.15	0.90	700	0.09	4.49
Strain B-6	250	0.16	1.12	640	0.12	7.19
Strain H-4	200	0.15	1.10	645	0.09	4.23
Strain X-11	0	0	0	730	0.05	3.29
Strain X-24	0	0	0	720	0.06	3.19
Strain X-26	0	0	0	710	0.04	3.10

RESULTS AND DISCUSSION

Growth and Production of Enzyme

Six bacterial strains were tested for the growth and production of cellulase and xylanase. In the culture conditions used, all strains could grow well on xylan but only the strains S-1, B-6, and H-4 could grow on avicel, whereas alkaliphilic strains X-11, X-24, and X-26 could not grow on it. The highest cellulase and xylanase amounts were produced by the strain B-6, when compared with the activities produced by the other *Bacillus* spp. (Table 1). The strains S-1, B-6, and H-4 produced CMCases and xylanases when grown on avicel or xylan. However, the strains X-11, X-24, and X-26 produced both enzymes when grown on xylan only. Most microorganisms are known to produce cellulase together with xylanase in order to degrade lignocellulosic materials [8, 35]. Among these strains tested, the strain B-6 is the best producer of both enzymes.

Enzyme-Binding Ability to Insoluble Substrates

To investigate the cellulose/xylan-binding abilities, bindings of xylanases of the bacterial strains to avicel and insoluble xylan were conducted. As shown in Table 2, the enzymes produced by all bacterial strains grown on avicel or insoluble xylan could bind to avicel. Therefore, these enzymes have cellulose-binding factors (CBFs). The enzymes have not only cellulose-binding factors but also xylan-binding factors (XBFs), as shown in Table 3. Most cellulolytic enzymes consist of distinct catalytic and non-catalytic cellulose-binding domains (CBDs), and it was already known that the tight binding of the enzyme to insoluble substrate was enhanced by the presence of the CBD for better activity towards insoluble substrate [19]. The removal of the CBD of the enzyme decreased its activity on insoluble but not on soluble substrate [13]. The catalytic domains of the cellulases and xylanases in a cellulosome are attached to a scaffoldin protein through specific docking regions. The scaffoldin has a CBD for binding to cellulose and some of the catalytic domains also have additional CBDs of their own [21, 3].

Adherence Test of Cells to Insoluble Substrates

Since the cellulosome has been shown to be responsible for cellular adherence to cellulose [1], bacterial cells were

Table 2. Binding of xylanase to avicel.

Strain (*Bacillus* sp.) and growth conditions	Xylanase activity (U/ml)			Binding to avicel (%)
	Culture supernatant	Unbound enzyme	Bound enzyme	
1. Strain S-1				
Avicel-grown	0.53	0.30	0.23	43.4
Insoluble xylan-grown	3.14	1.95	1.19	37.9
2. Strain B-6				
Avicel-grown	0.28	0.12	0.16	57.1
Insoluble xylan-grown	4.60	2.80	1.80	39.1
3. Strain H-4				
Avicel-grown	0.20	0.10	0.10	50.0
Insoluble xylan-grown	2.73	1.88	0.85	31.1
4. Strain X-11				
Insoluble xylan-grown	2.40	1.70	0.70	29.2
5. Strain X-24				
Insoluble xylan-grown	2.30	1.62	0.68	29.6
6. Strain X-26				
Insoluble xylan-grown	2.20	1.58	0.62	28.2

Table 3. Binding of xylanase to insoluble xylan.

Strain (*Bacillus* sp.) and growth conditions	Xylanase activity (U/ml)			Binding to insoluble xylan (%)
	Culture supernatant	Unbound enzyme	Bound enzyme	
1. Strain S-1				
Avicel-grown	0.53	0.27	0.26	49.1
Insoluble xylan-grown	3.14	1.70	1.44	45.8
2. Strain B-6				
Avicel-grown	0.28	0.10	0.18	64.3
Insoluble xylan-grown	4.60	2.23	2.37	51.5
3. Strain H-4				
Avicel-grown	0.24	0.12	0.12	50.0
Insoluble xylan-grown	2.73	1.68	1.05	38.5
4. Strain X-11				
Insoluble xylan-grown	2.40	1.32	1.08	45.0
5. Strain X-24				
Insoluble xylan-grown	2.30	1.47	0.83	36.1
6. Strain X-26				
Insoluble xylan-grown	2.20	1.36	0.84	38.2

