STUDY ON MULTIENZYME COMPLEX (CELLULOSOME/ XYLANOSOME) FROM BACTERIUM UNDER ANAEROBIC AND HIGH TEMPERATURE CONDITIONS

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ABSTRACT

This study described the screening and isolation of anaerobic thermophilic biomass-degrading bacteria species and elucidated the enzymatic complex produced from high potential biomass-degrading enrichment culture. Soil, decomposed wastes, and agricultural residue samples were collected from several places in Thailand. One hundred and fifty samples were cultivated in basal medium containing corn hull as the sole carbon source. One of enrichment cultures (namely NKP) showed the highest degrading corn hull. The stable existence NKP enrichment culture contained two different bacterial strains. Two bacteria strains, NKP-2 and NOI-1 were successfully isolated from NKP by utilized carbon sources of properties. Strain NKP-2 grew well on cellulose and xylan as sole carbon sources, but at a slower pace (2-5 weeks) on xylan whereas strain NOI-1 grew well on xylan as a carbon source but not cellulose. Therefore, the isolate strains can be separated in this study. Purified culture of both strains (namely strain NKP-2 and NOI-1) were identified by using 16S rRNA gene analysis. Strain NKP-2 showed high similarity (99%) with *Clostridium thermocellum*, whereas strain NOI-1 was identified as *Thermoanaerobacterium*

thermosaccharolyticum (99% similarity). Thus, pure isolated strains were named as *C. thermocellum* NKP-2 and *T. thermosaccharolyticum* NOI-1, respectively. Furthermore, the purity of the strain NOI-1 and NKP-2 were performed by using specific primer for each strain and indicated that each strains were pure culture. Both strains were anaerobic, thermophilic, Gram-positive, rod-shaped and spore-forming. The optimum temperature for growth of both strains was 60°C and optimum pH was 6.0. The enzymatic system of each isolated strains when cultivated individually on corn hulls demonstrated different cellulolytic and xylanolytic enzyme activities. *C. thermocellum* NKP-2 produced cellulose- and xylan-main chain cleaving enzymes such as carboxymethylcellulase (CMCase), avicelase and xylanase as major enzymes, whereas strain NOI-1 produced dominantly short- and side-chain cleaving enzymes such as cellobiohydrolase, β-glucosidase, β-xylosidase, α-L-arabinofuranosidase and acetyl esterase. The determination of plant cell wall polysaccharide degradation by combining cellulolytic enzymes from *C. thermocellum* NKP-2 and endocellulase-free xylanolytic enzymes from *T. thermosaccharolyticum* NOI-1 showed that the maximum of synergistic effect between endocellulase-free xylanolytic enzymes and cellulolytic enzymes was 2.8 at 3 hours for corn hull degradation. Moreover, corn hull utilization, cell growth, and fermentation products (ethanol, butanol, acetic acid, butyric acid, H_2 and CO_2) were found to be highly increased in the co-culture as compared with individual cultivation of each strain. The symbiotic behavior of co-culturing between both strains is one form of mutualism. In this case, synergistic biomass-degrading enzyme system helps them obtain sugar needed for living in their natural environment.

Interestingly, enzymatic system in term of multienzyme complex in *Thermoanaerobacterium* species has not been reported. Therefore, *T. thermosaccharolyticum* strain NOI-1 was characterized and elucidated. After cultivation of strain NOI-1 in basal medium containing oat spelt xylan as the sole carbon source, the culture supernatant was used as crude enzyme. The culture could produce crude enzyme that comprises xylanase, β-xylosidase, α-L-arabinofuranosidase, acetyl esterase, cellobiohydrolase and β-glucosidase, but could not produce endocellulase. The crude enzyme was active in broad ranges of pH and temperature, however the optimum condition was pH 6.0 and 60°C. Scanning electron microscopy (SEM) analysis revealed that the bacterial cells adhere to insoluble xylan and Avicel. The endocellulase-free

multienzyme complex was isolated from crude enzyme of the strain NOI-1 by affinity purification on cellulose and subsequently with Sephacryl S-300 gel filtration chromatography. The molecular mass of the multienzyme complex was estimated to be about 1,200 kDa. The multienzyme complex showed one protein on native-polyacrylamide gel electrophoresis (native-PAGE), one xylanase on native-zymogram, 21 proteins on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and 5 xylanases on SDS-zymogram. It was found that the multienzyme complex, which consists of xylanase, β-xylosidase, α-L-arabinofuranosidase, β-glucosidase and cellobiohydrolase effectively hydrolyzes oat spelt xylan and corn hull. This is the first report of an endocellulase-free multienzyme complex produced by *Thermoanaerobacterium* species.

Key words: Anaerobic thermophilic bacterium/ *Clostridium thermocellum*/ Cellulolytic-xylanolytic enzymes/ Co-culturing/ Endocellulase-free multienzyme complex/ Symbiotic behavior/ *Thermoanaerobacterium thermosaccharolyticum*

หัวข้อวิทยานิพนธ์	การศึกษาเอนไซม์เชิงซ้อน (เซลลูโลโซม/ไซลาโนโซม) จากแบคทีเรียภายใต้สภาวะปราศจากออกซิเจนและอุณหภูมิสูง
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สาขาวิชา	เทคโนโลยีชีวเคมี
สายวิชา	เทคโนโลยีชีวเคมี
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บทคัดย่อ

งานวิจัยนี้ศึกษาการแยกและคัดเลือกแบคทีเรียที่มีศักยภาพในการย่อยสลายสารชีวมวลและศึกษาคุณสมบัติของเอนไซม์เชิงซ้อนที่แบคทีเรียดังกล่าวผลิตขึ้นภายใต้สภาวะปราศจากออกซิเจนและอุณหภูมิสูง โดยทำการคัดแยกเชื้อจากแหล่งตัวอย่างต่างๆ ได้แก่ ดิน ของเสีย และเศษวัสดุเหลือใช้ทางการเกษตรต่างๆ ภายในประเทศไทย จากตัวอย่างจำนวนทั้งสิ้น 150 ตัวอย่างที่นำมาเพาะเลี้ยงในอาหาร basal medium ที่มีเปลือกข้าวโพดเป็นแหล่งคาร์บอนพบว่า NKP เป็นกลุ่ม enrichment culture ที่สามารถย่อยสลายเปลือกข้าวโพดได้สูงสุด และจากการตรวจสอบพบว่า NKP ประกอบด้วยแบคทีเรียสองชนิดอยู่ร่วมกัน (co-culture) ซึ่งสามารถแยกแบคทีเรียสองสายพันธุ์ได้แก่ สายพันธุ์ NKP-2 และสายพันธุ์ NOI-1 ออกจากตัวอย่าง NKP โดยอาศัยคุณสมบัติการใช้แหล่งคาร์บอนในการเจริญเติบโตได้ต่างกัน โดยพบว่าแบคทีเรียสายพันธุ์ NKP-2 สามารถเจริญเติบโตในอาหารที่มีเซลลูโลสเป็นแหล่งคาร์บอนได้เป็นอย่างดีแต่เจริญเติบโตในอาหารที่มีไซแลนเป็นแหล่งคาร์บอนได้ช้า (2-5 สัปดาห์) ในขณะที่แบคทีเรียสายพันธุ์ NOI-1 สามารถเจริญเติบโตในอาหารที่มีไซแลนเป็นแหล่งคาร์บอนได้เป็นอย่างดีแต่ไม่สามารถเจริญเติบโตในอาหารที่มีเซลลูโลสเป็นแหล่งคาร์บอนได้ ดังนั้นในการศึกษาครั้งนี้จึงสามารถแยกแบคทีเรียทั้งสองออกจากกันได้ หลังจากการคัดแยกได้แบคทีเรียบริสุทธิ์สองสายพันธุ์ ได้แก่ สายพันธุ์ NKP-2 และสายพันธุ์ NOI-1 เมื่อนำไปตรวจสอบ

ลำดับนิวคลีโอไทด์บริเวณ 16S rRNA พบว่าแบคทีเรียสายพันธุ์ NKP-2 คล้ายคลึงกับ *Clostridium thermocellum* ร้อยละ 99 และสายพันธุ์ NOI-1 คล้ายคลึงกับ *Thermoanaerobacterium thermosaccharolyticum* ร้อยละ 99 ดังนั้นสายพันธุ์ NKP-2 และสายพันธุ์ NOI-1 จึงถูกเรียกว่า *C. thermocellum* สายพันธุ์ NKP-2 และ *T. thermosaccharolyticum* สายพันธุ์ NOI-1 ตามลำดับ นอกจากนั้นแบคทีเรียทั้งสองสายพันธุ์ยังถูกตรวจสอบความบริสุทธิ์โดยอาศัย primer ที่มีความจำเพาะกับแต่ละสายพันธุ์ พบว่าทั้งสองสายพันธุ์มีความบริสุทธิ์ ตรวจสอบคุณลักษณะของแบคทีเรียทั้งสองสายพันธุ์ พบว่าแบคทีเรียทั้งสองเจริญเติบโตได้ดีภายใต้สภาวะปราศจากออกซิเจนและอุณหภูมิสูง เป็นแกรมบวก มีรูปร่างเป็นท่อน สร้างสปอร์ได้และเจริญเติบโตได้ดีที่สุดที่อุณหภูมิ 60 องศาเซลเซียสและพีเอช 6.0 จากนั้นทำการเพาะเลี้ยงแบคทีเรียทั้งสองสายพันธุ์แบบเดี่ยวในอาหารที่มีเปลือกข้าวโพดเป็นแหล่งคาร์บอน เมื่อทำการตรวจสอบเอนไซม์ที่ถูกผลิตขึ้น พบว่าทั้งสองสายพันธุ์ผลิตเอนไซม์เซลลูโลไลติกและไซลาโนไลติกได้ต่างกัน โดยสายพันธุ์ NKP-2 ผลิตเอนไซม์ที่ย่อยโครงสร้างหลักของสายเซลลูโลสและไซแลนเป็นหลักได้แก่ คาร์บอกซีเมททิลเซลลูเลส อะไวซิเลสและไซลาเนส ในขณะที่สายพันธุ์ NOI-1 ผลิตเอนไซม์ที่ย่อยโครงสร้างสายสั้นของเซลลูโลสและไซแลนและ/หรือ กิ่งก้านของไซแลนได้แก่ เซลโลไบโอไฮโดรเลส เบต้ากลูโคซิเดส เบต้าไซโลซิเดส แอลฟา-แอล-อะราบิโนฟูราโนซิเดส และอะซิติลเอสเทอเรส เมื่อศึกษาการทำงานร่วมกันของเอนไซม์จากทั้งสองสายพันธุ์ พบว่าการใช้เอนไซม์ผสมสามารถเพิ่มการย่อยสลายเปลือกข้าวโพดได้สูงขึ้นถึง 2.8 เท่าที่เวลา 3 ชั่วโมง นอกจากนี้ได้ทำการเพาะเลี้ยงแบคทีเรียทั้งสองสายพันธุ์ร่วมกัน พบว่าสามารถเพิ่มอัตราการย่อยสลายเปลือกข้าวโพด อัตราการเจริญเติบโต และผลิตภัณฑ์ที่ได้จากการหมัก (แอลกอฮอล์ บิวทานอล กรดอะซิติก กรดบิวทีริก ก๊าซไฮโดรเจนและก๊าซคาร์บอนไดออกไซด์) สูงขึ้นเมื่อเทียบกับการเพาะเลี้ยงแบคทีเรียทั้งสองสายพันธุ์แบบเดี่ยวๆ ดังนั้นแสดงให้เห็นว่าพฤติกรรมการอยู่ร่วมกันของแบคทีเรียทั้งสองสายพันธุ์นี้ จัดเป็นรูปแบบหนึ่งของการพึ่งพาอาศัยซึ่งกันและกัน โดยมีการทำงานร่วมกันในการย่อยสลายสารชีวมวลให้ได้น้ำตาลเพื่อนำมาใช้ในการดำรงชีวิตอยู่ในธรรมชาติ

สิ่งที่น่าสนใจจากการศึกษาก่อนหน้านี้ พบว่ายังไม่มีรายงานว่า *T. thermosaccharolyticum* สามารถผลิตเอนไซม์เชิงซ้อนได้ ดังนั้นงานวิจัยนี้จึงสนใจศึกษาเอนไซม์เชิงซ้อนจาก *T. thermosaccharolyticum* สายพันธุ์ NOI-1 โดยเพาะเลี้ยงในอาหารที่มีไซแลน (oat spelt xylan) เป็นแหล่งคาร์บอน เอนไซม์ที่ผลิตในน้ำเลี้ยงถูกใช้เป็นเอนไซม์หยาบเพื่อทำการศึกษาต่อไป และจากการตรวจสอบกิจกรรมของเอนไซม์พบว่าเอนไซม์หยาบแสดงกิจกรรมของเอนไซม์ไซลาเนส เบต้าไซโลซิเดส แอลฟา-แอล-อะราบิโนฟูราโนซิเดส อะซิติลเอสเทอเรส เซลโลไบโอไฮโดรเลส และเบต้ากลูโคซิเดส แต่ไม่พบกิจกรรมของเอนไซม์เอนโดเซลลูเลส เอนไซม์หยาบสามารถทำงานได้

ในช่วงพีเอชและอุณหภูมิกว้าง โดยทำงานได้ดีที่สุดที่พีเอช 6.0 และอุณหภูมิ 60 องศาเซลเซียสและเมื่อตรวจสอบด้วยกล้อง scanning electron microscope (SEM) พบว่าแบคทีเรียสายพันธุ์ NOI-1 สามารถยึดเกาะกับไซแลนที่ไม่ละลายน้ำและเซลลูโลส (อะไวเซล) ได้ดี ดังนั้นจึงทำการแยกเอนไซม์เชิงซ้อนออกจากเอนไซม์หยาบโดยใช้เทคนิค affinity chromatography ซึ่งยึดเกาะอย่างจำเพาะกับเซลลูโลสร่วมกับ Sephacryl S-300 gel filtration chromatography พบว่าเอนไซม์เชิงซ้อนที่แยกได้มีขนาดประมาณ 1,200 กิโลดาลตัน เมื่อตรวจสอบรูปแบบของโปรตีนและกิจกรรมของเอนไซม์โดยใช้เทคนิค native-PAGE, native-zymogram, SDS-PAGE และ SDS-zymogram พบว่ามีโปรตีนเพียงกลุ่มเดียวและแสดงกิจกรรมของเอนไซม์ไซลาเนสในรูปแบบธรรมชาติ (เมื่อตรวจสอบด้วยเทคนิค native-PAGE และ native-zymogram) ซึ่งโปรตีนกลุ่มนี้ประกอบด้วยโปรตีนหน่วยย่อยอย่างน้อย 21 ชนิด และไซลาเนสอย่างน้อย 5 ชนิด (เมื่อตรวจสอบด้วยเทคนิค SDS-PAGE และ SDS-zymogram ตามลำดับ) นอกจากนี้เอนไซม์เชิงซ้อนยังแสดงกิจกรรมของเอนไซม์เบต้าไซโลซิเดส แอลฟา-แอล-อะราบิโนฟูราโนซิเดส เบต้ากลูโคซิเดส และเซลโลไบโอไฮโดรเลส จากการตรวจสอบประสิทธิภาพการย่อย พบว่าเอนไซม์เชิงซ้อนที่แยกได้มีประสิทธิภาพสูงในการย่อยสลายไซแลนและเปลือกข้าวโพดซึ่งงานวิจัยนี้เป็นการรายงานครั้งแรกที่ศึกษาเกี่ยวกับเอนไซม์เชิงซ้อนที่ผลิตจากแบคทีเรียในจีนัส *Thermoanaerobacterium* โดยเอนไซม์เชิงซ้อนสายพันธุ์ NOI-1 ที่ผลิตขึ้นนี้จัดเป็นเอนไซม์เชิงซ้อนที่ปราศจากเอนโดเซลลูเลส

คำสำคัญ: การเพาะเลี้ยงร่วมกัน/ แบคทีเรียไม่ต้องการออกซิเจนและทนอุณหภูมิสูง/ พฤติกรรมการอยู่ร่วมกัน/ เอนไซม์เชิงซ้อนที่ปราศจากเอนโดเซลลูเลส/ เอนไซม์เซลลูโลไลติก-ไซลาโนไลติก/ *Clostridium thermocellum*/ *Thermoanaerobacterium thermosaccharolyticum*

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LIST OF ABBREVIATIONS

°C	= Degree celcious
CBD	= Cellulose-binding domain
CBMs	= Carbohydrate-binding modules
CE	= Carbohydrate esterase
CMC	= Carboxymethylcellulose
CMCase	= Carboxymethylcellulase
Coh	= Cohesin
d	= Day
Da	= Dalton
DNA	= Deoxyribonucleic acid
DP	= Degree of polymerization
g	= Gram
GH	= Glycosyl hydrolase
h	= Hour
HLD	= Hydrophillic domain
kDa	= Kilodalton
µg	= Microgram
µl	= Microliter
M	= Molar
mg	= Milligram
min	= Minute
mM	= Millimolar
nm	= Nanometer
OD	= Optical Density
PBS	= Phosphate buffer saline
PCR	= Polymerase Chain Reaction

CHAPTER 1 INTRODUCTION

1.1 Background

Lignocellulosic biomass is the most abundant material in the world. Its sources range from trees to agricultural residues. Long ago, these materials were used as firewood, building materials and animal food. Nowadays, lignocellulosic materials are not just used in their old ways, their applications have expanded into the fiber level as in pulp and paper products, used for fibers in the textile industry, used as an adhesive component in the composite industry (lignin) or used as an important source of renewable energy.

The most abundant and renewable biomass on earth is lignocellulose, which contains three major polymer groups: cellulose, hemicellulose and lignin. In component, cellulose and hemicellulose comprise 40-60% of component. Cellulose consists of long polymers of β-1,4-linked glucose units. It forms a crystalline structure, whereas the structure of hemicellulose is that of any of several heteropolymers contain many different sugar monomers such as xylose, arabinose, galactose, mannose, and glucose.

Lignocellulose-degrading enzymes have become increasingly important, since the development of efficient lignocellulose degradation methods and the conversion of sugars to valuable products such as butanol and amino acids and utilizable forms of energy such as ethanol and methane could lead to less dependence on imported petroleum as a fuel and chemical source. Biodegradation of lignocellulose the major component of cellulose and hemicellulose is a complex process that requires the coordination of several enzymes (cellulolytic and xylanolytic enzymes).

However, enzymatic degradation of lignocellulose into simple monomeric sugar is not easy due to their recalcitrant nature. Microorganisms meet this challenge with the aid of multienzyme system. Aerobic bacteria produce numerous individual, extracellular enzymes with binding modules for different cellulosic conformations. Specific enzymes act in synergy to elicit effective hydrolysis. In contrast, anaerobic bacteria produce a unique extracellular multienzyme complex, called cellulosome. The cellulosome is a large multienzyme complex used for the efficient degradation of plant cell

wallpolysaccharides and macromolecular machine, whose components interact in a synergistic manner to catalyze the efficient degradation of cellulose whereas xylanosome are discrete, multifunctional, multienzyme complexes. These complexes play an important role in the degradation of hemicelluloses. Though much knowledge has been accumulated about the cellulosome, little is known about the xylanosome

Some microorganisms are reported to produce enzyme systems containing multiactivities. For example, *Clostridium thermocellum*, *Clostridium josui*, and *Bacteroides* sp. P-1, thermophilic and anaerobic bacteria, produce a multienzyme complex (cellulosome/xylanosome) when grown on cellulose as the substrate. Thus, it is interesting to investigate and produce cellulosome and xylanosome which are active and stable at thermophilic and anaerobic conditions.

1.2 Objectives

1. To screen thermophilic cellulolytic-xylanolytic bacteria under anaerobicconditions
2. To purify and characterize the cellulosome/xylanosome
3. To apply cellulolytic-xylanolytic anaerobic bacteria for degradation of lignocelluloses under thermophilic conditions

1.3 Scopes

1. Isolation of anaerobic thermophilic cellulolytic-xylanolytic bacteria from soil samples and identification of the isolated strains by morphological properties and 16S rDNA sequencing analysis
2. Studies on biochemical properties of the isolated multienzyme complex from cell pellet and culture supernatant such as molecular mass, protein composition, enzymatic activities and enzymatic hydrolysis.
3. Application of cellulolytic-xylanolytic anaerobic bacteria degradation of lignocellulose under thermophilic conditions

1.4 Benefits

1. This research will provide the new knowledge of multienzyme complex produced by bacterium, under anaerobic and thermophilic conditions.
2. Understanding the multienzyme complex system from the new cellulolytic/xylanolytic bacterium.
3. This bacterium and its multienzyme complex have the ability to adsorb to the insoluble substrates to hydrolyze. It may enable to improve biodegradation of insoluble substrates such as agricultural wastes, avicel or xylan by multienzyme complex for the production of industrial chemical, fuel, food and animal feed.

CHAPTER 2 LITERATURE REVIEW

2.1 Biomass

2.1.1 Biomass overview

Biomass comprises all the living matter present on earth. It is derived from growing plants including algae, trees and crops or from animal manure (Bridgwater, 1999). The biomass resources are the organic matters in which the solar energy is stored in chemical bonds. It generally consists of carbon, hydrogen, oxygen and nitrogen. Sulfur is also present in minor proportions. Some biomass also consist significant amounts of inorganic species. Plants, via photosynthesis, produce carbohydrates which form the building blocks of biomass (Demirbas, 2001a). Biomass has always been a major source of energy for mankind from ancient times. Presently, it contributes around 10–14% of the world's energy supply (Putun et al., 2001). Biomass can be used in many ways to obtain energy. Most of the biomass energy is consumed in domestic purposes and by wood-related industries. It is burned by direct combustion to produce steam that drives the turbine/generator to produce electricity. Gasifiers are used to convert biomass into a combustible gas which is then used to drive a high efficiency, combined cycle gas turbine (Saxena et al., 2009).

In the past several years, the energy demand in Thailand has increased rapidly as results of the economic expansion and population growth. In 2011, the total final energy consumption in Thailand were 46.9% of petroleum products, 18% of electricity, 9.3% coal and lignite, 6.3% natural gas and 19.5% renewable energy. The renewable energy increased from the previous year at the rate 2.5%. Concerning biomass energy which currently is the second major energy source in Thailand still plays an importance energy source in households and rural industries. Thailand is an agro-industry base country. Therefore, there are varieties of agriculture products that their residue can be used as an energy source.

2.1.2 Biomass components

The importance of particular type of biomass depends on the chemical and physical properties of the large molecules from which it is made. The chemical structure and

major organic components in biomass are important in the development of processes for producing derived fuel and chemicals. Biomass contains varying amounts of cellulose, hemicellulose, and lignin as they mainly components (Fig. 2.1) (Bridgwater, 1999; Ratanakhanokchai et al., 2013). Cellulose is a glucose polymer containing linear chains of (1,4)-D-glucopyranose units, in which the units are linked 1–4 in the alpha configuration, with an average molecular weight of around 100,000. Alpha cellulose is a polysac-charide having the general formulae ($C_6H_{10}O_5$). Hemicelluloses are complex polysaccharides that exist in association with cellulose in the cell wall. It is a mixture of polysaccharides, composed almost entirely of sugars such as glucose, mannose, xylose and arabinose and methlyglucoronic and galaturonic acids, with an average molecular weight of <30,000. Lignins are highly branched, substituted, mononuclear aromatic polymers in the cell walls of the certain biomass, especially woody species, and are often adjacent to cellulose fibers to form a lignocellulosic complex (Drummond and Drummond, 1996). In biomass, cellulose is generally the largest fraction, about 40–50% by weight and hemicellulose about 20–40%. For example, the sugarcane bagasse contains cellulose (40–50%), hemicellulose (20–30%), lignin (20–25%) and ash (1.5–3.0%) (McKendry, 2002).

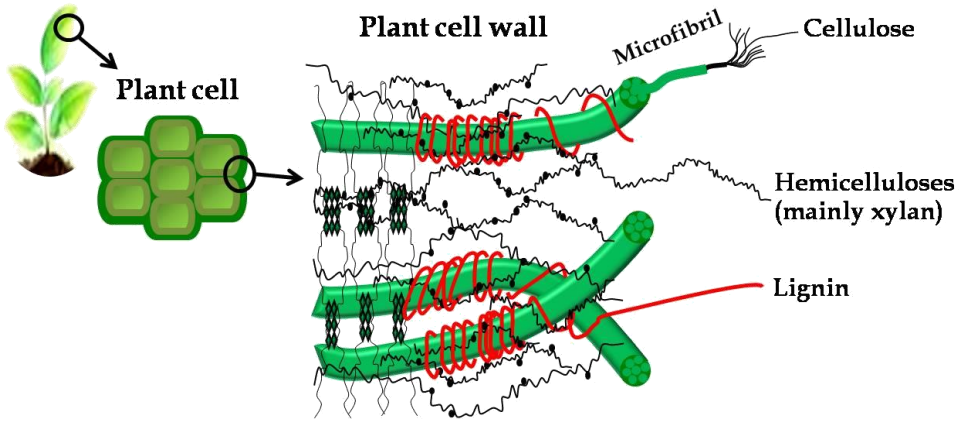


Figure 2.1 Structure of plant biomass (Ratanakhanokchai et al., 2013)

Biomass contains varying amounts of cellulose, hemicellulose, lignin and small amounts of lipids, proteins, simple sugars and starches. Biomass also contains inorganic constituents and a fraction of water. Among these compounds, cellulose, hemicellulose, and lignin are the three main constituents (Zhang et al., 2010). The combination of cellulose, hemicelluloses, and lignin are called ‘lignocellulose’, which comprises around half of the plant matter produced by photosynthesis and represents the most

abundant renewable organic resource on earth. Cellulose, hemicelluloses and lignin are strongly intermeshed in lignocelluloses and are chemically bonded by non-covalent forces or by covalent crosslinkages. Cellulose is the largest component of lignocellulosic materials, followed by hemicellulose and lignin. Whereas cellulose and hemicellulose are macromolecules constructed from different sugars; lignin is an aromatic polymer synthesized from phenylpropaniod precursors (Saidur et al., 2011). The composition and proportions of these compounds in different plants is illustrated in Table 2.1

Table 2.1 Lignocellulosic constituents of some biomass

Lignocellulosic residues	**Hemicellulose (%)**	**Cellulose (%)**	**Lignin (%)**	**Ash (%)**	**Reference**
Nut shells	25–30	25–30	30–40	NA	(Abbasi and Abbasi, 2010)
Corn Cobs	35	45	15	1.36	
Paper	0	85–99	0–15	1.1–3.9	
Rice Straw	24	32.1	18	NA	
Sorted Refuse	20	60	20	NA	
Leaves	80–85	15–20	0	NA	
Cotton seeds Hair	5–20	80–95	0	NA	
Waste paper	10–20	60–70	5–10	NA	
Waste water solids	NA	8–15	24–29	NA	
Sugar cane bagasses	27–32	32–44	19–24	4.5–9	
Barley straw	24–29	31–34	14–15	5–7	
Oat straw	27–38	31–37	16–19	6–8	
Rye straw	27–30	33–35	16–19	2–5	
Bamboo	15–26	26–43	21–31	1.7–5	
Rye grass (early leaf)	15.8	21.3	2.7	NA	
Rye grass (seed setting)	25.7	26.7	7.3	NA	
Orchard grass	40	32	4.7	NA	
Esparto grass	27–32	33–38	17–19	6–8	
Sabai grass	23.9	NA	22.0	6.0	
Elephant grass	24	22	23.9	6	
Bast fiber seed flax	25	47	23	5	
Bast fiber Kena	22–23	31–39	15–19	2–5	
Bast fiber Jute	18–21	45–53	21–26	0.5–2	

Table 2.1 Lignocellulosic constituents of some biomass (Continued)

Lignocellulosic residues	Hemicellulose (%)	Cellulose (%)	Lignin (%)	Ash (%)	Reference
Banana waste	14.8	13.2	14	11.4	
Hardwood stems	24–40	40–50	18–25	NA	
Softwood stems	25–35	45–50	25–35	NA	
Beech Wood	31.2	45.3	21.9	NA	(Demirbas, 2004)
Spruce Wood	20.7	49.8	27.0	NA	
Walnut Shell	22.7	25.6	52.3	NA	
Almond Shell	28.9	50.7	20.4	NA	
Sunflower shell	34.6	48.4	17.0	NA	(Demirbas, 2003a)
Ailanthus wood	26.6	46.7	26.2	NA	
Hazelnut kernel husk	15.7	29.6	53.0	NA	
Corn Cob	32.32	52.49	15.19	NA	(Demirbas, 2001b)
Corn straw	30.88	51.53	17.59	NA	
Olive cake	21.63	23.08	55.29	NA	
Newspaper	25–40	40–55	18–30	NA	(Demirbas, 1997)
Swine waste	28	6.0	NA	NA	
Solid cattle manure	1.4–3.3	1.6–4.7	2.7–5.7	NA	
Coastal Bermuda grass	35.7	25	6.4	NA	
Grasses	35–50	25–40	10–30	NA	
Hazelnut shell	29.9	25.9	42.5	1.3	
Hazelnut seed coat	15.7	29.6	53.00	1.40	
Soft Wood	24.4	45.80	28.00	1.7	
Hardwood	31.30	45.30	21.70	2.7	
Waste Material	29.2	50.60	24.70	4.50	
Tea Waste	19.90	30.20	40.00	3.40	
Wood Bark	29.80	24.80	43.80	1.60	
Wheat Straw	39.10	28.80	18.60	13.50	
Corn Stover	30.70	51.20	14.40	3.70	
Tobacco stalk	28.20	42.40	27.00	2.40	
Tobacco Leaf	34.40	36.30	12.10	17.2	
Olive Husk	23.6	24.0	48.4	4.0	
Spruce Wood	21.20	50.80	27.50	0.5	
Beech Wood	31.80	45.80	21.90	0.4	

Table 2.1 Lignocellulosic constituents of some biomass (Continued)

Lignocellulosic residues	Hemicellulose (%)	Cellulose (%)	Lignin (%)	Ash (%)	Reference
Ailanthus wood	26.60	46.70	26.20	0.5	
Biomass	20–40	40–60	10–25	NA	(Zhang et al., 2010)
Birch wood	25.70	40.00	15.70	NA	(Mohan et al., 2006)
Switch grass	32.10	37.10	17.20	NA	(Lemus et al., 2002)

2.1.3 Biomass conversion

Biomass can be converted to fuel by means of numerous processes. The actual choice of a process will depend on the type and quantity of available biomass feedstock, the desired energy carrier, environmental standards, economic conditions and other factors. For example, biomass can be directly burned as a fuel. However, this manner of use can be regarded as a source of very substantial pollution. Therefore, it is necessary to convert biomass into liquid or gaseous fuels which can replace oil. A large varietyof liquid and gaseous fuels can be derived from biomass as shown in Fig. 2.2 (Saidur et al., 2011). There are several methods available to convert biomass into useable form of energy. The foremost among them is thermal conversion where combustion, gasification, and pyrolysisare used to retrieve energy from the biomass. The next is biochemical conversion where microorganisms during fermentation, anaerobic digestion and esterification release energy from the biomass. Biochemical conversion is usually preferred for biomass with high water content. The last is the chemical conversion where various chemical reactions drawout energy from the biomass. Fig. 2.2 shows thermal and biochemical conversion of biomass.

2.1.3.1 Pyrolysis

Pyrolysis of biomass is thermal decomposition of the organic matters in the absence of oxygen. Pyrolysis is a relatively slow chemical reaction occurring at low temperatures to convert biomass to a more useful fuel such as hydrocarbon rich gas mixture and a carbon rich solid residue. The main products of biomass pyrolysis depend on the temperature, heating rate, particle size and catalyst used. Typical gas composition of woody biomass pyrolysis includes CO, CO_2, CH_4 and H_2 as major products along with

other organic compounds. Usually, fast pyrolysis yields more gases than solids (Sami et al., 2001).

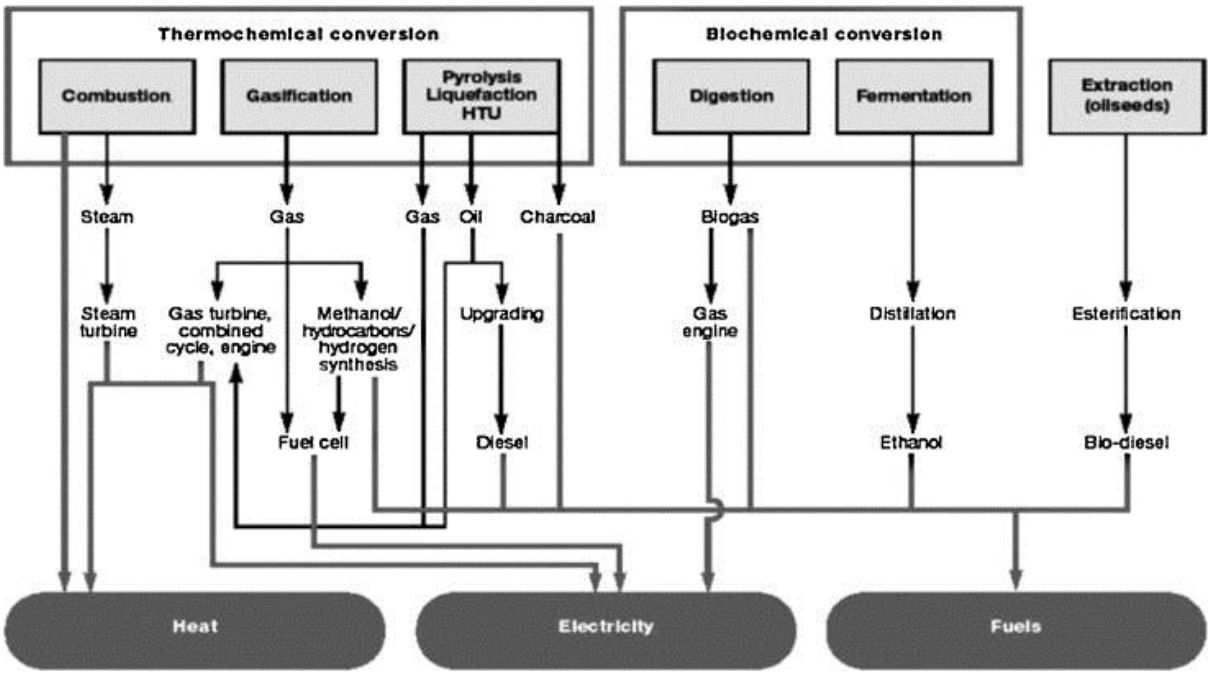


Figure 2.2 Main conversion options for biomass to secondary energy carriers (Balat et al., 2009; Caputo et al., 2005; Faaij, 2006; Saidur et al., 2011)

2.1.3.2 Gasification

Gasification is the thermo-chemical conversion of biomass into gaseous fuels by means of partial oxidation of the biomass at high temperatures. It can be used to produce a low (4–6 MJ/m^3) to high (4–6 MJ/m^3) caloric value gas. This process also allows for the production of methanol or hydrogen, each of which may have a good future as fuels (Demirbas, 2003b).

2.1.3.3 Direct combustion

Biomass can be burned directly in waste toenergy plants without any chemicals processing to produce steam for making electricity. Direct combustion and co-firing with coal for electricity production from biomass has been found to be a promising method in the nearest future. Also biomass can be burned to provide heat for industries and homes (Demirbas, 2005).

2.1.3.4 Fermentation

Fermentation is the process by which ethanol fuel can be produced. There are several types of process that can produce analcohol (ethanol) from various plants, especially corn. The two most commonly used processes involve using yeast to ferment the starch in the plant to produce ethanol which can be used as a fuel in the transportation sector. One of the newest processes involves using enzymes to break down the cellulose in the plant's fibers, allowing more ethanol to be made from each plant (Agarwal, 2007; Demirbas, 2009).

2.1.3.5 Anaerobic digestion

Digestion is the biochemical conversion of organic material to biogas, a mixture of mainly methane and carbon dioxide. The biomass is converted by bacteria in an anaerobic environment in absence of oxygen. Anaerobic digestion is a commercially proven technology and is widely used for treating wet organic wastes. Biogas can be used in many different applications. It can be upgraded to natural gas quality and applied in grids (Demirbas, 2005).

2.1.3.6 Chemical conversion

Biomass can be converted into gas or liquid fuels by using chemicals or heat. In India cow manure is converted to methane gas to produce electricity. Methane gas can be converted to methanol, a liquid form of methane (Faaij, 2006).

2.2 Plant cell wall

Plant cell walls represent the most abundant renewable resource on this planet. They are rich in mixed complex and simple biopolymers, which have opened the door to the development of wide applications in different technologic fields. In this regard the polymerization processes that allow the synthesis of the cell wall and their components in living models are relevant, as well as the properties of the polymers and their derivatives. Therefore this chapter outlines the basis of polymerization with a biological approach in the plant cell wall, highlighting the biological effects of plant cell wall derivatives and their current applications (Ochoa-Villarreal et al., 2012).

