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Chapter 16

Paenibacillus curdlanolyticus Strain B-6 Multienzyme Complex: A Novel System for Biomass Utilization

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Additional information is available at the end of the chapter

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1. Introduction

To develop a bio-based economy for sustainable economic growth, it is necessary to produce chemicals and fuels from renewable resources, such as plant biomass. Plant biomass contains a complex mixture of polysaccharides, mainly cellulose and hemicellulose (mainly xylan), and other polysaccharides (Aspinall, 1980). The hemicelluloses, as well as the aromatic polymer lignin, interact with the cellulose fibrils, creating a rigid structure strengthening the plant cell wall. Therefore, complete and rapid hydrolysis of these polysaccharides requires not only cellulolytic enzymes but also the cooperation of xylanolytic enzymes (Thomson, 1993). Many microorganisms that produce enzymes capable of degrading cellulose and hemicellulose have been reported and characterized. Two enzyme systems are known for their degradation of lignocellulose by microorganisms. In many aerobic fungi and bacteria, endoglucanase, exoglucanase, and ancillary enzymes are secreted individually and can act synergistically on lignocellulose. The most thoroughly studied enzymes are the glycosyl hydrolases of *Trichoderma reesei* (Dashtban et al., 2009). On the other hand, several anaerobic cellulolytic microorganisms such as *Clostridium thermocellum* (Lamed & Bayer, 1988), *C. cellulovorans* (Doi et al., 2003), *C. josui* (Kakiuchi et al., 1998) and *C. cellulolyticum* (Gal et al., 1997) are known to produce a cell-associated, large extracellular polysaccharolytic multicomponent complex called the cellulosome, in which several cellulolytic and xylanolytic enzymes are tightly bound to a scaffolding protein (core protein). Thus, the cellulosome provides for a large variety of enzymes and attractive enzymatic properties for the degradation of recalcitrant plant biomass. So far, anaerobic microorganisms have been identified as producing the multienzyme complex, cellulosome

(Doi & Kosugi, 2004; Demain et al., 2005). However, when compared with aerobic enzymes, production of those enzymes by anaerobic culture presents a high cost because of the high price of medium, slow rate of growth and low yield of enzyme, while only a little information has been reported on cellulosome-like multienzyme complex produced by aerobic bacteria (Kim & Kim, 1993; Jiang et al,, 2004; van Dyk et al., 2009). Therefore, the multienzyme complexes, cellulosomes, produced by aerobic bacteria show great potential for improving plant biomass degradation. A facultatively anaerobic bacterium, *P. curdlanolyticus* strain B-6, is unique in that it produces extracellular xylanolytic-cellulolytic multienzyme complex under aerobic conditions (Pason et al., 2006a, 2006b; Waeonukul et al., 2009b). In the following years, the characteristics, function, genetics and mechanism of the xylanolytic-cellulolytic enzymes system of this bacterium has been the subject of considerable research. In light of new findings in this field, this review will describe the state of knowledge about the multienzyme complex of strain B-6 and its potential biotechnological exploitations.

1.1. Composition of lignocellulosic biomass

Lignocellulosic biomass is composed mainly of plant cell walls, with the structural carbohydrates, cellulose and hemicelluloses and heterogeneous phenolic polymer lignin as its primary components (Fig. 1). However, their proportions vary substantially, depending on the type, the species, and even the source of the biomass (Aspinall et al., 1980; Pérez et al., 2002; Pauly et al., 2008).

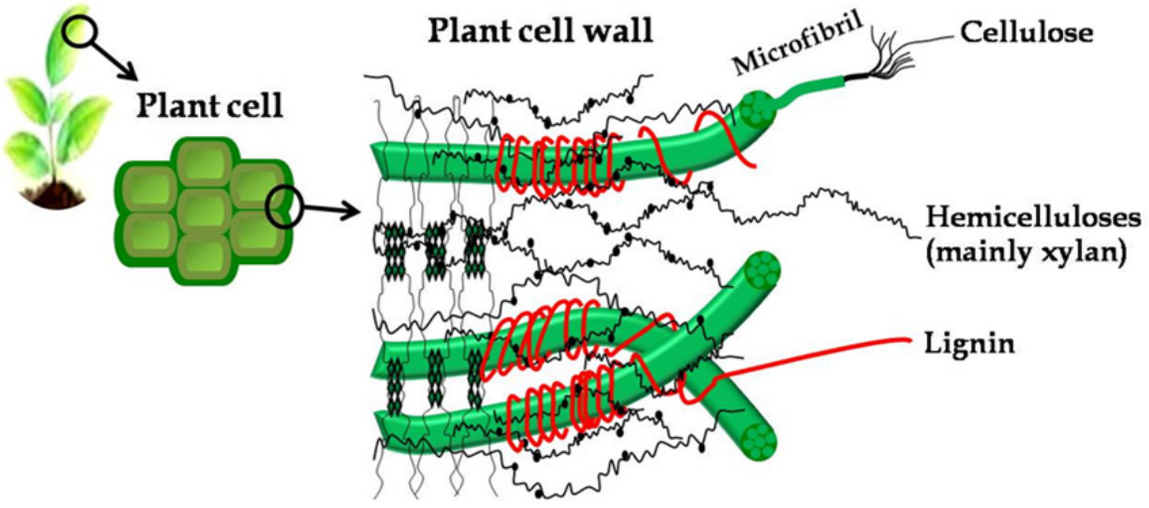

Figure 1. Structure of lignocellulosic plant biomass. (This figure is adapted from Tomme et al., 1995).

Cellulose: Cellulose, the main constituent of the plant cell wall, is a polysaccharide composed of linear glucan chains linked together by β-1,4-glycosidic bonds with cellobiose residues as the repeating unit at different degrees of polymerization, depending on resources. The cellulose chains are grouped together to form microfibrils, which are bundled together to form cellulose fibers. The cellulose microfibrils are mostly independent but the ultrastructure of cellulose is largely due to the presence of covalent bonds, hydrogen bonds

and Van der Waals forces. Hydrogen bonding within a cellulose microfibril determines 'straightness' of the chain but inter-chain hydrogen bonds might introduce order (crystalline) or disorder (amorphous) into the structure of the cellulose (Klemm et al., 2005). In the latter conformation, cellulose is more susceptible to enzymatic degradation (Pérez et al., 2002). In nature, cellulose appears to be associated with other plant compounds and this association may affect its biodegradation.

Hemicelluloses: Hemicelluloses are the second most abundant polymers and differ from cellulose in that they are not chemically homogeneous. Hemicelluloses are branched, heterogenous polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose) and acetylated sugars. They have lower molecular weight compared to cellulose and branches with short lateral chains that are easily hydrolysed (Saha, 2003; Scheller & Ulvskov, 2010). Hemicelluloses differ in composition. Hemicelluloses in agricultural biomass like straws and grasses are composed mainly of xylan, while softwood hemicelluloses contain mainly glucomannan. In many plants, xylans are heteropolysaccharides with backbone chains of 1,4-linked β-D-xylopyranose units. In addition to xylose, xylan may contain arabinose, glucuronic acid, or its 4-*O*-methyl ether, acetic acid, ferulic and *p*-coumaric acids. Hemicelluloses are bound via hydrogen bonds to the cellulose microfibrils in the plant cell wall, crosslinking them into a robust network. Hemicelluloses are also covalently attached to lignin, forming together with cellulose to form a highly complex structure.