tested for binding to avicel and insoluble xylan. Table 4 gives the results of such adherence test conducted on bacterial cells of six strains grown on avicel and insoluble xylan. The only the strain B-6, grown on avicel or insoluble xylan, could adhere to both avicel and insoluble xylan, whereas the other strains could not adhere to both of them. These results suggested that the strain B-6 had an essential component responsible for anchoring the cellulose- and xylan-binding factors to the cell surface. There have been reports on the adhesion of cellulosome-producing anaerobic bacterial cells, such as *C. thermocellum*, *Fibrobacter succinogens*, *Ruminococcus albus*, and *Bacteroides* sp. P-1,

Table 4. Adherence test of bacterial cells to avicel and insoluble xylan.

Strain (*Bacillus* sp.) and growth conditions	A_{400} for the following suspensions:		
	Control	Avicel added	Insoluble xylan added
Strain S-1			
Avicel-grown	0.224	0.231	0.233
Insoluble xylan-grown	0.348	0.349	0.350
Strain B-6			
Avicel-grown	0.418	0.301	0.251
Insoluble xylan-grown	0.553	0.478	0.140
Strain H-4			
Avicel-grown	0.371	0.373	0.372
Insoluble xylan-grown	0.420	0.429	0.420
Strain X-11			
Insoluble xylan-grown	0.476	0.481	0.480
Strain X-24			
Insoluble xylan-grown	0.419	0.427	0.425
Strain X-26			
Insoluble xylan-grown	0.418	0.420	0.420

to cellulose by means of protuberant structures on the cell surface, on which the multienzyme complex was designed for efficient binding and hydrolysis of the substrate [27, 29, 31]. The structural localization of cellulosome on the cell surface of the anaerobic bacterium *C. thermocellum* has been previously studied by SEM, which showed protuberant structures on the cell surface [2]. To confirm the presence of protuberant structures on the strain B-6 cell surface, cells harvested at the early-stationary phase (24 h) and late-stationary phase (36 h) of growth on xylan were analyzed by SEM. The surface of the cells at the early-stationary phase showed the adhesion of the cell to xylan (Fig. 1). This result is similar to the cellulosomal protuberances of *C. thermocellum*,

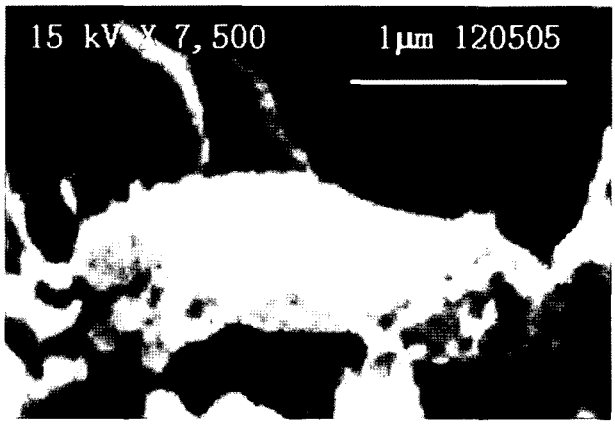

Fig. 1. SEM of the cell surface structure of the strain B-6.
SEM of the cell surface structure of the strain B-6 grown on xylan at the early-stationary phase (24 h). The sample was collected by filtration through a membrane filter (0.45 micron pore size) and dehydrated by a series of graded ethanol solutions and critical-point dried with a critical point dryer (780 Samdri). The preparation was coating with gold and examined with a JEOL JSM-5410LV scanning electron microscope.