Plant cell wall is a dynamic network highly organized which changes throughout the life of the cell. The new primary cell wall is born in the cell during cell division and rapidly increases in surface area during cell expansion. The middle lamella forms the interface between the primary walls of neighboring cells. Finally, at differentiation, many cells elaborate with the primary wall a secondary cell wall, building a complex structure uniquely suited to the function of the cell. The functions of the plant cell wall may be grouped by its contribution to the structural integrity supporting the cell membrane, sense extracellular information and mediate signaling processes (Hematy et al., 2009). The main components of the plant cell wall involve different polymers including polysaccharides, proteins, aromatic substances, and also water and ions. Particularly, the different biomechanical properties of the plant cell wall are mainly defined by the content of the polymers cellulose, hemicelulloses, lignin and pectins and their interactions (McCann and Carpita, 2008).

2.2.1 Plant cell wall compositions

Plant cell wall is a complex matrix of polysaccharides that provides support and strength essential for plant cell survival. Properties conferred by the cell wall are crucial to the form and function of plants. The main functions of the cell wall comprise the confer of resistance, rigidity and protection to the cell against different biotic or a biotic stresses, but still allowing nutrients, gases and various intercellular signals to reach the plasma membrane. The wall provides enough rigidity to support the heavy weight of high trees as large as 100 m height, but also is flexible and elastic allowing growth during expansion and differentiation. During growth, cell turgor pressure provides high tensile stress to the wall, enabling its enlargement due to the accumulation of polymers during a combination of stress relaxation cycles. The primary cell wall surrounds and protects the inner cell; it lies down the middle lamella during growth and expansion (McCann and Carpita, 2008). Plant cell wall is a dynamic and highly specialized network formed by a heterogeneous mixture of cellulose, hemicelluloses and pectins, and in some extent proteins and phenolic compounds (lignin) (McKendry, 2002).

2.2.1.1 Cellulose

Cellulose is the major polymeric component of plant matter, composed of repeating units of cellobiose, 4-*O*- (β-D-glucopyranosyl)-D-glucopyranose and is the most

abundant polysaccharide on the earth. The basic molecular structure is a linear polymer consisting of up to 10^4 D-glucose molecules that are arranged in fibrils. The fibrils consist of several parallel cellulose molecules stabilized by hydrogen bonds. Although individually the hydrogen bonds are relatively weak, collectively they become a strong associative force as the degree of polymerization increases. The fibrils are organized into a "paracrystalline" state, thus adding to the structural rigidity of cellulose. Over three-quarters of the cellulose structure is considered to exist in these enzymatically resistant, crystalline regions and the remainder comprises the relatively easily hydrolyzable "amorphous" areas (Fig. 2.3) (Bayer et al., 1998a).

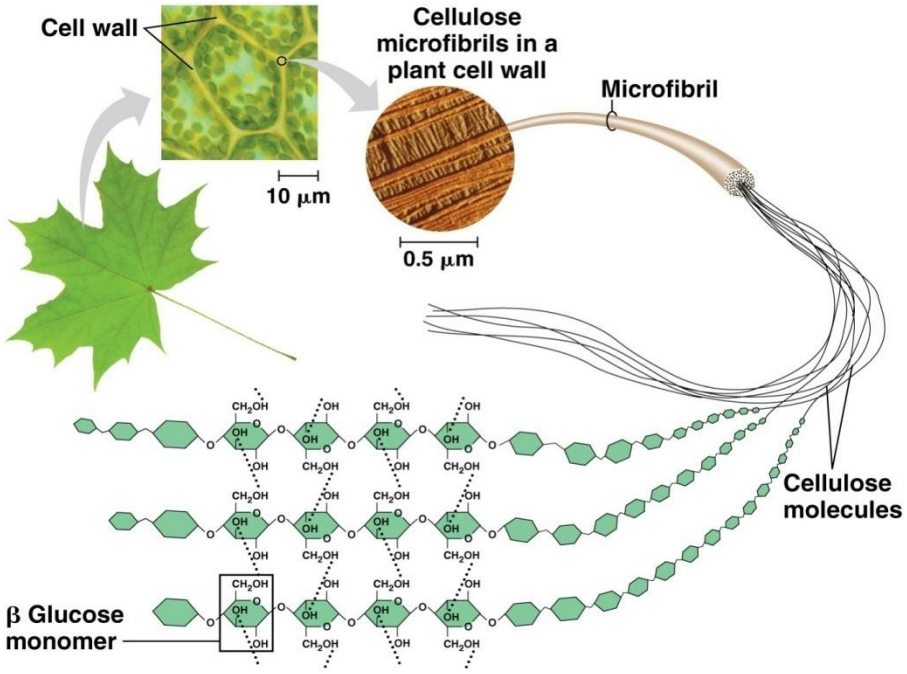


Figure 2.3 Microfibril cellulose in plant cell wall, Source: Cellulose and plant cell wall [Online], Available:http://www.thaigoodview.com/node/21065?page=0,4.[2013, July 23]

Microfibrils comprise two types of cellulose called cellulose Iα and Iβ. The Iα has a single-chain triclinic unit cell, whereas cellulose Iβ has two chain monoclinic unit cell. In both forms cellulose in parallel and the terminal glucose residues rotated 180° forming a flat ribbon in which cellobiose (two glucose molecules linked by a β-(1,4) bond) is the repeating unit (Somerville, 2006). Cellulose chains may align in parallel (Type I) or antiparallel (Type II) orientation to each other. Only the Type I conformation is known to naturally occur in plants; however, concentrated alkaline

treatments may cause Type II cellulose to form during harsh extraction procedures. The cellulose chains may form the Type Iα or Type Iβ conformation depending on the extent of staggering of the chains in relation to each other. Probably the interaction of cellulose microfibrils with hemicelluloses may affect the ratio of Type Iα to Type Iβ cellulose (Eichhorn and Davies, 2006). The microfibrilar disposition allows the existence of micro spaces between the microfibrils that are fulfilled by matricial polysaccharides according to the age and tissue type.

2.2.1.2 Hemicelluloses

Hemicelluloses, the second most common polysaccharides in nature, represent about 20–35% of lignocellulosic biomass. Xylans are the most abundant hemicelluloses. In recent years, bioconversion of hemicellulose has received much attention because of itspractical applications in various agro-industrial processes, such as efficient conversion of hemicellulosicbiomass to fuels and chemicals, delignification of paperpulp, digestibility enhancement of animal feedstock, clarification of juices, and improvement in the consistency of beer (Wong et al., 1988).

Hemicelluloses are heterogeneous polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose), and sugar acids. Unlike cellulose, hemicelluloses are not chemically homogeneous. Hemicelluloses are polysaccharides in plant cell walls that have β-(1, 4)-linked backbones with an equatorial configuration. Hemicelluloses include xyloglucans, xylans, mannans and glucomannans, and β-(1,3, 1,4)-glucans. These types of hemicelluloses are present in the cell walls of all terrestrial plants, except for β-(1,3, 1,4)-glucans, which are restricted to Poales and a few other groups. The detailed structure of the hemicelluloses and their abundance vary widely between different species and cell types. The most important biological role of hemicelluloses is their contribution to strengthening the cell wall by interaction with cellulose and, in some walls, with lignin (Scheller and Ulvskov, 2010).

Xylans of many plant materials are heteropolysaccharides with homopolymeric backbone chains of 1,4-linked β-D-xylopyranose units. Besides xylose, xylans may contain arabinose, glucuronic acid or its 4-*O*-methyl ether, and acetic, ferulic, and *p*-coumaric acids. The frequency and composition of branches are dependent on the source of xylan (Bochicchio and Reicher, 2003; Saha, 2003). The backbone consists of

O-acetyl, α-L-arabinofuranosyl, α-1,2-linked glucuronic or 4-*O*-methylglucuronic acid substituents. However, unsubstituted linear xylans have also been isolated from guar seed husk, esparto grass, and tobacco stalks (Biely, 1985; Eda et al., 1976). Xylans can thus be categorized as linear homoxylan, arabinoxylan, glucuronoxylan, and glucuronoarabinoxylan. Xylans from different sources, such as grasses, cereals, softwood, and hardwood, differ in composition. About 80% of the xylan backbone is highly substituted with monomeric side-chains of arabinose or glucuronic acid linked to*O*-2 and/or *O*-3 of xylose residues, and also by oligomeric side chains containing arabinose, xylose, and sometimes galactose residues (Fig. 2.4) (Saulnier et al., 1995). The heteroxylans, which are highly cross-linked by diferulic bridges, constitute a network in which the cellulose microfibrils may be imbedded. Structural wall proteins might be cross-linked together by isodityrosine bridges and with feruloylated heteroxylans, thus forming an insoluble network (Hood et al., 1991). In softwood heteroxylans, arabinofuranosyl residues are esterified with*p*-coumaric acids and ferulic acids (Mueller-Harvey et al., 1986). In hardwood xylans, 60-70% of the xylose residues are acetylated (Timell, 1967). The degree of polymerization of hard-wood xylans (150-200) is higher than that of softwoods (70-130). However, composition and distribution of the substitutions is wide variable according to the plant cell species (Fig.2.5-2.6)

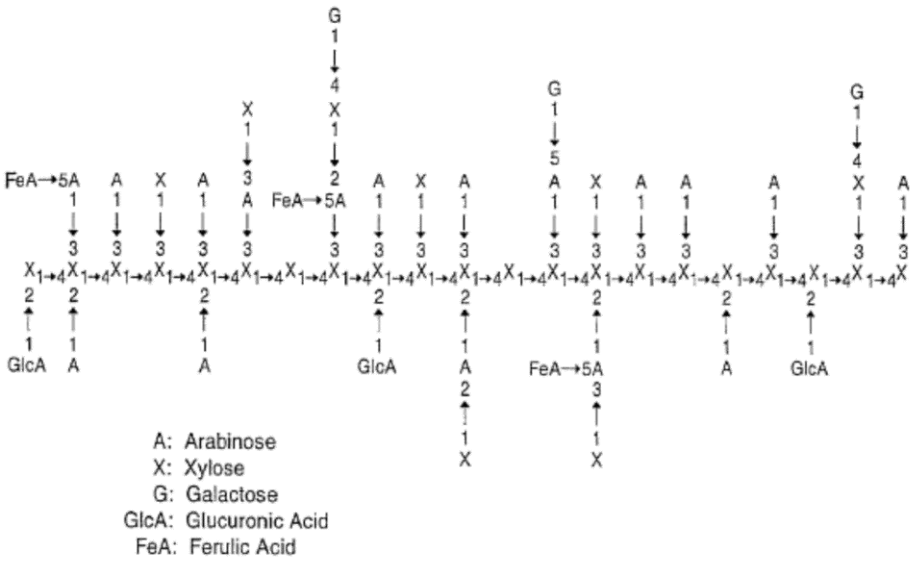


Figure2.4 Schematic structure of corn fiber heteroxylan (Saulnier et al., 1995).

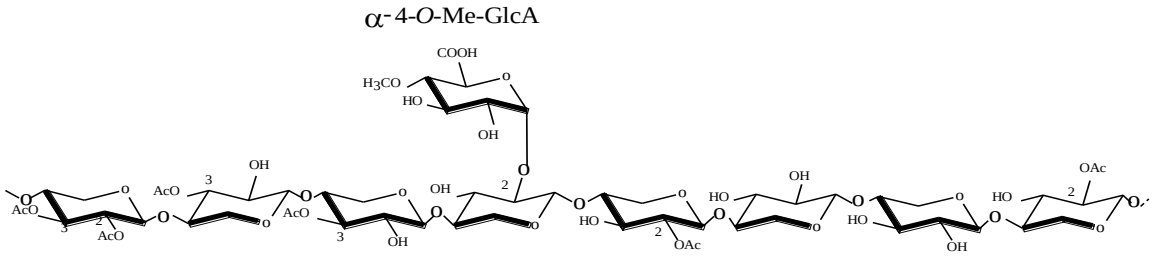


Figure 2.5 Composition of *O*-acetyl-4-*O*-methylglucuronoxylans (hardwoods xylan) (Sunna and Antranikian, 1997). Numbers indicate the carbon atom at which substitutions take place. Ac: Acetyl group; α-4-*O*-Me-GlcA: α-4-*O*-methylglucuronic acid

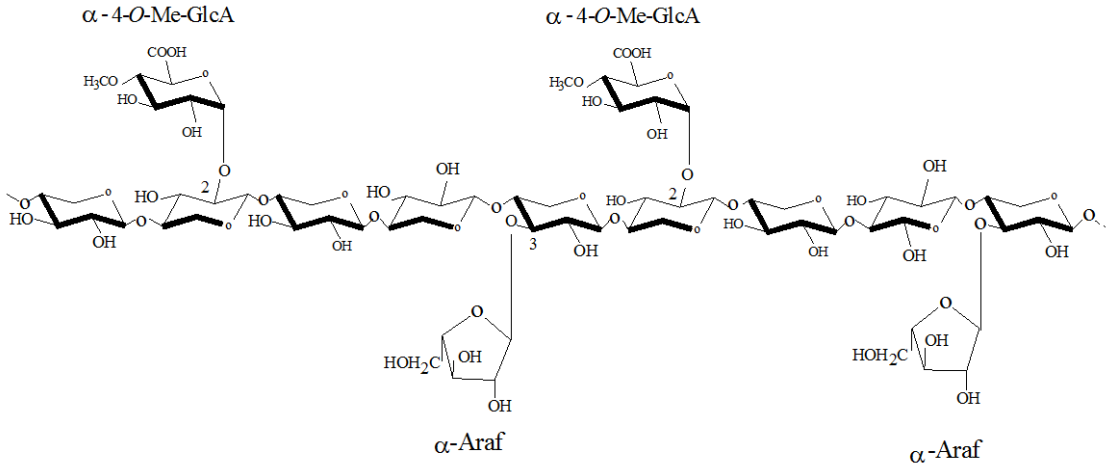


Figure 2.6 Composition of arabino-4-*O*-methylglucuronoxylans (soft wood xylan) (Sunna and Antranikian, 1997). Numbers indicate the carbon atom at which substitutions take place. α-Araf: α-Arabinofuronose; α-4-*O*-Me-GlcA: α-4-*O*-methylglucuronic acid.

Xyloglucan (XyG) is the most abundant hemicellulose in primary cell walls found in every land plant species that has been analyzed. XyG are branched with α-D-xylose linked to C-6 of the backbone. The most frequently xyloglucan structure in dicotyledonous flowering plants is the repeating heptamer integrated by four glucans residues with α-D-xylose substituents in three constitutive glucans of the backbone, followed by a single unsubstituted glucan residue (Fig. 2.7). The presence of this repeating heptamer block is an indicator of the presence of XyG polysaccharides in dicots species (Ochoa-Villarreal et al., 2012). Beside the XyG residues, it may contain

β-D-galactose and in less proportion L-fucose-α-(1,2)-D-galactose; in all cases the galactose residues are acetylated. The fact that all the substituents of xyloglucans are conserved denotes a highly biosynthesis control. On the other hand, in graminaceous monocots, XyG consist of 1 or 2 adjacent α-(1,6)-linked xylose residues with approximately 3 unsubstituted β-(1,4)-linked glucose backbone (Kato and Matsuda, 1981). Despite the structural variability found in the species,the functions of the XyG in plants growth and development are hypothesized to be conserved among all species of flowering plants (Caffall and Mohnen, 2009).

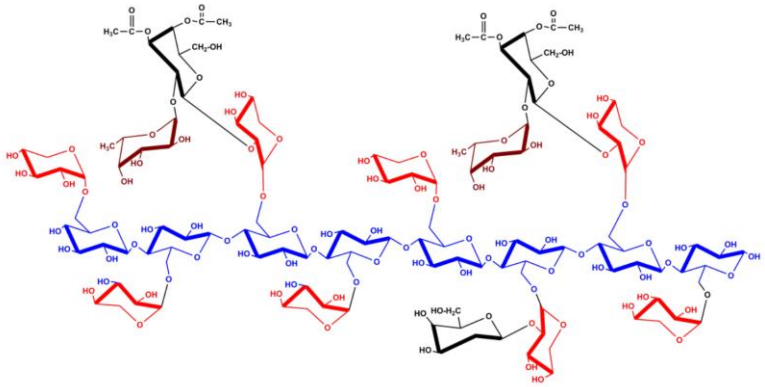
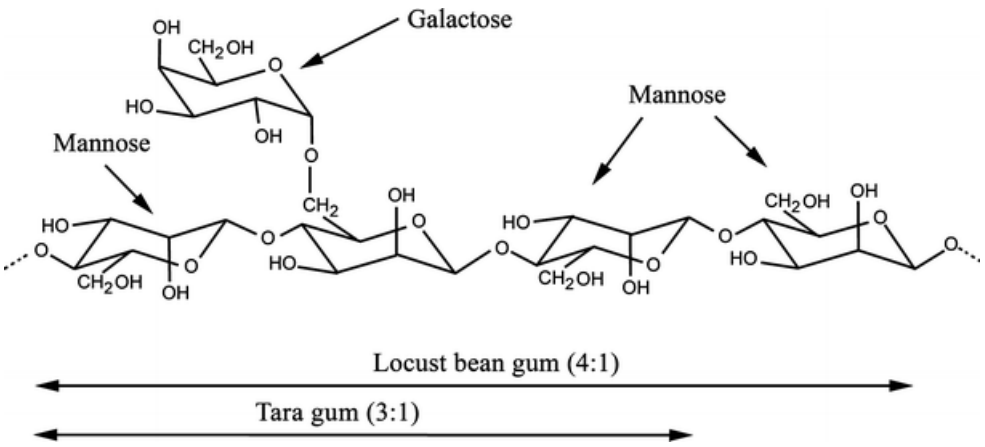


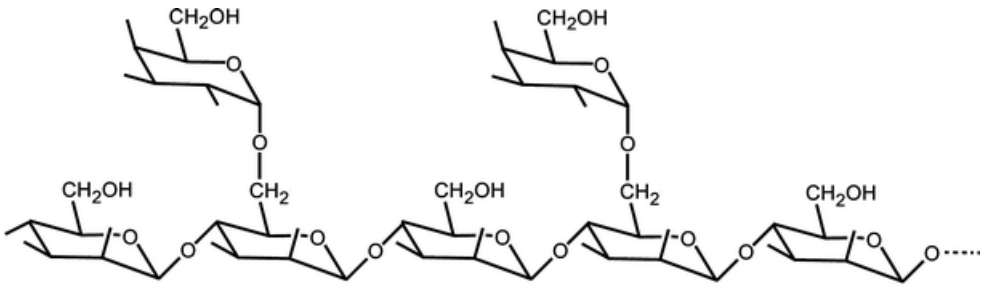
Figure 2.7 Structure of xyloglucan; principal component of the hemicelluloses
The heptamer block is shown (glucan$_4$-xylose$_3$). In blue backbone β-D-glucans; in red α-D-xylose; in black α-D-galactose and in brown α-L-fucose residues (Ochoa-Villarreal et al., 2012).

The mannan, an important component of the hemicelluloses family, can be classified in four subfamilies: linear mannan, glucomannan, galactomannan, and galactoglucomanan (De O. Petkowicz et al., 2001). Each of these polysaccharides presents a β-1,4-linked backbone containing mannose or a combination of glucose and mannose residues. In addition, the mannan backbone can be substituted with side chains of α-1,6-linked galactose residues. Mannans are the major constituents of the hemicelluloses fraction in softwoods and show wide spread distribution in plant tissues (De O. Petkowicz et al., 2001). In plants, they present a structural role, acting as hemicelluloses that bind cellulose. In addition, they also display a storage function as nonstarch carbohydrate reserves in endosperm walls and vacuoles of seeds and vacuoles in vegetative tissues (Moreira and Filho, 2008). Differences in the distribution of D-galactosyl units along

the mannan structure are found in galactomannans from different sources. The Fig. 2.8 shown structure of mannans (locust bean gum, tara gum and guar gum).



(A)



(B)

Figure 2.8 The structure of locust bean gum, tara gum (A) and guar gum (B) (Moreira and Filho, 2008; Sittikijyothin et al., 2005)

2.2.1.3 Pectins

Pectin is a polymeric material having carboxylic groups esterified with methanol. It can be divided in two regions ‘‘smooth region’’ and ‘‘hairy region’’ (Fig. 2.9). The degree of esterifications varies depending on its source. It mostly consist of three structurally well-characterized polysaccharide motifs: homogalacturonan (HGA), rhamnogalacturonan I and rhamnogalacturonan II (RG I and RG II). These three polysaccharides form a network, which has a considerable potential for modulation of its structures by action of cell wall degrading enzymes. Homogalacturonan represents

the backbone chain of the pectin molecule, containingα-1,4-linked residues of D-galacturonic acid, which can be methylated at the *O*-6position. RGI is located in the highly branched are a containing large number of side chains of α -1,2-linked residues of L-rahmnopyranose whereas the more complex RG II structure has been identified in the primary cell wall of some plants. It is believed to play a role as a signal molecule in plant cell wall development rather than being a structural polymer. The pectin molecule is branched at the rhamnogalacturonan part by side chains like arabinans, galactans or arabinogalactans which are linked by β-(1,4) linkages to rhamnose. In the main side chains, the arabinose units areα-(1,5) linked and the galactose units are joined by β-(1,4) linkages. Apart from theseneutral sugars, the side chains of pectins can also contain xylopyranose, D-glucopyranose and L-fucopyranose, whereas in RG II,D-apiose, 2-*O*-methyl-D-xylose and 2-*O*-methyl-L-fucose are present. InRG I the galacturonic acid residues are often acetylated at the C_2 or C_3 position, but acetylation was found also in the homogalacturonan region. Generally, pectins have variable compositions. D-Galacturonic acid residues form most of the molecules in blocks of ‘smooth’ and ‘hairy’ regions. The molecule does not adopt a straight conformation in solution but is extended and curved (worm-like) with a large amount of flexibility. The ‘hairy’ regions of pectins are even more flexible and may have pendant arabinogalactans. The carboxylate groups tend to expand the structure of pectins as a result of their charge unless they interact through divalent cationic bridging (their pKas are of about 2.9) ensuring considerable negative charge under most circumstances. Methylation of these carboxylic acid groups forms their methyl esters, which take up a similar space but are much more hydrophobic and consequently have a different effect on the structuring of the surrounding water. The properties of pectins depend on the degree of esterification, which is normally about 70% (Yadav et al., 2009).

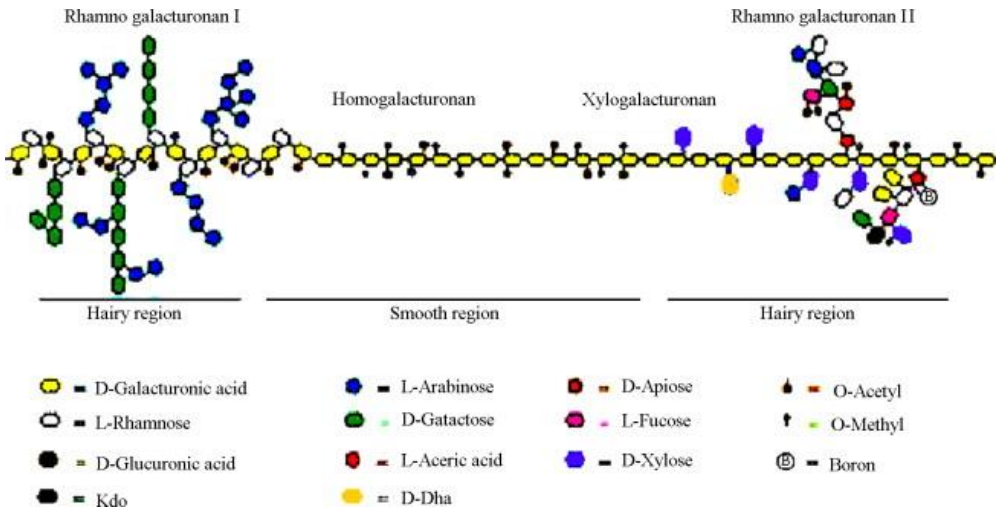


Figure 2.9 Structure of pectin (Yadav et al., 2009)

2.2.1.4 Lignin

Lignin, a valuable resource for chemicals and energy, is a main component of wood, together with cellulose and hemicellulose. It is the second large source of organic raw material, constituting about 4-35 wt% of most biomass, 16-25 wt% of hardwoods and 23-35 wt% of softwoods (Chakar and Ragauskas, 2004). As the most abundant natural aromatic polymer (Lora and Glasser, 2002), lignin has a highly branched three-dimensional phenolic structure including three main phenylpropane units, namely *p*-coumaril, coniferyl and sinapyl (Fig. 2.10). Softwood lignin contains relatively fewer sinapyl units and consists mainly of guaiacyl structures, while hardwood lignin contains guaiacyl-syringyl structures.As a by-product of the paper industry, lignin is most often used by paper mills as a fuel for the recovery of its energy content. However, due to the very large generated quantities, lignin is increasingly considered as a potential source of chemicals, and studies on its thermal degradation receive much interest. "Pyrolytic lignin", the organic phase obtained from the pyrolysis of wood or of other biomass resources, consists of a brown tar containing high molecular weight compounds derived from lignin, while the water-soluble fraction, accounting for 60-70 wt% of the whole oil, contains lower molecular weight substances (Pandey and Kim, 2011).

Lignin could be also used to prepare sorbents, activated carbons or carbon fibres with very high surface areas and pore volumes (Baklanova et al., 2003; Gonzalez-Serrano et al., 2004; Hayashi et al., 2000). Pure lignin has a lower adsorption capacity, compared

to lignin-based activated carbons, being primarily responsible for the microporosity of the activated carbons obtained from lignocellulosic precursors. When prepared at lower temperatures, lignin carbon is more stable than the cellulose one, due to its higher content of aromatic structures (Xie et al., 2009).

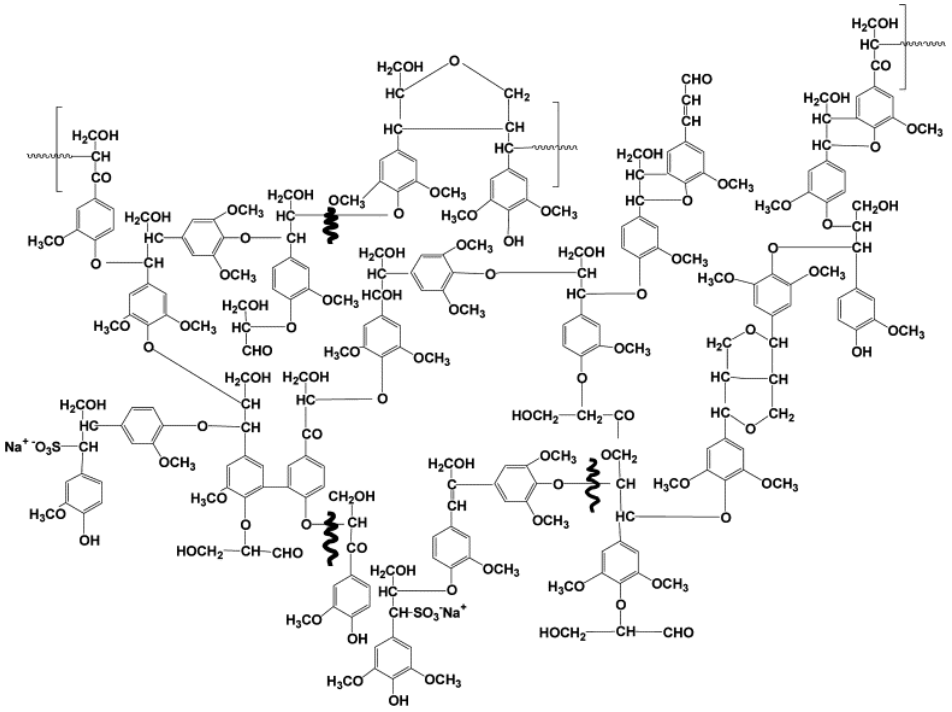


Figure 2.10 Partial structure of hardwood lignin (Mohan et al., 2006).

2.2.2 Plant cell wall polysaccharide biodegradation

Plant cell wall degrading enzymes have become increasingly important, since the development of efficient biomass degradation methods and the conversion of sugars to valuable products such as butanol and amino acids and utilizable forms of energy such as ethanol and methane could lead to less dependence on imported petroleum as a fuel and chemical source. Several biological methods for plant cell wall polysaccharide recycling based on the enzymology of cellulose, hemicelluloses, and pectin degradation have been developed. To date, processes that use plant cell wall degrading enzymes or microorganisms could lead to promising, environmentally friendly technologies. Efficient conversion of this material by engineered enzymes and/or microorganisms would be highly desirable. The rate limiting step in biomass degradation is the conversion of the cellulose and hemicellulose polymers to sugars.

2.2.2.1 Cellulose biodegradation

Cellulose is a homopolysaccharide composed of β-D-glucopyranose units, linked by β-(1,4)-glycosidic bonds. Cellobiose is the smallest repetitive unit of cellulose and can be converted into glucose residues. The cellulose-hydrolysing enzymes (i.e. cellulases) are divided into three major groups: endoglucanases, cellobiohydrolases (exoglucanases), and β-glucosidases. The endoglucanases catalyse random cleavage of internal bonds of the cellulose chain, while cellobiohydrolases attack the chain ends, releasing cellobiose. β-Glucosidases are only active on cello-oligosaccharides and cellobiose, and release glucose monomers units from the cellobiose, for instance (Fig.2.11).

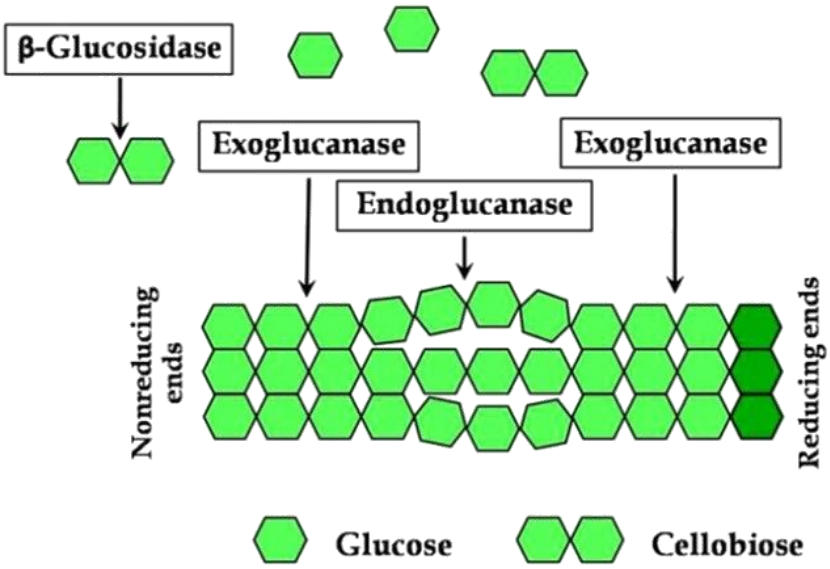


Figure 2.11 Enzyme systems involved in the degradation of cellulose (Ratanakhanokchai et al., 2013)

Bioconversion of cellulose into fermentable sugars is a biorefining area that has invested enormous research efforts, as it is a prerequisite for the subsequent production of bioenergy. Sugars and starch comprise the feedstock for 90% of the produced ethanol today, but the most prevalent forms of sugar in nature are cellulose and hemicellulose. Lignocellulosic biomass can be converted to ethanol by hydrolysis and downstream fermentation processing. This process is much more complicated than just fermentation of C6 sugar and is still far from being cost effective as compared to the production of bioethanol from starch or sugar crops. In hydrolysis, the cellulosic part of the biomass is converted into sugars, and fermentation converts these sugars to ethanol.

Lignocellulosic biomass consists of 10–25% lignin, which contains no sugar, and therefore impossible to convert into sugars. Lignin is therefore a residue in ethanol production, and it represents a big challenge to convert it into a value-added product (Kumar et al., 2008).

2.2.2.2 Hemicellulose biodegradation

Hemicellulose is the second most abundant renewable biomass and accounts for 25-5% of lignocellulosic biomass (Saha, 2000). Hemicelluloses are heterogeneous polymers built up by pentoses (D-xylose, D-arabinose), hexoses (D-mannose), hardwood contained mainly xylans, while in softwood glucomannans are most common. There are various enzymes responsible for the degradation of hemicelluloses. In xylan degradation, for instance, endo-1,4-β-xylanase, β-xylosidase, α-glucuronidase, α-L-arabinofuranosidase and acetylxylan esterase all act on the different heteropolymers available in nature (Fig. 2.12). In glucomannan degradation, β-mannanase, and β-mannosidase cleave the polymer backbone (Fig. 2.12). Like cellulose, hemicellulose is also an important source of fermentable sugars for biorefining applications. Xylanases are being produced and used as additives in feed for poultry and as additives to wheat flour for improving the quality of baked products at the industrial scale (Niehaus et al., 1999).

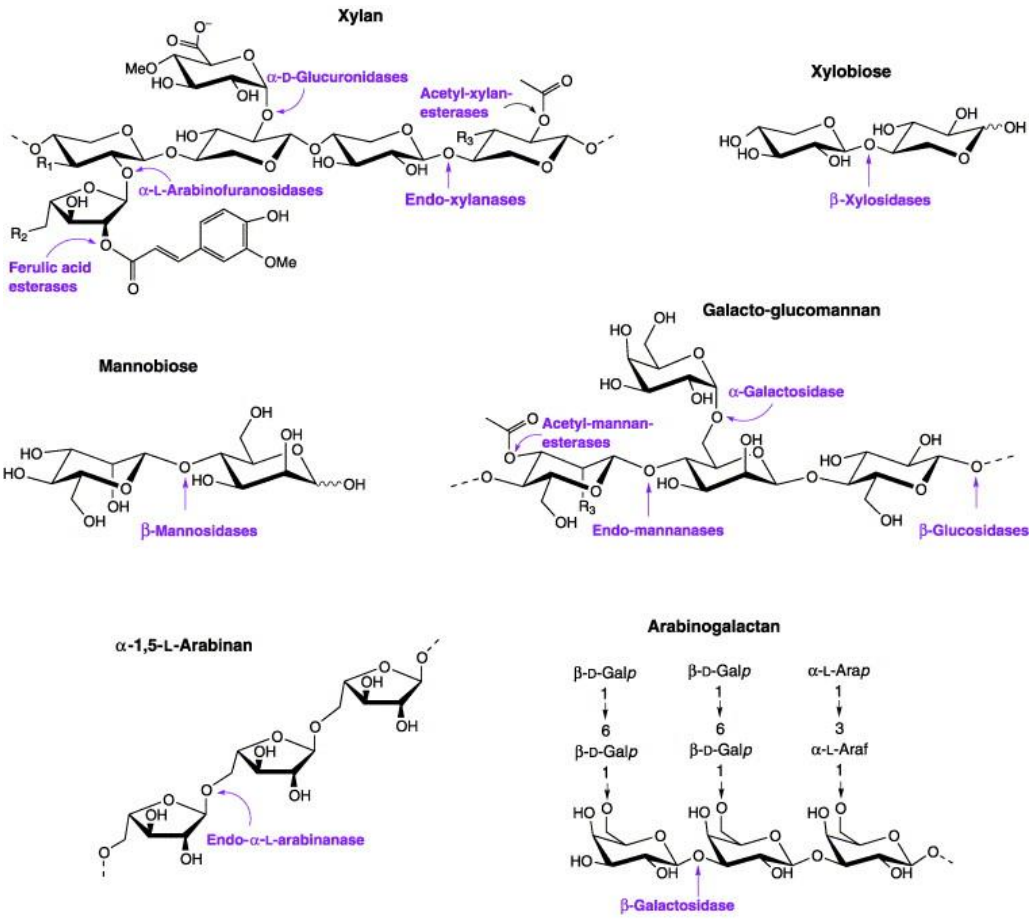


Figure 2.12 The basic structural components found in hemicellulose and thehemicellulases responsible for their degradation (Shallom and Shoham, 2003).

2.2.2.3 Pectinbiodegradation

Pectins are the third main structural polysaccharide group of plant cell walls, abundant in sugar beet pulp andfruits, e.g. citrus and apple fruit, where it can form up to half of the polymeric content of the cell wall (Brummell, 2006). Pectin has found widespread commercial use, especially in the textile industry and inthe food industry as a thickener, texturizer, emulsifier, stabilizer, filler in confections, dairy products, and bakery products, etc (Liu et al., 2006). Despite these applications, pectins are similar to cellulose and hemicelluloses, common waste materials that can be converted to soluble sugars, ethanol and biogas. Many enzymes are involvedin pectin degradation (Fig. 2.13). They may be acting either by hydrolysis or by trans-elimination; the latter performed by lyases. Pectin-degrading enzymes i.e. polymethylgalacturonase, (endo-) polygalacturonase pectindepolymerase, pectinase, exopolygalacturonase, and exopolygalacturanosidase hydrolyse the polygalacturonic acid chain of the pectin polymer by the addition ofa water molecule α-L-rhamnosidases hydrolyse rhamnogalacturonan in the pectic backbone. α-L-arabinofuranosidases hydrolyse the L-

arabinose side-chains and endo-arabinase act on arabinan side-chains in pectin.These two enzymes operate synergistically indegrading branched arabinan to yield L-arabinose. Polysaccharide lyases (PL) cleave the galacturonic acidpolymer by elimination and comprise e.g. polymethyl-galacturonate lyase (pectin lyase), polygalacturonatelyase (pectate lyase)and exopolygalacturonate lyase (pectate disaccharide-lyase) (Jayani et al., 2005).