Lignin: Lignin is the third most abundant polymer in nature. It is present in plant cell walls and confers a rigid, impermeable, resistance to microbial attack and oxidative stress. Lignin is a complex network formed by polymerization of phenyl propane units and constitutes the most abundant non-polysaccharide fraction in lignocelluloses (Pérez et al., 2002; Sánchez, 2009). The three monomers in lignin are *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol; they are joined through alkyl–aryl, alkyl–alkyl and aryl–aryl ether bonds. Lignin embeds the cellulose thereby offering protection against microbial and enzymatic degradation. Furthermore, lignin is able to form covalent bonds to some hemicelluloses, e.g. benzyl ester bonds with the carboxyl group of 4-*O*-methyl-D-glucuronic acid in xylan. More stable ether bonds, also known as lignin carbohydrate complexes, can be formed between lignin and arabinose, or between galactose side groups in xylans and mannans.

1.2. Biodegradation of lignocellulosic biomass

Several biological methods for lignocellulose recycling based on the enzymology of cellulose, hemicelluloses, and lignin degradation have been developed. To date, processes that use lignocellulolytic enzymes or microorganisms could lead to promising, environmentally friendly technologies. The relationship between cellulose and hemicellulose in the cell walls of higher plants is much more intimate than was previously thought. It is possible that molecules at the cellulose-hemicellulose boundaries, and those within the crystalline cellulose, require different enzymes for efficient hydrolysis.

Cellulase: Cellulases responsible for the hydrolysis of cellulose are composed of a complex mixture of enzymes with different specificities to hydrolyze the β-1,4-glycosidic linkages (Fig. 2A). Cellulases can be divided into three major enzyme activity classes (Goyal et al., 1991; Rabinovich et al., 2002). These are endoglucanases or endo-1-4-β-glucanase (EC 3.2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). Endoglucanases, are thought to initiate attack randomly at multiple internal sites in the amorphous regions of the cellulose fiber, which opens-up sites for subsequent attack by the cellobiohydrolases. Cellobiohydrolases remove cellobiose from the ends of both sides of the glucan chain. Moreover, cellobiohydrolase can hydrolyze highly crystalline cellulose. β-glucosidase hydrolyzes cellobiose and in some cases short chain cellooligosaccharides to glucose.

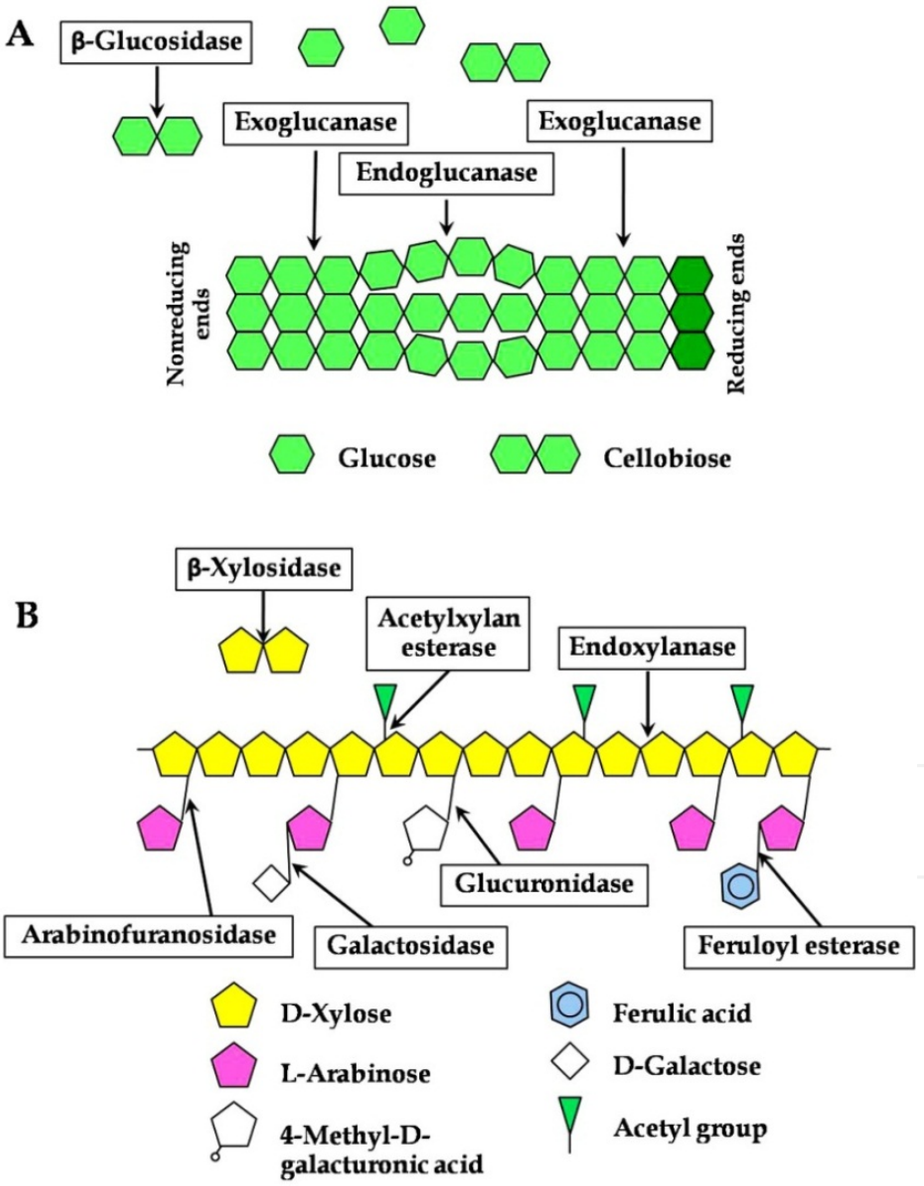

Figure 2. Enzyme systems involved in the degradation of cellulose (A) and xylan (B). (This figure is adapted from Aro et al., 2005).

Hemicellulase: Xylan is the main carbohydrate found in hemicelluloses. Its complete degradation requires the cooperative action of a variety of hydrolytic enzymes (Fig. 2B). Xylanases are frequently classified according to their action on distinct substrates: endo-1,4-β-xylanase (endoxylanase) (EC 3.2.1.8) generates xylooligosaccharides from the cleavage of xylan while 1,4-β-xylosidase (EC 3.2.1.37) produces xylose from xylobiose and short chain xylooligosaccharides. In addition, xylan degradation needs accessory enzymes, such as α-L-arabinofuranosidase (EC 3.2.1.55), α-4-*O*-methyl-D-glucuronidase (EC 3.2.1.39), acetyl xylan esterase (EC 3.1.1.72), ferulic acid esterase (EC 3.1.1.73), and *p*-coumaric acid esterase (EC 3.1.1.-), acting synergistically, to efficiently hydrolyze wood xylans. In the case of acetyl-4-*O*-methylglucuronoxylan, which is one of the most common hemicelluloses, four different enzymes are required for degradation: endo-1,4-β-xylanase, acetyl esterase (EC 3.1.1.6), α-glucuronidase, and β-xylosidase. The degradation of *O*-acetyl galactoglucomannan starts with the rupture of the polymer by endomannanase (EC 3.2.1.78). Acetylglucomannan esterase (EC 3.1.1.-) removes acetyl groups, and α-galactosidase (EC 3.2.1.22) eliminates galactose residues. Finally, β-mannosidase (EC 3.2.1.85) and β-glucosidase break down the endomannanase-generated oligomeric β-1,4 bonds (Thomson, 1993; Li et al., 2000; Pérez et al., 2002).