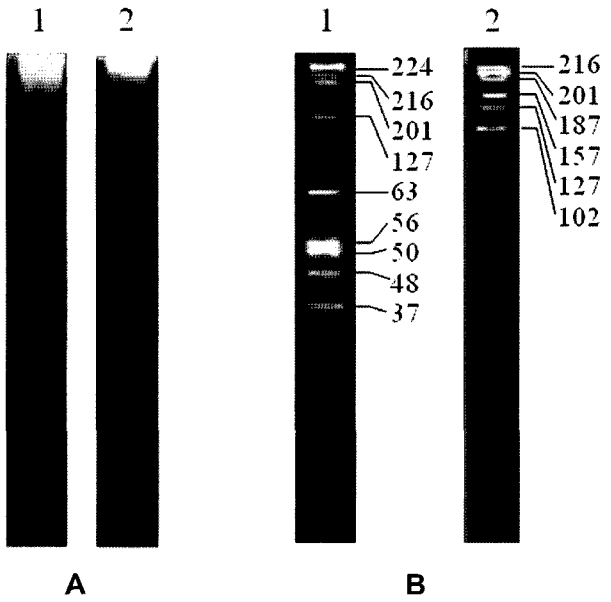

Fig. 2. Zymograms of native-PAGE (**A**) and (**B**) SDS-PAGE of xylanases and CMCases of the strain B-6.

The bacteria were grown on Berg's mineral salts medium containing 0.5% xylan. The cultures were incubated in a rotary incubator at 200 rpm and 37°C and harvested at the late-stationary phase (36 h). After centrifugation at 12,000 ×*g* for 10 min at 4°C, the culture supernatants were concentrated by using a rapid flow filtration capsule with a 10-kDa cutoff membrane (Minimate TFF capsule W/100Ka Omega) and used as samples to analyze the zymograms of native-PAGE (**A**) and SDS-PAGE (**B**). Zymograms of native-PAGE and SDS-PAGE were performed in 10% polyacrylamide gels without or with SDS, by the method of Ratanakhanokchai *et al.* [30]. Lanes 1 and 2 are zymograms for xylanases and CMCases, respectively.

whereas the surface of the cells at the late-stationary phase lacked such structures because the multienzyme complex was released into the medium from the cell surface [2].

Multiple Cellulases and Xylanases

The presence of multiple cellulases and xylanases in the culture supernatants of all bacteria was revealed by zymograms analysis. It showed that after growth on xylan, all those strains except the strain B-6 had only 2 or 3 xylanase bands, whereas the strains S-1 and H-4 showed 1 or 2 cellulase bands. However, the strain B-6 grown on xylan had 9 xylanases and 6 cellulases (Fig. 2), indicating that the strain B-6 produced multiple cellulases and xylanases.

Zymograms of the Strain B-6

It has been reported that at the late-stationary phase, the multienzyme complex on the protuberances on the cell surface was released into the culture medium [2]. Thus, the culture supernatant of the strain B-6 at this phase was analyzed by zymograms. It was found to consist of a high molecular weight band of xylanase and CMCase on zymograms of native-PAGE (Fig. 2A) and that protein band was dissociated into major and minor components by treatment with boiling in SDS solution, showing more than 9 and 6 subunits of xylanases and CMCases, respectively (Fig. 2B). The result confirmed that the high molecular weight product might be a multienzyme complex associated with at least 9 xylanases and 6 CMCases. In *C. thermocellum*, the cellulosome consisted of many different types of glycosyl hydrolases, including cellulases, hemicellulases, and carbohydrate esterases, which served to promote their synergistic action [21].

Therefore, the results revealed that among the bacteria tested, only the strain B-6 produced a multienzyme complex, cellulosome or xylanosome. The strain B-6 was thus selected and its multienzyme complex will be further studied for its role in the mechanism related to cellulases and xylanases degradation of insoluble polysaccharides and industrial waste materials.

Identification of the Bacterium

The bacterium strain B-6 was a facultative, spore-forming, Gram-positive, motile, rod-shaped organism and produced catalase. Thus, this bacterium was identified as a member of the genus *Bacillus* according to *Bergey's Manual of Systematic Bacteriology* [11]. The bacterium was also identified by 16S rRNA gene sequence analysis. The use of a specific PCR primer designed for differentiating the genus *Paenibacillus* from other members of the *Bacillaceae* showed that this strain had the same amplified 16S rRNA gene fragment as a member of the genus *Paenibacillus*. Base on these observations, this strain was transferred to the genus *Paenibacillus* [32]. The 16S rDNA sequence of this strain had 1,424 base pairs and 97% similarity with *Paenibacillus curdlanolyticus* [15]. Therefore, it was tentatively identified as *Paenibacillus curdlanolyticus* B-6.

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