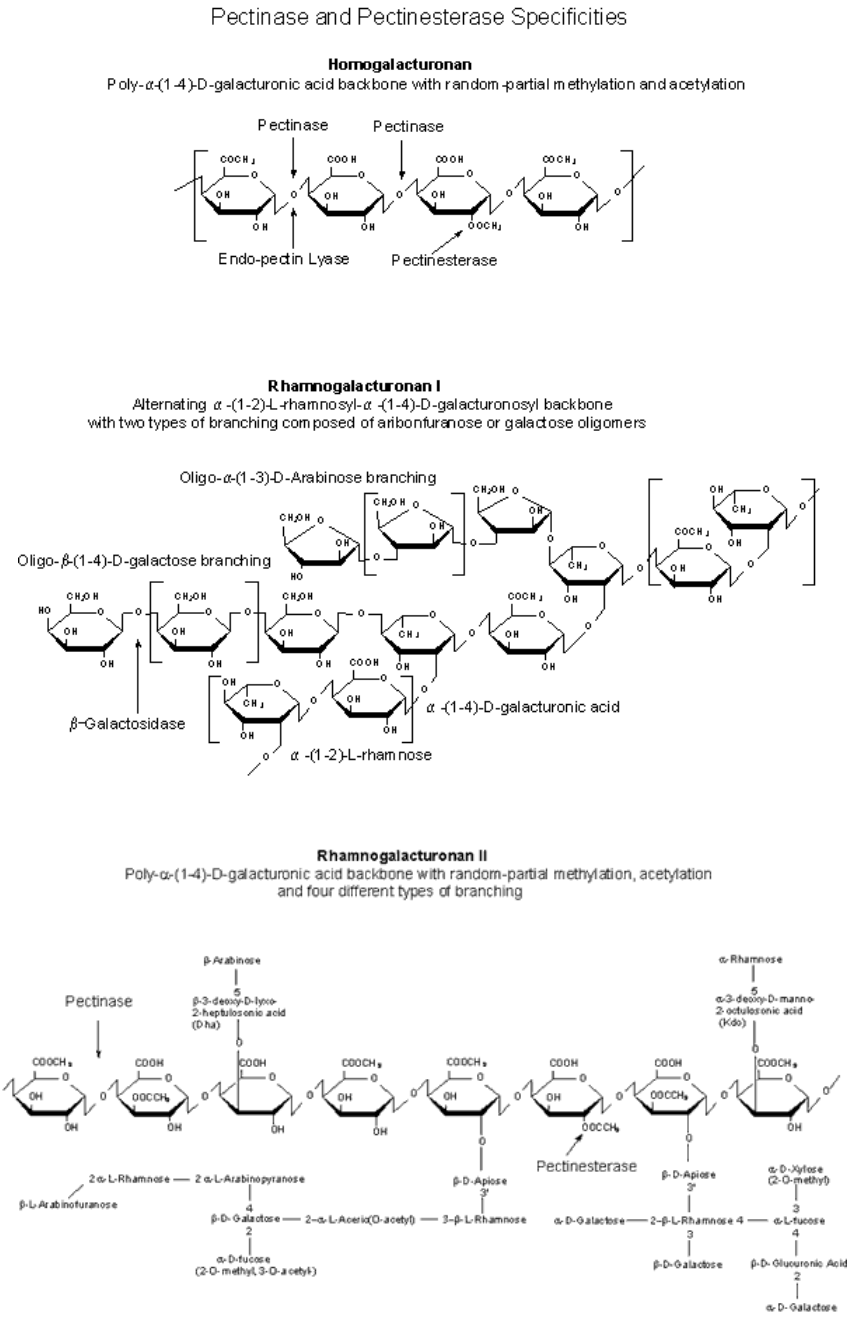


Figure 2.13 Structure of pectin and enzymatic sites for pectin lyase
Source: Structure of pectin and enzymatic sites for pectin lyase [Online], Available: http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/learning-center/carbohydrate-analysis/carbohydrate-analysis-iii.html [2013, July 23]

However, the degradation of plant cell walls by microorganisms has an important role in the carbon cycle of the earth. Most plant cell walls are composed of approximately 15–40% cellulose, 30–40% hemicelluloses and pectin, and 20% lignin. These components are degraded enzymatically to yield smaller oligomers and eventually

glucose, pentoses and other (Doi and Kosugi, 2004). Cellulose and hemicelluloses are the main structural component of plant cell walls and that are the most abundant carbohydrate polymer in nature but it is extremely difficult to degrade. Two types of enzyme systems for the degradation of plant cell walls have been observed in microorganisms. In the case of aerobic fungi, andaerobic and anaerobic bacteria, several individual cellulolytic-xylanolytic enzymes are secreted that can act synergistically to attack plant cell walls.

2.2.3 Microorganisms producing plant cell wall polysaccharide degrading enzymes

Plant cell wall has evolved mechanisms over millennia to protect the structural forms of polysaccharides from which their cell walls are comprised. Therefore, only a small fraction of microorganisms possess the ability to degrade cellulose efficiently. Fungi and bacteria are the dominant micro organisms responsible for plant cell wall degradation in the biosphere. These microbes show significant diversity in their surviving environments and can be found in mesophilic as well as thermophilic ecosystems where plant matter is abundant, such as forest and pasture soils, hot spring pool and decaying plant debris (Table 2.2-2.3). In these decay communities, degradation of the plant cell wall is accomplished by complex suites of hydrolytic enzymes that all plant cell walldegrading microbes secrete outside of their cell wall (Yang et al., 2011).

Table 2.2 Representative cellulolytic microbes isolated from diverse natural ecosystems (Himmel et al., 2010).

Aerobes			
Bacteria		**Fungi**	
Species	**Source**	**Species**	**Source**
Mesophilic bacteria		**Mesophilic fungi**	
Bacillus brevis	Termite gut	*Aspergillus nidulans* *A. niger*	Soil, wood rot
Cellulomonas fimi	Soil	*Agaricus bisporus*	Compost
Cellvibrio japonicus	Soil	*Coprinus truncorum*	Soil, compost
Cytophaga hutchinsonii	Soil, compost	*Geotrichum candidum*	Soil, compost
Paenibacillus polymyxa	Compost	*Penicillium chrysogenum*	Soil, wood rot
Pseudomonas fluorescens *P. putida*	Soil, sludge	*Phanerochaete chrysosporium*	Compost
Saccharophagus degradans	Rotting marsh grass	*Trichocladium canadense*	Soil
Sorangium cellulosum	Soil	*Hypocrea jecorina*	Soil, rotting canvas
Thermophilic bacteria		**Thermophilic fungi**	
Acidothermus cellulolyticus	Hot spring	*Chaetomium thermophilum*	Soil
Thermobifida fusca	Compost	*Corynascus thermophilus*	Mush compost
Paecilomyces thermophila	Soil, compost	*Thielavia terrestris*	Soil

Table 2.2 Representative cellulolytic microbes isolated from diverse natural ecosystems (Continued)

Anaerobes			
Bacteria		**Fungi**	
Species	**Source**	**Species**	**Source**
Mesophilic bacteria		**Mesophilic fungi**	
Acetivibrio cellulolyticus	Sewage	*Neocallimastix patriciarum*	Rumen
Bacteroides cellulosolvens	Sewage	*Orpinomyces joyonii*	Rumen
Clostridium cellulolyticum	Compost	*Orpinomyces* PC-2	Rumen
Clostridium cellulovorans	Wood fermenter	*Piromyces equi*	Rumen
Clostridium josui	Compost	*Piromyces* E2	Feces
Clostridium papyrosolven	Mud (freshwater)		
Clostridium phytofermentans	Soil		
Fibrobacter succinogenes	Rumen		
Prevotella ruminicola	Rumen		
Ruminococcus albus	Rumen		
Ruminococcus flavefaciens	Rumen		
Thermophilic bacteria			
Anaerocellum thermophilum	Hot spring		
Caldicellulosiruptor saccharolyticus	Hot spring		
Clostridium thermocellum	Sewage, soil, manure		
Clostridium stercorarium	Compost		
Thermotoga maritima	Mud (marine)		
Rhodothermus marinus	Hot spring		

Table 2.3 Microorganisms having xylanolytic abilities (Beg et al., 2001)

Bacteria	• *Acidobacterium capsulatum* • *Bacillus* sp. W–1 • *Bacillus circulans* WL–12 • *Bacillus stearothermophilus* T–6 • *Bacillus* sp. BP-23 • *Bacillus* sp. BP-7 • *Bacillus polymyxa* CECT 153 • *Bacillus* sp. K-1 • *Bacillus* sp. NG-27 • *Bacillus* sp. SPS-0 • *Bacillus* sp. AR-009 • *Bacillus* sp. NCIM 59 • *Cellulomonas fimi* • *Cellulomonas* sp. N.C.I.M 2353
Fungi	• *Acrophialophora nainiana* • *Aspergillus niger* • *Aspergillus kawachii* IFO 4308 • *Aspergillus nidulans* • *Aspergillus fischeri* Fxn1 • *Aspergillus sojae* • *Aspergillus sydowii* MG 49 • *Cephalosporium*sp. • *Fusarium oxysporum* • *Geotrichum candidum* • *Paecilomyces varioti* • *Penicillium purpurogenum* • *Thermomyces lanuginosus* DSM 5826 • *Thermomyces anuginosus*–SSBP • *Trichoderma harzianum* • *Trichoderma reesei*
Yeast	• *Aureobasidiumpullulans* Y-2311–1 • *Cryptococcus albidus* • *Trichosporon cutaneum* SL409

Table 2.3 Microorganisms having xylanolytic abilities (Continued)

Actinomycete	• *Streptomyces*sp. EC 10 • *Streptomyces*sp. B–12–2 • *Streptomyces*sp.T7 • *Streptomycesthermoviolaceus* OPC–520 • *Streptomyceschattanoogensis* CECT 3336 • *Streptomycesviridisporus*T7A • *Streptomyces*sp. QG-11-3 • *Thermomonosporacurvata*

Table 2.4 Microorganisms having pectinolytic enzymes (Yadav et al., 2009)

Microorganisms	
• *Aspergillus sojae*	• *Aspergillus ficuum*
• *Erwinia aroideae*	• *Fusarium oxysporum*
• *Aspergillus niger*	• *Bacillus* sp. PN 33
• *Aspergillus japonicus*	• *Cystofilobasidium capitatum*
• *Alternaria mali*	• *Penibacillus amylolyticus*
• *Penicillium paxilli*	• *Penicillium canescens*
• *Aspergillus oryzae*	• *Penicillium expansum*
• *Colletotrichum lindemuthianum*	• *Penicillium italicum*
• *Pseudomonas marginalis*	• *Penicillium viridicatum*
• *Rhizoctonia solani*	• *Pythium splendens*
• *Rizopus oryzae*	• *Pseudomonas fluorescens*
• *Aspergillus flavus*	

2.2.4 Plant cell wall polysaccharide degrading enzymes

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 domains require different enzymes for efficient hydrolysis. Each polymer is degraded by a variety of microorganisms which produce a battery of enzymes that work synergitically. If so, this may help to explain why cellulolytic microorganisms typically synthesize a range of different cellulase with apparently overlapping specificities and why some xylanases carry substrate-binding domains with affinity for cellulose. In any event, a

cellulolytic and xylanolytic microbe is not in pure culture, but exists in a mixture with other bacterial and/or fungal species. One or more stains serve as the central polymer degraders, which give their respective simple sugar and other degradation product.

Due to its great abundance in nature, the difficulty in its enzymatic breakdown and release pure cellobiose and glucose in accomplishing the latter process, the major cellulose-degradation strain plays a major and critical role in this ecosystem. Cellulose and hemicellulose are connected by β-1,4 bond and no single enzyme is able to hydrolyze them. Most organisms capable of degrading cellulose and xylan synthesize a range of isoenzymes with very similar substrate specificity. Enzyme mechanism generally depends on single molecules fitting in their substrate pocket. The polymer degraders are assisted by other satellite microbes that contribute to the purging of the microenvironment of the polymer breakdown products (i.e., oligosaccharides and simple sugars), end-products (organic acid, molecular hydrogen), and toxic by-product. The more hydrolysis is the more enzymes to work together, synergistic effect (Sun et al., 1998; Tenkanen et al., 1995; Varrot et al., 1999). The enzyme-catalyzed hydrolysis of cellulose to soluble sugar that can intern serve as substrates for fermentation to fuel and chemicals. As the significant and underutilized resources, the hemicellulose fraction of cellulosic substances has recently received attention as a carbohydrate substrate for fermentation to alternative fuels and other biobased products. The application of acid hydrolysis for the release of fermentable xylose has resulted in limited yields as well as the production of inhibitors of fermentation so enzyme for depolymerizing lignocellulose is interesting.

The enzyme for degrade lignocellulosic substance is glycosyl hydrolase. *O*-Glycoside hydrolases (EC 3.2.1.-) are a widespread group of enzymes which hydrolyse the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. The IUB-MB Enzyme nomenclature of glycoside hydrolases is based on their substrate specificity and occasionally on their molecular mechanism; such a classification does not reflect (and was not intended to) the structural features of these enzymes. Later, a more complete classification system was introduced which allowed the classification of not only xylanases, but glycosidases in general (EC 3.2.1.x), and which has now become the standard means for the classification of these enzymes. This system is based on primary structure comparisons of the catalytic

domains only and groups of enzymes in families of related sequences. A classification of glycoside hydrolases in families based on amino acid sequence similarities has been proposed a few years ago. There is a direct relationship between sequence and folding similarities. The classification was updated to 132 families of glycoside hydrolase families exist (see the carbohydrate–active enzyme CAZY server at http://www.cazy.org/fam/acc_GH.html), with approximately one-third of these families being polyspecific, i.e., contain enzymes with diverse substrate specificities.

2.2.3.1 Cellulolytic enzymes

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. Cellulases can be divided into three major enzyme activity classes (Cabezas et al., 2012). 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 hydrolyze cellobiose and in somecases short chain cellooligosaccharides to glucose (Bayer et al., 1998a).

2.2.3.1.1 Endoglucanases (EG, EC 3.2.1.4)

The endo-β-(1,4)-glucanases or β-(1,4)-D-glucan-4-glucanohydro-lases (EC 3.2.1.4), which act randomly on soluble and insoluble β-(1,4)-glucan substrates and are commonly measured by detecting the reducing groups released from carboxymethylcellulose.

2.2.3.1.2 Exoglucanase (EC 3.2.1.91)

The exo-β-(1,4)-D-glucanases, including both the β-(1,4)-D-glucan glucohydrolases (EC 3.2.1.74), which liberate d-glucose from β-(1,4)-D-glucans and hydrolyze D-cellobiose slowly, and β-(1,4)-D-glucan cellobiohydrolase (EC 3.2.1.91), which liberates D-cellobiose in a ‘processive’ manner (successive cleavage of product) from β-(1,4)-glucans

2.2.3.1.3 β-Glucosidase (EC 3.2.1.21)

The β-D-glucosidases or β-D-glucoside glucohydrolases (EC 3.2.1.21) which act torelease D-glucose units from cellobiose and soluble cellodextrins, as well as an array of glycosides.The above classification scheme is not entirely rigid and a few enzymes have properties that do not fit one of the above definitions.

2.2.3.2 Xylanolytic enzymes

Hemicellulose is also highly variablein its structure, and although the actual number of different chemical bonds is limited, they can bepresented in different structural surroundings. The efficient degradation of the polymer requires the concertedaction of many enzymes that have to work synergistically. Total biodegradation of xylan requires endo-β-1,4-xylanase, β -xylosidase, and several accessory enzymes, such as α-L-arabinofuranosidase, α-glucuronidase, acetylxylan esterase, ferulic acid esterase, and *p*-coumaric acid esterase, which are necessary for hydrolyzing various substituted xylans. Table 2.5 lists the enzymes involved in the degradation of xylan and their modes of action. The endoxylanase attacks the main chains of xylans, and β-xylosidase hydrolyzes xylooligosaccharides to xylose. The α-L-arabinofuranosidase and α-glucuronidase remove the arabinose and 4-*O*-methyl glucuronic acid substituents, respectively, from the xylan backbone. The esterases hydrolyze the ester linkagesnbetween xylose units of the xylan and acetic acid (acetylxylan esterase) or between arabinose side chain residues and phenolic acids, such as ferulic acid (ferulic acid esterase) and *p*-coumaric acid (*p*-coumaric acid esterase) (Saha, 2003).

Table 2.5 Enzymes involved in the hydrolysis of complex heteroarabinoxylans (Saha and Bothast, 1999)

Enzyme	Mode of action
Endo-xylanase	• Hydrolyzes mainly interior β-1,4-xylose linkages of the xylan backbone
Exo-xylanase	• Hydrolyzes β-1,4-xylose linkages releasing xylobiose
β-Xylosidase	• Releases xylose from xylobiose and short chain xylooligosaccharides
α-L-Arabinofuranosidase	• Hydrolyzes terminal nonreducing α-arabinofuranose from arabinoxylans
α-Glucuronidase	• Releases glucuronic acid from glucuronoxylans
Acetylxylan esterase	• Hydrolyzes acetylester bonds in acetyl xylans
Ferulic acid esterase	• Hydrolyzes feruloylester bonds in xylans
q-Coumaric acid esterase	• Hydrolyzes *q*-coumaryl ester bonds in xylans

2.2.3.2.1 Xylanase

Xylanases (EC 3.2.1.8) hydrolyze theβ-1,4 bond in the xylan backbone, yielding short xylooligomers. Most known xylanases belong to GH families 10 and 11 (over 300 gene sequences are known), and about 20 more xylanase genes are distributed between families 5, 8 and 43. Two unique xylanase structures have recently been published: a repre-sentative GH8 xylanase from the Antarctic bacterium *Pseudoalteromonas haloplanktis* (Collins et al., 2002; Van Petegem et al., 2003) and an ultrahigh resolution (0.89 A˚) crystal structure of a GH10 xylanase from *Thermoascus aurantiacus*. When the structure of the GH8 xylanase (60% activity at 48 °C) was compared with that of a thermostable GH8 endoglucanase (active and stable at 80 °C), it appeared to have less salt bridges and an increased number of hydrophobic residues exposed to the surrounding. The structure of the *T. aurantiacus* GH10 xylanase is one of the highest resolution structures of a $(\beta/\alpha)_8$ fold (TIM barrel), which allowed the identification of alternate arrangements mediated by buried water molecules. The TIM barrel, one of the most common tertiary folds of protein, is composed of a central barrel formed by parallel β strands surrounded by seven or eight α helices, which shield the barrel from the solvent. Regarding substrate specificity of xylanases, usually, the presence of 4-*O*-

methyl-glucuronic acid and arabinofuranose side chains hinder the binding and hydrolysis of xylan (Biely et al., 1997). Hulbertet al. (Hurlbert and Preston, 2001), however, characterized a xylanase from *Erwinia chrysanthemi* D1, which needs the 4-*O*-methyl-glucuronic acid substitution for recognition and cleavage of the β-1,4-xylosidic bond.

2.2.3.2.2 α-L-Arabinofuranosidases

α-L-arabinofuranosidases (EC 3.2.1.55) and α-L-arabinanases (EC 3.2.1.99) hydrolyze arabinofuranosyl containing hemicelluloses and are found in GH families 3, 43, 51, 54 and 62. Some of these enzymes exhibit broad substrate specificity, acting on arabinofuranoside moieties at *O*-5, *O*-2 and/or *O*-3 as a single substituent, as well as from *O*-2 and *O*-3 doubly substituted xylans, xylooligomers and arabinans (Saha, 2000). The crystal structure of the α-L-arabinanase from *C. japonicus* is the first three-dimensional structure of a GH43 enzyme (Nurizzo et al., 2002b). This enzyme has a five-bladed β-propeller fold, and it is the first enzyme known to display this topology. The structure of an active-site mutant (Asp158→Ala) in complex with α-1,5-L-arabinotriose allowed the identification of the catalytic and substrate-binding residues. In the retaining GH51 α-L-arabinofuranosidase, the catalytic residues were identified by combining detailed kinetic analysis and the azide rescue methodology (Debeche et al., 2002; Shallom et al., 2002).

2.2.3.2.3 α-D-Glucuronidases

α-D-glucuronidases cleave the α-1,2-glycosidic bond of the 4-*O*-methyl-D-glucuronic acid side chain of xylans, and are found exclusively in family 67. The first crystal structure for a GH67 glycosidase was solved for the α-glucuronidase from *C. japonicas* (Nurizzo et al., 2002a). The structure reveals three domains, the central one of which is a $(\beta/\alpha)_8$ barrel, accommodating the active site. These enzymes do not hydrolyze synthetic substrates and therefore the identification of their catalytic residues by mutagenesis and kinetic analysis is problematic. However, crystal structures of complexes of the enzyme with the reaction products xylotriose and glucuronic acid allowed the assignment of the catalytic residues of this inverting enzyme. The results are consistent with the appropriate catalytic mutants of α-glucuronidases from both *C.*

japonicas and *Geobacillus stearothermophilus* T-6 (Nurizzo et al., 2002a; Zaide et al., 2001).

2.2.3.2.4 β-Xylosidase

β-Xylosidases (EC 3.2.1.37) are exo-type glycosidasesthat hydrolyze short xylooligomers into single xyloseunits, and are found in families 3, 39, 43, 52 and 54. The spatial similarity between D-xylopyranose andL-arabinofuranose leads to bifunctional xylosidase–arabinosidase enzymes, found mainly in families3, 43 and 54 (Mai et al., 2000). A detailed kinetic analysis of GH39 xylosidases provided the assignment of the catalyticacid/base residue (Bravman et al., 2001; Vocadlo et al., 2002b). Vocadlo et al. (Vocadlo et al., 2002a) suggested that in the β-xylosidase from *Thermoanaerobacterium saccharolyticum*, there is a case of 'reverse protonation',in which there are two carboxylic acids functioning togetheras the acid/base catalyst. This kind of unique mechanismwas previously suggested for the GH11 xylanase from*Bacillus circulans* (Joshi et al., 2000).

2.2.3.2.5 Hemicellulolytic esterases

Hemicellulolytic esterases include acetyl xylan esterases (EC 3.1.1.72) hydrolyze the acetyl substitutions on xylose moieties, and feruloyl esterases (EC 3.1.1.73), which hydrolyze the ester bond between the arabinose substitutions and ferulic acid. This latter ester bond is involved in crosslinking xylan to lignin. The crystal structures of the CE1 feruloyl esterase modules from two cellulosomal xylanases of *C. thermocellum* were published almost simultaneously (Prates et al., 2001; Schubot et al., 2001). Both structures display a $(\beta/\alpha)_8$ fold, with a classical Ser–His–Asp catalytic triad. The structure of complexes with their substrate also allowed the identification of the binding pocket and the residues comprising the 'oxyanion hole'.

2.2.3.2.6 β-Mannanases

β-Mannanases (EC 3.2.1.78) hydrolyze mannan based hemicelluloses and liberate short β-1,4-manno-oligomers, which can be further hydrolyzed to mannose by β-mannosidases (EC 3.2.1.25). There are currently about 50 β-mannanase gene sequences in GH families 5 and 26, and about 15 β-mannosidase gene sequences in families 1, 2 and 5. Interestingly, β-mannosidases are also found in higher animals and are involved in lysosomal degradation of glycoproteins. Deficiency in these enzymes in humans and

ruminants (termed β-mannosidosis) leads to mental retardation and skeletal abnormalities. The first crystal structure of a GH26 β-mannanase from *Cellvibrio japonicus* (formerly known as *Pseudomonas cellulosa*) displayed the basic features expected from a clan GH-A member: a $(\beta/\alpha)_8$fold, with the two catalytic residues located at the ends ofβstrands 4 and 7 (Hogg et al., 2001). The use of the mechanism based inhibitor 2,4- dinitrophenyl 2-deoxy-2-fluoro-β-mannotrioside, together with the acid/base mutant of this enzyme, enabled the trapping of the Michaelis complex and covalent intermediate complex (Ducros et al., 2002). Although there are three-dimensional structures for representatives of all the GH families in which β-mannosidases are classified, there are still no high resolution structures solved for β-mannosidases. Using a combination of small angle X-ray scattering (SAXS) and poor-resolution crystallographic data, the structure of the molecular envelope of the GH2β-mannosidase from *Trichoderma reesei* was elucidated (Aparicio et al., 2002). In another study, the substrate specificities of two GH1 enzymes,β-mannosidase andβ-glucosidase, were analyzed by constructing several reciprocal replacements of two active site conserved residues (Kaper et al., 2002): in the β-glucosidase, both substitutions increased the specificity for mannosides, whereas in the β-mannosidase, one of the replacements resulted in improved catalysis towards glucosides. In combination with inhibition studies, it was concluded that the mutated residues are directly involved in the stabilization of the transition states, and also participate in the ground state binding of substrates with the equatorial C2-hydroxyl

However, hemicellulases are either glycoside hydrolases or carbohydrate esterases. Our understanding of the structure/function relationship of these enzymes has advanced considerably from the combination of high resolution crystal structures and in depth catalytic analysis. Several new crystal structures and novel specificities have been described in the past year. The structure of hemicellulase and hemicellulose-binding modules are shown in Fig. 2.14. The classifications of hemicellulolytic enzymes into families are shown in Table 2.6 and the occurrence of the main hemicellulase genes in representative microorganisms is shown in Table 2.6.

Table 2.6 The hemicellulolytic enzymes, their classification into glycosyl hydrolase (GH) and carbohydrate esterase (CE) families (Shallom and Shoham, 2003).

Enzymes	**Substrates**	**EC**	**Family**	
Endo-β-1,4-xylanase	β-1,4-xylan	3.2.1.8	GH	5, 8, 10, 11, 43
Exo-β-1,4-xylosidase	β-1,4-xylooligomers Xylobiose	3.2.1.37	GH	3, 39, 43, 52, 72
α-L-Arabinofuranosidase	α-Arabinofuranosyl (1→2) or (1→3) xylooligomers α-1,5-arabinan	3.2.1.55	GH	3, 43, 51, 72, 62
Endo-α-1,5-arabinanase	α-1,5-arabinan	3.2.1.99	GH	43
α-Glucuronidase	4-*O*-methyl-α-glucuronic acid (1→2) xylooligomers	3.2.1.139	GH	67
Endo-β-1,4-mannanase	β-1,4-mannan	3.2.1.78	GH	5, 26
Exo-β-1,4-mannosidase	β-1,4-mannooligomers mannobiose	3.2.1.25	GH	1, 2, 5
α-Galactosidase	α-galactopyranose (1→6) Mannooligomers	3.2.1.22	GH	4, 2, 6, 57, 73
β-Glucosidase	β-glucopyranose (1→4) Mannopyranose	3.2.1.21	GH	1, 3
Endo-galactanase	β-1,4-galactan	3.2.1.89	GH	53
Acetyl xylan esterase	2- or 3-*O*-acetyl xylan	3.1.1.72	CE	1, 2, 3, 4, 5, 6, 7
Acetyl mannan esterase	2- or 3-*O*-acetyl mannan	3.1.1.6		
Ferulic and p-cumaric acid esterases		3.1.1.73	CE	1

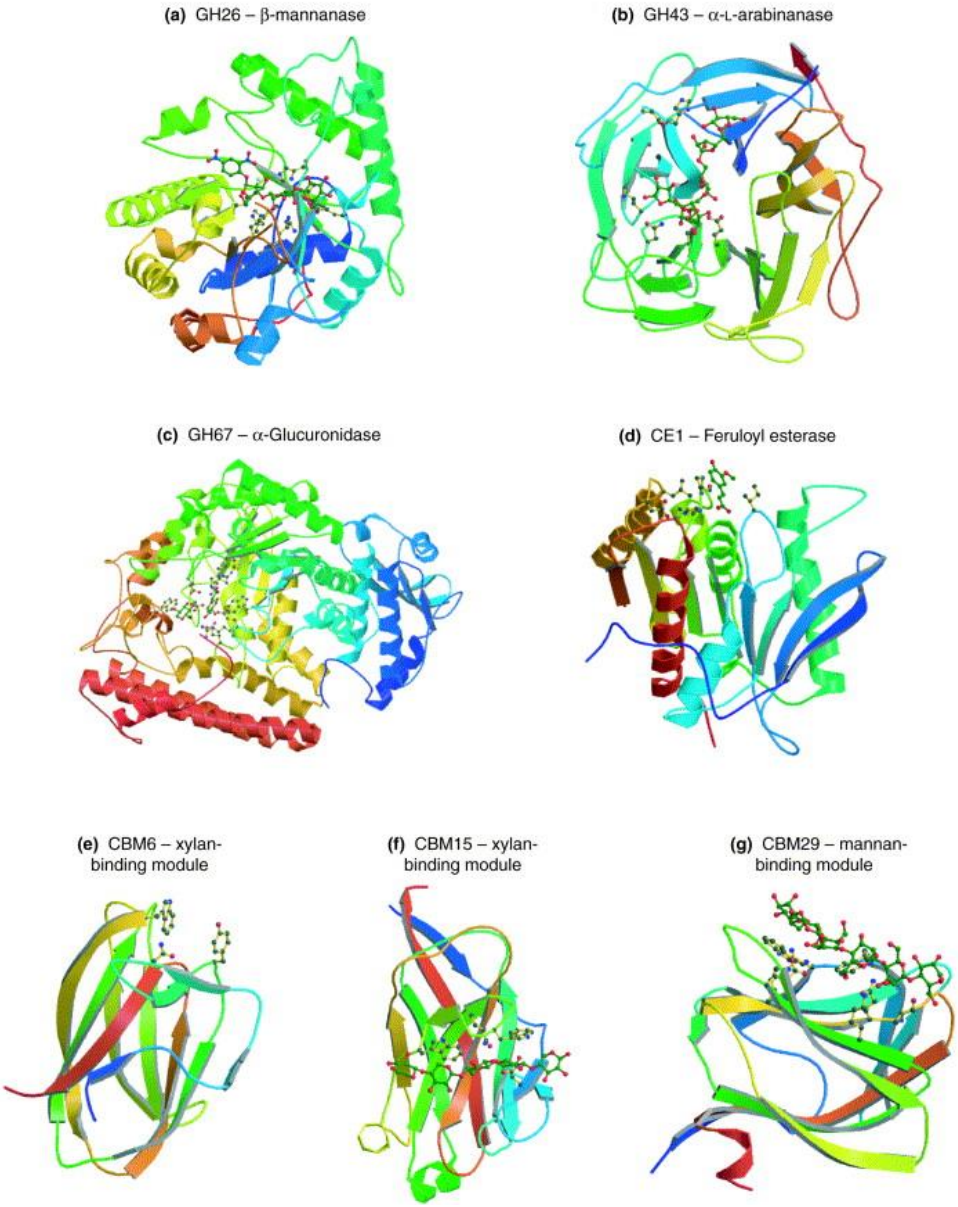


Figure 2.14 Overall folds of recently solved structures of hemicellulases and hemicellulose-binding modules.The proteins are colored fromthe amino (blue) to the carboxyl (red) termini. Ligands and bindingsite residues are in ballandstick representation, oxygen atoms, red; nitrogenatoms, blue; carbon atoms of the ligands, green; carbon atoms and backbone of the protein residues, gray and yellow, respectively.(a) The β-mannanase from *Cellvibrio japonicus*: the E212A in the Michaelis complex with the mechanism based inactivator 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-mannotrioside (PDB code 1GVY). (b) The α-L-arabinanase from *Cellvibrio japonicus*: the D158A mutant in complex with several overlapping α-1,5-L-arabinotriose molecules (PDB code 1GYE).(c) The α-D-glucuronidase from *Cellvibrio*

japonicusin complex with the reaction products glucuronic acid and xylotriose (PDB code 1GQL). (d) The feruloyl esterase module of xylanase Z from *Clostridium thermocellum*: the Ser172→Ala mutant in complex with the reaction product ferulic acid (PDB code 1JT2). (e) The xylan-binding module CBM6 derived from the *Clostridium thermocellum* xylanase 11A (PDB code 1GNY). (f) The xylan-binding module CBM15 derivedfrom the *Cellvibrio japonicas*xylanase Xyn10C, in complex with xylopentaose (PDB code 1GNY). (g) The cellulose/mannan-binding module CBM29-2 from the cellulosomal noncatalytic protein NCP1 of *Piromyces equi*, in complex with mannohexaose (PDB code 1GWL). The figures were prepared using MOLSCRIPT and Raster3D (Shallom and Shoham, 2003).

2.2.3.3 Pectinolytic enzymes

There is a group of pectinolytic enzymes broadly known as pectinases which are involved in pectin degradation. These enzymes have been reviewed a number of times. Base donmode of action and preferred substrate these enzymes can be briefly classified as mentioned below (Table 2.7). Due to the great diversity in the structure of pectins they can be categorized into enzymes acting on the ‘‘smooth regions’’ composed of homogalacturonan and enzymes acting on the ‘‘hairy region’’ composed of rhamnogalacturonan and side chains. The groups of enzymes which are involved in the degradation of hairy region of pectins are rhamnogalacturonan hydrolase (RG hydrolase), rhamnogalacturonan lyase, rhamnogalacturonan rhamnohydrolase (RG rhamnohydrolase), rhamnogalacturonan galactohydrolase (RG galactouronohydrolase). These enzymes have rarely been studied (Suykerbuyk et al., 1995) and need extensive studies on their structures and functions. There are however other accessory enzymes involved in degradation of side chains of pectins which include α-arabinofuranosidase (E.C. 3.2.1.55), endoarabinase (E.C. 3.2.1.99), β-galactosidase (E.C. 3.2.1.23), endogalactanase (E.C. 3.2.1.89) and feruloyl and *p*-coumaroyl esterases. The group of enzymes which are involved in the degradation of ‘‘smooth region’’ (homogalacturonan) include deesterifying enzymes i.e. pectinmethyl esterases (PME, E.C. 3.1.1.11) and pectin acetyl esterase (PAE, E.C. 3.1.1.6) which removes methoxyl and acetyl residues of pectin producing polygalacturonic acid. The other subclass of homogalacturonan degrading group are broadly termed as depolymerases which break the α-1,4-linkages either by hydrolysis i.e. Polygalacturonases (PG, E.C. 3.2.1.15) or via transelimination mechanism namely pectate lyases (PL, E.C. 4.2.2.2) and pectin lyases

(PNL, E.C. 4.2.2.10) (Table 2.7). The main role that these enzymes (pectinases) play are basically to degrade pectin that occurs as structural polysaccharides in the middle lamella and primary cell wall of young plant cells(Yadav et al., 2009).

Table 2.7 Classification of pectinolytic enzymes (Yadav et al., 2009).

Type of pectinases	E.C. no.	Substrate	Mode of action	Product
1. Esterases				
(a) PME	3.1.1.11	Pectin	Hydrolysis	Pectic acid + methanol
(b) PAE	3.1.1.6	Pectin	Hydrolysis	Pectic acid + ethanol
2. Depolymerases				
(a) Hydrolases				
(i) Endo PG	3.2.1.15	Pectic acid	Hydrolysis	Oligogalacturonates
(ii) Exo PG	3.2.1.67	Pectic acid	Hydrolysis	Monogalacturonates
(b) Lyases				
(i) Endo PL	4.2.2.2	Pectic acid	Transelimination	Unsaturated oligogalactouronates
(ii) Exo PL	4.2.2.9	Pectic acid	Transelimination	Unsaturated digalactouronates
(iii) Endo PNL	4.2.2.10	Pectin	Transelimination	Unsaturated methyloligogalactouronates

PME, pectin methyl esterase; PAE, pectin acetyl esterase; PG, polgalacturonase; PL, pectate lyase; PNL, pectin lyase

2.2.5 Application of plant cell wall polysaccharide degrading enzymes

2.2.5.1 Application of cellulolytic enzymes in various industries

Cellulases are being commercially produced by several industries globally and are widely being used in food, animal feed, fermentation, agriculture, pulp and paper, and textile applications. With modern biotechnology tools, especially in the area of microbial genetics, novel enzymes and new enzyme applications will become available for the various industries. Summary of microbial cellulases find applications in various industries as shown in Table 2.8.