1.3. Multienzyme complex cellulosome

The enzyme systems for the lignocellulose degradation by microorganisms can be generally regarded as non-complexed or complexed enzymes (Lynd et al., 2002). In the case of aerobic fungi and bacteria, the cellulase enzymes are free and mostly secreted. In such organisms, by the very nature of the growth of the organisms, they are able to reach and penetrate the cellulosic substrate and, hence, the secreted cellulases are capable of hydrolyzing the substrate. The enzymes in these cases are not organized into high molecular weight complexes and are called non-complexed (Fig. 3A). The polysaccharide hydrolases of the aerobic fungi are largely described based on the examples from *Trichoderma, Penicillum, Fusarium, Humicola, Phanerochaete*, etc., where a large number of the cellulases are encountered (Dashtban et al., 2009; Sánchez, 2009). In contrast, various cellulases and hemicellulases from several anaerobic cellulolytic microorganisms, are tightly bound to a scaffolding protein, as core protein and organized to form structures on the cell surfaces; these systems are called complexed enzymes or cellulosomes (Fig. 3B). The cellulosome is thought to allow concerted enzyme activities in close proximity to the bacterial cell, enabling optimum synergism between the enzymes presented on the cellulosome. Concomitantly, the cellulosome also minimizes the distance over which hydrolysis products must diffuse, allowing efficient uptake of these oligosaccharides by the host cells (Bayer et al., 1994; Schwarz, 2001; Lynd et al., 2002).

Biotechnological applications in terms of hydrolysis efficiency for complexed enzyme systems might have an advantage over non-complexed enzyme systems. The high efficiency of the cellulosome has been attributed to (i) the correct ratio between catalytic domains that optimize synergism between them, (ii) appropriate spacing between the individual

components to further favor synergism, (iii) the presence of different enzymatic activities (cellulolytic or hemicellulolytic enzymes) in the cellulosome that can remove "physical hindrances" of other polysaccharides in heterogeneous plant cell materials (Lynd et al., 2002), and (iv) the presence of carbohydrate-binding modules (CBMs) that can increase the rate of hydrolysis by bringing the cellulosome into intimate and prolonged association with its recalcitrant substrate (Shoseyov et al., 2006). Thus, the complexed enzyme system, cellulosome, may provide great potential for the degradation of plant biomass.

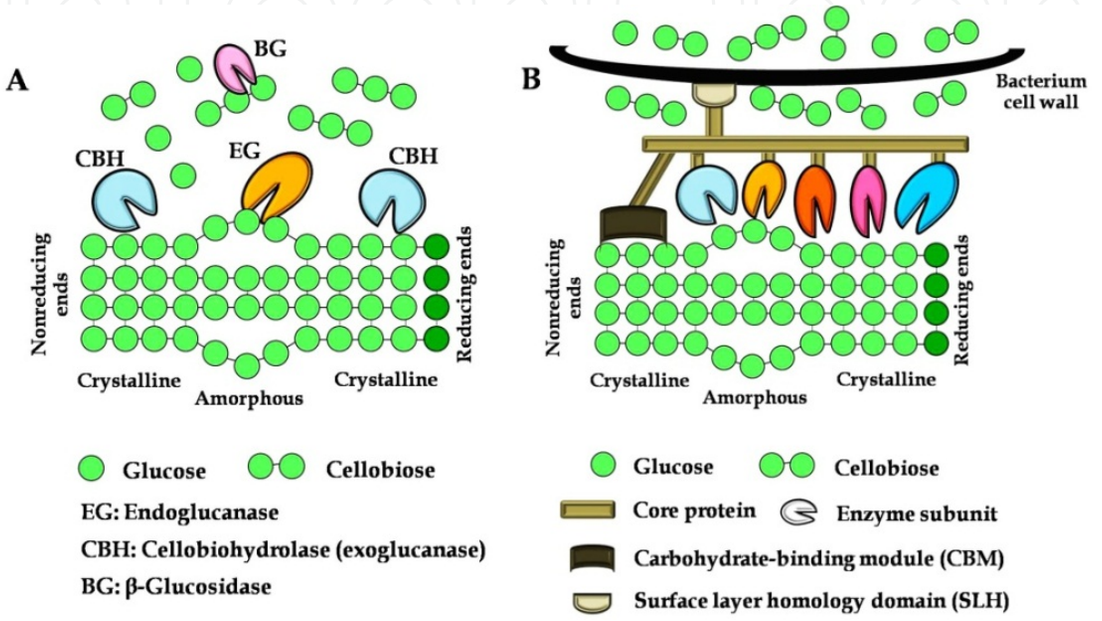

Figure 3. Simplified schematic of the hydrolysis of amorphous and microcrystalline celluloses by non-complexed (A) and complexed (B) cellulase systems. (This figure is adapted from Lynd et al., 2002).

The cellulosome was first identified in 1983 from the anaerobic, thermophilic, spore-forming *Clostridium thermocellum* (Lamed et al., 1983). The cellulosome of *C. thermocellum i*s commonly studied along with cellulosomes from the anaerobic mesophiles, *C. cellulovorans* (Doi et al., 2003), *C. josui* (Kakiuchi et al., 1998) and *C. cellulolyticum* (Gal et al., 1997). All cellulosomes share similar characteristics, they all contain a large distinct protein, referred to as the scaffoldin which allows binding of the whole complex to microcrystalline cellulose via CBM. Also, the cellulosome scaffoldin expresses type I cohesins which allow binding of a wide variety of cellulolytic and hemicellulolytic enzymes within the complex via the expression of complementary type I dockerins on enzymes. Similarly, at the C-terminal the scaffoldin expresses type II cohesins, which allow the binding of the cellulosome to the cell through type II dockerins on surface layer homology proteins (SLH) (Fig. 4).

Cellulosomes are produced mainly by anaerobic bacteria, mostly from the class clostridia, and some anaerobic fungi such as genus *Neocallimastix* (Dalrymple et al., 1997), *Piromyces* (Teunissen et al., 1991) and *Orpinomyces* (Li et al., 1997). However, evidence suggests the presence of cellulosomes or cellulosome-like multienzyme complexes in a few aerobic microorganisms (Table 1). It is speculated that several other cellulolytic bacteria may also produce cellulosomes not yet described.

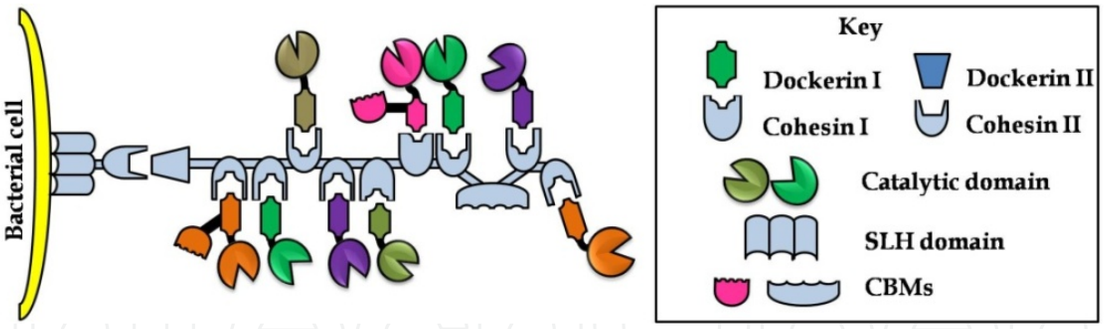

Figure 4. Simplified schematic of general cellulosome components and connection with cell surface based on knowledge of *Clostridium* cellulosome. (This figure is adapted from Bayer et al., 1994).