2.2.5.1.1 Pulp and paper industry.

Interest in the application of cellulases in the pulp and paper industry has increased considerably during the last decade (Mai et al., 2004). The mechanical pulping processes such as refining and grinding of the woody raw material lead to pulps with high content of fines, bulk, and stiffness. While in contrast, biomechanical pulping using cellulases resulted in substantial energy savings (20–40%) during refining and improvements in hand sheet strength properties (Bhat, 2000; Pere et al., 2001). Mixtures of cellulases (endoglucanases I and II) and hemicellulases have also been used for biomodification offiber properties with the aim of improving drainage and beatability in the paper mills before or after beating of pulp (Dienes et al., 2004). Mansfield et al. (Mansfield et al., 1996) studied the action of a commercial cellulase preparation on different fractions of Douglasfir kraft pulp and observed that the cellulase treatment decreased the defibrillation reducing the fibre coarseness.While endoglucanases have the ability to decrease the pulpviscosity with a lower degree of hydrolysis, cellulases have also been reported to enhance the bleachability of softwood kraft pulp producing a final brightness score comparable to that of xylanase treatment (Kuhad et al., 2011). Cellulases alone, or used in combination with xylanases, are beneficial for deinking of different types of paperwastes. Most applications proposed so far use cellulases and hemicellulases for the release of ink from the fiber surface by partial hydrolysis of carbohydrate molecules (Chander Kuhad et al., 2010). It has been postulated that improvements in dewatering and deinking of various pulps result in the peeling of the individual fibrils and bundles, which have high affinity for the surrounding water and ink particles (Kibblewhite et al., 1995). The main advantages of enzymatic deinking are reduced or eliminated alkali usage, improved fiber brightness, enhanced strength properties, higher pulp freeness and cleanliness, and reduced fine particles in the pulp (Chander Kuhad et al., 2010; Kuhad et al., 2010). Moreover, deinking using enzymes at acidic pH also prevents the alkaline yellowing, simplifies the deinking process, changes the ink particle size distribution, and reduces the environmental pollution. Although enzymatic deinking can lower the need for deinking chemicals and reduce the adverse environmental impacts of the paper industry, the excessive use of enzymes must be avoided, because significant hydrolysis of the fines could reduce the bond ability of the fibers (Karnis, 1995). Interestingly, the use of cellulases in improving the drainage has also been pursued by several mills with the objective to increase the production rate. Enzyme treatmentsre move some of the fines or peel off fibrils on the fiber surface and

dissolved and colloidal substances, which often cause severe drainage problems in paper mills. In this aspect, cellulases have shown considerable improvement inthe overall performance of paper mills (Bhat, 2000). Enzymatic treatment also destabilizes the lipophilic extractives in the filtrates and facilitates their attachment to thermo mechanical pulping fibers. These enzymes are also used in preparation of easily biodegradable cardboard, manufacturing of softpaper including paper towels and sanitary paper, and removal of adhered paper (Buchert et al., 1998).

2.2.5.1.2 Textile industry

Cellulases are the most successful enzymes used in textile wet processing, especially finishing of cellulose based textiles, with the goal of improved hand and appearance (Hebeish and Ibrahim, 2007; Karmakar and Ray, 2011). Cellulases have been successfully used for the biostoning of jeans and biopolishing of cotton and other cellulosic fabrics. During the biostoning process, cellulases act on the cotton fabric and break offthe small fiber ends on the yarn surface, thereby loosening the dye, which is easily removed by mechanical abrasion in the wash cycle. The advantages in there placement of pumice stones by a cellulose based treatment include less damage of fibers, increased productivity of the machines, and less work intensive and environment benign (Sukumaran et al., 2005; Uhlig and Linsmaier-Bednar, 1998). The biopolishing is usually carried out duringthe wet processing stages, which include desizing, scouring, bleaching, dyeing, and finishing. The acidic cellulases improve softness and water absorbance property of fibres, strongly reduce the tendency for pill formation, and provide a cleaner surface structure with less fuzz (Sreenath et al., 1996). Cellulase preparations rich in endoglucanases are best suited for biopolishing enhancing fabric look, feel, and color without needing any chemical coating of fibers. The action of cellulases removes short fibers, surface fuzziness, creates a smooth and glossy appearance, and improves colorbrightness, hydrophilicity and moisture absorbance, and environmentally friendly process (Bhat, 2000). Similarly, endoglucanase activity rich cellulase is also proved better for biofinishing. Most cotton or cotton blended garments, during repeated washing, tend to be comefluffy and dull, which is mainly due to the presence ofpartially detached microfibrils on the surface of garments.The use of cellulases can remove these microfibrils and restore a smooth surface and original color to the garments (Hebeish and Ibrahim, 2007; Ibrahim et al., 2011).

2.2.5.1.3 Bioethanol industry

Enzymatic saccharification of lignocellulosic materials such as sugarcane bagasse, corncob, rice straw, *Prosopis juliflora*, *Lantana camara*, switch grass, saw dust, and orest residues by cellulases for biofuel production is perhaps the most popular application currentlybeing investigated (Gupta et al., 2011a; Sukumaran et al., 2005). Bioconversion of lignocellulosic materials into useful and higher value products normally requires multistep processes (Ghosh and Singh, 1993; Wyman et al., 2005). These processes include; pretreatment (mechanical, chemical, orbiological), hydrolysis of the polymers to produce readily metabolizable molecules (e.g., hexose and pentose sugars), bioconversion of these smaller molecules to support microbial growth and/or produce chemical products, and these paration and purification of the desired products. The utility cost of enzymatic hydrolysis may be low compared with acid or alkaline hydrolysis because enzyme hydrolysisis usually conducted at mild conditions (pH 4–6 andtemperature 45-50°C) and does not have corrosion issues (Chander Kuhad et al., 2010; Gupta et al., 2011a).Technologies are currently available for all steps in the bioconversion of lignocellulosics to ethanol and otherchemical products (Kuhad et al., 1997a; Kuhad and Singh, 1993; Sun and Cheng, 2002). However, some of these technologies must be improved to produce renewable biofuel and other by products at prices, which can compete with more conventional production systems. Not only the recalcitrance of the substrate, but also several other factors thatalso limit cellulase efficiency during the hydrolysis process including end product inhibition, thermal deactivation ofthe native protein, nonspecific binding to lignin, and irreversible adsorption of the enzymes to the heterogeneoussubstrate (Mosier et al., 2005; Taniguchi et al., 2005). To reduce the enzyme cost in the production of fuel ethanol from lignocellulosic biomass, two aspects are widely addressed: optimization of the cellulase production and development of a more efficient cellulose based catalysis system. Protein engineering and directed evolution are powerfultools that can facilitate the development of more efficient thermophilic cellulases (Baker et al., 2005). Strategies for recycling andreusage of the enzymes may also be used to reduce enzymatic hydrolysis costs (Lee et al., 1995; Mosier et al., 2005; Sun and Cheng, 2002). Among different strategies to recover and reuse the cellulases are concentration of the cellulose fraction by ultrafiltration to remove sugars and other small compounds that may inhibit the action of the enzymes and recycling of immobilized enzymes, which enables separation of the enzymes from the process flow (Dourado et al., 2002; Mosier et al.,

2005; Tu et al., 2007). However, the recycling techniques are mostly tested at laboratory scale. Therefore, the ability to scale up the techniques, the robustness, and feasibility still needs to be demonstrated.

2.2.5.1.4 Wine and brewery industry

Microbial glucanases andrelated polysaccharides play important roles in fermentation Processes to produce alcoholic beverages including beers andwines (Bamforth, 2009; Sukumaran et al., 2005). These enzymes can improve bothquality and yields of the fermentedproducts. Glucanasesare added either during mashing or primary fermentation tohydrolyze glucan, reduce the viscosity of wort, and improvethe filterability (Bamforth, 2009). In wine production, enzymes such as pectinases, glucanases, and hemicellulases play an important role byimproving color extraction, skin maceration, must clarification, filtration, and finally the wine quality and stability. β-Glucosidases can improve the aroma of wines by modifying glycosylated precursors. Macerating enzymes alsoimprove pressability, settling, and juice yields of grapes usedfor wine fermentation. A number of commercial enzyme preparations are now available to the wine industry. The main benefits of using these enzymes during wine making include better maceration, improved color extraction, easy clarification, easy filtration, improved wine quality, and improved stability. Beer brewing is based on the action of enzymes activated during malting and fermentation. Malting of barley depends on seed germination, which initiates the biosynthesis and activation of α-and β-amylases, carboxypeptidase, and β-glucanase that hydrolyze the seed reserves. A range of improved enzymes like cellulase and pectinase that would be exogenously added to the process are expected to enhance the productivity of existing brewing processes in future (Bamforth, 2009).

2.2.5.1.5 Food processing industry

Cellulases have a wide range of potential applications in food biotechnology as well. The production of fruit and vegetable juices requires improved methods for extraction, clarification, and stabilization. Cellulases also have an important application as a part of macerating enzymes complex (cellulases, xylanases, and pectinases) used for extraction and clarification of fruit and vegetable juices to increase the yield of juices (De Carvalho et al., 2008; Minussi et al., 2002). The macerating enzymes are used to improve cloud stability and texture and decrease viscosity of the nectars and purees

from tropical fruits such as mango, peach, papaya, plum,apricot, and pear (Bhat, 2000; De Carvalho et al., 2008; Sukumaran et al., 2005). Texture, flavor, and aroma properties of fruits and vegetables can be improved by reducing excessive bitterness of citrus fruits by infusionof enzymes such as pectinases and β-glucosidases (Baker and Wicker, 1996; Rai et al., 2007; Youn et al., 2004). Enzyme mixtures containing pectinases, cellulases, and hemicellulases are also used for improved extraction of oliveoil. Use of macerating enzymes not only improves the cloud stability and texture of nectars and purees, but also decreases their viscosity rapidly. Thus, the macerating enzymes,composed of mainly cellulase and pectinase, play a key role in food biotechnology, and their demand will likely increase forextraction of juice from a wide range of fruits and vegetables (Dourado et al., 2002). Furthermore, infusion of pectinases and β-glucosidases has also shown to alter the texture, flavor, and other sensory properties such as aroma and volatile characteristics of fruitsand vegetables (Bhat, 2000; Humpf and Schreier, 1991; Karmakar and Ray, 2011; Marlatt et al., 1992).

2.2.5.1.6 Animal feed industry.

Applications of cellulases andhemicellulases in the feed industry have received considerable attention because of their potential to improve feed value and performance of animals (Dhiman et al., 2002). Pretreatment ofagricultural silage and grain feed by cellulases or xylanases can improve its nutritional value. The enzymes can also eliminate antinutritional factors present in the feed grains, degrade certain feed constituents to improve the nutritional value, and provide supplementary digestive enzymes such as proteases, amylases, and glucanases. For instance, thedietary fiber consists of nonstarch polysaccharides such as arabinoxylans, cellulose, and many other plant components including resistant dextrins, inulin, lignin, waxes, chitins, pectins, β-glucan, and oligosaccharides, which can act as antinutritional factor for several animals such as swine (Ali et al., 1995b). In this case, the cellulases effectively hydrolyse the antinutritional factor, cellulose in the feed materials into easily absorbent ingredient thus improve animal health and performance. β-Glucanases and xylanases have been used in the feed of monogastric animals to hydrolyze nonstarch polysaccharides such as β-glucans and arabinoxylans. Cellulases, used as feed additives alone or with proteases, can significantly improvethe quality of pork meat. Glucanases and xylanases reduce viscosity of high fibre rye and barley based feeds in poultry and pig. These enzymes can also cause weight gain in chickens and piglets by improving digestion and absorption of

feed materials (Bhat, 2000; Karmakar and Ray, 2011; Shrivastava et al., 2011). Most low quality feedstuffs contain higher concentrations of cellulose, small amounts of protein and fat and relatively high ash contents when compared with high quality feedstuffs. Cellulases can be used to improve silage production for cattle feeding, which involves enhancement of the digestibility of grasses containing large amounts of potentially total digestible nutrients and energy values together with only small amounts of water soluble carbohydrates. The forage diet of ruminants, which contains cellulose, hemicellulose, pectin, and lignin, is more complex than the cereal based diet of poultry and pigs. Enzyme preparations containing high levels of cellulase, hemicellulase, and pectinase have been used to improve the nutritive quality of forages (Graham and Balnavel, 2008; Lewis et al., 1996). Nevertheless, the results with the addition of enzyme preparations containing cellulase, hemicellulase and pectinase to ruminant diet are some what inconsistent. Animal feedstock production processes generally include heat treatments that inactivate potential viral and microbial contaminants. Application of thermophilic cellulase in feedstock production has the potential to reduce pathogens aswell as to enhance digestibility and nutrition of the feed, thereby facilitating a combination of heat treatment and feed transformation in a single step (Karmakar and Ray, 2011). Cellulases have a positive effect on the caecal fermentation processes by increasing the production of propionic acid, whichact as a bacteriostatic material and thus can decrease the colonization of pathogenic bacteria (Bolduan et al., 1988; Pazarlioglu et al., 2005).

2.2.5.1.7 Agricultural industries.

Various enzyme preparations consisting of different combinations of cellulases, hemi-cellulases, and pectinases have potential applications in agriculture for enhancing growth of crops and controlling plant diseases (Bhat, 2000). Plant or fungal protoplasts producsing microbial hydrolases can be used to produce hybrid strains with desirable properties. Cellulases and related enzymes from certain fungi are capable of degrading thecell wall of plant pathogens in controlling the plant disease (Bhat, 2000). Fungal β-glucanases are capable of controlling diseases by degrading cell walls of plant pathogens. Many cellulolytic fungi including *Trichoderma* sp., *Geocladium* sp., *Chaetomium* sp., and *Penicillium* sp. are known to play a keyrole in agriculture by facilitating enhanced seed germination,rapid plant growth and flowering, improved root system andincreased crop yields (Harman and Kubicek, 1998). Although these fungi

have both direct (probably through growth promoting diffusible factor) and indirect (by controlling the plant disease and pathogens) effects on plants, it is not yet clearhow these fungi facilitate the improved plant performance. It has been reported that β-1,3-glucanase and N-acetyl-glucosaminidase from *T. harzianumstrain* P1 synergistically inhibited the spore germination and germ tube elongation of *B. cinerea* (Lorito et al., 1994). Moreover, the exoglucanase promoters of *Trichoderma* are used for the expression of the different proteins, enzymes and antibodies in large amount. The exoglucanase promoters of *Trichoderma* have been usedfor the expression of chymosin and other proteins: glucoamylase, lignin peroxidase, and laccase (Harkki et al., 1989; Saloheimo et al., 1989; Saloheimo and Niku-Paavola, 1991). Cellulolytic fungi applications such as *Aspergillus*, *Chaetomium* and *Trichoderma*, and actinomycetes have shown promising results. Therefore using exogenous cellulase may be a potential means to accelerate straw decomposition and increase soil fertility (Abdulla and El-Shatoury, 2007; Bowen and Harper, 1990; Fontaine et al., 2004; Tiwari et al., 1987).

2.2.5.1.8 Olive oil extraction

In recent years, extraction ofolive oil has attracted the interest of international market because of its numerous health claims. Extraction of oliveoil involves (1) crushing and grinding of olives in a stoneor hammer mill; (2) passing the minced olive paste through a series of malaxeurs and horizontal decanters; (3) high speed centrifugation to recover the oil (Harman and Kubicek, 1998). To produce highquality olive oil, freshly picked, clean, and slightly immaturefruits have been used under cold pressing conditions (De Faveri et al., 2008; Harman and Kubicek, 1998). However, high yields have been obtained with fullyripened fruit, when processed at higher than ambient temperatures, but this resulted in oil with high acidity, rancidity, and poor aroma (Harman and Kubicek, 1998). Hence, an improved method for the extraction of high quality olive oil was needed to meet the growing consumer demand. The commercial enzyme preparation, Olivex (a pectinase preparation withcellulase and hemicellulase from*Aspergillus aculeatus*), was the first enzyme mixture used to improve the extraction of olive oil. Furthermore, the use of macerating enzymes increased the antioxidants in extravirg in olive oiland reduced the induction of rancidity. The main advantages of using macerating enzymes during olive oilextraction are (1) increased extraction (up to 2 kg oil per100 kg olives) under cold processing conditions; (2) better centrifugal fractionation of the oily must; (3) oil with high levels of antioxidants and

vitamin E; (4) slow induction of rancidity; (5) overall improvement in plant efficiency; (6) low oil content in the waste water. Likewise, the macerating enzymes could play a prominent role in theextraction of oils from other agricultural oilseed crops. These enzymes can also be used during olive paste malaxation. It is necessaryto underline that the selected enzymes are naturally presentinside the olive fruit, but they are strongly deactivated during the critical pressing step, probably because of oxidation phenomena (Chiacchierini et al., 2007). So, the replacement of these enzymes is expected to be appropriate in relation with the role they play in determining the final product quality (De Faveri et al., 2008; Hurlbert and Preston, 2001).

2.2.5.1.9 Carotenoid extraction.

Carotenoids are the main group of coloring substances in nature being responsible for many plant colors fromred to yellow. There is a continuously growing market for carotenoids as food colorants due totheir desirable properties, such as their natural origin, null toxicity, and high versatility, providing both lipo- and hydrosoluble colorants with colors ranging from yellow tored. In addition, provitamin A activity, a role inlipid oxidation, and anticarcinogenic properties are very important biological functions of these pigments. Usually a combination of cellulolytic and pectinolytic enzymes accelerates the rate of hydrolysis for achieving complete liquefaction. Cellulase randomly splits cellulose chains into glucose whereas commercial pectinase preparations from *Aspergillus niger* have pectinesterase (PE), polygalacturonase (PG), and pectin lyase (PL) activity.The use of pectinase and cellulase enzymes disrupts the cell wall of orange peel, sweet potato and carrot, and releases the carotenoids in the chloroplasts and in cell fluids (Cinar, 2005).

2.2.5.1.10 Detergent industry.

Use of cellulases along with protease and lipase in the detergents is a more recent innovation in this industry (Kuhad and Singh, 1993). Cellulase preparations capable of modifying cellulose fibrils can improve color brightness,feel, and dirt removal from the cotton blend garments. The industrial application of alkaline cellulases as a potential detergent additive is being actively pursued with a view to selectively contact the cellulose within the interior of fibers and remove soil in the interfibril spaces in the presence of themore conventional detergent ingredients (Kuhad and Singh, 1993; Sukumaran et al., 2005). Nowadays liquid laundry detergent containing anionic or

nonionicsurfactant, citric acid or a water soluble salt, protease, cellulose, and a mixture of propanediol and boric acid or its derivative has been used to improve the stability of cellulases. As most of the cellulose fibers in the modern textile industry enzymes are used increasingly in the finishing of fabrics and clothes are arranged as long, straight chains of some small fibers can protrude from the yarn or fabric. The cellulases are applied to remove these rough protuberances for a smoother, glossier, and brighter colored fabric (Karmakar and Ray, 2011).

2.2.5.1.11 Wastemanagement.

The wastes generated from forests,agricultural fields, and agro-industries contain a large amount of unutilized or underutilized cellulose, causing environmental pollution (Milala et al., 2005). Nowadays, these socalled wastes are judiciously utilized to produce valuable products such as enzymes, sugars, biofuels, chemicals, cheap energy sources for fermentation, improved animal feeds, and humannutrients (Gupta et al., 2011b; Gupta et al., 2009; Karmakar and Ray, 2011; Kuhad et al., 1997b).

Table 2.8 Applications of cellulases in various industries (Kuhad et al., 2011).

Industry	Applications
Agriculture	• Plant pathogen and disease control; generation of plant and fungal protoplasts; enhanced seed germination and improved root system; enhanced plant growth and flowering; improved soil quality; reduced dependence on mineral fertilizers.
Bioconversion	• Conversion of cellulosic materials to ethanol, other solvents, organic acids and single cell protein, and lipids; production of energy-rich animal feed; improved nutritional quality of animal feed; improved ruminant performance; improved feed digestion and absorption; preservation of high quality fodder.
Detergents	• Cellulase-based detergents; superior cleaning action without damaging fibers; improved color brightness and dirt removal; remove of rough protuberances in cotton fabrics; antiredeposition of ink particles.
Fermentation	• Improved malting and mashing; improved pressing and color extraction of grapes; improved aroma of wines;improved primary fermentation and quality of beer; improved viscosity and filterability of wort; improvedmustclarification in wine production; improved filtration rate and wine stability.
Food	• Release of the antioxidants from fruit and vegetable pomace; improvement of yields in starch and proteinextraction; improved maceration, pressing, and color extraction of fruits and vegetables; clarification of fruitjuices; improved texture and quality of bakery products; improved viscosity fruit purees; improved texture,flavor, aroma, and volatile properties of fruits and vegetables; controlled bitterness of citrus fruits.
Pulp and Paper	• Coadditive in pulp bleaching; biomechanical pulping; improved draining; enzymatic deinking; reduced energyrequirement; reduced chlorine requirement; improved fiber brightness, strength properties, and pulp freenessand cleanliness; improved drainage in paper mills; production of biodegradable cardboard, paper towels, andsanitary paper.
Textile	• Biostoning of jeans; biopolishing of textile fibers; improved fabrics quality; improved absorbance propertyof fibers; softening of garments; improved stability of cellulosic fabrics; removal of excess dye from fabrics;restoration of colour brightness.
Others	• Improved carotenoids extraction; improved oxidation and colour stability of carotenoids; improved olive oilextraction; improved malaxation of olive paste; improved quality of olive oil; reduced risk of biomass waste;production of hybrid molecules; production of designer cellulosomes.

2.2.5.2 Application of xylanolytic enzymes in various industries

Xylanolytic enzymes from microorganism have attracted great deal of attention in the last decade, particularly because of their biotechnological potential in various industrial processes (Bajpai, 1999; Kuhad and Singh, 1993; Niehaus et al., 1999; Wong and Saddler, 1992), such as food, feed, and pulp and paper industries (Table 2.9). Xylanases have shown an immense potential for increasing the production of several useful products in a most economical way.

2.2.5.2.1 Bioethanol production

The process of ethanol production from lignocellulosic biomass includes delignification of plant biomass and hydrolysis of cellulose and hemicellulose to monosaccharides (Beg et al., 2001). The hydrolysis process can be performed by treatment with acids at high temperatures or by enzyme action. The acidic hydrolysis requires significant energy consumption and acidresisting equipment which makes the process more expensive. However, enzymatic hydrolysis does not have these disadvantages. Because of the complex composition of lignocellulosic biomass, the synergistic action of several enzymes viz. endoglucanases, (EC3.2.1.4), β-glucosidases (EC 3.2.1.21), endo-1, 4-β-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37) is required for complete hydrolysis (De Vries and Visser, 2001). In some cases, endo-1,4-β-xylanase has been reported to be a bifunctional enzyme having endo-1,4-β-xylanase aswell as cellulase activity. Bifunctionality of endo-1,4-β-xylanase could result in more efficientand cheaper saccharification process of the agricultural residues, municipal and industrial wastes used for bioethanol production as it can degrade both cellulose and xylan residues. Saccharification of the cellulose and hemicellulose in biomass results in sugarrich liquid streams useful for the production of a variety of value added products including ethanol,furfural, and various functional biopolymers (Fuller et al., 1995). An increased possibility of fermentation ofboth hexoses and pentoses sugars present in lignocelluloses into methanol has also beenreported (Roehr et al., 2001).

2.2.5.2.2 Animal feedstocks

It has been found that addition of endo-1,4-β-xylanase in animal feed stimulates animal growth rates by improving digestibility and improving the quality of animal litter (Biely, 1985; Damiano et al., 2003). The endosperm cell walls of cereal grains are enriched in polysaccharides that are usually in the form of arabinoxylans mixed with

linked β-glucans, celluloses, mannans, and galactans (Longland et al., 1995). Of these, arabinoxylans and β-glucans constitute major parts. For example, wheat, triticale, and rye are enriched in arabinoxylan whereas oats and barley contain more β-glucans(Bonnin et al., 1998). Generally, the viscous properties of the polysaccharides make them difficult to digest by domestic animals. Therefore, inclusion of xylanase in wheat or rye based diets or β-glucanase in barley based diets is an important measure to enhance the availability of the polysaccharides (Annison, 1992; Salih et al., 1991). Endo-1,4-β-xylanase and 1,3-1,4-β-glucanase have been widely applied for breakdown of the internal β-1,4-linkages of 1,4- β-D-xylan backboneand specific cleavage of 1,4-β-D-glucosidic bonds adjacent to β-1,3-linkages in the mixed linked β-glucans, respectively (Collins et al., 2005). Therefore, endo-1,4-β-xylanase thins out the gut contents and allows increased nutrient absorption and increased diffusion of the pancreatic enzymes. Endo-1,4-β-xylanase also converts hemicellulose to sugars and thereafter, nutrients trapped within the cell walls are released and chickens get sufficient energy from lesser feed. The barn is cleaner due to more thoroughly digested feed and the chicken waste is drier and lesser sticky. In addition, chicken eggs are cleaner due to dry laying area (Planas, 2000).

2.2.5.2.3 Production of xylooligosaccharides in paper industries

A recent and exciting application of xylanases is the production of xylooligosaccharides (XOs) (Tan et al., 2008), these are newly developed functional oligosaccharides that feature many beneficial biomedical and health effects, such as the stimulation of human intestinal *Bifidobacteria* growth (Yang et al., 2005). Currently, XOs are produced mainly by enzymatic hydrolysis due to the high specificity, the negligible substrate loss, and side product generation (Tan et al., 2008). Pulp and paper industry is one of the major polluting industries in the developed as well as developing countries (Battan et al., 2007). Xylan degradation requires the interaction of several enzymatic activities, including endo-1,4-β-xylanase, xylosidase and arabinosidase. The end products of this degradation are xylose, arabinose, and methyl-glucuronic acid containing oligosaccharides. Xylooligosaccharides are sugar oligomers showing potential for practical applications in a variety of fields, including pharmaceuticals, feed formulations, agricultural purposes and food applications (Vazquez et al., 2001). As additives for functional foods, XOs have prebiotic action showing positive biological effects such as improvement in the intestinal function by increasing the number of

healthy *Bifidobacteria* (Fooks and Gibson, 2002; Rycroft et al., 2001). These xylooligosaccharides if used as dietary supplements may be beneficial to gastrointestinal health and may reduce the risk of colon cancer. As food ingredients, XOs have an acceptable odor, and are non-carcinogenic and have low-calorie value, allowing their utilization in antiobesity diets. In food processing, XOs show advantages over insulin in terms of resistance to both acids and heat, allowing their utilization in low pH juices and carbonated drinks (Modler, 1994).

2.2.5.2.4 Pulp fiber morphology

After comparing micrographs of soft wood sulphate pulp with that of the same pulp after xylanase prebleaching and alkali extraction, Pekarovicova et al. (Pekarovicova et al., 1992) found that there is no marked change in the shape of fiber after xylanase prebleaching. However, flattening of the fiber arise after alkaline extraction, confirming that the lignin extraction from the cell wall results in its collapse. Another report on application of xylanases for bagasse sulfite pulp pretreatment also confirmed the formation of ‘peels’ and ‘cracks’ of fiber surfaces (Agnihotri et al., 2012).

2.2.5.2.5 Need for cellulase free endo-1,4-β-xylanase

The public concerned on the impact of pollutants from paper and pulp industries, whichuse chlorine as the bleaching agent act as strong driving force in developing biotechnology aided techniques for novel bleaching i.e biobleaching. The cellulases easily result in the hydrolysis of cellulose, which should be the main recovered product in paper industry. However, the enzyme preparations from microorganisms producing higher levels of endo-1, 4-β-xylanases with tenuous or no cellulase activity can be applied in paper industry because the loss of pulp viscosity is at the minimum level (Sharma and Kumar, 2013).

2.2.5.2.6 Biobleaching

Endo-1, 4-β-xylanases have also been applied to biobleaching processes, as their removal of the xylan layer enhances the bleaching effect of chemical reagents (Birijlall et al., 2011; Ko et al., 2011; Valls and Roncero, 2009). Therefore, numerous biobleaching studies using endo-1, 4-β-xylanases or laccases have been reported (Chauhan et al., 2006; Fillat et al., 2010). A few studies have assessed both enzymes in sequence or jointly (Bajpai et al., 2006; Valls and Roncero, 2009). Chlorination of pulp

does not show any decolorizing effect, and in fact, the color of the pulp may increase with chlorination and it is the oxidative mechanism which aids pulp bleaching. At low pH, the main reaction of chlorine is chlorination rather than oxidation. Therefore, chlorine selectively chlorinates and degrades lignin compounds rather than the carbohydrates moieties (eg. hemicelluloses–xylan) in the unbleached pulp. Enzymatic hydrolysis of the reprecipitated and relocated xylans on the surface of the fibers apparently renders the structure of the fiber more permeable (Sharma and Kumar, 2013).

2.2.5.2.7 Kraft process

Removal of residual lignin from kraft pulp is physically and chemically restricted by hemicelluloses. Lignin has been reported to link with hemicelluloses and there are reports regarding the isolation of lignin carbohydrate complexes from the kraft pulp (Laine et al., 2004).

2.2.5.2.8 Bread fluffier

Xylanases are used as additives in the baking industry to increase the elasticity of the gluten network. Elasticity improves handling and stability of the dough. Thereafter, the enzyme gets denatured and inactivated during bread baking. Addition of xylanases to wheat flour for bread making changes arabinoxylans resulting in nearly 10% more voluminous loaf (Basinskiene et al., 2006). Arabinoxylans are highly branched xylans present in wheat and rye flour. Several xylanases from bacterial and fungal sources are used in baking industries (Pariza and Johnson, 2001). Xylanase action also increases crumb softness after storage. It is important to note that due to differences insubstrate specificities, action patterns, interactions with inhibitors and kinetics capabilities, all xylanases are not effective in baking (Basinskiene et al., 2006). On the comparison of the abilities of different xylanases isolated from *Aspergillus oryzae, Humicola insolens* and *Trichoderma reesei* to improve the quality of bread made from wheat flour, it was shown that the most effective isxylanase from *A. oryzae* (Basinskiene et al., 2006). However, maximum antistaling effect was observed with xylanase from *T. reesei*. Jiang et al. (Jiang et al., 2005) isolated axylanase from thermophilic bacteria, *Thermomyces lanuginosus* CAU44 and showed itsapplication in bread making.

2.2.5.2.9 Fruit juice and beer clarification

Endo-1,4-β-xylanase helps in increasing juice yield from fruits or vegetables and also in the maceration process. Besides, it reduces the viscosity of the fruit juice improving its filterability (Biely, 1985). Endo-1, 4-β-xylanase also improves extraction of more fermentable sugar from barley and therefore useful for making beer, as well as processing the spent barley for animal feed. In addition, added endo-1, 4-β-xylanase reduces the viscosity of the brewing liquid, improving its filterability (Kulkarni et al., 1999).

2.2.5.2.10 Improving silage

It is known that endo-1,4-β-xylanase and cellulase treatment of forages produces a better quality silage that improves the subsequent rate of plant cell wall digestion by ruminants.There is a considerable amount of sugar sequestered in the xylan of plant biomass. As a result of endo-1,4-β-xylanase treatment, there is increased nutritive sugar and that is useful for digestion in cow and other ruminants. It is also known that endo-1,4-β-xylanase also produces compounds which are the nutritive source for many ruminal microflora (Kulkarni et al., 1999).

2.2.5.2.11 Bioconversion

For bioconversion process that converts lignocellulose to fermentative products, maximal utilization of the various polymeric sugars is desirable. Present knowledge clearly suggests that “complete” xylanolytic (and cellulolytic) systems are required to achieve maximum hydrolysis of complex substrates to yield monomeric residues. In certain bioconversion processes, total hydrolysis of substrate may not be required or desirable at the hydrolysis phase. For example, fermentative organisms such as *Klebsiellapneumonae* can utilize disaccharides such as xylobiose (Yu et al., 1985). Furthermore, hydrolysis limitations associated with product inhibition can be relieved by using a method such as sequential co-culture or simultaneous saccharification and fermentation (Yu et al., 1985; Yu and Saddler, 1985). Finally,the release of acetyl, glucuronosyl, or phenolic residues, or all the three, during hydrolysismay interfere with fermentation.

2.2.5.2.12 Bioenergy

Chiranjeevi et al. (Chiranjeevi et al., 2012) optimized the influential parameters for the production of holocellulases (mixture of cellulases and xylanases) from *Cladosporium cladosporioides* under submerged cultivation system. They discussed that efficient holocellulases cocktail plays a significant role in commercialization of biorefinery, textile, detergent for mulation and paper manufacturing industries. Song et al. (Song et al., 2012) reported that wheat straw is an abundant co-product of the agri-food industry and is considered to be a primary source of lignocellulosic biomass for second generation bio-refining. They engineered GH11 xylanase by mutating at position 111 using a directed evolution strategy in order to develop better biomass degrading ability in it. However, they also pointed out that enzyme engineering alone can't overcome the limits imposed by the complex organization of the plant cell wall and the lignin barrier. Cavka et al. (Cavka et al., 2011) investigated the possibility to utilize fiber sludge,waste fibers from pulp mills and lignocellulose based biorefineries for combined production of liquid biofuel and biocatalysts. They showed the potential of converting waste fiber sludge to liquid biofuel and enzymes as co-products in lignocellulose based biorefineries. A most thermal and acid stable xylanase called as extreme xylanase has been discovered in a catalogued bacteria, *Alicyclobacillus acidocaldarius* originally isolated from Nymph Creek inYellowstone National Park's Norris Geyser basin. The enzyme is stable and active at temperatures ranging from hot tap water to nearly boiling, and in acidic conditions ranging from battery acid to acid rain. The enzyme is capable of efficiently converting hemicelluloses and cellulose components of biomass into energy rich sugars and these sugars can be used to make fuels and high value chemicals (Sharma and Kumar, 2013).

Table 2.9 Commercial preparations of xylanases (Beg et al., 2001; Corral and Villasenor-Ortega, 2006; Haltrich et al., 1996)

Company	Product	Strain and mode of fermentation	Applications
Alltech ,Inc,(USA)	Allzym PT	*Aspergillus niger*(SmF)	Upgrading animal feed.
Alltech ,Inc,(USA)	Fibrozyme	*Aspergillus niger*& *Trichodermaviride*(SSF)	Upgrading animal feed.
Amano Pharmaceutical Co,Ltd(Japan)	Amano 90	*Aspergillus niger*(SSF)	Pharmaceutical, food and feedindustry.
A/S	Resinase	N.c	Cellulose and paper industry
Biocon,(India)	Bleachzyme F	N.c	Pulp bleaching
Biotec	Ecosane	*Trichoderma reesei*(SmF)	Animal feed
Clariant(UK)	Cartazyme	*Termomonospora fusca*	Pulp bleaching
Ciba –Geiby, Ltd(Switzerland)	Irgazyme40	*Trichoderma longibrachiatum*(SmF)	Pulp and paper industry and animal feed
DaniscoIngredients (Denmark)	Grindazym PF & Grindazym GP 5000	*Aspergillus niger*(SmF)	Supplementation of poultry and piglet food
Gamma Chemie GmbH(Germany)	Gammafeed X Gammazym X4000L	*Trichoderma longibrachiatum* (SmF) *Trichodermareesei*(SSF)	Production of wheat starch,baking and brewing industry. Feed and brewing industry
Genecor International Europe Ltd, (Finland)	Multifect XL	*Trichoderma longibrachiatum* (SmF)	Food industry
Hankyo Bioindustry Co.Ltd(Japan)	Xylanase250 Hemicellulase 100	*Trichoderma viride*(SSF) *Aspergillus niger*(SSF)	Baking industry & for macerating vegetables and fruits. Improving the filtration speed of saccharified cereal solutions and fruit juices
IogenCorp (Canada)	Xylanase GS35	*Trichoderma reesei*(SmF)	Pulp bleaching,pulp cleaningand animal feed processing.
Novozymes (Denmark)	Bio-feed-plus Novozym 431 Pulpzyme	*Humicola insolens*(SmF) *Trichoderma longibrachiatum* (SmF) *Bacillus* sp.	Animal feed Animal feed Cellulose and paper industry

Table 2.9 Commercial preparations of xylanases (Continued)

Company	Product	Strain and mode of fermentation	Applications
Primalco Ltd Biotec(Finland)	Ecopulp X-200	*Trichoderma reesei* (SmF)	Improve the bleachability of softwood & hardwood kraft pulps
Quest International (Ireland)	Bioxylanase	*Trichoderma reesei*(SmF)	Brewing and animal feed industry
Rohm GmbH (Germany)	Rohalasa 7118 Vernon 191	*Aspergillus* sp. &*Trichoderma* sp.(SmF) *Aspergillus* sp.&*Trichoderma* sp.(SmF)	Reduction of viscosity in starch processing. Baking industry
Seikagaku Corporation (Japan)	No commercial name	*Trichoderma* sp. (SmF)	Structure studies of carbohydrates
Shin Nihon Chemical (Japan)	Sumizyme X	*Trichoderma koningii* (SSF)	Manufacturing of mushroom and vegetable extracts, enzymatic peeling of cereals and baking industry.
Solvay Enzymes GmbH&Co. (Germany)	Solvay pentosanasa	*Trichoderma reesei* (SmF)	Starch and baking industry
Stern Enzym GmbH & Co. (Germany)	Sternzym HC46 Sternzym HC40	*Trichoderma reesei* (SmF) *Aspergillus niger* (SSF/SmF)	Bakery industry, animal feed, hydrolysis of plant raw materials.

SSF: Solid stated fermentation

SmF: Submerged fermentation

N.c: Not cited.

2.2.5.3 Application of pectinolytic enzymes in various industries

Over the years, pectinases have been used in several conventional industrial processes, such as textile, plantfiber processing, tea, coffee, oil extraction, treatment of industrial wastewater, containing pectinacious material, etc (Ricard and Reid, 2004; Viikari et al., 2001). They are yet to be commercialized.

2.2.5.3.1 Fruit juice extraction

The largest industrial application of pectinases is in fruit juice extraction and clarification. Pectins contribute to fruit juice viscosity and turbidity. A mixture of pectinases and amylases is used to clarify fruit juices. It decreases filtration time up to 50% (Blanco et al., 1999). Treatment of fruit pulps with pectinases also showed an increase in fruit juice volume from banana, grapes and apples (Kaur et al., 2004). Pectinases in combination with other enzymes, viz., cellulases, arabinases and xylanases, have been used to increase the pressing efficiency of the fruits for juice extraction (Gailing et al., 2000). Vacuum infusion of pectinases has a commercial application to soften the peel of citrus fruits for removal. This technique may expand in future to replace hand cutting for the production of canned segments (Baker and Wicker, 1996). Infusion of free stone peaches with pectin methylesterase and calcium results in four times firmer fruits. This may be applied to pickle processing where excessive softening may occur during fermentation and storage (Baker and Wicker, 1996).

2.2.5.3.2 Textile processing and bioscouring of cotton fibers

Pectinases have been used in conjunction with amylases, lipases, cellulases and hemicellulases to remove sizing agents from cotton in a safe and ecofriendly manner, replacing toxic caustic soda used for the purpose earlier. Bioscouring is a novel process for removal of noncellulosic impurities from the fiber with specific enzymes. Pectinases have been used for this purpose without any negative side effect on cellulose degradation (Hoondal et al., 2002).

2.2.5.3.3 Degumming of plant bast fibers

Bast fibers are the soft fibers formed in groups outside the xylem, phloem or pericycle, e.g. Ramie and sunn hemp. The fibers contain gum, which must be removed before its use for textile making (Hoondal et al., 2002). The chemical degumming treatment is

polluting, toxic and non-biodegradable. Biotechnological degumming using pectinases in combination with xylanases presents an ecofriendly and economic alternative to the above problem (Kapoor et al., 2001).

2.2.5.3.4 Retting of plant fibers

Pectinases have been used in retting of flax to separate the fibers and eliminate pectins (Hoondal et al., 2002).

2.2.5.3.5 Waste water treatment

Vegetable food processing industries release pectin, containing wastewaters as by-product. Pretreatment of these wastewaters with pectinolytic enzymes facilitates removal of pectinaceous material and renders it suitable for decomposition by activated sludge treatment (Hoondal et al., 2002).

2.2.5.3.6 Coffee and tea fermentation

Pectinase treatment accelerates tea fermentation and also destroys the foam forming property of instant tea powders by destroying pectins. They are also used in coffee fermentation to remove mucilaginous coat from coffee beans (Fowler et al., 1997).

2.2.5.3.7 Paper and pulp industry

During paper making, pectinase can depolymerise pectins and subsequently lower the cationic demand of pectin solutions and the filtrate from peroxide bleaching (Ricard and Reid, 2004; Viikari et al., 2001).

2.2.5.3.8 Animal feed

Pectinases are used in the enzyme cocktail, used for the production of animal feeds. This reduces the feed viscosity, which increases absorption of nutrients, liberates nutrients, either by hydrolysis of non-biodegradable fibers or by liberating nutrients blocked by these fibers, and reduces the amount of faeces (Hoondal et al., 2002).

2.2.5.3.9 Purification of plant viruses

In cases where the virus particle is restricted to phloem, alkaline pectinases and cellulases can be used to liberate the virus from the tissues to give very pure preparations of the virus (Salazar and Jayasinghe, 1991).

2.2.5.3.10 Oil extraction

Citrus oils such as lemon oil can be extracted with pectinases. They destroy the emulsifying properties of pectin, which interferes with the collection of oils from citrus peel extracts (Scott, 1978).

2.2.5.3.11 Improvement of chromaticity and stability of red wines

Pectinolytic enzymes added to macerated fruits before the addition of wine yeast in the process of producing red wine resulted in improved visual characteristics (colour and turbidity) as compared to the untreated wines. Enzymatically treated red wines presented chromatic characteristics, which are considered better than the control wines. These wines also showed greater stability as compared to the control (Revilla and Jose, 2003).

2.3 Prokaryotic cellulase enzyme system

The cellulolytic bacteria produce a variety of different cellulases and related enzymes, which together convert the plant cell wall polysaccharides to simple soluble sugars that can subsequently be assimilated. The complement of cellulases and hemicellulases that are synthesized by a given bacterium for this purpose is referred to as its "cellulase system." Different bacteria exploit different strategies for the ultimate degradation of their substrates. The given strategy is reflected by the complement and type of enzymes produced by a given bacterium. The bacterial cellulase system may be characterized by free enzymes, cell-bound enzymes, multifunctional enzymes, multienzyme complex, or any combination of the latter (Bayer et al., 2006)

2.3.1 Free enzymes

As mentioned earlier in this chapter, the free enzymes in their simplest form comprise a catalytic module alone with no accessory domains or modules. Such enzymes often specialize in degrading soluble oligosaccharide breakdown products. Alternatively, such single-modular enzymes may rely on an intrinsic association with insoluble polysaccharide substrate such as cellulose, perhaps related to the active site of theenzyme. A higher order level of organization and activity are free

enzymes composed ofa polypeptide chain that includes both a catalytic domain together with a CBM. This basic bi-modular arrangement can be further extended by the inclusion of additional types of modules or repeating units of the same module, all of which serve to modulate.

2.3.2 Cell-bound nzymes

Some enzymes are connected directly to the cell wall. Many Gram-positive bacteria have a surface layer protein (SLP) that surrounds the exterior cell wall. This layer of proteins is attached to secondary cell wall polymers in the rigid cellwall layer (Doi and Kosugi, 2004). The surface layer homology (SLH) domains of several extracellular enzymes are homologous to regions of the SLP and it is believed that, like the SLP, SLH domains also attach to secondary cellwall polymers and bind these SLH-containing enzymes to the cell surface (Fig. 2.15). This arrangement may have evolved to provide a more economic degradation of insoluble substrates and to reduce competition with other bacteria for the soluble products, subject to diffusion in the media. As opposed to free enzymes, diffusion of an attached enzyme would itself be prevented.

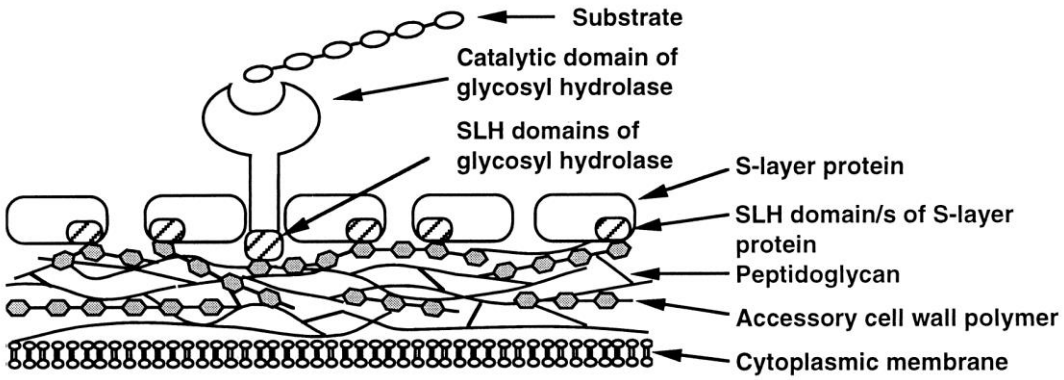


Figure 2.15 Hypothetic model for the attachment of exocellular proteins from *Thermoanaerobacterium thermosulfurigenes* EM1 to the cell envelope via their SLH domains.The SLH domainsof the S-layer as well as the SLH domains of exocellular glycosyl hydrolases interact with secondary cell wall polymers, which are covalently linked to the underlying peptidoglycan layer (Brechtel and Bahl, 1999)

Examples of enzymes, which are bound to the cell surface via an SLH module include, a family-5 cellulase and family-13 amylase-pullulanase from *Bacillus*, a family-10 xylanase from *Caldicellulosiruptor* (Saul et al., 1990), a family-5 endoglucanase from *Clostridium josui*, a family-16 lichenase and family-10 xylanase from *Clostridium thermocellum* (Jung et al., 1998), and a variety of enzymes (family-10 xylanases, a family-5 mannanase and a family-13 amylasepullulanase) from different species of *Thermoanaerobacter* (Matuschek et al., 1996). The modular architecture of these enzymes may be particularly complicated, containing several different modules in a single polypeptide chain, thus forming extremely large enzymes sometimes comprising over 2,000 amino acids (Fig. 2.16).

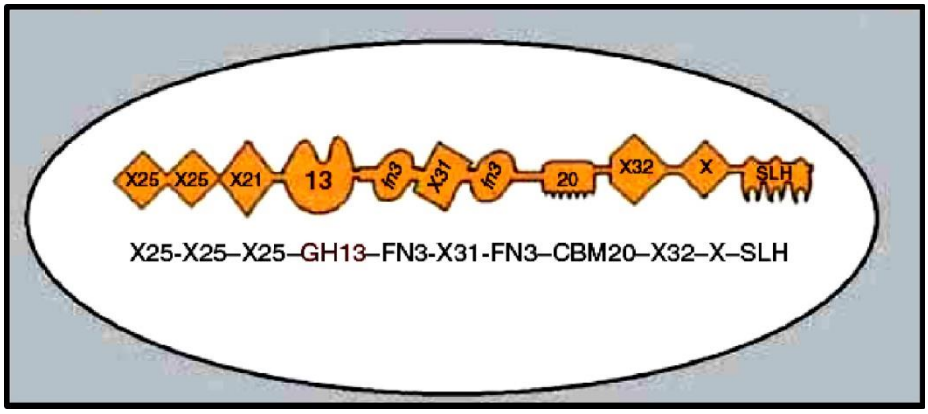


Figure 2.16 A very large, cell surface enzyme from *Thermoanaerobacter thermosulfurogenes*. The 1861-residue enzyme contains an SLH module, which is believed to mediate the attachment of the enzyme to the cell surface in Gram-positive bacteria. The enzyme contains a multiplicity of modules, which apparently serve to regulate the hydrolytic action of its single family-13 catalytic module with the complex substrate. Several X domains of unknown function may either represent as yet undescribed catalytic functions, carbohydrate binding activities or structural entities (Bayer et al., 2006).

2.3.3 Multi-functional enzymes

Some cellulases exhibit a more complex architecture in that more than one catalytic domain and/or CBM may be included in the same protein. Examples of such enzymes are the very similar cellulases from *Anaerocellum thermophilum* (Zverlov et al., 1998)

and *Caldocellum saccharolyticum* (Teo et al., 1995), both of which contain a family-9 and a family-48 catalytic domain. Other paired catalytic domains include those from family 44 and either family 5 or 9. Such an arrangement might indicate a close cooperation between two particular catalytic domains, which may lead to synergistic action on the cellulosic substrate, thus portending on a smaller scale the advent of cellulosomes.

Like the cellulases, xylanases also tend to exhibit a modular structure, being composed of multiple domains joined by linker sequences. Family-10 and -11 xylanases may be linked in the same polypeptide chain either to each other, to catalytic domains from families 5, 16 and 43 or to carbohydrate esterases (Flint et al., 1993; Laurie et al., 1997). One particularly interesting combination of multifunctional catalytic modules that appear in the same polypeptide chain is a typical xylanase together with a feruloyl esterase. Such a combination would allow the rapid cleavage of hemicellulose from the lignin in natural systems, i.e., the plant cell wall. In this manner, the xylan chain would be severed by the xylanase component and the lignin-xylan association would be disconnected simultaneously by the feruloyl acid esterase.

Indeed, some xylanases are extremely complex in their modular architecture. In addition to multiple catalytic modules, these enzymes often contain several different types of CBMs. The modular proximity of the xylanase shown in Fig. 2.17 would presumably indicate that the two CBM22s would modulate the action of the family-10 catalytic module, and the C-terminal CBM6 would facilitate the catalysis by the family-43 module. Together, the two catalytic modules would act synergistically to degrade susceptible plant cell wall components. In this context, the complex architecture of a xylanase would reflect the complex chemistry of its substrate and the neighboring polymers of its immediate environment in the plant cell wall.

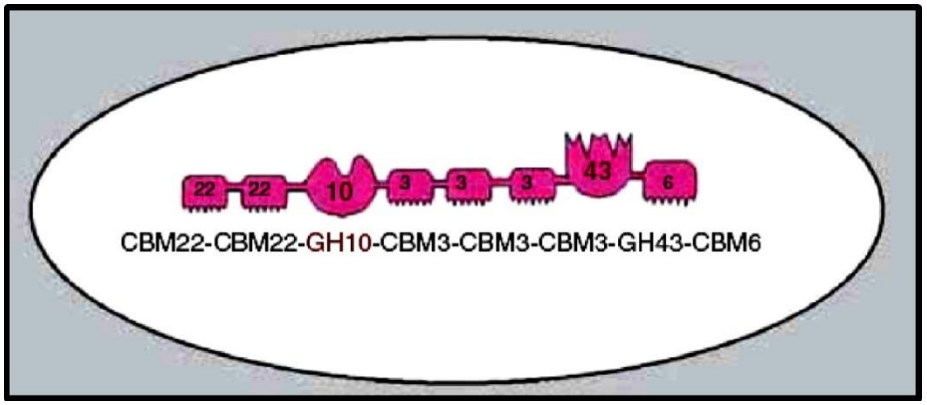


Figure 2.17 A very large, multimodular xylanase from *Caldicellulosiruptor* sp.. The 1,795 residue enzyme contains 8 separate modules, including 2 catalytic modules from families 10 (invariably a xylanase) and 43 (frequently an arabinofuranosidase). These are modulated by numerous carbohydrate binding modules, which include 3 from family 3 (likely for binding to crystalline cellulose), 2 from family 22 (newly classified and shown to function in xylan binding and one from family 6 (Bayer et al., 2006).

2.3.4 Multienzyme complexes

For years, it was believed that microbial cellulase systems consisted solely of numerous types of free cellulases that act synergistically on the insoluble substrate. Many cellulase systems, particularly in aerobic microorganisms, seem to be characterized by free enzymes. The rumen is a highly anaerobic environment that represents one of the most active sites for breakdown of plant cell wall material in nature. In recent years, multienzyme complexes, have been identified and described in many anaerobic, thermophilic, cellulolytic bacteria. The bacterium adhesion to cellulose is accomplished by means of a discrete multifunctional, multicomponent cell surface protein complex, known as the cellulosome which is exquisitively designed for efficient binding and hydrolysis of the substrate (Lamed et al., 1983). The production of the multienzyme complex may have a number of advantages for the effective hydrolysis of cellulosic substances:

1. Synergism is optimized by the correct ratio between the components, which is determined by the composition of the complex.

2. Non-productive adsorption is avoided by the optimal spacing of components working together in synergistic fashion.
3. Competitiveness in binding to a limited number of binding sites is avoided by binding the whole complex to a single site through a strong binding domain with low specificity.
4. A halt in hydrolysis on depletion of one structural type of multienzyme complex at the site of adsorption is avoided by the presence of other enzymes with different specificity.

The multienzyme complexes attach both to cell envelope and to the substrate, mediating the proximity of the cell to the cellulose (Ohara et al., 2000). Thus multienzyme complex is efficient to hydrolyze the complex (Fig. 2.18)

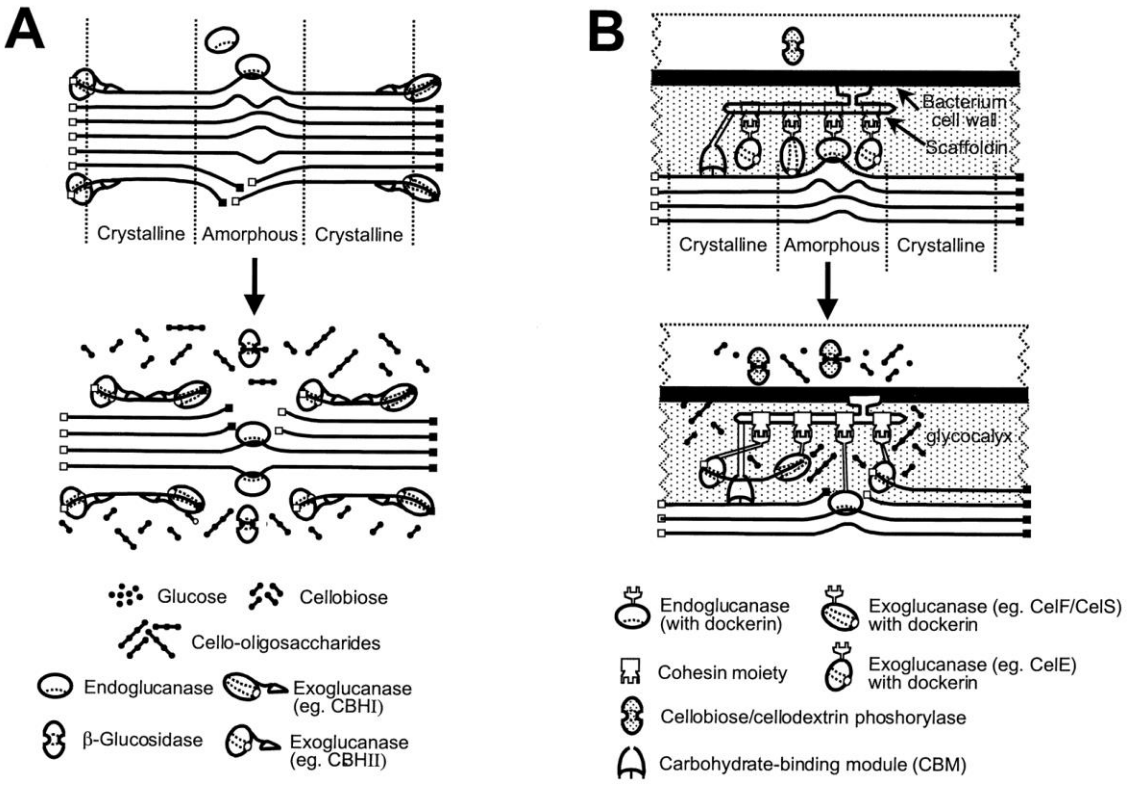


Figure 2.18 Schematic representation of the hydrolysis of amorphous and microcrystalline cellulose by noncomplexed (A) and complexed (B) cellulase systems. The solid squares represent reducing ends, and the open squares represent nonreducing ends. Amorphous and crystalline regions are indicated. Cellulose, enzymes, and hydrolytic products are not shown to scale (Lynd et al., 2002).

2.4 Concept of multienzyme complex

The cellulosome is an extracellular supramolecular machine that can efficiently degrade crystalline cellulosic substrates and associated plant cell wall polysaccharides. The cellulosome arrangement can also promote adhesion to the insoluble substrate, thus providing individual microbial cells with a direct competitive advantage in the utilization of the soluble hydrolysis products (Shoham et al., 1999).

The enzymes in these cases are not organized into high molecular weight complexes and are called non-complexed (Fig. 2.19A) (Ratanakhanokchai et al., 2013). 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; Sanchez, 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. 2.19B) (Ratanakhanokchai et al., 2013).

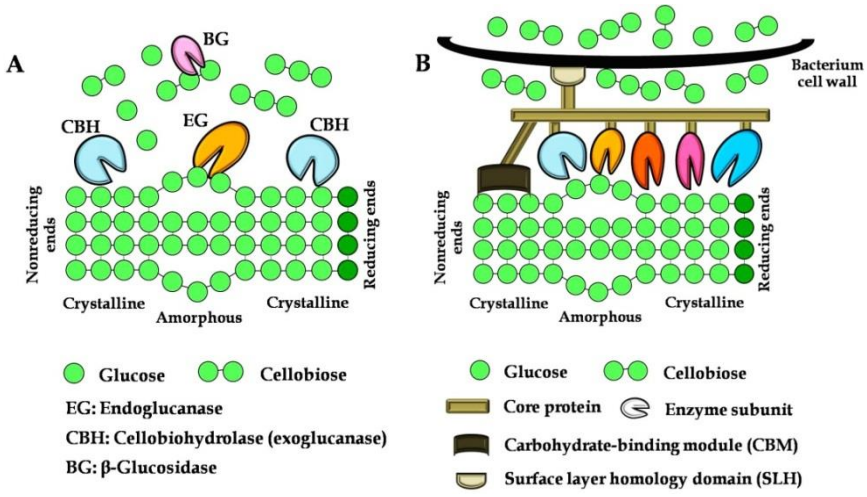


Figure 2.19 Simplified schematic of the hydrolysis of amorphous and microcrystalline celluloses by non-complexed (A) and complexed (B) cellulase systems (Ratanakhanokchai et al., 2013)

The cellulosome are produced by anaerobic bacteria such as *Clostridium*, *Acetivibrio*, *Bacteroides*, and *Ruminococcus*. These microorganisms are found in various environmental niches, including soil, wood chip piles, sewage, and rumens. Cellulosomes may be the largest extracellular enzyme complexes found in nature, since polycellulosomes have been reported to be as large as 100 MDa, although the individual cellulosomes range in mass from about 650,000 Da to 2.5 MDa (Doi et al., 2003).

Cellulosomes were first observed by Bayer and Lamed as large protuberances on the surface of *Clostridium thermocellum* and the complex was found to consist of a nonenzymatic scaffolding protein to which were attached a number of enzymatic subunits (Bayer et al., 1985; Lamed et al., 1983).The cellulosome was first identified in 1983 from the anaerobic, thermophilic, spore- forming *Clostridium thermocellum* (Lamed et al., 1983). The cellulosome of *C. thermocellum* is 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 onenzymes. 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. 2.20).

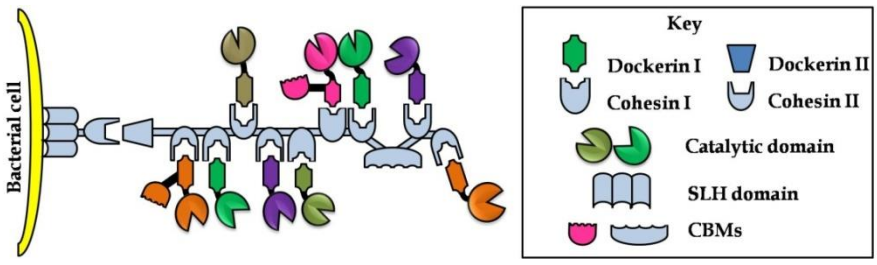


Figure 2.20 Simplified schematic of general cellulosome components and connection with cell surface based on knowledge of *Clostridium* cellulosome (Ratanakhanokchai et al., 2013).

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 2.10). It is speculated thatseveral other cellulolytic bacteria may also produce cellulosomes not yet described.

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.

Table 2.10 Cellulosome and cellulosome-like multienzyme complexes from anaerobic and aerobic microorganisms (Ratanakhanokchai et al., 2013)

Anaerobic	
Microorganisms	**Source**
Bacteria	
• *Acetivibrio cellulolyticus*	Sewage
• *Amorocellulobacter alkalithermophilum*	Soil
• *Bacteroides cellulosolvens*	Sewage
• *Bacteroides* sp. strain P-1	Anaerobic digester
• *Butyrivibrio fibrisolvens*	Rumen
• *Clostridium acetobutylicum*	Soil
• *Clostridium cellobioparum*	Rumen
• *Clostridium cellulolyticum*	Compost
• *Clostridium cellulovorans*	Fermenter
• *Clostridium josui*	Compost
• *Clostridium papyrosolvens*	Paper mill
• *Clostridium thermocellum*	Sewage soil
• *Eubacterium cellulosolvens*	Rumen
• *Ruminococcus albus*	Rumen
• *Ruminococcus flavefaciens*	Rumen
• *Tepidimicrobium xylanilyticum* BT14	Soil
• *Thermoanaerobacterium thermosaccharolyticum* NOI-1	Soil (This study)
Fungi	
• *Neocallimastix patriciarum*	Rumen
• *Orpinomyces joyonii*	Rumen
• *Orpinomyces* PC-2	Rumen
• *Piromyces equi*	Rumen
• *Piromyces* E2	Faeces

Table 2.10 Cellulosome and cellulosome-like multienzyme complexes from anaerobic and aerobic microorganisms (Continued)

Aerobic	
Microorganism	**Source**
Bacteria	
• *Bacillus circulans* F-2	Potato starch granules
• *Bacillus licheniformis* SVD1	Bioreactor
• *Paenibacillus curdlanolyticus* B-6	Anaerobic digester
• *Sorangium cellulosum*	Soil
Actinomycetes	
• *Streptomyces olivaceoviridis* E-86	Soil
Fungi	
• *Chaetomium sp.* Nov. MS-017	Rotted wood

2.4.1 Cellulosome component

The cellulosome complex contains many different types of glycosyl hydrolases, all of which are bound to a major polypeptide called scaffoldin (also known as the cellulosome-integrating protein, CipA). The multiple roles of scaffoldin, namely the cellulose binding and cell anchoring functions, as well as its role in the organization of the enzyme subunits in the cellulosome complex, were recognized in the early stages of cellulosome research (Bayer et al., 1985). Similarly, early research also showed that scaffoldin promotes the activity of a cellulosomal enzyme subunit (Wu et al., 1988). Scaffoldin contains many functional modules that dictate its various activities. These modules include a single cellulose binding domain, or CBD, and nine very similar repeating domains, termed cohesins, which interact with the cellulosomal enzymes. The scaffoldin of the *C. thermocellum* cellulosome has an additional domain that allows it to attach to the cell surface. The cellulosomal enzymes are also modular in nature. In addition to a definitive catalytic module, they all possess an additional domain, called a dockerin, that binding tightly with the cohesins of the scaffoldin.The cohesin–dockerin interaction therefore governs the assembly of the complex, while the interaction of the complex with cellulose is mediated by the scaffoldin borne CBD (Shimon et al., 1997; Tavares et al., 1997; Tormo et al., 1996). A schematic view of the cellulosome and its interaction with cellulose and the cell surfaceis presented in Fig. 2.21

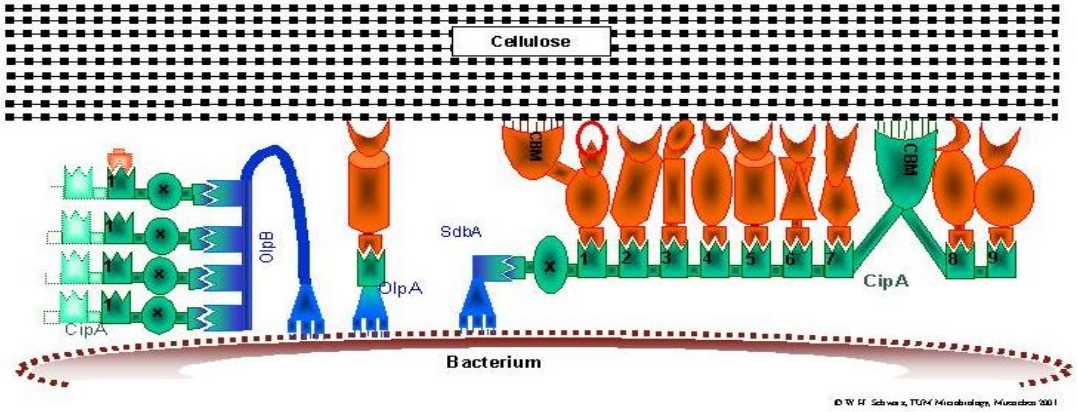


Figure 2.21 Schematical presentation of the cellulosome component of *Clostridium thermocellum*. CipA protein is drawn in green, catalytical components in orange, cell wall binding components in blue, the bacterial cell in brown and the cellulose microfibril in black. The S-layer is punctuated. The cohesin modules are numbered 1 to 9. The binding of the enzymes to specific positions (cohesins) is hypothetical, as is the linear orientation of the scaffoldin. The scaffoldins (CipA) bound to OlpB are only sketched partially (Bayer et al., 2006).

2.4.1.1 Scaffolding protein (scaffoldin)

The cellulosome consists of a scaffolding protein called CbpA (Shoseyov and Doi, 1990; Shoseyov et al., 1992), CipA (Gerngross et al., 1993; Kakiuchi et al., 1998; Sabathe and Soucaille, 2003), or CipC (Pages, 1999) complexed with a number of cellulosomal enzymes. The scaffolding proteins, also called “scaffoldins” (Bayer et al., 1994), are large nonenzymatic proteins that usually contain a number of cohesion domains (Coh) and cellulose-binding domain (CBD) or carbohydrate-binding modules (CBMs); however, hydrophilic domains, dockerin II domains, the enzyme coding domain, and a number of unidentified domains whose functions remain unknown have also been observed in some of the scaffoldins (Fig. 2.22).

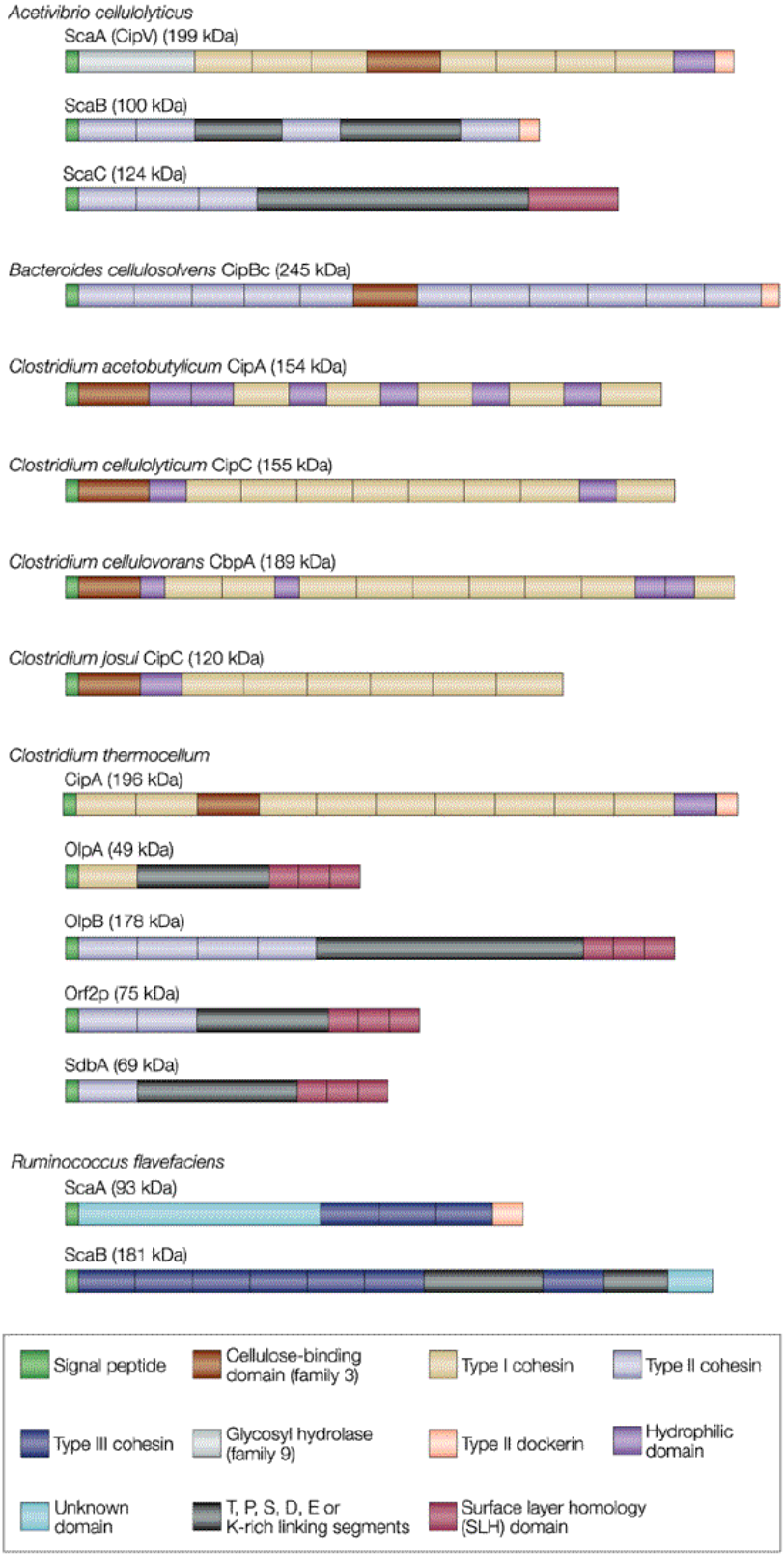


Figure 2.22 The modular structure of scaffoldins from various microorganisms (Doi and Kosugi, 2004)

2.4.1.1.1 Cohesin

Cohesin modules are the major building blocks of scaffoldins which are responsible for organizing the enzyme subunits into the cellulosome. The number of cohesins present in various scaffoldins varies, as Fig. 2.21. There is a considerable degree of amino acid homology between cohesins from different species. However, the cohesins appear to be quite specific in their interactions with the dockerin domains that are present in all cognate cellulosomal enzymes. This was demonstrated when the cohesins present in CipA of *Clostridium thermocellum* did not interact with the dockerins present in *Clostridium cellulolyticum* enzymes and vice versa (Pages, 1997). This high specificity in cohesin-dockerin interaction has allowed the development of an interesting test system for synergy.

2.4.1.1.2 Carbohydrate-binding modules (CBMs)

The carbohydrate binding module (CBM) of scaffoldins binds the cellulosome tightly to the cellulose substrate (Boraston et al., 1999). The CBD appears to bind the cellulosome to the crystalline form of cellulose more efficiently than to amorphous forms of cellulose (Goldstein et al., 1993). The CBDs are variable and are divided into a number of families based on their amino acid sequences and occur not only in scaffoldins, but as domains of various cellulosomal and noncellulosomal cellulases (Boraston et al., 1999). In the context of the CBMs illustrated in Fig. 2.22, the family 3CBMs are capable of binding crystalline cellulose.

The CBMs contain from 30 to about 200 amino acids and exist as a single, double, or triple domain in one protein. Their location within the parental protein can be both C- or N-terminal and is occasionally centrally positioned within the polypeptide chain. The three-dimensional (3D) structures of representative members of 23 CBM families have been deciphered (Table 2.11). Data from these structures indicate that CBMs from different families are structurally similar and that their carbohydrate binding capacity can be attributed, at least in part, to several aromatic amino acids that constitute the hydrophobic surface. The CBMs, by bringing the biocatalyst into intimate and prolonged association with its recalcitrant substrate, increase the rate of catalysis.

Table 2.11 CBM structures (Boraston et al., 2004).