Anaerobic			Aerobic		
Microorganism	Source	Ref.	Microorganism	Source	Ref.
Bacteria			***Bacteria***		
Acetivibrio cellulolyticus	Sewage	Ding et al., 1999	*Bacillus circulans* F-2	Potato starch granules	Kim and Kim, 1993
Amorocellulobacter alkalithermophilum	Soil	Watthanalarm-lort et al., 2012	*Bacillus licheniformis* SVD1	Bioreactor	van Dyk et al., 2009
Bacteroides cellulosolvens	Sewage	Ding et al., 2000	*Paenibacillus curdlanolyticus* B-6	Anaerobic digester	Pason et al., 2006b
Bacteroides sp. strain P-1	Anaerobic digester	Ponpium et al., 2000	*Sorangium cellulosum*	Soil	Hou, et al., 2006
Butyrivibrio fibrisolvens	Rumen	Berger et al., 1990			
Clostridium acetobutylicum	Soil	Sabathé et al.,2002			
Clostridium cellobioparum	Rumen	Lamed et al., 1987	***Actinomycetes***		
Clostridium cellulolyticum	Compost	Pagès et al., 1997	*Streptomyces olivaceoviridis* E-86	Soil	Jiang et al., 2004
Clostridium cellulovorans	Fermenter	Sleat et al., 1984			
Clostridium josui	Compost	Kakiuchi et al., 1998	***Fungi***		
Clostridium papyrosolvens	Paper mill	Pohlschröder et al., 1994	*Chaetomium* sp. Nov. MS-017	Rotted wood	Ohtsuki et al., 2005
Clostridium thermocellum	Sewage soil	Lamed et al., 1983			
Eubacterium cellulosolvens	Rumen	Blair and Anderson, 1999b			
Ruminococcus albus	Rumen	Ohara et al., 2000			
Ruminococcus flavefaciens	Rumen	Ding et al., 2001			
Tepidimicrobium xylanilyticum BT14	Soil	Phitsuwan et al., 2010			

Anaerobic			Aerobic		
Microorganism	Source	Ref.	Microorganism	Source	Ref.
Thermoanaerobacterium thermosaccharolyticum NOI-1	Soil	Chimtong et al., 2011			
Fungi					
Neocallimastix patriciarum	Rumen	Dalrymple et al., 1997			
Orpinomyces joyonii	Rumen	Qiu et al., 2000			
Orpinomyces PC-2	Rumen	Borneman et al., 1989			
Piromyces equi	Rumen	Teunissen et al., 1991			
Piromyces E2	Faeces	Teunissen et al., 1991			

Table 1. Cellulosome and cellulosome-like multienzyme complexes from anaerobic and aerobic microorganisms. (This table is adapted from Doi & Kosugi, 2004).

2. Novel multienzyme complex system from *P. curdlanolyticus* strain B-6

Efficient enzymatic degradation of lignocellulosic biomass 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. Multienzyme complexes, cellulosomes from anaerobic cellulolytic microorganisms, are dedicated to hydrolyzing lignocellulosic substances efficiently because of a large variety of cellulases and hemicellulases in complexes, useful enzymatic properties, and binding ability to insoluble cellulose and/or xylan via CBMs (Bayer et al., 2004; Doi and Kosugi, 2004; Schwarz et al., 2001; Shoham et al., 1999). When compared with aerobic enzymes, they produce several individual enzymes, but microorganisms are not binding to insoluble substrates. However, *P. curdlanolyticus* B-6 was found to produce a multienzyme complex under aerobic conditions (Pason et al., 2006a, 2006b). Little information has been reported on cellulosome-like multienzyme complexes produced by aerobic bacterium (Kim & Kim, 1993; Jiang et al., 2004; van Dyk et al., 2009). Therefore, the multienzyme complex produced by strain B-6 is critical for improving plant biomass degradation.

2.1. Selection of multienzyme complex-producing bacteria under aerobic cultivation

Among several *Bacillus* strains, isolated from various sources and cultivated under aerobic conditions, *P. curdlanolyticus* strain B-6 shows important evidences for multienzyme complex producing bacterium (Pason et al., 2006a) as follows: high production of cellulase and xylanase, presence of CBMs that have ability to bind to insoluble substances, adhesion of bacterial cells to insoluble substances, and production of multiple cellulases and

xylanases in the form of a high molecular weight complex. Thus, strain B-6 exhibits great promise bacterium in the production of multienzyme complex under aerobic conditions. Some properties of bacterial cells and cellulase and xylanase from strain B-6 compared with other *Bacillus* spp. are shown in Table 2.

Strain (*Bacillus* sp.) and growth condition	Specific activity (U/mg protein)		Enzyme binding ability to insoluble substances (%)		Adhesion of cells to insoluble substances (%)		Zymogram analysis	
	CMCase	Xylanase	Avicel	Xylan	Avicel	Xylan	CMCase band	Xylanase band
1. Strain B-6								
Avicel grown	0.16	1.12	57.1	64.3	28.0	39.9	11	13
Xylan grown	0.12	7.19	39.1	51.5	13.6	74.7	9	15
2. Strain H-4								
Avicel grown	0.15	1.10	50.0	50.0	0	0	2	2
Xylan grown	0.09	4.23	31.1	38.5	0	0	1	3
3. Strain S-1								
Avicel grown	0.15	0.90	43.4	49.1	0	0	3	2
Xylan grown	0.09	4.49	37.9	45.8	0	0	2	3
4. Strain X-11								
Avicel grown	0	0	0	0	0	0	0	0
Xylan grown	0.05	3.29	29.2	45.0	0	0	0	2
5. Strain X-24								
Avicel grown	0	0	0	0	0	0	0	0
Xylan grown	0.06	3.19	29.6	36.1	0	0	0	2
6. Stain X-26								
Avicel grown	0	0	0	0	0	0	0	0
Xylan grown	0.04	3.10	28.2	38.2	0	0	0	2

Table 2. Production of carboxymethyl cellulase (CMCase) and xylanase by *Bacillus* strains; binding ability of enzymes to insoluble substances; adherence of bacterial cells to insoluble substances; and zymograms analysis in culture supernatant.

P. curdlanolyticus 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 (Sneath, 1986). 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*. Based on these observations, it is reckoned that this strain was transferred to the genus *Paenibacillus* (Shida et al., 1997). The 16S rDNA sequence of this strain had 1,424 base pairs and 97% similarity with *Paenibacillus curdlanolyticus* (Innis & Gelfand, 1990).