Family	Protein	PDB code
CBM1	Cellulase 7A (*Trichoderma reesei*)	1CBH
CBM2	Xylanase 10A (*Cellulomonas fimi*)	1EXG
	Xylanase 11A (*Cellulomonas fimi*)	2XBD
	Xylanase 11A (*Cellulomonas fimi*)	1HEH
CBM3	Scaffoldin (*Clostridium cellulolyticum*)	1G43
	Scaffoldin (*Clostridium thermocellum*)	1NBC
	Cellulase 9A (*Thermobifida fusca*)	1TF4
CBM4	Laminarinase 16A (*Thermotoga maritima*)	1GUI
	Cellulase 9B (*Cellulomonas fimi*)	1ULO; 1GU3
	Cellulase 9B (*Cellulomonas fimi*)	1CX1
	Xylanase 10A (*Rhodothermus marinus*)	1K45
CBM5	Cellulase 5A (*Erwinia chrysanthemi*)	1AIW
	Chitinase B (*Serratia marcescens*)	1E15
CBM6	Xylanase 11A (*Clostridium thermocellum*)	1UXX
	Xylanase 11A (*Clostridium stercorarium*)	1NAE
	Xylanase 11A (*Clostridium stercorarium*)	1UY4
	Endoglucanase 5A (Cellvibrio mixtus)	1UZO
CBM9	Xylanase 10A (*Thermotoga maritima*)	1I8A
CBM10	Xylanase 10A (*Cellvibrio japonicus*)	1QLD
CBM12	Chitinase Chi1 (*Bacillus circulans*)	1ED7
CBM13	Xylanase 10A (*Streptomyces olivaceoviridis*)	1XYF
	Xylanase 10A (*Streptomyces lividans*)	1MC9
	Ricin toxin B-chain (*Ricinus communis*)	2AAI
	Abrin (*Abrus precatorius*)	1ABR
CBM14	Tachycitin (*Tachypleus tridentatus*)	1DQC
CBM15	Xylanase 10C (*Cellvibrio japonicus*)	1GNY
CBM17	Cellulase 5A (*Clostridium cellulovorans*)	1J83
CBM18	Agglutinin (*Triticum aestivum*)	1WGC
	Antimicrobial peptide (*Amaranthus caudatus*)	1MMC
	Chitinase/agglutinin (*Urtica dioica*)	1EIS
CBM20	Glucoamylase (*Aspergillus niger*)	1ACO
	β-amylase (*Bacillus cereus*)	1CQY
CBM22	Xylanase 10B (*Clostridium thermocellum*)	1DYO
CBM27	Mannanase 5A (*Thermotoga maritima*)	1OF4
CBM28	Cellulase 5A (*Bacillus sp. 1139*)	1UWW
CBM29	Non-catalytic protein 1 (*Pyromyces equi*)	1GWK
CBM31	Xylanase 26A (*Alcaligenes* sp.)	2COV
CBM32	Sialidase 33A (*Micromonospora viridifaciens*)	1EUU
	Galactose oxidase (*Cladobotryum dendroides*)	1GOF
CBM34	*α*-Amylase 13A (*Thermoactinomyces vulgaris*)	1UH2
	Neopullulanase (*Geobacillus stearothermophilus*)	1JOH
CBM36	Xylanase 43A (*Paenibacillus polymyxa*)	1UX7

In recent years, the 3D structures of representative members from 22 CBM families have been resolved. Data from these structures indicate that CBMs from different families are structurally similar and that their binding to cellulose can be attributed, at least in part, to their hydrophobic surface, which is composed of several aromatic amino acids. CBMs are classified into seven "fold families," (Table 2.12, Fig. 2.23) based on their 3D structures and functional similarities, into three types. The classification of these CBM types relative to the fold family and sequence families are shown in Table 2.13. Type A CBMs have a flat hydrophobic surface that interacts with adjacent chains on the surface of crystalline cellulose (Fig. 2.24), whereas Type B CBMs have a cleft that accommodates the single glycan chains of poly- or oligo-saccharide ligands (Fig. 2.25). Both Type A and Type B CBMs show relatively high affinity. In contrast, Type C CBMs have surface pockets and indentations that accommodate oligosaccharide ligands, and they exhibit the lectin-like property of binding optimally to mono-, di- or tri-saccharides (Fig.2.26). The structure-function relationship is discussed extensively in a review by Boraston et al. (Boraston et al., 2004)

Table 2.12 CBM fold families (Boraston et al., 2004).

Fold Family	Fold	CBM families
1	β-Sandwich	2, 3, 4, 6, 9, 15, 17, 22, 27, 28, 29, 32, 34, 36
2	β-Trefoil	13
3	Cysteine knot	1
4	Unique	5, 12
5	Oligosaccharide binding (OB) fold	10
6	Hevein fold	18
7	Unique; contains hevein-like fold	14

Table 2.13 CBM types (Boraston et al., 2004)

Type	Fold family	CBM families
A	1, 3, 4, 5	1, 2a, 3, 5, 10
B	1	2b, 4, 6, 15, 17, 20, 22, 27, 28, 29, 34, 36
C	1, 2, 6, 7	9, 13, 14, 18, 32

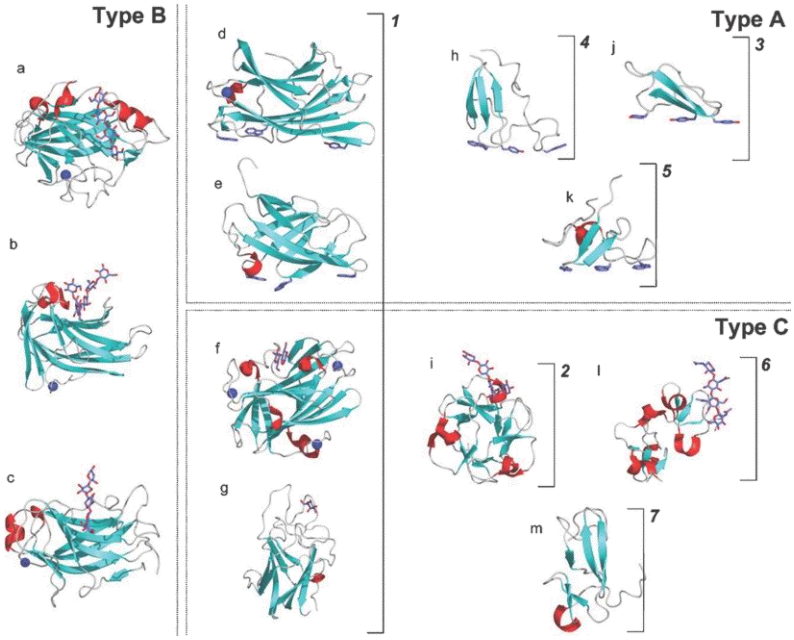


Figure 2.23 The big picture: CBMs, shown as ribbon structures, grouped as fold families and functional types Red boxes surround examples of CBMs belonging to the functional Types A, B, and C. Brackets with numbers indicate examples of CBMs belonging to fold families 1–7. CBMs shown are as follows: (**a**) family 17 CBM, *Cc*CBM17, from *Clostridium cellulovorans* in complex with cellotetraose (PDB code 1J84); (**b**) family 4 CBM, *Tm*CBM4-2, from *Thermotoga maritima* in complex with laminariohexaose (PDB code 1GUI); (**c**) family 15 CBM, *Cj*CBM15, from *Cellvibrio japonicus* in complex with xylopentaose (PDB code 1GNY); (**d**) family 3 CBM, *Ct*CBM3, from *Clostridium thermocellum* (PDB code 1NBC); (**e**) family 2 CBM, *Cf*CBM2, from *Cellulomonas fimi* (PDB code 1EXG); (**f**) family 9 CBM, *Tm*CBM9-2, from *Thermotoga maritima* in complex with cellobiose (PDB code 1I82); (**g**) family 32 CBM, *Mv*CBM32, from *Micromonospora viridifaciens* in complex with galactose (PDB code 1EUU); (**h**) family 5 CBM, *Ec*CBM5, from *Erwinia chrysanthemi* (PDB code 1AIW); (**i**) family 13 CBM, *Sl*CBM13, from *Sreptomyces lividans* in complex with xylopentaose (PDB code 1MC9); (**j**) family 1 CBM, *Tr*CBM1, from *Trichoderma reesi* (PDB code 1CBH); (**k**) family 10 CBM, *Cj*CBM10, from *Cellvibrio japonicus* (PDB code 1E8R); (**l**) family 18 CBM from *Urtica dioca* in complex with chitotriose (PDB code 1EN2); (**m**) family 14 CBM, tachychitin, from *Tachypleus tridentatus* (PDB code 1DQC) (Boraston et al., 2004).

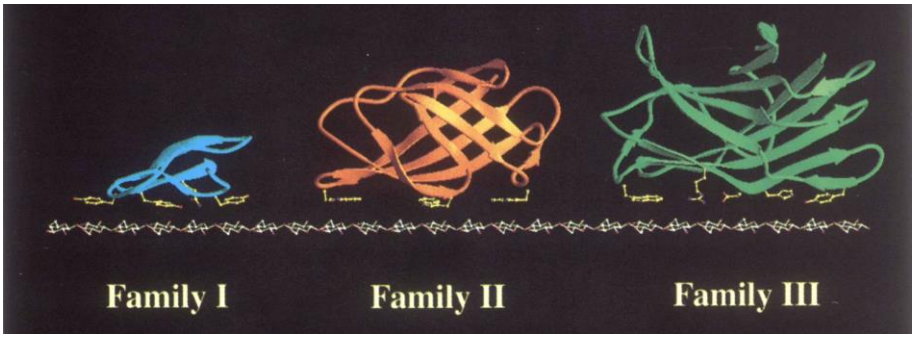


Figure 2.24 Model for the interaction of families 1, 2 and 3 CBMs from Type A CBMs with cellulose (Tormo et al., 1996).

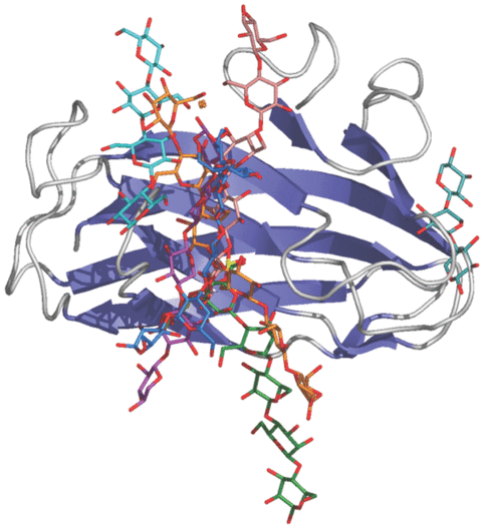


Figure 2.25 Schematic diagram of the family 4 CBM from the N-terminus of Cel9B from *Cellulomonas fimi* is shown as a representative *β*-sandwich Type B CBM (Boraston et al., 2004)

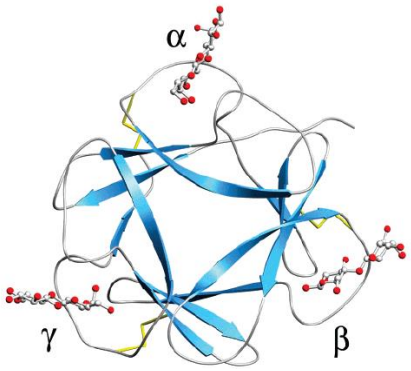


Figure 2.26 Schematic diagram of family 13 CBM from *Sreptomyces lividans* with lactose moieties bound in all three subsites of the trefoil fold Ttype C CBM(Notenboom et al., 2002)

2.4.1.1.3 Hydrophilic domains (HLDs)

The various scaffoldins also contain domains the functions of which have not been clearly identified or which are present somewhat uniquely in the scaffoldin. The presence of hydrophilic domains (HLDs) has been observed in a number of scaffoldins and there are up to four of them in *Clostridium cellulovorans* CbpA (Shoseyov and Doi, 1990). It has been proposed that these HLDs or surface layer homology domains (SLHs) in *Clostridium cellulovorans* play a role in binding of the cellulosome to the cell surface (Kosugi et al., 2002b; Tamaru and Doi, 1999). This idea is based on data from the SLHs present in endoglucanase E (EngE) (Tamaru and Doi, 1999) (Fig. 2.27). During growth, there is a tight association between the cell and the cellulose substrate. This association is facilitated by the cellulosome, 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 the cellulosomal enzymes.

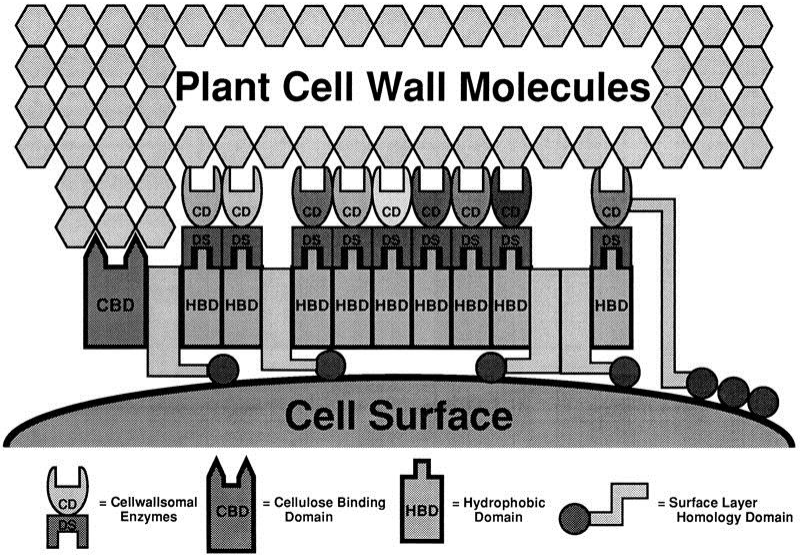


Figure 2.27 Hypothetical model for attachment of EngE to the CbpA of *C. cellulovorans* and the cell surface. The drawing is not to scale. Because of its similarity to the S-layer protein and the presence of a duplicated sequence (DS or dockerin), the three SLH domains of the N terminus of EngE integrate into the lattice of the S-layer, while the C terminus of EngE is bound to CbpA through its DS. Also, CbpA integrates itself into the lattice of the S-layer through its four SLH domains. CD, catalytic domain (Tamaru and Doi, 1999).

2.4.1.1.4 Other domains of scaffoldin

The other domains of scaffoldin include a different type of dockerin, called dockerin II, in the *Clostridium thermocellum* scaffoldin CipA (Kataeva et al., 1997). The dockerin II binds to cohesin II domains found in proteins SdbA, OlpA, and OlpB, which also contain SLHs. These three proteins can bind cellulosomes through the dockerin II-cohesin II interaction, and these complexes can then bind to the cell surface binding complex that attaches the cellulosome to the surface of the *Clostridium thermocellum* cell through their SLH domains (Fujino et al., 1993; Leibovitz and Beguin, 1998; Leibovitz et al., 1997; Lemaire et al., 1998; Lemaire et al., 1995). The dockerin II domain has not been observed in any of the scaffoldins of mesophilic cells. Thus, a different mechanism may exist for attaching the cellulosome to the surface of mesophilic bacteria. Thus, in this case, the binding of the cellulosome to the cell surface does not depend on SLH domains and illustrates the existence of another type of cellulosome-cell association mechanism.

In the case of *Ruminococcus flavefaciens*, the scaffoldin-type ScaA contains three cohesin domains, a C-terminal dockerin, and a unique N-terminal X domain (Ding et al., 2001). ScaA does not contain an identifiable CBM and yet is able to bind to cellulose. Thus, it appears that either a novel CBM has not been identified for ScaA, or some other factor is involved in binding ScaA to the substrate. It is proposed that ScaA interacts with a surface bound ScaB protein and that the cellulosome bound to the cell surface by this interaction.

An unusual situation exists with *Acetivibrio cellulolyticus*, since its scaffoldin not only serves as a scaffolding protein for a number of enzymatic subunits but also contains a family 9 glycosyl hydrolase enzymatic domain that can function to degrade cellulose (Ding et al., 1999). Thus, the properties of scaffoldins suggest that they not only serve as scaffolding proteins to bind cellulosomal enzymes, but some have evolved or combined multiple functions, including cell surface binding functions and even enzymatic activity. It is likely that as more scaffoldins are characterized, diverse structures and functions will be found.

2.4.1.2 Enzyme subunits (cellulase and related enzyme)

The cellulosomal enzymes include cellulases, hemicellulases, pectinase, chitinase and many ancillary enzymes that can degrade plant cell wall materials. A list of cellulosomal enzymes representing several glycosyl hydrolase familiesis presented in Table 2.14. Some 26 cellulosomal enzymes have been identified for *C. thermocellum* (Bayer et al., 1998a). Some of the enzymes work in concert to facilitate the degradation of the main polymers, for example, xylans and mannans. These include both cellulosomal and non-cellulosomal enzymes that remove various groups from the xylan and mannan backbones.

The endoglucanases, which cleave cellulose internally, primarily belong to glycosyl hydrolase families 5 and 9, and the exoglucanases, which can attack cellulose from either the reducing or non-reducing ends, belong to family 48. The family 9 endoglucanases are versatileas they not only cleave cellulose molecules internally, but also proceed in a processive manner along the chain from the cleavage site, and could be important enzymes in the cellulosome (Gaudin et al., 2000).

C. cellulovorans, *C. acetobutylicum* and *C. cellulolyticum* contain large gene clusters as well as unlinked genes that encode cellulosomal enzymes (Belaich et al., 2002; Nolling, 2001; Tamaru et al., 2000). The organization of the gene clusters in these species is related and the clusters might have evolved from a common set of genes. In the case of the *C. cellulovorans*gene cluster, transposase gene is located at its 3′end, indicating that lateral gene transfer might have occurred (Tamaru et al., 2000). By contrast, the genes encoding the *C. thermocellum* cellulosomal enzymes are scattered through out the chromosome (Guglielmi and Beguin, 1998) and no clusters of cellulosomal enzyme genes have been observed, except for several genes involved in binding the cellulosome to the cell surface (Fujino et al., 1993).

Table 2.14 Cellulosomal enzymes of clostridia (Doi and Kosugi, 2004)

Cellulosomal enzymes	Function	Molecular mass (kDa)	Modular structure
Clositridium acetobutylicum			
CelA	Endoglucanase	54	GH5-DS1
CelE	Endoglucanase	96	CBD3-Ig-GH9-DS1
CelF	Exoglucanase	81	GH48-DS1
CelG	Endoglucanase	77	GH9-CBD3-DS1
CelH	Exoglucanase	80	GH9-CBD3-DS1
CelL	Endoglucanase	60	GH9-DS1
EngA	Endoglucanase	67	GH44-DS1
ManA	Mannanase	47	GH5-DS1
CAC0919	Sialidase	91	GH74-DS1
CAC3469	Endoglucanase	110	(SLH)3-GH5-X-DS1
Clositridiumcellulolyticum			
CelA	Endoglucanase	50	GH5-DS1
CelC	Endoglucanase	51	GH8-DS1
CelD	Endoglucanase	63	GH5-DS1
CelE	Endoglucanase	97	CBD4-Ig-GH9-DS1
CelF	Exoglucanase	78	GH48-DS1
CelG	Endoglucanase	80	GH9-CBD3-DS1
CelH	Endoglucanase	83	GH9-CBD3-DS1
CelJ	Endoglucanase	85	GH9-CBD3-DS1
CelM	Endoglucanase	58	GH9-DS1
ManK	Mannanase	48	DS1-GH5
RglY	Rhamnogalacturonan lyase	75	GPL11-DS1

Table 2.14 Cellulosomal enzymes of clostridia (Contnued)

Cellulosomal enzymes	Function	Molecular mass (kDa)	Modular structure
Clostridium thermocellum			
CbhA	Cellobiohydrolase	138	CBD4-Ig-GH9-X-X-CBD3-DS1
CelA	Endoglucanase	53	GH8-DS1
CelB	Endoglucanase	64	GH5-DS1
CelD	Endoglucanase	72	Ig-GH9-DS1
CelE	Endoglucanase	90	GH5-DS1-CE2
CelF	Endoglucanase	82	GH9-CBD3-DS1
CelG	Endoglucanase	63	GH5-DS1
CelH	Endoglucanase	102	GH26-GH5-CBD11-DS1
CelJ	Endoglucanase	178	X-Ig-GH9-GH44-DS-X
CelK	Endoglucanase	101	CBD4-Ig-GH9-DS1
CelN	Endoglucanase	82	GH9-CBD3-DS1
CelO	Endoglucanase (Cellobiohydrolase)	75	CBD3-PT-GH5-DS1
CelP	Endoglucanase	58	GH9-DS1
CelQ	Endoglucanase	80	GH9-CBD3-DS1
CelS	Exoglucanase	83	GH48-DS1
CelT	Exoglucanase	65	GH9-DS1
CseP	Unknown	62	UN-DS1
ChiA	Chitinase	55	GH18-DS1
LicB	Lichenase	38	GH16-DS1
ManA	Mannanase	67	CBD4-GH26-PT-DS1
XynA (XynU)	Xylanase	74	GH11-CBD4-DS1-CE4
XymB (XynV)	Xylanase	50	GH11-CBD6-DS1
XynC	Xylanase	70	X-GH10-DS1
XynD	Xylanase / feruloyl esterase	70	CBD22-GH10-DS1
XynY	Xylanase / feruloyl esterase	120	CBD22-GH10-CBD22-DS1-CE1
XynZ	Xylanase / feruloyl esterase	92	CE1-CBD6-DS1-GH10

Table 2.14 Cellulosomal enzymes of clostridia (Contnued)

Cellulosomal enzymes	Function	Molecular mass (kDa)	Modular structure
Clositridiumcellulovorans			
EngB	Endoglucanase	49	GH5-DS1
EngE	Endoglucanase	110	(SLH)3-GH5-X-DS1
EngH	Endoglucanase	79	GH9-CBD3-DS1
EngK	Endoglucanase	97	CBD4-Ig-GH9-DS1
EngL	Endoglucanase	58	GH9-DS1
EngM	Endoglucanase	96	CBD4-Ig-GH9-DS1
EngL	Endoglucanase	80	CBD2-GH9-DS1
ExgS	Exoglucanase	80	GH48-DS1
ManA	Mannanase	47	DS1-GH5
PelA	Pectate lyase	94	X-CBD2-GPL9-DS1
XynA	Xylanase / acetyl xylan esterase	57	GH11-DS1-CE4
Clositridiumjosui			
CelB	Endoglucanase	51	GH8-DS1
CelE	Endoglucanase	81	GH9-CBD3-DS1
CelD	Exoglucanase	80	GH48-DS1
AgaA	α-Galactosidase	52	GH27-DS1

*The modular structures of cellulosomal subunits are indicated by abbreviations: CBD, cellulose-binding domain; CE, carbohydrate esterase family; DS1, dockerin domain type I; GH, glycosyl hydrolase; GPL, polysaccharide lyase family 9 (pectate lyase); Ig, immunoglobulin-like module; PT, proline-rich linker; SLH, surface layer homology domain; UN, unknown domain; X, unknown domain containing a hydrophilic domain. Modified with permission from REF. 10 © (2003) American Society for Microbiology.

2.4.2 Cohesin-dockerin interaction

The cohesion-dockerin interaction is crucial for cellulosome assembly. All cellulosomal enzymes contain dockerin domains that interact with the cohesins that are present on the scaffoldins (Tokatlidis et al., 1991). There is species specificity in this interaction; for example, the dockerins that are found in *Clostridium cellulolyticum* cellulosomal enzymes do not interact with the cohesins that are found in *C. thermocellum* scaffoldins and viceversa (Pages, 1997). This specific interaction has been analysed by mutational studies of the cohesion and dockerin domains, and X-ray crystallographic studies of the cohesin domain and the cohesion-dockerin complex. The interaction between cohesins and dockerins was postulated to reside in four specific amino acid residues in the dockerin domain (Miras et al., 2002; Pages, 1997). This prediction was validated by the fact that mutating these four residues resulted in a change in the cohesion-dockerin

recognition specificity (Mechaly et al., 2000). The association between dockerin and cohesin domains was shown to be largely dependent on hydrophobic interactions (Schaeffer et al., 2002). The three-dimensionalstructure of the dockerin-cohesin complex indicates that the cohesion-dockerin interaction is mediated mainly by hydrophobic interactions between one ofthe ‘faces’ of the cohesin and α-helices 1 and 3 of the dockerin (Fig. 2.28).

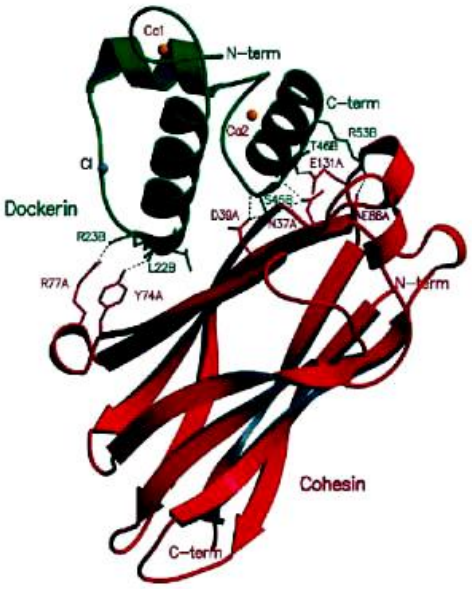


Figure 2.28 Three-dimensional crystal structure of the *C. thermocellum* cohesin-dockerin heterodimer. The complex is formed between a scaffoldin derived cohesion module and an enzyme-derived dockerin domain. The key interacting residues are shown as stick models (Bayer et al., 2004).

2.4.3 Bacterial adhesion to cellulose

Bacterium attacks an insoluble substrate by maintaining a physical association with the substrate (i.e., an adhesion) or via less specific forces (e.g., charge-mediated or hydrophobic interactions). Sometimes, the initial adhesion is superceded by more intensive secondary interactions that lead to a firmer colonization of the organism. The adherence of a bacterium to its substrate would in the ory impart spatial advantages in the subsequent degradation of the substrate (Bayer et al., 1998b; Lamed, 1987). The primary event in the degradation of cellulose is the tight adhesion of the cellulolytic bacterium to its substrate. Adhesion of the bacterium brings the cell into close proximity to the substrate and concentrates the hydrolytic enzymes on the cellulose surface (Fig. 2.29).

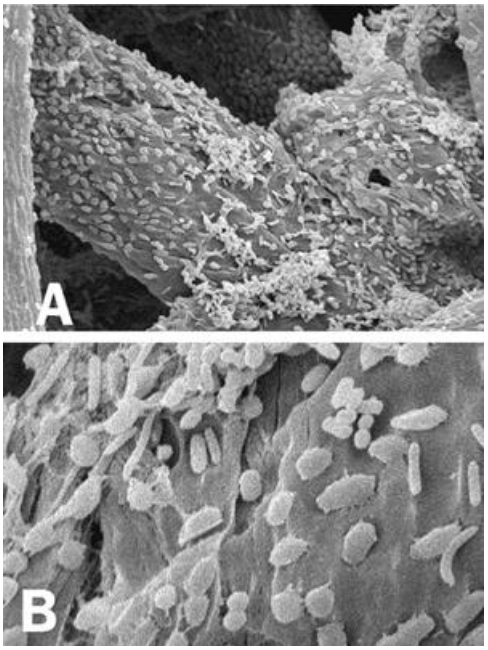


Figure 2.29 Adherence of mixed rumen bacteria to plant material. A: Scanning electron micrograph of adherence to plant cell wall. B: Close examination of bacterial cells reveals protuberances that are likely adherence factors that bind the cells to the plant surface (Krause, 2003).

Such bacteria have been shown to display a new type of surface organelle that appears to harbor the relevant cellulose-adhesion factors. The interaction of *C. thermocellum* with cellulose is shown schematically in Fig. 2.30. The complex is arranged on the cell surface as polycellulosomal protuberance-like organelles. These protuberances comprise multiple copies of the cellulosome, associated with an interior matrix that contains fibrous material (Bayer et al., 1998a). The protuberances are associated with the cell surface, at intervals, on a layer of exocellular anionic material (Bayer and Lamed, 1986; Lemaire et al., 1998). Upon binding to cellulose, these organelles undergo a dramatic conformational change to form elongated fibres between the substrate and the cell surface. These fibres might direct the soluble products from the insoluble substrate to the cell permeases. The attachment of the cellulosome to the cell surface is mediated by a unique type of cohesion-dockerin interaction. The carboxy-terminus of scaffoldin contains a type-II dockerin that fails to bind to its own type-I cohesins but in-stead interacts with complementary type-II cohesins of cell-surface anchoring proteins (Leibovitz and Beguin, 1996; Salamitou et al., 1994). These anchoring proteins also contain an SLH (S-layer homology) module (Sleytr and Beveridge, 1999), believed to

be associatedwith the cell surface of Gram-positive bacteria. Thus, the SLH module interacts with the cell surface, and the type-II cohesin, in turn, interacts with scaffoldin via its type-II dockerin, thereby incorporating the cellulosome into the cell surface

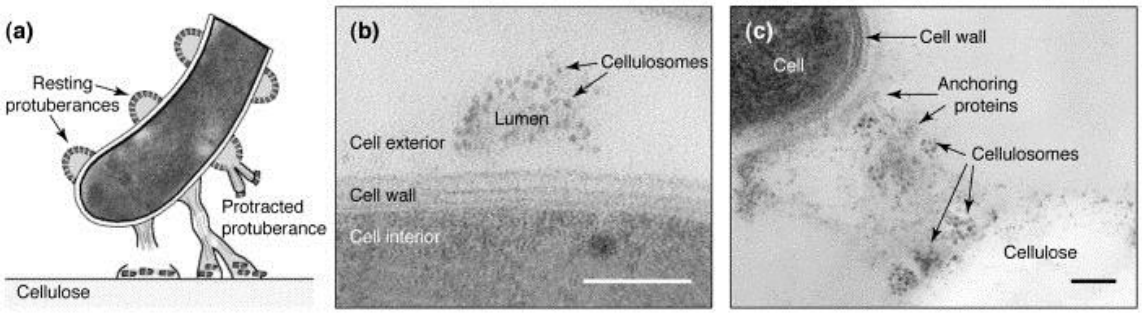
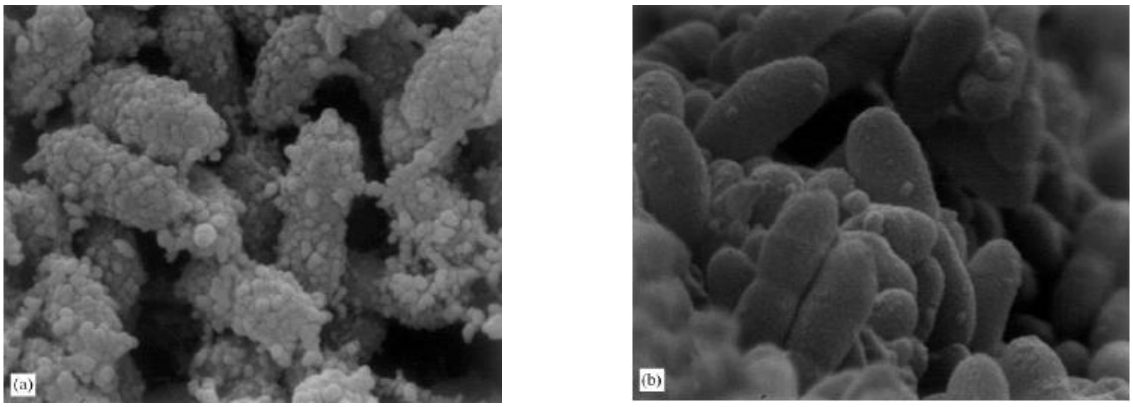


Figure 2.30 Ultrastructure of the *Clostridium thermocellum* cell surface. (a) Diagrammatic representation of a typical cell bound to cellulose. (b) Transmission electron micrographof a resting polycellulosomal protuberance. (c) Transmission electron micrograph ofa protracted polycellulosomal protuberance. The cellulosome is mainly associated with the cellulose surface and connected tothe cell via extended fibrous material, believed to comprise the anchoring proteins. Scale bars = 100 nm (Bayer et al., 1998b).

2.4.4 Carbon source regulation of cellulosome production

The regulation of cellulosomal gene expression has been examined at the microscopic, physiological and transcription levels. The earliest microscopic studies demonstrated the presence of protuberances, which contain polycellulosomes (Lamed et al., 1983). In a study using scanning electron microscopy, the protuberances were observed from cellulose-grown cells, but not glucose-, fructose-, cellobiose- or CMC-grown cells (Blair and Anderson, 1999b). The formation of protuberances took about 4 hours when *Clostridium cellulovorans* cells were grown on cellulose. Within 5 minutes of the addition of the soluble sugars glucose, cellobiose or methylglucose, the protuberances could no longer be detected. This indicated that the dissociation of the protuberances was rapid and that the presence of soluble sugars was responsible. An early transcription study on a cellulosomal gene, *engB*, indicated that it was transcribed as a single transcription unit and that the relative amount of *engB* mRNA was much higher

in cellulose-grown cells than in cellobiose-grown cells (Attwood et al., 1994). The regulation of cellulose-inducible system *Eubacterium cellulovorans* is similar to *Clostridium cellulovorans* (Blair and Anderson, 1999a). Scanning electron micrographs of representative cells of *Eubacterium cellulovorans* are shown in Fig. 2.31 and 2.32.



A B

Figure 2.31 Scanning electron micrographs (x22000) of representative cells of *Eubacterium cellulosolvens* incubated in media containing cellulose (A) or glucose (B). Cultivation in cellobiose, maltose, fructose, or CMC gave results similiar to that of glucose-grown cells. Reproduced at 80% (Blair and Anderson, 1999a).

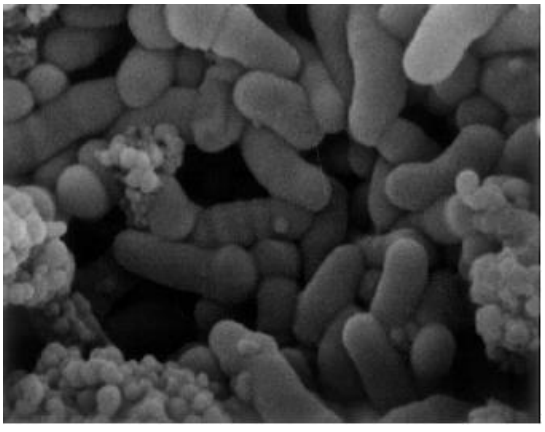


Figure 2.32 Scanning electron micrograph (x22000) of representative cells of cellulose-grown *Eubacterium cellulosolvens* immediately following the addition of a competing substrate (glucose, cellobiose, fructose, maltose, 2-deoxyglucose, or CMC) to the medium (reproduced at 80%) (Blair and Anderson, 1999a).

The growth medium has been shown to affect both subunit structure and function of the cellulosome. When cells are grown on different substrates, such as glucose, cellobiose, xylan, mannan or pectin, and their cellulosomes are fractionated by anion-exchange chromatography, fractions are obtained that differ in subunit composition and enzymatic activity (Ali et al., 1995a; Bayer et al., 1985; Han et al., 2003b). This implies that the cell responds to different substrates by expressing cellulosomal genes, which results in a population of cellulosomes with activities that are directed towards the available substrate. So, the growth substrate has a significant effect on cellulosome synthesis and subunit composition.

As the cellulosome comprises a scaffoldin protein and a large number of enzymatic subunits, it is of interest to determine how their genes are regulated, whether there is coordinate expression of the genes to form this multisubunit enzyme complex, and whattype of promoter region controls their expression. Transcription studies of the *Clostridium cellulovorans* large cellulosomal gene cluster indicated that there are several operons within the gene cluster and that there is coordinate expression of several of the operons (Han et al., 2003b). The promoters for the genes were similar to those found for Sigma-A RNA polymerases of Gram-positive bacteria. When cells were grown on different substrates such as glucose, xylan, mannan or pectin and their mRNA analysed, abundant expression was observed for most of the genes in the cellulosomal gene cluster as well as for cellulosomal genes unlinked to the cluster, and moderate or low levels of expression were observed when various monosaccharides were the substrates. The xylanase and pectate lyase genes were specifically induced in the presence of xylan and pectin, respectively. The results indicated that cellulases and hemicellulases were coordinately expressed, that cellulase expression was regulated by a catabolite-repression-like mechanism, and that the presence of hemicelluloses influenced cellulose utilization by the cell (Han et al., 2003b). Analysis of the transcription of cellulosomal genes that were unlinked to the large cellulosomal gene cluster indicated that most were monocistronic and could be expressed coordinately with the genes in the large gene cluster (Han et al., 2003a). Previous studies with *Clostridium thermocellum* also concluded that a catabolite-repression-like mechanism was controlling the expression of cellulosomal genes (Mishra et al., 1991).

2.4.5 Biotechnological uses of cellulosomes

There is much interest in exploiting the properties of cellulosomes for practical purposes (Bayer et al., 1994). The specific cohesion-dockerin interaction, the strong cellulose binding property of the CBD domain, the potential for transforming non-cellulose degraders to cellulose degraders and the construction and use of 'designer' cellulosomes for specific degradative activities are important properties of the cellulosome that can be used in biotechnology.

2.4.5.1 Practical application of the CBM

In recent years the practical use of CBMs has been established in different fields of biotechnology, and the number of published articles and patents is constantly on the rise. Three basic properties have contributed to CBMs being perfect candidates for many applications: (i) CBMs are usually independently folding units and therefore can function autonomously in chimeric proteins; (ii) the attachment matrices are abundant and inexpensive and have excellent chemical and physical properties; and (iii) the binding specificities can be controlled, and therefore the right solution can be adapted to an existing problem. Utilization of CBMs has been extensively reported and reviewed in the literature (Levy and Shoseyov, 2002; Shoseyov et al., 2006; Tomme, 1998), and their use has been described in several patents. Therefore, this section summarizes only the basic principles of CBM application, along with recent developments.

2.4.5.2 Bioprocessing

Bioprocessing is the major application for CBMs, given that large scale recovery and purification of biologically active molecules continue to be challenges for many biotechnological products. Biospecific affinity purification (affinity chromatography) has become one of the most rapidly developing divisions of immobilized affinity ligand technology because they can potentially replace low yield multistep purification procedures with a single highly selective adsorption step that simultaneously concentrates and purifies the product.

To date, numerous affinity tag systems have been developed. Although each of these affinity tag systems has been shown to offer particular advantages, none has found widespread use in production-scale applications, due in part to the high cost of the associated affinity matrix that arises from the complex chemical modifications required

to cross-link the solid support or to graft the affinity-tag receptor to the resin surface. The relatively low tolerance of many of these affinity resins to repeated processing and sanitization cycles also limits their use at the production scale. Cellulose or related polysaccharides have a number of advantages which makes it an ideal matrix for large scale affinity purposes; it is cheap, has excellent physical properties, is inert and has a low, nonspecific affinity for proteins. The wide use of CBD as an affinity tag in expression and purification is illustrated in Fig. 2.33.

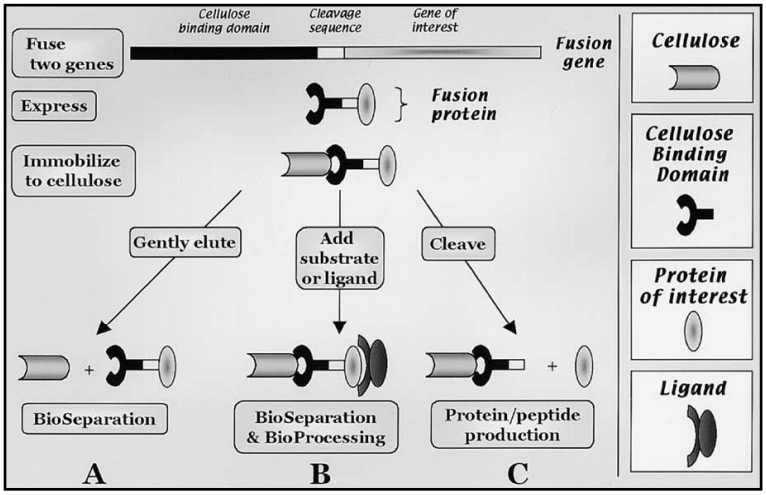


Figure 2.33 CBM-based expression and purification of recombinant proteins. Protein expression and purification via CBM involves several steps. (1) Gene fusion between cbm and a gene of interest. (2) Transformation of ligated plasmid vector into a prokaryotic or eukaryotic expression system. (3) Overexpression of the recombinant protein. (4) Purification by immobilization of CBM-tagged protein on cellulose. (5) Reconstitution of the target protein by (A) gentle elution of target protein from cellulosic matrix, (B) addition of a ligand or a substrate, or (C) proteolytic cleavage of the engineered sequence located between the CBM and the target(Levy and Shoseyov, 2002).

2.4.5.3 Protein engineering with CBMs

Several studies have shown the potential of CBMs for modifying the characteristics of several enzymes. The basic approach in CBM engineering was to replace or add a CBM in order to improve hydrolytic activity. Addition of a CBM derived from cellobiohydrolase II of *Trichoderma reesei* to *Trichoderma harzianum* chitinase resulted in increased hydrolytic activity of insoluble substrates (Limon et al., 2001).

Replacement of the CBM of endo-1,4-β-glucanase from *Bacillus subtilis* with the CBM of exoglucanase I from *Trichoderma viride* conferred higher binding, with enhanced hydrolytic activity on the microcrystalline cellulose (Kim et al., 1998). Similar results were obtained with other glycoside hydrolase.

2.4.5.4 Diagnostics

Recently Siegel and Shoseyov (Siegel and Shoseyov, 2001) developed a system based on CBM, which enables rapid detection of pathogenic microbes in food samples (illustrated in Fig. 2.34). In this method, CBM is conjugated to a bacteria binding protein such as an epitope specific monoclonal antibody and is loaded on to a cellulosic matrix (e.g., cotton gauze) that acts as a bacterial cell concentrator (Fig. 2.34A). The structure of the cotton gauze enables passage of relatively large volumes of liquids so sufficient bacteria can be isolated, even from dilute samples. The bacteria can then be further enriched with a short growing period (Fig. 2.34B) or eluted from the loaded matrix, all the while maintaining a very low bacterial background (Fig. 2.34C). The eluted bacteria can be utilized for enumeration and/or classification. The advantage of CBMs in diagnostics can be attributed to the wealth of different cellulosic matrices that possess very low nonspecific binding to proteins.

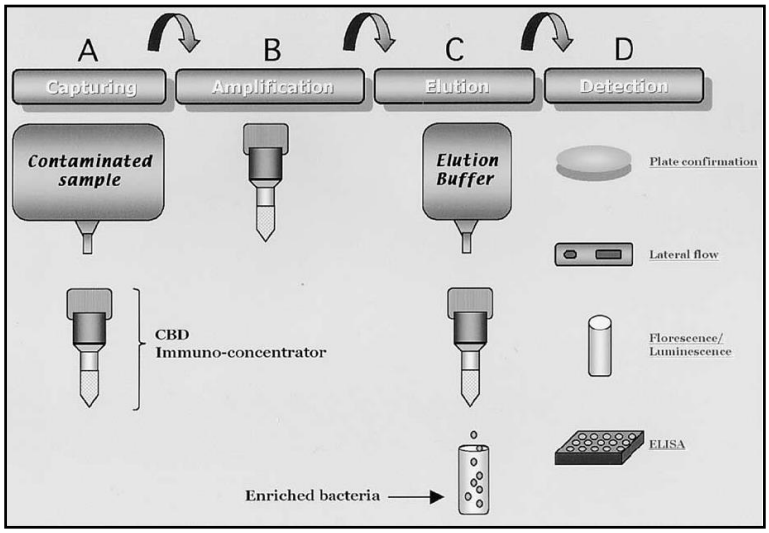


Figure 2.34 CBM-based pathogen detection system.The method involves conjugation of CBM to a bacteria-binding protein that is subsequently loaded onto a cellulosic matrix column (e.g., cotton gauze). This column acts as a bacterial cell concentrator (A). If the anticipated concentration is insufficient, brief growth period prior to elution from

the loaded matrix can be applied to increase bacterial count (B). The resultant isolated bacterial flora contains a very low undesirable background (C). The eluted bacteria can be further analyzed quantitatively or classified into types by means of ELISA, lateral flow detection, or plating onto selective or differential media (D) (Levy and Shoseyov, 2002).

2.4.5.5 Cell immobilization

Cell immobilization technology has many applications in biotechnology. The applications range from ethanol production and phenol degradation to mammalian cell attachment and whole-cell diagnostics. Several industrial technologies have been developed to immobilize cells; however, they have serious drawbacks. Hollow fibers are expensive and undergo a steady decline in filtration rate. Covalent immobilization results in loss of cell viability while cell entrapment is affected by a high degree of mass transfer resistance between the cell and its surroundings.

Whole-cell immobilization to cellulosic material was first demonstrated when *E. coli* surface-anchored CBD, derived from *Clostridium fimi*, was attached to cellulose (Francisco et al., 1993). In this study, recombinant *E. coli* cells expressed surface-exposed CBM that enabled high affinity and specific immobilization onto the cellulose surface. Subsequently, it was shown that immobilization via CBM_{Cex} derived from *Clostridium fimi* provided a monolayer of cells on different cellulosic supports.

2.4.5.6 Bioremediation

Heavy metals are major contributors to pollution; therefore, efficient removal systems are required. Recently, Xu et al. (Xu et al., 2002) reported the cloning and expression of a recombinant protein composed of a CBM fused to a synthetic phytochelatin. The immobilized sorbent was shown to be highly effective in removing cadmium at the level of parts per million.Atrazine is a commonly used pesticide that is persistent in water, is mobile in soil, and is among the most frequently detected pesticides in groundwater. Therefore, its removal or detoxification from industrial wastewater is required before its disposal. Recently Kauffmann et al. (Kauffmann et al., 2000) reported a novel method for enzymatic removal of atrazine from water. Atrazine chlorohydrolase (AtzA) was fused to a CBM and immobilized on cellulose. The active cellulose-AtzA resin was then

used to dechlorinate atrazine. Hydroxyatrazine is an unregulated compound and is not leached from the soil.

2.4.5.7 Industrial applications

Cellulose is a major component of numerous commercial products, several of which are capable of being recycled. Therefore, CBMs can be used for the targeting of functional molecules to materials containing cellulose. The commercial potential of CBMs in this context was first realized for denim stonewashing, where cellulases were used as an alternative to the original abrasive stones (Cavaco-Paulo et al., 1998; Kalum and Andersen, 2000). The presence of CBM allowed for the targeting of the enzyme onto the garment. The final product was fabric or a garment with a ‘‘stone-washed’’ or ‘‘worn’’ look exhibiting localized variation in color density.

Another textile associated CBM application used in numerous laundry powders is fabric targeting of recombinant enzymes that do not possess a native affinity for the cellulosic fibers (e.g., amylases, proteases, lipases, and oxidoreductases). This can be achieved by recombinant enzyme technology, where fusion to CBMs with a desired enzyme is achieved. Additional substances can also be targeted to cellulosic fabrics. Fragrance-bearing particles conjugated to CBMs can be added to laundry powder, hence reducing the amount of fragrance needed in the product (Berry et al., 2001).

2.4.6 Designer cellulosomes-construction of function-specific cellulosomes

Designer cellulosomes comprise recombinant chimaeric scaffoldin constructs and selected dockerin-containing enzyme hybrids as a conceptual platform for promoting synergistic action among enzyme components (Fig. 2.35). This approach enables the precise incorporation of complementary dockerin-containing components into a designer cellulosome by simply mixing them in solution together with the chimeric scaffoldin, thus controlling the composition and architecture of the resultant complexes. This approach will eventually be appropriate for general use as a molecular Lego for application in biotechnology and nanotechnology.

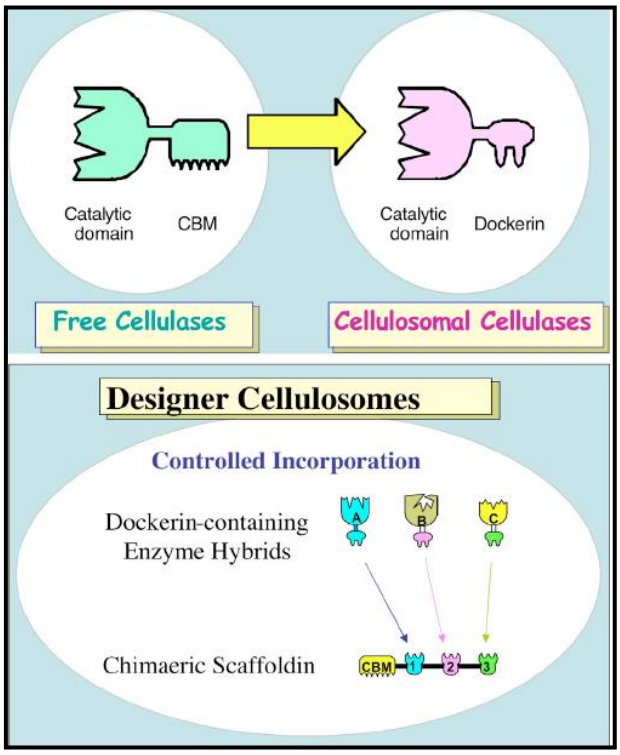


Figure 2.35 Designer cellulosome, an engineered system, designed to convert a free cellulase system into a cellulosomal one, by replacing the enzymes' CBM with a dockerin module. Since the different enzymes are appended with dockerins of divergent specificity, we can control their incorporation into precise positions of the artificial chimaeric scaffoldin by virtue of their selective interaction with matching cohesions (Bayer et al., 2006).

The sequence specificity in cohesion-dockerin interactions has been exploited to construct 'mini-cellulosomes', which contain 'mini-scaffoldins'with either species specific cohesins or cohesins from different species (Pages, 1997). A mini-scaffoldin with species-specific cohesins will bind enzymes only from that species. Mini-scaffoldins that are constructed to contain cohesins from two or more different species will bind cognate enzymes from those species. These mini-cellulosomes have been used to study phenomena such as cohesion-dockerin interactions cellulosomal enzyme synergy, the synergy with neighbouring enzymes, the effect of the CBM on enzyme activity and the potential for metabolic pathway engineering (Fierobe et al., 2002; Fierobe et al., 2001; Mechaly et al., 2000). An example of a mini-cellulosome constructed from a mini-scaffoldin and enzymes is illustrated in Fig.2.36.

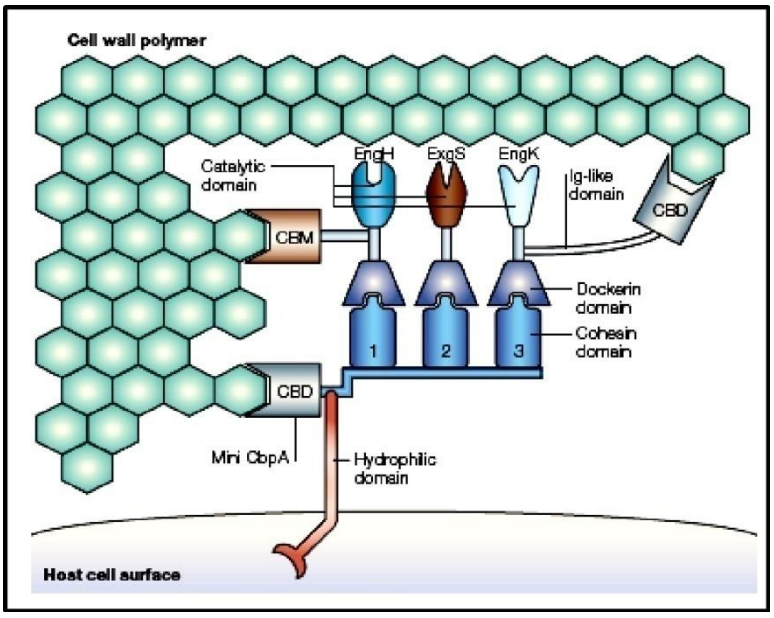


Figure 2.36A model of a designer mini-cellulosome. Mini-cellulosomes contain 'miniscaffoldins', which can either contain one particular cohesin, and thus bind one particular enzyme, or a variety of cohesins and thus bind a variety of enzymes. This mini-scaffoldin has a cellulose-binding domain (CBD), one hydrophilic domain, and three cohesin domains (labelled 1, 2 and 3) to which three different cellulosomal enzymes are attached through their dockerin domains (Doi and Kosugi, 2004).

Mini-cellulosomes from *Clostridium thermocellum* have been used to demonstrate the one-to-one stoichiometric relationship between a cohesin and a dockerin-containing endoglucanase and the enhancing effect of the CBD on cellulosome activity (Kataeva et al., 1997). Mini-cellulosomes constructed from only *Clostridium cellulovorans* components were used to investigate the synergy between cellulases (Murashima et al., 2002), and hemicellulases (Murashima et al., 2003), and between a cellulosomal enzyme and non-cellulosomal enzymes (Kosugi et al., 2002a; Morag et al., 1990). In all cases, synergy was observed, indicating that the synergy between the enzymes in cellulosomes makes the cellulosome structure more effective in attacking the substrate. The synergy observed between cellulosomes and noncellulosomal enzymes also suggests that the maximum effectiveness in degrading natural substrates requires the interaction of cellulosomes and non-cellulosomal degradative enzymes (Kosugi et al., 2002a; Morag et al., 1990).

A scaffoldin with three different cohesins that can bind three enzymes by their cognate dockerins has been constructed. These researchers hope to show that this designer minicellulosome can organize three tandem enzymes into a metabolic pathway that is capable of converting a substrate into a desired product by 'enzyme channelling' in a similar manner to that found *in vivo*. This could lead to the future development of artificial metabolic pathways with controlled activities for the synthesis of any desired product.

2.4.7 Improving cellulosomal properties

A full understanding of the function of the cellulosome should lead to the synthesis of a maximally efficient cellulosome with specified properties. As well as maximizing the synergy both between the cellulosomal enzymes and between cellulosomes and non-cellulosomal enzymes, another approach would be to modify and improve the properties of the enzymes. The creation of a recombinant cellulase with greater heat stability has been reported (Murashima et al., 2002). In this case, DNA shuffling was carried out between two highly homologous endoglucanases from *Clostridium cellulovorans*. One of the enzymes, EngB, is a cellulosomal enzyme whereas the other enzyme, EngD, is noncellulosomal. Three recombinants were obtained that had improved stability at 55ºC compared with the parental enzymes, which were stable up to 45ºC. Not only were the recombinant enzymes more stable at the higher temperature, but also they were as active as the parental EngB. Thus by using DNA shuffling, the further improvements or modifications of cellulosomal enzymes are possible.

2.5 Bioconversion of plant cell wall polysaccharide by microbial combination

The degradation of plant cell walls by microorganism is of major economic importance in the developed as well as developing world. Microorganism fermentation is unique in that efficient plant cell wall degradation relies on the cooperation between microorganisms that produce plant cell wall degrading enzymes. Increasing the efficiency with which the microorganism degrades fiber has been the subject of extensive research for at least the last 10 years.In nature, plant cell wall polysaccharide is degraded with the cooperation of many microorganisms, mainly including diverse

fungal and bacterial genera producing a variety of cellulolytic and hemicellulolytic enzymes under aerobic and anaerobic conditions (Kumar et al., 2008). The biodegradation of plant cell wall polysaccharide through the use of microbial co-cultures or complex communities has been proposed as a highly efficient approach for biotechnological application, since it avoids the problems of feedback regulation and metabolite repression posed by isolated single strains (Haruta et al., 2002; Soundar and Chandra, 1987; Torre and Campillo, 1984). Symbiosis between cellulolytic and non-cellulolytic microorganisms has been reported to promote cellulose degradation by mixed cultures (Pohlschroeder et al., 1994; Valaskova et al., 2009). Bioconversion of cellulosic substrates into first precursor products, such as glucose, is a complex process. It requires the synergistic action of all three enzymatic components i.e. endo/exo-glucanase and β-glucosidase. The ability of major cellulolytic members of microbial strains including fungi or bacteria identified so far produced limiting levels of one or the other enzymatic components. For assistance, *Trichoderma reesei*, a cellulolytic fungus, was reported to have lower levels of glucosidase, whereas, *Aspergillus niger*fungi have limited levels of the endoglucanase component (Madamwar and Patel, 1992; Maheshwari et al., 1994). Therefore, attempts have been made to increase the levels of the enzymatic components either by genetic manipulation (Kuhad et al., 1994) or by co-cultivation approach (Srivastava et al., 1987). Co-cultivation of the cellulolytic organisms complementing the desired cellulolytic component has been attempted for achieving an increased rate of plant cell wall polysaccharide bioconversion. *Trichoderma reesi* Qm 9123 and *Aspergillus niger* were co-cultured for cellulase production using paper mill sludge as a cellulosic substrate (Maheshwari et al., 1994). Similarly Gupte and Madamwar (Gupte and Madamwar, 1997) cultivated *Aspergillus ellipticus* and *A. fumigatusand* reported improved hydrolytic activities as compared to separate cultures in a solid state fermentation system. Improved enzyme levels were also achieved by Madamwar and Patel (Madamwar and Patel, 1992) when *Trichoderma reesei* was co-cultured with using bagasse, corn cobs and saw dust, as the substrates in solid state fermentation. Haruta and co-workers obtained a structurally stable and complex plant cell wall polysaccharide degrading microbial consortium from successive enrichment culture from rice straw compost with a high activity on various cellulosic materials, including rice straw, paper, and cotton (Haruta et al., 2002). On the other hand, it would be much simpler to genetically modify an excellent cellulase producer that produces ethanol, acetate, and lactate so that mainly ethanol is produced. This

approach has the added benefit of addressing the more serious economic problem, that of product selectivity, rather than the less important problem (i.e., for a thermophile) of ethanol sensitivity. *C. thermocellum* is the organism of choice for such an effort, since it is the most thoroughly described cellulolytic ethanol producing thermophile. Studies (Lynd, 1989) revealed that continuous cultures of *C. thermocellum* grown on pretreated hardwood can achieve essentially complete hydrolysis in a 12-h residence time. Because *C. thermocellum* is capable of utilizing only the hexoses and not the pentose sugars generated from cellulose and hemicellulose, the use of mixed-culture (i.e., dual-culture) systems is of great interest. *C. thermocellum* has been cultivated with thermophilic, anaerobic bacteria that are capable of utilizing pentose as well as hexose sugars, i.e., *Clostridium thermosaccharolyticum* (Saddler and Chan, 1984; Venkateswaren and Demain, 1986), *Clostridium thermohydrosulfuricum* (Ng et al., 1981; Saddler and Chan, 1984), *Thermoanaerobacter ethanolicus* (Wiegel and Ljungdahl, 1981) and *Thermoanaerobium brockii* (Lamed and Zeikus, 1980). One such direct process was studied at M.I.T. (Venkateswaren and Demain, 1986), and involved a mixture of *C. thermocellum* and *C. thermosaccharolyticum* (McBee, 1950). This combination forms closely associated, syntrophic, and very stable dual cultures (Wiegel, 1980).

Studies on microbial consortia and their mixed enzyme systems could thus provide an important basis for understanding complex interactions on plant cell wall polysaccharide degradation in nature and can be a platform for biotechnological application involving biomass degradation in composting, anaerobic digestion, enzymatic biomass saccharification, and also the recently introduced concept on direct microbial conversion of plant cell wall polysaccharide to products in the absence of added saccharolytic enzymes (Lynd et al., 2005). The functional and structural stabilities of microbial consortia are considered to be important factors in their plant cell wall polysaccharide degradation capability (Fig. 2.37), and thus their potential for biotechnological application (Kato et al., 2004; Kato et al., 2005).

With the increasing demands for energy and the shrinking energy resources, the utilization of plant cell wall polysaccharide for the production of biofuel offers a renewable alternative. Apart from biofuels, other value added products such as fermentable sugars, organic acids, solvents and drink softeners etc. may also be produced from plant cell wall polysaccharide using appropriate technologies.

Theoretically this is all quite possible; however, technologically it is not an easy task because of various technological gaps. Cellulosic bioconversion is a multi-step process requiring a multi-enzyme complex for effcient bioconversion into fermentable sugars and the co-cultivation of organisms has the ability to produce different components of cellulase complexes in adequate quantity but have been tried with only limited success due to induced end product/ feedback inhibition.

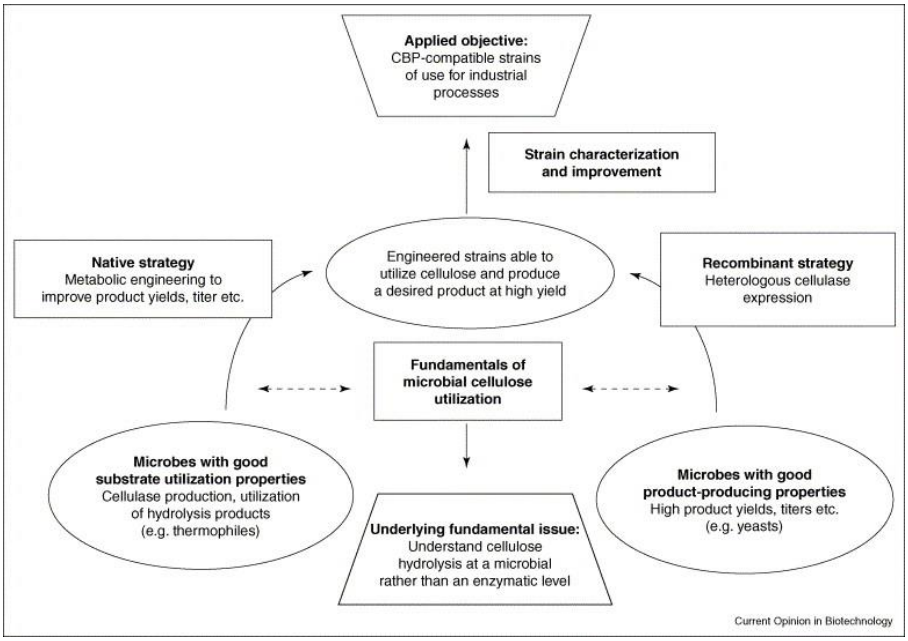


Figure 2.37 Organism development strategies and related fundamentals (Lynd et al., 2005)

CHAPTER 3 MATERIALS AND METHODS

3.1 Culture medium

Basal medium (BM) was used for screen and isolated the bacteria. The medium was adjusted to pH 7.0 and prepared anaerobically, the medium contained (per liter) 1.5 g of K_2HPO_4, 2.9 g of KH_2PO_4, 2.1 g of urea, 4.5 g of yeast extract, 0.001 g of resazurin, 0.5 g of cystein, and 0.2 ml of a mineral salt solution. The mineral salt solution contained (per liter) 250 g of $MgCl_2 \cdot 6H_2O$, 37.5 g of $CaCl_2 \cdot 2H_2O$, and 0.3 g of $FeSO_4 \cdot 6H_2O$. The solid medium contained 15 g of agar per liter.

3.2 Carbon sources

L-Arabinose, D-fructose, D-galactose, D-glucose, D-mannose, D-rhamnose, D-xylose, trehalose and raffinose were purchased from Merck (Darmstadt, Germany). Carboxymethylcellulose (CMC), cellobiose, birchwood xylan (BWX) and oat spelt xylan (OSX) were from Sigma (Saint Louis, MO, U.S.A.). Lactose, maltose, sucrose, dextran, pectin, starch and Avicel were from Fluka (Buchs, Switzerland).

Corn hulls were collected from Lampang Province, Thailand. It was firstly cut using scissors to obtain a piece size (1 cm × 1 cm) and then washed and dried to a constant weight before use in this study. Ball mill corn hulls were prepared by Planetary Ball Mill PM 100 (Retsch, Germany) same as ball mill cellulose that was previously described by Mariko *et al*. (Mariko et al., 2004) with minor modifications.

3.3 Corn hull component analysis

Corn hulls were analyzed components. Lignin, acid detergent fiber (ADF), and neutral detergent fiber (NDF), were determined using the AOAC standard method. The cellulose percentage was calculated indirectly from ADF and lignin, whereas the hemicellulose percentage was calculated indirectly from NDF and ADF (AOAC., 1997).

3.4 Preparation of insoluble xylan

Insoluble xylan was prepared by the method of Irwin et al. (Irwin et al., 1994). Five grams of OSX was suspended in 100 ml of distilled water. The mixture was adjusted to pH 10.0 with 1 N NaOH and stirred at room temperature for 1 h. After that, the mixture was centrifuged at 3,000×*g*for 10 min. The pellet was suspended and adjusted to pH 7.0 with 1 M acetic acid. The suspension was centrifuged in the above conditions and washed twice with 10 volume of distilled water. The pellet was filtered with Whatman No. 1 paper and dried

3.5 Sampling procedure

One hundred and fifty soil samples were taken from farm sites and land fields in Thailand such as QP factory (Ratchaburi), Siam pulp and paper (Ratchaburi), Thai-Ken paper (Prachinburi), Palm factory (Chonburi), Animal feed (Phetchaburi), Mushroom farm (Nakornprathom), Cassava field (Chonburi), Bagasse field (Chonburi), Bangkhuntein (Bangkok), Banana and bamboo field (Phetchaburi). They were kept anaerobically in polyethylene bags, then taken to the laboratory at ambient temperature and stored at 4 ˚C until used.

3.6 Screening and isolation of thermophilic anaerobic biomass degrading strains

Approximately 0.1 g of each soil sample was transferred to Hungate tubes containing 10 ml of BM and 0.5% (w/v) corn hull as a carbon source prepared under oxygen-free nitrogen. The cultures were incubated at 60 ˚C to observe the bacterial growth. The screening step was carried out several times to obtain promising candidates. The sample showing the effective corn hull degradation was enriched. The sample was transferred into ball mill corn hull agar plates for isolation. Then, the ball mill corn hull agar plates were kept in anaerobic jars and incubated at 60 ˚C. The single colony was picked up and streaked onto new plate. Two kinds of colony characteristics (given namely NKP-2 and NOI-1) appeared on the ball mill corn hull agar plates, even though the single colony was picked and streaked. After that, they were streaked on cellulose powder agar plates. One of them (namely NOI-1) could not grow on the cellulose powder agar plate.

Therefore, strain NOI-1 was isolated using the roll tube technique as described earlier containing xylan as a sole carbon source for several times until there was a single pattern appearance. In the case of strain NKP-2, it also was repeatedly streaked on a cellulose powder agar plate more than 5 times until there was a single pattern appearance. The pure isolates, NKP-2 and NOI-1, were kept in appropriate culture broths containing cellulose powder and xylan as the sole carbon source, respectively.

3.7 Isolation of genomic DNA

The isolated strains NKP-2 and NOI-1 were grown in 0.2 % (w/v) cellobiose culture medium for 1 day (OD 600= 0.8) at pH 7.0 and 60 ˚C under an anaerobic condition which were used to extracted genomic DNA. The genomic DNA was followed using a Qiamp DNA Stool kit (Qiagen) according to the manufacturer's protocol.

3.8 Detection of contamination by PCR Assay

To detect contamination of *Clostridium thermocellum*, PCR amplification was performed using Taq polymerase (Promega) and was amplified with primers Cth-P (5'-AACTGCAGTCGAGCGGGGATATACGGAAG-3') and Cth-E (5'-AAGAATTCCTTCGTCCCCAATCAAAGAAG-3'). Whereas primers ThmV1 and ThmR1 was used for the detection of the *Thermoanaerobacterium* spp. [ThmV1 (5'-GAAGGGAGTACTACGGTAC-3') and ThmR1 (5'-TATGGTACCGTCATTTCTTT-3')] (Dotzauer et al., 2002). The PCR amplification conditions followed the method of Erbezniket al. (Erbeznik et al., 1997) and Dotzauer et al. (Dotzauer et al., 2002) for *C. thermocellum* and *Thermoanaerobacteriumspp*., respectively.

3.9 16S rRNA gene analysis and phylogenetic analysis

The 16S rRNA gene was amplified by PCR using following primers: 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1,492R (5'-GGTTACCTTGTTACGACTT-3'). The PCR protocol used was belonging to Qiagen manufacturer's protocol. The determined sequence was compared with references available in the GenBank/EMBL database using the BLAST program (Thompson et al., 1994).

The phylogenetic tree based on 16S rRNA gene sequences of strain NOI-1 was aligned and edited nucleotides by using CLUSTAL W, version 1.81 and then was constructed by using the neighbour-joining method in MEGA, version 3.1 (Kumar and Singh, 2001). The confidence values of branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1,000 resamplings.

3.10 Nucleotide sequence accession number

The GenBank/EMBL/DDBJ accession number for the 16s rRNA gene sequence of strain NKP-2 and NOI-1 are JX508848 and FJ546341, respectively.

3.11 Bacterial strains and growth condition

C. thermocellum NKP-2 and *T. thermosaccharolyticum* NOI-1 were cultivated anaerobically at 60°C at pH 7.0 in basal medium 0.5% carbon sources. Whereas, *Bacillus* sp. strain K-1, used in this study, was isolated from a wastewater treatment plant of a pulp and paper manufacturer that was cultivated aerobically at 37°C in Berg's mineral salt medium containing 0.5% birchwood xylan. This medium was adjusted to pH 10.5 with 1% Na_2CO_3 after autoclaving.

For studied symbiotic of behavior of co-culturing, batch cultivations of each mono- and co-culture were carried out in a serum vial that consisted of BM medium with 1.0% (w/v) corn hulls under anaerobic conditions at 60 °C. An inoculation volume of 14 ml (0.22 g/l of total cell) of bacteria suspension at exponential growth phase was transferred into 86 ml BM medium for all cultivation. For the co-culture, the *C. thermocellum* NKP-2/ *T. thermosaccharolyticum* NOI-1 ratio was 5:2, which was a suitable condition (data not shown). All cultures were inoculated from freshly prepared cultures at the exponential growth phase. Three replicates of culture vials were used at each experimental sampling point. Zero hour samples were collected immediately after inoculation and used as controls. Bacterial grown cultures were centrifuged at 8,000×g for 15 min. The culture supernatants at the stationary growth phase were collected for the enzyme activities and product determinations. The cell-corn hull complex (pellet) was washed 3 times with phosphate-buffer saline (PBS), pH 7.0 and suspended in 0.5% (v/v) Tween 80 at 4 °C for 30 min with occasional stirring and removal of residual corn

hulls (Lamed et al., 1983). The cell suspension was used for measuring cell growth by monitoring the optical density (600 nm) and the remaining corn hull was quantified by a gravimetric determination after being dried at 80 °C until a constant weight was reached.

3.12 Effect of temperature on cell growth

The both bacteria strains were grown in the above condition at temperatures ranging from 37°C to 80°C and measured the remained substrate by dry weight for cell growth.

3.13 Characterization of *T. thermosaccharolyticum* NOI-1

The morphology was determined by scanning electron microscope (Smibert and Krie, 1994). The surfaces of the cells grown on xylan harvested at the exponential and the early declining growth phases were analyzed by scanning electron microscope (SEM). The cell samples were fixed on specimen with 2.5% glutaraldehyde and 1.0% osmium tetroxide, dehydrated by a series of graded ethanol solutions, and critical point dried with liquid CO_2. The samples were then coated with platinum and examined with a JEOL JSM-35 scanning electron microscope.

The growth temperature range of the strain was examined by monitoring the optical density (600 nm). The cultures were incubated at 37°C to 75°C in BM medium containing 0.5% (w/v) of xylose as a carbon source at pH 6.0. For the effect of pH, the isolated strain was grown at 60°C at different pH from 4.0 to 8.5. In the case of carbon utilization, the isolated strain was incubated in BM containing 0.5% (w/v) of various carbon sources at 60°C and pH 6.0.

3.14 Adhesion of bacterial bells to insoluble substances

The adhesion of xylan-grown strain NOI-1 cells to Avicel was performed (Ponpium et al., 2000). At the exponential phase of growth, cells were harvested by centrifugation at 8,000 $\times g$ for 7 min. Cell suspensions were collected and washed three times with phosphate buffered saline (PBS; 0.15 M sodium chloride in 0.1 M sodium phosphate buffer, pH 7.0). Each washed cell suspension was adjusted with PBS to 0.2 of an optical density at 400 nm and then brought to a total volume of 3 ml with 1 ml of 20% Avicel

and 1 ml of PBS. The suspension was mixed for 40 seconds and allowed to settle at room temperature for 60 min. The turbidity of the suspension was measured at 400 nm and compared with the suspension of an identical cell suspension wherein PBS was substituted for the Avicel suspension.The xylan adhesion was also conducted according to the above procedure, using insoluble OSX.

$$\text{Cell binding ability } (\%) = \left(\frac{\text{A-B}}{\text{A}}\right) \times 100$$

where; $A = OD_{400}$ of initial turbidity of the suspension

$B = OD_{400}$ of residual turbidity after settle at various time

3.15 Fermentation products analysis

Fermentation products of mono- and co-culture between the strain NKP-2 and NOI-1 on corn hull cultivation at stationary phase were analyzed using gas chromatography (GC) (Shimadzu model GC-2014) equipped with a flame ionization detector. The column was a DB-WAX column (30 m × 0.32 mm × 0.5 μm). The column temperature was maintained at 170 °C and the temperatures at the detector and injection block were maintained at 230 °C.

Biogas composition from mono- and co-culture between the strain NKP-2 and NOI-1 on corn hull cultivation at stationary phase were measured using GC (model Shimadzu GC-2014 equipped with Porapak N and Porapak Q columns and a thermal conductivity detector) and using argon as a carrier gas. The operational temperatures at the injection port, the column oven and detector were 80, 100 and 120 °C, respectively. One milliliter of the gas phase from the culture was injected directly into the GC column.

3.16 Protein determination

Protein concentrations were measured as described by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard.

3.17 Enzyme production

The strain NOI-1 was grown in the serum vials containing the BM and 1% (w/v) OSX and incubated at 60°C. The culture supernatant was concentrated by using an ultrafiltration with a 10-kDa-cut off membrane and then, it was used as the crude enzyme (extracellular protein). In the case of pellet-bound fraction, the pellets were washed three times with PBS by centrifugation. Then, the enzyme was eluted with 2% (v/v) triethylamine (TEA). The eluate was dialyzed against 5 mM phosphate buffer (pH 7.0) and assayed for xylanase activity.

The crude enzyme from *C. thermocellum* NKP-2 was prepared as described to the above procedure using Avicel as the carbon source.

Bacillus firmus K-1 (formerly known as *Bacillus* sp. strain K-1), which produces free endocellulase-free xylanolytic enzymes was grown on Berg's mineral salts medium, as described previously (Ratanakhanokchai et al., 1999). The crude enzyme from the strain K-1 was used to compare the hydrolysis of xylan with the strain NOI-1.

3.18 Enzyme assays

All assays were studied in triplicate. The xylanase activity was measured by determining the amount of reducing sugar released from BWX. The reaction mixture consisted of 1% xylan in 10 mM phosphate buffer, pH 6.0 and enzyme to give a final volume of 0.5 ml. The reaction mixture was incubated at 60 ^{o}C for 15 min. The release of reducing sugars was determined by Somogyi-Nelson methods(Nelson, 1944)using xylose as the standards. One unit (U) of enzyme activity was defined as the amount of enzyme producing 1 µmol of reducing sugar in 1 minnute under the assay condition. The CMCase activity was measured under the same conditions as described above using CMC as a substrate and using glucose as the standards. For, avicelase activity was measured under the same conditions but incubation time was 60 minute and one unit (U) of avicelase activity was defined as the amount of enzyme producing 1 µmol of reducing sugar in 1 hour under this condition.

The β-xylosidase assay mixture consisted of 1.0 ml of 0.9 mM *p*-nitrophenyl-β-D-xylo pyranoside in 10 mM phosphate buffer, pH 6.0 and enzyme (0.1 ml) to give a final

volume of 1.1 ml. The reaction mixture was incubated at 60 ^{o}C for 15 min and then 2.0 ml of 0.4 M sodium carbonate was added to terminate the reaction. The amount of *p*-nitrophenol released was measured by monitoring the optical density at 405 nm (Kyu et al., 1994).