2.2. Characteristics of *P. curdlanolyticus* B-6 multienzyme complex

During growth of *P. curdlanolyticus* B-6 on Berg's mineral salt medium containing 0.5% xylan as carbon sources, the protein concentration in the medium was low up to the late stationary growth phase. CMCase and xylanase activities could be detected in the culture medium after the late exponential phase (Pason et al., 2006b). At the declining growth phase, the extracellular xylanase and CMCase rapidly increased due to the release of enzymes from the cell surfaces into the culture medium. These phenomena were different from the growth patterns of other aerobic bacteria, which grew and produced extracellular enzymes into culture supernatant immediately, but similar to those of the anaerobic bacteria which produced multienzyme complexes (cellulosomes) around the cell surfaces and adhered to these substrates and secreted into culture supernatant later (Bayer & Lamed, 1986; Lamed & Bayer, 1988). The observation of cell surfaces at the late exponential growth phase by scanning electron microscopy (SEM) revealed that the cells adhered to xylan (Fig. 5A), similar to the cells of the cellulosome producing anaerobic bacterium, *C. thermocellum*, which is a cell associated entity that mediates the adhesion of the bacterium to cellulose (Lamed et al., 1987; Mayer et al., 1987), whereas the surface of the cells of strain B-6 at the late stationary growth phase lacked such structures because the multienzyme complex was released into the medium from the cell surfaces (Fig. 5B). In addition, the pattern of multienzyme complex in the culture medium at the late stationary growth phase was determined. Native-polyacrylamide gel electrophoresis (native-PAGE) exhibited a high molecular weight band at the top of the gel (Fig. 6, lane 1). This protein band was dissociated into major and minor components through treatment by boiling in sodium dodecyl sulphate (SDS) solution, showing at least 18 proteins with molecular masses in the range of 29 to 280 kDa (Fig. 6, lane 2). Among those protein bands, at least 15 bands showed xylanase activities (Fig. 6, lane 3) and at least 9 bands showed CMCase activities (Fig. 6, lane 4) on zymograms. These multiple cellulases and xylanases are assembled into the high molecular weight complexes and released from the cell surfaces into medium at the late stationary growth phase. 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 (Lamed et al., 1983). These evidences confirm that the strain B-6 can produce xylanolytic-cellulolytic enzyme system that exists as multienzyme complex under aerobic conditions.

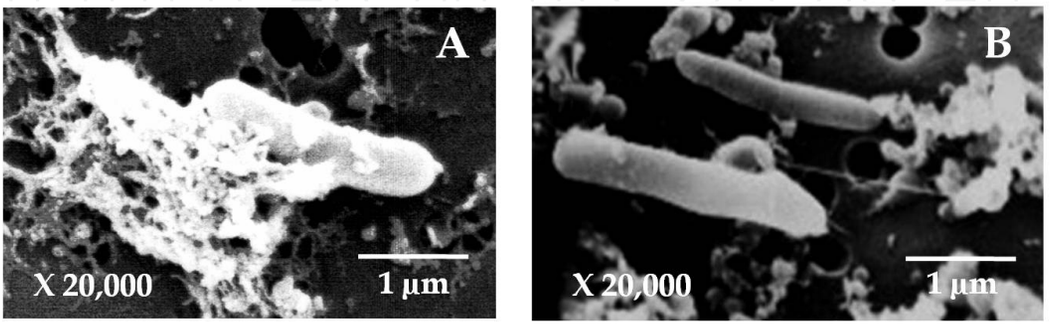

Figure 5. SEM of the cell surfaces of *P. curdlanolyticus* B-6 harvested at the late exponential growth phase showing adhesion of cell to xylan (A) and the cell harvested at the late stationary growth phase showing no adhesion of cell to xylan (B).

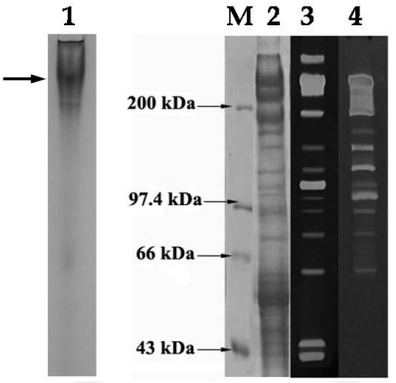

Figure 6. Proteins and enzymes patterns of multienzyme complex in culture supernatant at the late stationary growth phase; Native-PAGE (lane 1), SDS-PAGE (lane 2), and zymograms analysis of xylanase activity (lane 3), and CMCase activity (lane 4).

2.3. Effect of carbon sources on the induction of multienzyme complex in *P. curdlanolyticus* B-6

The effect of polymeric substances such as cellulose, xylan, corn hull, and sugarcane bagasse, and of soluble sugars such as L-arabinose, D-galactose, D-glucose, D-xylose, and cellobiose on the induction of multienzyme complexes in a facultatively anaerobic bacterium, *P. curdlanolyticus* B-6, was investigated under aerobic conditions (Waeonukul et al., 2008; 2009b). Cells grown on each carbon source adhered to cellulose. Hence strain B-6 cells from all carbon sources must have an essential component responsible for anchoring the cells to the substrate surfaces. Native–PAGE, SDS–PAGE, zymograms analysis, and enzymatic assays revealed that many proteins having xylanolytic and cellulolytic activities from *P. curdlanolyticus* B-6 grown on each carbon source were produced as multienzyme complex into the culture supernatants. These results indicated that strain B-6 produced multienzyme complexes when grown on both polymeric substances and soluble sugars. However, the subunits expressed in the multienzyme complex of strain B-6 depended on the carbon sources. These observations are consistent with previous reports that the enzymatic activities and enzyme compositions of the cellulosomes of *C. thermocellum* (Bayer et al., 1985; Bhat et al., 1993; Nochur et al., 1993), *C. cellulolyticum* (Mohand-Oussaid et al., 1999), and *C. cellulovorans* (Kosugi et al., 2001; Han et al., 2004; 2005) and the xylanosome of *S. olivaceoviridis* E-86 (Jiang et al., 2004) were affected by carbon sources in the media.

Many investigators have reported that the synthesis of cellulosome assemblies requires the presence of crystalline cellulose under anaerobic conditions, and that synthesis hardly occurs in growth on glucose or other soluble carbohydrates (Nochur et al., 1992; Blair & Anderson; 1999a; Bayer 2004; Doi & Kosugi, 2004). Some strains of *C. thermocellum* (Bayer et al., 1985; Bhat et al., 1993), however, can induce cellulosome synthesis when grown on cellobiose. *P. curdlanolyticus* B-6 differs from most cellulosome-producing microorganisms in that it produces multienzyme complex when grown on both polymeric substances and soluble sugars under aerobic conditions. Therefore, the mechanism of multienzyme complex formation by strain B-6 must be different from that of other microorganisms.

3. The feature of *P. curdlanolyticus* B-6 multienzyme complex

Recently, the structures and mechanisms for assembly of multienzyme complexes, cellulosomes, in anaerobic cellulolytic microorganisms are clear (Bayer et al., 2004, 2007; Doi & Kosugi, 2004). Generally, the key feature of the cellulosome is a scaffoldin that integrates the various catalytic subunits into the complex by self-assembly by cohesion-dockerin interaction. However, the structure and mechanism of the multienzyme complex produced by a facultatively anaerobic bacterium, such as *P. curdlanolyticus* B-6 is still unknown. In order to describe features of the multienzyme complex system produced by strain B-6, the multienzyme complex was purified by four kinds of chromatography (cellulose affinity, gel filtration, anion-exchange and hydrophobic-interaction chromatographys) (Fig. 7).

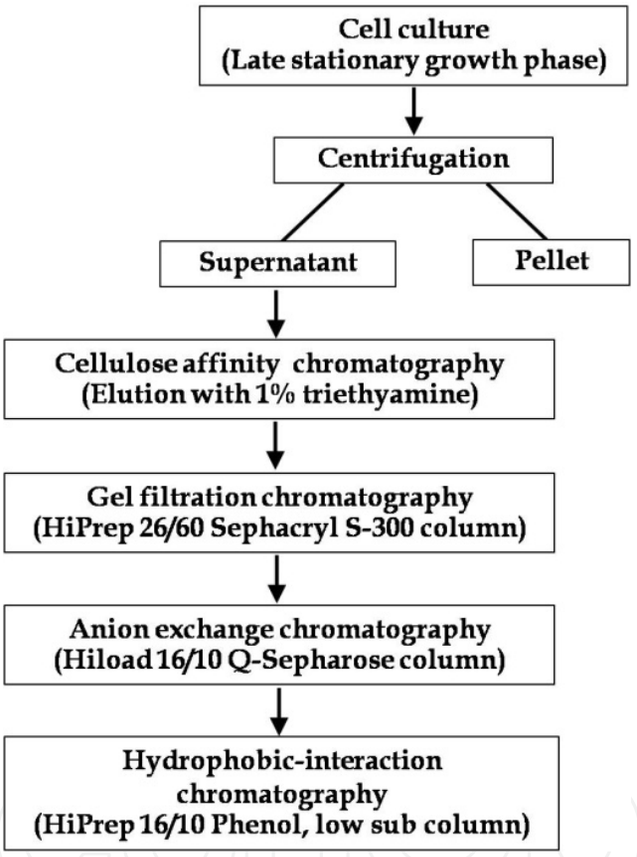

Figure 7. Isolation and purification of multienzyme complex of *P. curdlanolyticus* strain B-6.

The multienzyme complex of *P. curdlanolyticus* strain B-6 with molecular mass of 1,450 (G1) was isolated from culture supernatant at the late stationary growth phase through cellulose affinity and Sephacryl S-300 gel filtration chromatographys (Pason et al., 2006b). Basically, the individual cellulosomes from anaerobic bacteria show 600 kDa to 2.1 MDa complexes size and show cohesion-dockerin domain as a signature protein (Bayer et al., 2004; Doi & Kosugi, 2004). While, multienzyme complexes from aerobic microorganisms, were range in mass from about 468 kDa to 2 MDa (with contained 5-12 protein subunits) (Table 3) and has no report of cohesion-dockerin domain. Here, the multienzyme complex produced by strain B-6 under aerobic conditions was the first report on characterization.

Multienzyme complex	Mol. Mass (kDa)	Protein subunits	Ref.
Aerobic microorganisms			
Paenibacillus curdlanolyticus B-6	1450	11	Pason et al.,2006b
Bacillus circulans F-2	669	7	Kim and Kim, 1993
Bacillus licheniformis SVD1	2000	12	van Dyk et al., 2009
Sorangium cellulosum	1000-2000	10	Hou et al., 2006
Streptomyces olivaceoviridis E-86	1200	5	Jiang et al., 2004
Chaetomium sp. Nov. MS-017	468	12	Ohtsuki et al., 2005
Anaerobic microorganisms			
Clostridium acetobutylicum	665	11	Sabathé et al.,2002
Clostridium cellulolyticum	600	14	Gal et al., 1997
Clostridium cellulovorans	900	10	Shoseyov & Doi 1990
Clostridium josui	700	14	Kakiuchi et al., 1998
Clostridium popyrosolvens	600	15	Pohlschröder et al., 1994
Clostridium thermocellum	2100	14	Lamed et al., 1983
Ruminococcus albus	1500	15	Ohara et al., 2000

Table 3. Molecular weights and protein subunits of multienzyme complexes from aerobic and anaerobic microorganisms.

Elucidation of the purified multienzyme feature of *P. curdlanolyticus* strain B-6 was followed by anion-exchange and hydrophobic-interaction chromatographys (Pason et al., 2010). The complex G1 from gel filtration chromatography (1,450 kDa) was purified by anion-exchange chromatography and showed at least five large protein complexes or aggregates, namely F1-F5. Among the fractions obtained from anion-exchange chromatography, F1 was apparently the most suited fraction to study on the organization and function of the multienzyme system of strain B-6 because F1 formed one clear band on the top of native PAGE, had the highest xylanase activity, and its subunit composition was clearly shown on SDS-PAGE. In the final step, complex F1 was separated to one major complex (H1) and two minor protein components (H2 and H3) by hydrophobic-interaction chromatography. The multienzyme complex (H1) was composed of a 280 kDa protein with xylanase activity, a 260 kDa protein that is a truncated form on the C-terminal side of the 280 kDa protein, two xylanases of 40 and 48 kDa, and 60 and 65 kDa proteins having both xylanase and CMCase activities (Fig. 8). The two components (280 and 40 kDa) of the multienzyme complex has characteristics similar to the cellulosome of *C. thermocellum* in that it is composed of a scaffolding protein and a catalytic subunit (Bayer et al., 1998; Demain et al., 2005). The 280 kDa protein resembled the scaffolding proteins of the multienzyme complex based on its migratory behavior in polyacrylamide gels and as a glycoprotein. The 280 kDa protein and a 40 kDa major xylanase subunit are the key components of multienzyme complex of the strain B-6.

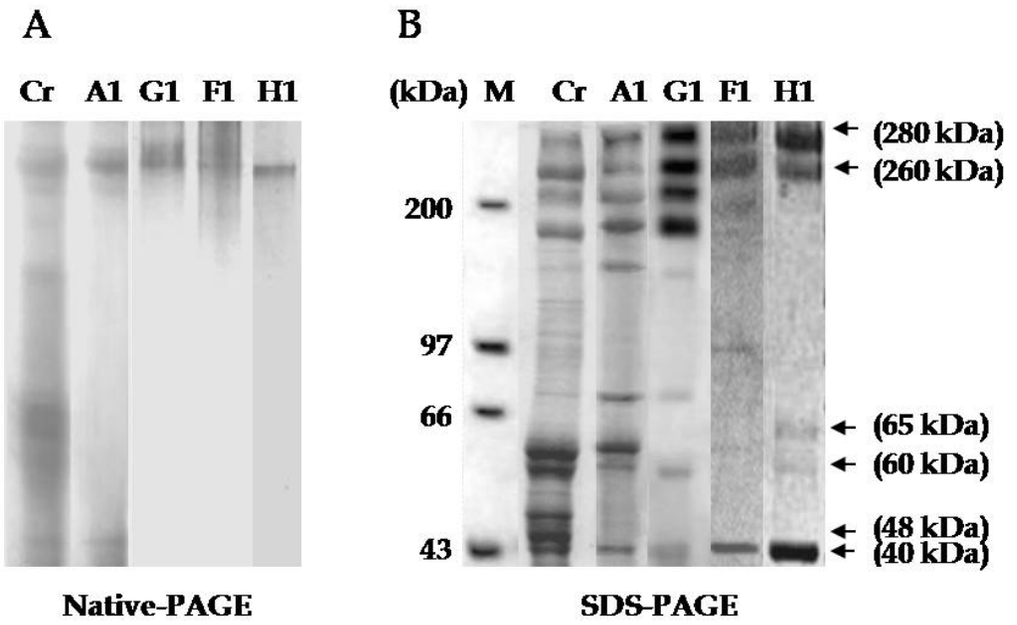

Figure 8. Native-PAGE (A) and SDS-PAGE (B) in isolated complex from culture supernatant at the late stationary growth phase (lane Cr), affinity column (lane A1), gel filtration column (lane G1), anion-exchange column (lane F1) and hydrophobic-interaction column (lane H1). All samples contained 200 μg of protein.

These apparently propose that *P. curdlanolyticus* B-6 produced multienzyme complex, which consisted of many subunit compositions. The large protein (280 kDa) may function as a scaffoldin-like protein that allowed the enzyme subunits, majority is 40 kDa, binding to form a multienzyme complex. The key components, 280 and 40 kDa, are identified in the next topic.

4. Molecular structure of important xylanases

P. curdlanolyticus B-6 produces an extracellular xylanolytic-cellulolytic multienzyme complex mainly comprised of xylanases under aerobic conditions. To understand the xylanase system, a genomic library of the strain B-6 was constructed and screened for high xylanase activity. Recently, six xylanase genes, *S1* (Pason et al., 2010), *xyn10A* (Waeonukul et al., 2009a), *xyn10B* (Sudo et al., 2010), *xyn10C* (unpublished data), *xyn10D* (Sakka et al., 2011) and *xyn11A* (Pason et al., 2010) were cloned, and the translated products were characterized (Table 4).

Enzyme	Modular structure	GH family	Mol. Mass (kDa)	GenBank accession No.
S1	SLH SLH No homology sequence	U	91	-
Xyn10A	CBM22 CBM22 CBM22 GH10 CBM9 SLH SLH SLH	10	142	EU418764
Xyn10B	GH10	10	40	AB570291
Xyn10C	GH10	10	35	AB688987
Xyn10D	GH10 Fn3 CBM3	10	61	AB600191
Xyn11A	GH11 CBM36	11	40	FJ956758

Abbreviations: CBM, carbohydrate-binding module; Fn, fibronectin homology module; GH, glycosyl hydrolase; SLH, surface layer homology domain; U, unhomology sequence

Table 4. Modular structure xylanases of *P. curdlanolyticus* strain B-6.

S1 protein: From the early research, the 280 kDa subunit (S1) plays a role of scaffoldin in assembling the enzyme complex and shows xylanase activity (Pason et al., 2010). The *S1* gene consists of 2,589 nucleotides and encodes 863 amino acids with a molecular weight of 91,000 Da, indicating that the 280 kDa subunit is highly glycosylated. Sequence analysis revealed that S1 did not have significant homology with any proteins in the databases except for two surface layer homology (SLH) domains in its N-terminal region. Surprisingly, the recombinant S1 exhibits xylanase activity, and cellulose- and xylan-binding ability, suggesting that the S1 should be a novel xylanase and CBM(s) with new functions (unpublished data).

Xylanase Xyn10A: The *xyn10A* gene consists of 3,828 nucleotides encoding a protein of 1,276 amino acids with a predicted molecular weight of 142,726 Da. Xyn10A is a multidomain enzyme comprised of nine domains in the following order: three family-22 CBMs, a family-10 catalytic domain of glycosyl hydrolases (GH), a family-9 CBM, a glycine-rich region, and three SLH domains. Xyn10A can effectively hydrolyze insoluble xylan and natural biomass without pretreatment such as sugarcane bagasse, corn hull, rice bran, rice husk and rice straw. Xyn10A binds to various insoluble polysaccharides such as cellulose, xylan and chitin. The SLH domains functioned in Xyn10A by anchoring this enzyme to the cell surfaces of *P. curdlanolyticus* B-6. Removal of the CBMs from Xyn10A strongly reduced the ability of binding and plant cell wall hydrolysis. Therefore, the CBMs of Xyn10A play an important role in the hydrolysis of native biomass materials (Waeonukul et al., 2009a).

Xylanase Xyn10B: The *xyn10B* gene consists of 1,047 nucleotides encoding a protein of 349 amino acids with a predicted molecular weight of 40,480 Da. Xyn10B consists of only a family-10 catalytic of GH. Xyn10B is an intracellular endoxylanase (Sudo et al., 2010).

Xylanase Xyn10C: The *xyn10C* gene consists of 957 nucleotides and encodes 318 amino acid residues with a predicted molecular weight of 35,123 Da. Xyn10C is a single module enzyme consisting of a signal peptide and a family-10 catalytic module of GH (unpublished data).

Xylanase Xyn10D: The *xyn10D* gene consists of 1,734 nucleotides and encodes 577 amino acid residues with a calculated molecular weight of 61,811 Da. Xylanase Xyn10D is a modular enzyme consisting of a family-10 catalytic module of the GH, a fibronectin type-3 homology (Fn3) module, and family-3 CBM, in that order, from the N terminus. The CBM3 in Xyn10D has an affinity for cellulose and xylan, and plays an important role in hydrolysis of arabinoxylan and native biomass materials (Sakka et al., 2011).

Xylanase Xyn11A: The *xyn11A* gene consists of 1,150 bp and encodes a protein of 385 amino acids with a molecular weight of 40,000 Da. Xyn11A is composed of two major functional domains, a catalytic domain belonging to family-11 GH and a CBM classified as family-36. A glycine- and asparagine-repeated sequences existed between the two domains. Xyn11A has been identified to be one of the major xylanase subunit in the multienzyme complex of strain B-6 (Pason et al., 2010).

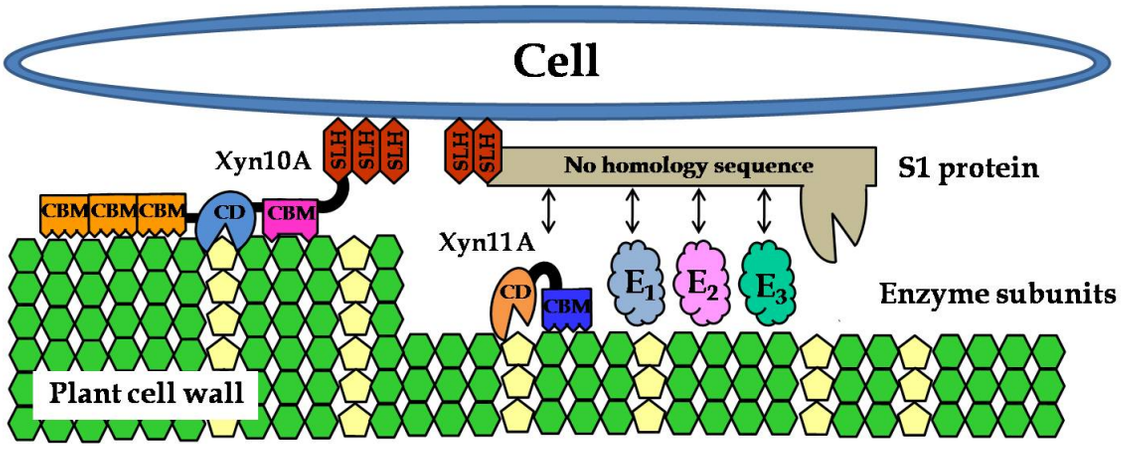

Figure 9. Simplified schematic view of the interaction between the *P. curdlanolyticus* B-6 multienzyme complex system and its substrate, and its connection to the cell surface via an associated anchoring protein. (Abbreviations: CBM, carbohydrate-binding module; CD, catalytic domain, E_n, enzyme subunit; SLH, surface layer homology domain).

Based on both biochemical and molecular biological findings, a simplistic schematic view of the enzyme system from *P. curdlanolyticus* B-6 and its interaction with substrate and cell surface was created and presented in Fig. 9. In this assessment, the S1 protein did not have significant homology with any proteins in the databases except for two S-layer homology domains in its N-terminal region. However, the S1 protein that exhibits xylanase activity and cellulose- and xylan-binding ability, and contains cell anchoring function, seems remarkable. The multifunctional protein S1 is also responsible for forming the enzyme subunits into the complex and anchoring the complex into cell surface via the SLH domain. The interaction between S1 protein and enzyme subunits should be a mechanism distinct from the cohesion-dockerin interaction known in cellulosome of anaerobic microorganisms, since cohesion- or dockerin– like sequences were not observed in the S1 protein or the major

xylanase subunit, Xyn11A. In addition, strain B-6 also produces cell bound multimodular xylanase Xyn10A that contains the numerous CBMs and SLH domains. Xyn10A can bind to the plant cell wall through CBM, whereas the catalytic module (GH10) is able to access its target substrate. Thus, the CBM greatly increases the concentration of the enzyme in the vicinity of the substrate, leading to the observed increase in polysaccharide hydrolysis. Besides, the presence of the functional CBMs and SLH domains in Xyn10A allows the cells to attach to substrate. Although, the overall structure of the enzyme complex system of the strain B-6 is not entirely clear, the enzyme complex has unique characteristics distinct from multienzyme complex cellulosome of anaerobic microorganisms. However, the mechanism for complex formation, interaction between the S1 protein as scaffoldin and enzyme subunits, needs to be further investigated.

5. Biotechnological uses of *P. curdlanolyticus* B-6 multienzyme complex

Biological conversion of lignocellulosic materials has been proposed as a renewable and sustainable route for the production of value-added products (Bayer et al., 2007, Doi et al., 2003). There is much interest in exploiting the properties of multienzyme complexes for practical purposes. The facultative bacterium, *P. curdlanolyticus* strain B-6 produces a unique extracellular multienzyme system under aerobic conditions that effectively degrade cellulose and hemicellulose by gaining access through the protective matrix surrounding the cellulose microfibrils of plant cell walls. Therefore, the multienzyme complex from strain B-6 is a promising enzyme which can potentially be used in many applications, such as enhancing extraction and production of value-added bioproducts by saccharification of cell wall components and application for construction of the modular enzymes creation (Fig. 10).

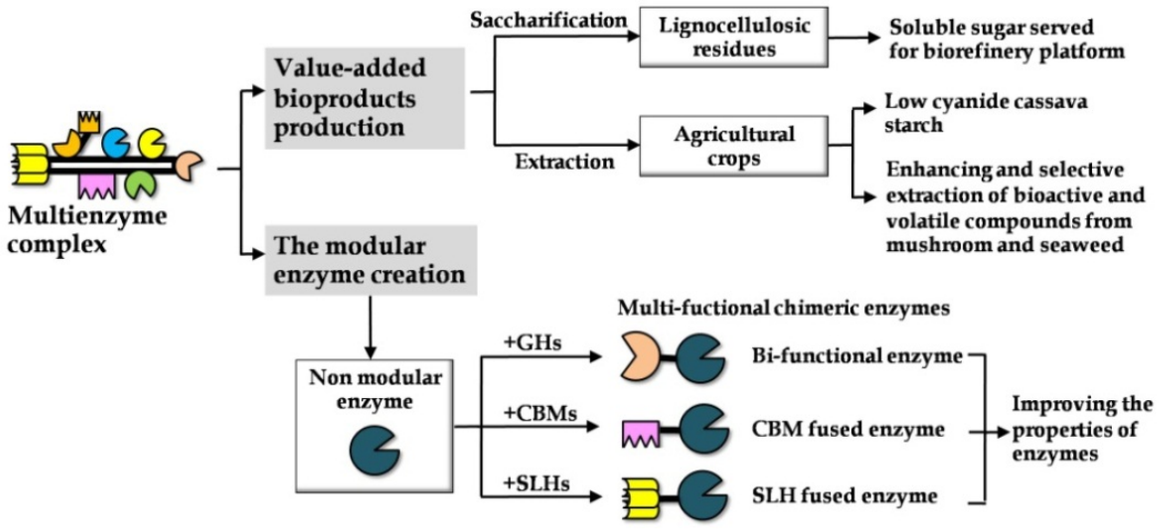

Figure 10. The multienzyme complex of *P. curdlanolyticus* strain B-6 for biotechnological applications.

Biological treatment and saccharification using microorganisms and their enzymes selectively for degradation of lignocellulosic residues has the advantages of low energy consumption, minimal waste production, and environmental friendliness (Schwarz, 2001). The catalytic components of the multienzyme complex release soluble sugars, simple 5- and 6-carbon, from lignocellulose providing the primary carbon substrates, which can be subsequently converted into fuels by microorganisms. For enzyme saccharification, the close proximity between cellulolytic and xylanolytic enzymes is key to concerted degradation of the substrate, whereby the activities of the different enzymes facilitate the activities of their counterparts by promoting access to appropriated portions of the rigid insoluble substrates, since the release of sugar products was high. The synergistic action of the combination of enzymes by different modes of actions (xylanases and cellulases) and the presence of xylan- or cellulose-binding ability on lignocellulose enhanced soluble sugars released from the plant cell walls. In practicality, the multienzyme complex produced from *P. curdlanolyticus* B-6 allows access to lignocellulosic substrate and produces reducing sugar more than non-complexed enzymes from fungi (*T. viride* and *Aspergillus niger*) when the same cellulase activity (0.1 unit) was applied for degradation of corn hull and rice straw residues (unpublished data). In addition, the multienzyme complex of strain B-6 has been used to improve the extraction of plant food such as making low-cyanide-cassava starch by using multienzyme complex to enhance linamarin released by allowing more contact between linamarase and linamarin (Sornyotha et. al., 2009). Also, extraction of volatile compounds such as sea food-like flavor from seaweed, served for food supplement. Consequently, enzymatic treatment has advantages for the preparation of β-glucan and acidic α-glucan-protein complex from the fruiting body of mushroom, *Pleurotus sajor-caju* because the specificity of the multienzyme complex and gentle conditions allow for the recovery of high purity glucans in their native forms with minimal degradation (Satitmanwiwat et al., 2012a,b).

Typically, most plant cell wall degrading enzymes are composed of a series of separate modules (modular enzymes). These domains may fold and function in an independent manner and are normally separated by short linker. *P. curdlanolyticus* B-6, produces a number of glycosyl hydrolase (GHs) families and CBM families which have different substrates recognition affinity and increase amorphous regions of cellulose by H-bond elimination. Interestingly, modular architecture created by chimeric proteins creation with various tandem CBMs, GHs, and SLH-specific, should make it possible to construct effective lignocellulosic degrading enzymes, strongly binding, targeting enzyme to their substrates and bacterial cell surfaces for enhancing a variety of substrates hydrolysis. The strong carbohydrate-binding property of the cellulose-binding domain and xylan-binding domain, specific degradative activities exhibit important properties of the lignocellulosic material degrading enzymes that can be used in biotechnology.

6. Conclusion

A facultatively anaerobic bacterium *P. curdlanolyticus* strain B-6, isolated from an anaerobic digester fed with pineapple wastes, is unique in that it produces extracellular xylanolytic-

cellulolytic multienzyme complex capable of efficient degradation of plant biomass materials under aerobic conditions. The production of strain B-6 multienzyme complex under aerobic conditions has several advantages: (i) a simple process, (ii) low price of medium, (iii) high growth rate, (iv) large quantities of extracellular enzymes yields, and (v) safe use with regard to health and environmental aspects. Thus, strain B-6 and its multienzyme complex is a promising tool for an industrial process employing direct hydrolysis for the bioconversion of cellulose as well as hemicellulose in biomass. This review shows that strain B-6 multienzyme complex is a novel enzymatic system known at the biochemical, genetic, and mechanism level. It also stresses that some points still need to be further investigated, mainly (i) the elucidation of scaffolding protein functions, (ii) the characterization of others key enzyme subunits, (iii) the assembly mechanism of the multienzyme complex, (iv) improvement of the efficiency in degradation of biomass of the multienzyme complex, and (v) improvement of the production of the multienzyme complex. The latter will certainly represent a challenge for future research.

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