β-Glucosidase and α-L-arabinofuranosidase activities were measured under the same condition as β-xylosidase activity as mentioned above except for the substrates. For the β-glucosidase assay, 1 mM *p*-nitrophenyl-β-D-glucopyranoside was used as the substrate, and for the α-L-arabinofuranosidase assay, 0.83 mM *p*-nitrophenyl-α-L-arabinofuranoside was used. The amount of *p*-nitrophenol released was measured by monitoring the optical density at 405 nm.

The acetyl esterase assay mixture consisted of 1 ml of 1 mM *p*-nitrophenylacetate and 0.5 ml enzyme to give a final volume of 1.5 ml. The substrate, dissolved in 50% (vol/vol) methanol, was prepared immediately prior to use. After 15 min of incubation at 60°C and then 1.0 ml of 0.05 M Tris-HCl buffer pH 7.0 was added to terminate the reaction. The amount of *p*-nitrophenol released was measured by monitoring the optical density at 405 nm.

The cellobiohydrolase activity was determined using the method of Kohring et al. (Kohring et al., 1990) with p-nitrophenyl β-D-cellobioside as the substrate. The reaction mixture consisted of 0.5 ml of 0.5 mM *p*-nitrophenyl β-D-cellobioside in 10 mM phosphate buffer, pH 6.0 and 0.5 ml of enzyme. The reaction mixture was incubated at 60 ^{o}C for 15 min. After incubated, 0.1 ml of the mixture was added to 2.0 ml of 1.0 M sodium carbonate. The amount of *p*-nitrophenol released was measured by monitoring the optical density at 405 nm.

β-Xylosidase, β-glucosidase, α-L-arabinofuranosidase, acetyl esterase and cellobiohydrolase activities were expressed as micromoles of *p*-nitrophenol released per min per milliliter of enzyme solution.

3.19 Gel electrophoresis and zymograms

Native-PAGE and SDS-PAGE were performed on a 10% polyacrylamide gel by the method of Laemmli (Laemmli, 1970). After electrophoresis, the proteins were stained with Coomassie brilliant blue R-250. The molecular weight standards used were from a high-molecular-weight calibration kit (Pierce).

Native-PAGE and SDS-PAGE zymograms were prepared for detection of endoglucanase and xylanase activities, as described previously by Ratanakhanokchai et al. (Ratanakhanokchai et al., 1999) with minor modification. 10% polyacrylamides gel zymograms without or with SDS were obtained by copolymerization of 0.1% (w/v) CMC or xylan. For SDS-PAGE zymogram, after elctrophoresis, the gel was soaked with 2% (v/v) triton X-100 with gentle shaking for 30 min for 2 times in order to remove SDS and renature the proteins. The native-PAGE and SDS-PAGE zymograms were then incubated with 0.1 M phosphate buffer at 60 ˚C for 10 min. Then, the gels were stained for residual carbohydrate with 0.1% (w/v) Congo red solution for 30 min at room temperature with mild shaking and destained with 1M NaCl. The clear bands would present on the gel and fixed the gel with 5% (v/v) acetic acid.

3.20 Effect of pH and temperature on enzyme activity and stability

The optimum pH of enzyme was measured at various pH under the standard assay conditions as described above. The reaction pH were adjusted with 50 mM various buffers such as acetate buffer (pH 4.0 to 5.5), phosphate buffer (pH 5.5-7.0), Tris-HCl buffer (pH 7.0 to 9.0), Na_2CO_3-$NaHCO_3$ buffer (pH 9.0 to 10.0), $NaHCO_3$-NaOH buffer (pH 9.6 to 11.0) and Na_2HPO_4-NaOH buffer (pH 10.9 to 12.0). The stability of the enzyme was determined by incubating at 60°C for 60 min in buffer solutions of different pH values (10 mM) as mention above without the substrate and the residual activity was measured by the standard assay method.

The optimum temperature for xylanase activity was determined by incubating the enzyme at different temperatures (37–90°C). For thermal stability determination, the xylanase was incubated at different temperatures for 60 min. After cooling the treated enzymes on ice, the residual xylanase activity was measured according to the standard assay method.

3.21 Binding of enzyme to insoluble substances

The binding ability assay was conducted by adding the enzyme to 2% insoluble xylan or Avicel in 50 mM sodium phosphate buffer, pH 7.0 (SPB) with occasional stirring for 60 min at 4°C. Then, the mixture was centrifuged and the amount of enzyme remained in the supernatant was determined by measuring the protein content in the supernatant. The amount of enzyme bound to the insoluble substances estimated from the difference between the amounts of protein before and after incubation. The ratio (%) of the protein bound to insoluble substances to the total protein added into assay mixture was defined as relative (Pason et al., 2006).

$$\%\ \text{Protien Binding ability} = \left(\frac{\text{Total protein-Unbound protein}}{\text{Total protein}}\right) \times 100$$

3.22 Isolation of multienzyme complex

The multienzyme complex was isolated from the crude enzyme preparation by affinity purification on Avicel and gel filtration chromatography. All purification steps were carried out at 4°C. After the bacterium was grown on OSX, the culture was harvested at the stationary growth phase (3 days) by centrifugation, and the supernatant (1,000 ml) was concentrated (50-fold) by using an ultrafiltration with a 10-kDa-cut off membrane. To collect the cellulose-binding proteins, which are mainly multienzyme complex, the cellulose-binding proteins were prepared by affinity purification on cellulose.The Avicel, suspended in SPB overnight, was packed in a column (1.00 cm ×5.00 cm) and equilibrated with the same buffer. The crude enzyme (10 mg protein) was applied on the column. The column containing the cellulose-binding proteins was washed four times with a large amount of the same buffer until proteins were not found in the eluate, and then eluted with 2% (v/v) triethylamine with a flow rate of 2 ml/min. The eluate was collected and dialyzed. The concentrated cellulose-binding proteins preparation (3 mg) was applied onto a Sephacryl S-300 high-resolution (Amersham Biosciences, Piscataway, NJ, USA) column (0.67 cm × 50.00 cm), which was equilibrated with SPB and eluted with the same buffer with a flow rate of 0.3 ml/min.

3.23 Hydrolysis of xylan and biomass

Biomass, such as corn hull, corn cob, rice straw, rice husk and sugarcane bagasse were ground (40 mesh) and wash several times in warm distilled water to remove any reducing sugars remaining in these residues. Pure xylans such as OSX, BWX, and larch wood xylan (LWX) and all biomass residues were studied hydrolysis. Each material (1% w/v) was hydrolyzed with the enzyme at pH 7.0 (50 mM sodium phosphate buffer) and 60 °C. After incubation, samples were taken and the amount of reducing sugars produced was determined using the Somogyi-Nelson method.

For crude enzyme from candidated bacterium, biomass was hydrolzed with 1.0 U of xylanase at above condition for 1 hour.

The crude enzyme of strain NOI-1, the hydrolysis of pure commercial xylans was determined. All substrates were hydrolyzed with crude enzyme (1.0 U xylanase). After appropriate incubation, the samples were taken and the amount of reducing sugar produced was determined by the Somogyi-Nelson method(Somogyi, 1952). Enzymatic hydrolysis products at last time (60 min) were analyzed by Thin-layer chromatography (TLC) (Ratanakhanokchai et al., 1999).

Comparision of OSX and cornhull hydrolysis with crude enzyme and isolated multienzyme complex of strain NOI-1 and crude enzyme from *Bacillus* sp. strain K-1 were studied. For OSX hydrolysis, after certain period of incubation, the samples were taken and the amount of reducing sugar produced was determined by the Somogyi-Nelson method whereas corn hull was hydrolyzed under the same condition as OSX for 1 h.

3.24 Thin layer chromatography

Soluble sugar of grown culture broth at the stationary growth phase by individual mono- and co-cultures of *C. thermocellum* NKP-2 and *T. thermosaccharolyticum* NOI-1 was analyzed by thin layer chromatography using aluminum sheet silica gel 60, F_{254} (Merck, Darmstadt, Germany). A mixture of *n*-butanol/acetic acid/distilled water (5:2:3 by volume) was used as a developing agent. The spray agent contained 1 g α-diphenylamine dissolved in a solution of aniline/phosphoric acid/acetone (1.0:7.5:50.0

by volume) (Ratanakhanokchai et al., 1999). The series of xylose (X_1-X_6) and glucose (G_1-G_5) from Megazyme (Wicklow, Ireland) were used as the standard. For product hydrolysis of pure xylans with crude enzyme of strain NOI-1 was analyzed under the same conditions as described above.

3.25 Combination of cellulolytic enzyme *C. thermocellum* NKP-2 andxylanolytic enzymes from *T. thermosaccharolyticum* NOI-1

The combination of crude enzyme from *C. thermocellum* NKP-2 and *T. thermosaccharolyticum* NOI-1 was tested for synergism at 60 °C in 0.01 M phosphate buffer (pH 7.0) containing 1.0% substrate. To determine the relationship between reaction period and synergy degree, corn hull was degraded by a mixture of crude enzyme from *C. thermocellum* NKP-2 and *T. thermosaccharolyticum* NOI-1 equal amounts of xylanase 0.05 U/mg of protein for 1 to 5 h at 60°C. The theoretical activity indicates the sum of individual activities crude enzyme from *C. thermocellum* NKP-2 and *T. thermosaccharolyticum* NOI-1. The synergy degrees (the activity of combination of crude enzyme from both strains was divided by the corresponding theoretical activities) (Murashima et al., 2003)

$$\text{Degree of synergy} = \frac{RS_{\text{combinded enzymes}}}{RS_{\text{individual of NKP-2}} + RS_{\text{individual of NOI-1}}}$$

where; RS = Released reducing sugar from hydrolysis reaction

The combination of cellulolytic enzymes from *C. thermocellum* NKP-2 and endo cellulase-free xylanolytic enzymes from *T. thermosaccharolyticum* NOI-1 was tested for synergism at 60 °C in 0.01 M phosphate buffer (pH 7.0) containing 0.5% substrate. The effect of enzymes from both strains on different substrates, that were determined by using different plant cell wall materials, such as corn hull, corn cob, rice straw, rice husk, cassava peel, cassava pulp and sugarcane bagasses. They were simultaneously incubated with different plant cell wall materials for 3 h at 60°C. Relationships between ratio and synergism of cellulolytic-xylanolytic enzymes from *C. thermocellum* NKP-2 and *T. thermosaccharolyticum* NOI-1 on degradation of corn hull was studied by various mixed enzyme ratios and each of them was adjusted to contain equal amount of xylanase activity (0.05 U/mg of protein) and both were combined in differently

proportions to maintain a constant sum of xylanase activity (0.1 U/mg of protein). Control experiments were also conducted with each enzyme alone, and the hydrolysis products were analyzed for reducing sugar. The degree of synergism for the combination of both enzymes was calculated as the reducing sugars released by the combination of both enzymes divided by the sum of reducing sugars released by each enzyme alone.

CHAPTER 4
STUDY ON COEXISTENCE OF TWO BACTERIAL STRAINS

4.1 Stable coexistence of cellulolytic and non-cellulolytic bacteria on biomass degrading enzyme production

4.1.1 Introduction

Lignocelluloses represent the most abundant biomass on earth which made up of three major polymer: cellulose, hemicellulose and (Kuhad and Singh, 1993). Among the major components of lignocellulose, cellulose and hemicellulose comprise 40-60% of plant cell walls(Doi and Kosugi, 2004). However, the hydrolysis of cellulose is extremely difficult, since cellulose fiber is well protected by a matrix of hemicellulose and lignin. In addition, its strong inter-chain hydrogen-bonding network and higher-order structure in plants contribute to biomass recalcitrance, further complicating the hydrolysis activity(Himmel et al., 2007).Thus, the synergism of enzymatic system for the efficient degradation of these polymers is noteworthy. Up to date, effort in finding effective enzymatic systems from various cellulolytic and/or xylanolytic microorganisms are still ongoing. Many microorganisms such as fungi, aerobic and anaerobic microorganism, that produce cellulosic and hemicellulosic enzymes have been reported and characterized(Lynd et al., 2002). The biodegradation of plant cell wall polysaccharide through the use of microbial co-cultures or complex communities has been proposed as a highly efficient approach for biotechnological application, since it avoids the problems of feedback regulation and metabolite repression posed by using only a single strains(Haruta et al., 2002; Soundar and Chandra, 1987; Torre and Campillo, 1984). Symbiotic interaction between cellulolytic and non-cellulolytic microorganisms in promoting cellulose degradation has been reported (Pohlschroeder et al., 1994; Valaskova et al., 2009).Recently, in our laboratory (Enzyme Technology Laboratory, King Mongkut's University of Technology Thonburi), a stable biomass degrading bacteria (designated as NKP following sample source for screening and isolation) capable of degrading various biomass and produce biomass-degrading enzymes was determined. To date, an anaerobic, thermophilic and cellulolytic bacterium, *C. thermocellum* NKP-2 and non-cellulolytic bacterium, *T. thermosaccharolyticum* NOI-1 have been successfully isolated from the NKP. In this

study, we report the discoveries of biomass degrading enzyme produced by a consortium of bacteria (NKP) and its properties. In addition, we will also describe the isolation of two bacteria (cellulolytic and non-cellulolytic bacteria).

4.1.2 Results and discussion

4.1.2.1 Composition of the corn hull

Biomass signifies an abundant, renewable and underutilized global source of carbon. Thailand, being an agricultural country, has wide variety of crops such as rice, cassava, sugarcane, corn, palm and others., whereby crop harvesting and processing produce high quantity of agricultural residues, such as rice straw, rice husk, corn stover, corn cobs, corn hull, cassava pulp, sugarcane bagasse, and others, which causes environmental problems. In terms of total production, corn is the third most important agriculture products in Thailand, after rice and cassava (source: Office of Agricultural Economics, Thailand; http://www.oae.go.th). In this experiment, corn hulls were collected from Lampang Province, Thailand and its composition was determined.Results show the composition (% dry matter) of cellulose, hemicellulose, lignin and other materials of the corn hull were 34.15, 45.83, 14.12 and 5.9%, respectively. Plant cell wall polysaccharides are a heterogeneous complex of carbohydrate polymers (such as cellulose and hemicellulose) and lignin. Many physiochemical, structural and compositional factors will thus, hinder the enzymatic digestibility these lignocellulosic materials(Abbasi and Abbasi, 2010; Demirbas, 1997; Saidur et al., 2011).

4.1.2.2 Enrichment of bacterial mixed culture able to grow on biomass (corn hull) and cellulose

For the primary isolation of biomass degrading microbial, more than 150 samples of soil, agricultural residues and wastes were individually cultivated in BM7 medium containing 0.5% corn hull as the sole carbon source. Results shows that most of the sample used could degrade and ferment the corn hull. Subsequently, these samples were subcultured under selective enrichment conditions, in order to establish a structurally stable microbial consortium with high corn hull degrading capability. The most active bacteria community in degrading corn hull (>50%) with largest clearing zone appear on cellulose agar plate were chosen and were shown in table 4.1 and Fig. 4.1.

Table 4.1 Growth and clear zone formation of anaerobic thermophilic biomass degrading bacteria phylotype a pH 7.0 and 60 °C

Phylotype	Growth on Corn Hull	Growth on Cllulose
NKP	+++++ (1-2 days)	+++++ (CZ, 1.5 cm)
NKP-13	+++++ (1-2 days)	+++++ (CZ, 1.0 cm)
QP-10	++++ (2-3 days)	+++ (CZ, 0.6 cm)
QP-15	++++ (2-3 days)	+++ (CZ, 0.6 cm)
QP-18	++++ (2-3 days)	++++ (CZ, 0.7 cm)
SPP-4	+++ (3-4 days)	++ (CZ, 0.3 cm)
SPP-21	+++ (3-4 days)	++ (CZ, 0.3 cm)
BT-10	+++ (3-4 days)	+ (CZ, 0.0 cm)
BT-14	+++ (3-4 days)	+ (CZ, 0.0 cm)
CHA-8	+++ (3-4 days)	+ (CZ, 0.0 cm)

+; positive growth, CZ; clear zone

A B C

Figure 4.1 Degradation pattern on corn hull and cellulose by NKP; control corn hull (A), residues of corn hull after cultivation for 7 days (B), clear zone on cellulose agar plate (C).

4.1.2.3 Properties of mixed bacteria, NKP

In this experiment, the NKP shows to be capable to grow at temperatures ranging from 45°C to 65°C, with optimum temperatures for growth from 60°C to 65°C. No cell growth was observed at temperature below 37°C and above 65°C. Growth of NKP was also examined in serum vials containing basal medium with 1% Avicel as a carbon source. The pH of the medium was initially adjusted to 7.0. The culture was sterilized, then treated with a stream of nitrogen gas and the vials were sealed with rubber stoppers and incubated at temperatures ranging from 37°C to 80°C for 5 days. Remaining Avicel (dry weight) was measured after the incubation period using a suction filtration method. Filter papers (42.5 mm Whatman no. 1) were dried at 80 °C for 120 minutes and the tare weight was recorded. The remaining Avicel was oven dried at 80 °C for 24-48 hours until no further reduction in weight. NKP was able to grow at temperatures from 50 °C to 65 °C with optimum temperature of 60 °C at pH 7.0 (Fig. 4.2).

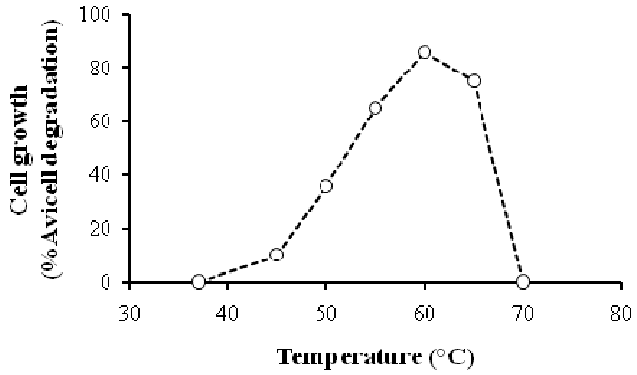


Figure 4.2 Growth of the NKP on BM media contained 1% Avicel at 37 °C to 80 °C. The growth of NKP was determined by reduction in dry weight of Avicel (%Avicel degradation).

Fermentation of microcrystalline cellulose (Avicel) will result in the production of ethanol and organic acids. The fermented products were analyzed by chromatography (GC). Results shows that ethanol (27 mM), acetic acid (46 mM) and butyric acid (14 mM) were the main fermentation end-products obtained when NKP was grown on Avicel under anaerobic conditions for 7 days(Fig. 4.3).

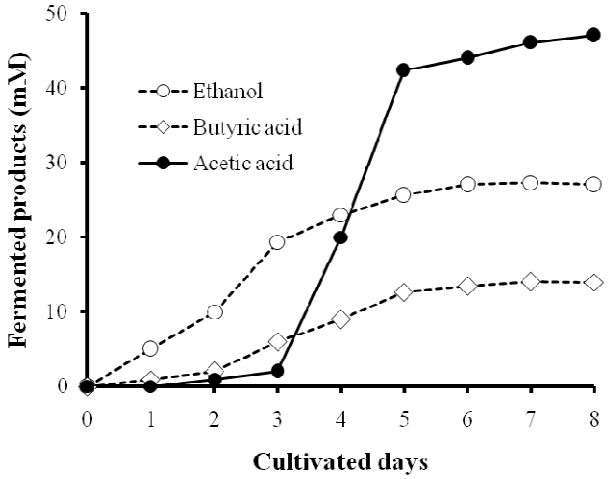


Figure 4.3 Time courses of fermented products from the NKP on Avicel cultivation at 60 °C under anaerobic condition

4.1.2.4 Plant cell wall degrading enzyme system and properties

Pant cell wall degrading enzyme activity profiles of NKP in the crude extracellular enzymes were analyzed. NKP grown in a BM medium containing Avicel as a carbon sources will produce cellulolytic-xylanolytic enzymes. The time course of carbon source reduction and enzyme production was shown in Fig. 4.4. Samples were collected at different time frame and analyzed for enzyme activities, and remaining Avicel. Results indicate that Avicel content in the culture medium was rapidly reduced from day 2 to 5, while the crude enzymes (CMCase, avicelase and xylanase) activity increases slowly due to culture broth. However, marked increase in CMCase and xylanase activities were observed after 5 days cultivation, while avicelase activity was increase slowly. Thus, rate of Avicel degradation was attributable to the continued action of enzyme activity.

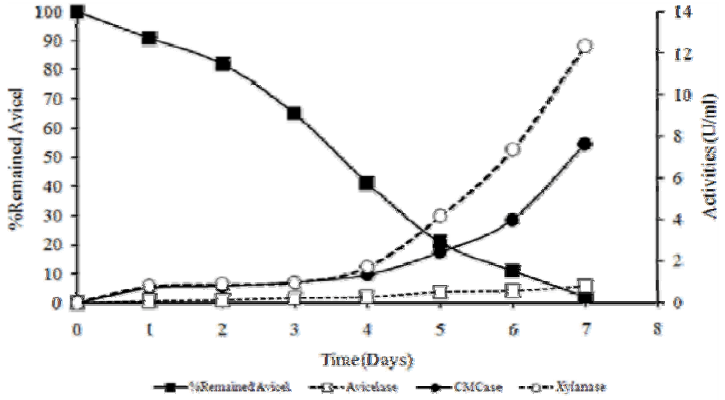


Figure 4.4 Profiles of remained Avicel and enzyme production by the NKP at pH 7.0 and 60 ˚C under an anaerobic condition.

The enzyme activities in culture supernatant were determined after 7 days of incubation (Table 4.2). The results showed that avicelase, CMCase, cellobiohydrolase, β-glucosidase, xylanase, β-xylosidase, α-L- arabinofuranosidase and acetyl esterase were all detected. Compared to that of cellulosome from *C. thermocellum* strains ATCC27405 and JW20, cellulolytic-xylanolytic enzymes of the NKP showed higher specific activities for all assayed glycoside hydrolases, except for avicelase. It is known that most microorganisms would produce cellulases together with xylanases to degrade the cellulosic materials (Biely, 1985). *C. thermocellum* is a cellulose-degrading bacterium that expressed a set of cellulolytic enzymes(Demain et al., 2005; Lynd et al., 2002)but produces less xylan-debranching enzymes, β-xylosidase and β –glucosidase(Duong et al., 1983; Tachaapaikoon et al., 2011). Since NKP showed higher enzyme activity than *C. thermocellum* strains, the NKP was selected as the best active bacterial phylotype and was used for further study.

Table 4.2 Comparison of cellulolytic-xylanolytic activities from the NPKwith cellulosome of *C. thermocellum* from strains JW20 and ATCC 27405.

Enzymes	Specific activities (U/ mg of protein)		
	NKP	*C. thermocellum**	
		Strain JW20	Strain ATCC 27405
Avicelase	0.28	0.34	0.56
CMCase	2.72	1.10	1.10
Cellobiohydrolase	4.73	ND	ND
β-Glucosidase	10.40	<0.001	<0.001
Xylanase	4.40	1.36	1.70
β-Xylosidase	3.34	<0.001	<0.001
α-L-Arabinofuranosidase	1.02	<0.001	<0.001
Acetyl esterase	4.22	ND	ND

ND, not detected

*, (Tachaapaikoon et al., 2011)

For detection of CMCase and xylanase activities from culture supernatant, zymogram analysis was used (Fig. 4.5). For analysis of CMCase activity, native-PAGE zymogram displayed one large protein band (high molecular weight) and more than 7 bands on SDS-PAGE zymogram. Similarly, native-PAGE zymogram of xylanase activity displayed one large protein bandbut with 11 protein bands on the SDS-PAGE zymogram. These results indicated that high molecular weight bands present on native-PAGE zymograms in both cases were broken down into small subunits after boiling in SDS solution buffer. Hence, this result presumed that the NKP could produce multienzyme complex composed of at least 7 CMCases and 11 xylanases. These cellulases and xylanases work synergistically in biomass degradation. Nevertheless, a multienzyme complex has been identified and described in an anaerobic, thermophilic, and cellulolytic bacterium, *C. thermocellum*that consisted of at least 14 proteins with numerous cellulase activities(Lamed et al., 1983)and more recently, in other bacteria and fungi(Bayer et al., 1998a; Chen and Brown Jr, 1998).

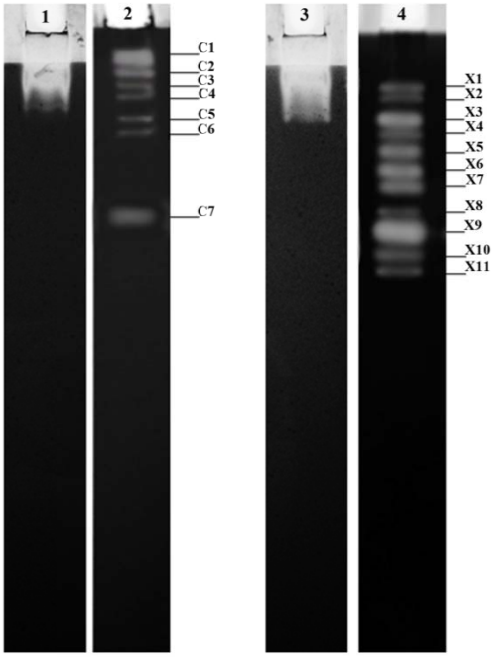


Figure4.5 Zymogramanalysis for CMCase and xylanase activities in culture supernatants of day 7 culture produced by the NKP at pH 7 and 60 °C under anaerobic condition. Lane 1; native zymograms for CMCase activity, lane 2; SDS zymogram for CMCase activities, lane 3; native zymograms for xylanase activity, lane 4; SDS zymogram for xylanase activities

The effect of temperature (40°C to 90 °C) on crude extracellular enzymes activity and stability of NKP was studied and is shown in Fig. 4.6. Both enzyme exhibited its optimum activity at 60 °C and remained stable at 60 °C for 60 min. Nevertheless, the thermostability of CMCase and xylanase showed high stability (above 80%) at 70 °C.Thisallow of these enzyme to be used in industrial applications that require enzymes to be stable at a high temperature. In biorefining, renewable resources such as agricultural crops or wood are utilized for extraction of intermediates or for direct bioconversion into chemicals, commodities and fuels (Fernando et al., 2006). Thermostable enzymes have an obvious advantage as catalysts in these processes.

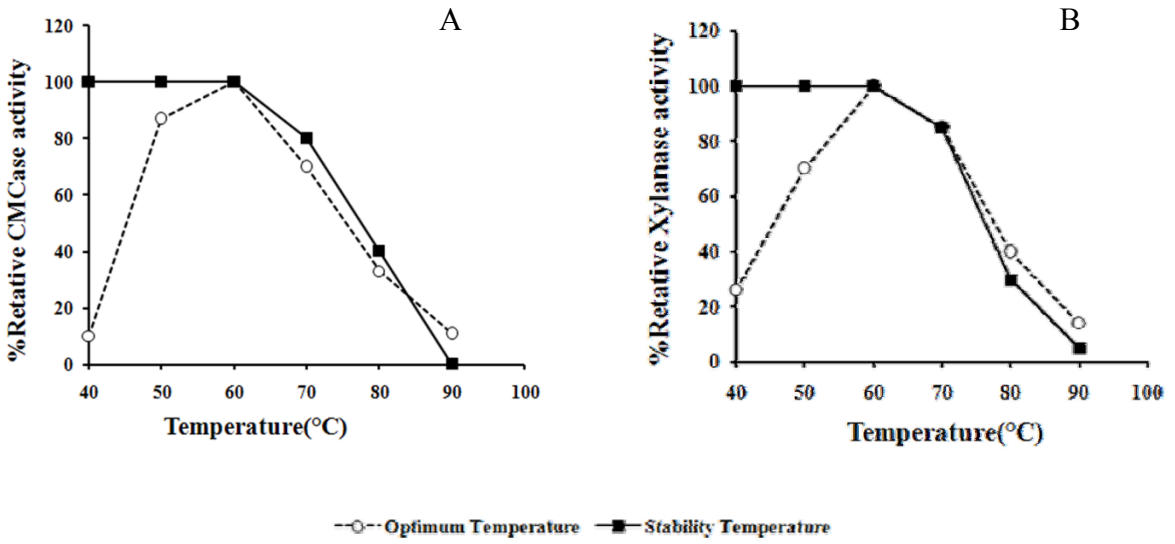


Figure 4.6 Effects of temperature on activity and stability of CMCase (A) and xylanase (B) of crude extracellular enzymes from the NKP.

Hydrolysis of biomass by crude extracellular enzymes from the NKP was investigated. Corn hull, corn cob, rice hull, rice husk and sugarcane bagasses were ground and used as substrates. Crude enzyme from day 7 was used to hydrolyse the substrates at 60 ˚C and pH 7 for 9 hours. At the end of incubation, reducing sugar content was measured (Fig. 4.7). Results indicates that the cellulolytic-xylanolytic enzymes produced by the NPK could hydrolyze those biomass efficiently even though these biomass exist as tightly packed and complex structures in nature. Among the biomass, corn hulls were hydrolyzed the most as indicated by the highest amount of reducing sugar produce at the end of incubation. Apart from corn hulls, the other biomass were also hydrolyzed but at a lower efficiency. It has been reported that the lignin contents, compositions and structures of plant materials varied between different plant variety, and could be the factors affecting enzymatic degradation of biomass as well as the different in degree of hydrolysis observed in this study(Mansfield et al., 1999; Waeonukul et al., 2009).

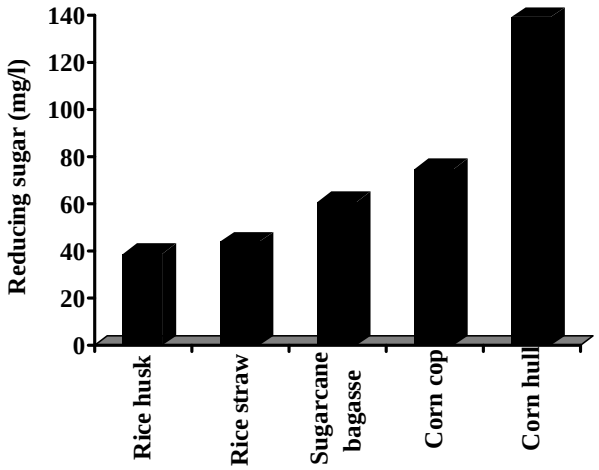


Figure 4.7 Reducing sugar content of biomass after hydrolysis with crude extracellular enzymes from the NKP for 1 hour (0.6 U of CMCase, 1.0 U of xylanase).

In conclusion, NKP is an efficient producer of cellulolytic-xylanolytic enzymes such as avicelase, CMCase, cellobiohydrolase, β-glucosidase, xylanase, β-xylosidase, α-L-arabinofuranosidase and acetyl esterase. Characterization of the crude enzyme reveals that it is a multienzyme complex composed of at least 7 types of CMCases and 11 types of xylanases. These enzymes were thermostable in nature and were able to hydrolyze the biomass efficiently. These results of this study, whilst encouraging and highlighted the potential of NKP as a cellulolytic-xylanolytic enzymes producer, it serve as a preliminary for further studies related to the use of these microorganisms for maximum production of enzymes required for biomass degradation. In nature, plant cell wall polysaccharide degradation involve the action of diverse fungal and bacterial genera, who produces a variety of cellulolytic and hemicellulolytic enzymes under aerobic and anaerobic conditions (Kumar et al., 2008). The biodegradation of plant cell wall polysaccharide through the use of microbial co-cultures or complex communities has been proposed as a highly efficient approach for biotechnological application. Symbiotic relationship between cellulolytic and non-cellulolytic microorganisms has been reported to promote cellulose degradation by mixed microbial cultures (Pohlschroeder et al., 1994; Valaskova et al., 2009).In conjunction to the above statement, NKP need to be isolated and the characteristics of each of the pure cultures should be investigated in detail. The roles and relationships among the members of the community in situ were evaluated based on these results.

4.2 Isolation and characterization of cultivable members of NKPand study of behavior symbiosis on corn hull cultivation

4.2.1 Introduction

In the previous section, it has been demonstrated that the NKP represent a group of biomass degrading bacterial strain which their coexistence is efficient in degrading corn hull. This brings forth the requirement to isolate and characterize of each of the members in the cultures, follow by the study of the roles and relationships among the members of the community. In this study, we found that NKP contains two kinds of microorganisms, *C. thermocellum* NKP-2 and *T. thermosaccharolyticum* NOI-1. The selection of bacteria possessing high corn hull degrading ability was performed. After a series of enrichment and isolation on a ball mill corn hull agar plate, two morphologically distinct colonies of anaerobic bacteria were observed. However, purification of each strain by the single colony isolation technique (on ball mill corn hull) were not successful, thus, follow up purification were done by using different carbon sources, after which were streak on cellulose and xylan agar plate. One of the strains (NOI-1) grows only on xylan agar plate, whereas the strain NKP-2 could grow on both plates. However, growth of NKP-2 on xylan agar plate was slow. Thus, strain NOI-1 was isolated using roll tube technique containing xylan as a sole carbon source whereas the strain NKP-2 was isolated using roll tube technique containing cellulose as a sole carbon for several times until single pattern appearance according to the anaerobic technique of Hungate(Hungate, 1969). Strain NKP-2 was then tested for contaminants by strain NOI-1 using molecular technique. A pair of primers, ThmV1 and ThmR1 which are specific for *Thermoanaerobacterium*spp. was used for amplification (Dotzauer et al., 2002). For verification of strain NOI-1, Cth-P and Cth-E primers which are specific for *C. thermocellum* was used instead(Erbeznik et al., 1997).Corn hull degradation by individual culture was significantly lower when compared to the degradation by a mixed culture. It has been reported that *C. thermocellum* and *T. thermosaccharolyticum* have symbiotic mutualism(Liu et al., 2008; Saddler and Chan, 1984).Therefore, the symbiotic relationship by co-culturing *C. thermocellum* and *T. thermosaccharolyticum* on corn hulls in terms of their enzymatic system were analyzed.

4.2.2 Results and Discussion

4.2.2.1 Isolation, purification and identification of cultivable members of NKP

NKP is an efficient corn hull degrading mixture of bacterial strains isolated from soil samples obtained from farm sites was enriched using corn hulls as a carbon source. The NKP culture was isolated using ball mill corn hull agar plate that were kept in anaerobic jars and incubated at 60 □C. Single colony was picked and streaked onto a new plate. Two morphology different colonies were observed on the ball mill corn hull agar plates, after which were streaked on cellulose and xylan powder agar plates since cellulose and xylan are the major polysaccharide component in plant cell wall (Kuhad and Singh, 1993).Strain NOI-1 grew well on xylan agar plate but not on cellulose powder agar plate. Thus, strain NOI-1 was isolated using the xylan roll tube technique. Strain NOI-1 was transferred by serial dilution into xylan agar medium tube. This step was repeated until a pure culture was obtained. The isolated colony that showed the highest xylanase activity was selected for further study.

In the case of strain NKP-2, it also was streaked on a cellulose powder agar plate several times until there was a single pattern observed. The pure isolates, NKP-2 and NOI-1, were kept in appropriate culture broths containing cellulose powder and xylan as the sole carbon source, respectively. Cellulolytic bacterium (NKP-2) and non-cellulolytic bacterium (NOI-1) were isolated from NKP, whichwas efficient in degrading corn hull symbiotically. Subsequently, both strains were isolated by their biochemical properties. The strain NKP-2 was able to growth on xylan as a carbon source slowly (2-5 weeks) whereas the strain NOI-1 unable grown on cellulose as a carbon source. Therefore, both strains were isolated based on their ability to grow on different carbon source. Table 4.3 shows some properties of NKP, NKP-2 and NOI-1.

Table 4.3 Characterization of coexistence bacteria (NKP) and cultivable members of its, cellulolytic bacterium strain NKP-2 and non-celluloltic bacterium strain NOI-1

	NKP	NKP-2	NOI-1
% Corn hull degradation (at 7 days)	55%	15%	12%
Growth on corn hull	+++++	+++	++
Growth on cellulose	+++++	+++++	-
Growth on xylan	+++++	-*	+++++
Growth condition	60 °C, pH 6.0	60 °C, pH 6.0	60 °C, pH 6.0

+, positive growth; -, negative growth; *, at 7 days on xylan cultivation

Freier et al., (1988) have reported that some microorganisms can survive in several places in nature symbiotically, just like the interaction between cellulolytic bacteria and non-cellulolytic bacteria (Freier et al., 1988).These bacteria within the community depend on each other for nutrient flow, carbon digestion, removal of toxic metabolites or pH balance and mechanisms have been developed to assist or protect these relationships.

Identification of both isolated strains by using 16S rRNA gene (NKP-2 and NOI-1) showed high similarity with *C. thermocellum* and *T. thermosaccharolyticum*(> 99% identity), respectively. Verification of the pure strain from contaminant was performed using a modified PCR assay in which a set of primer specific for (Thm V1 and Thm R1) specific for *Thermoanaerobacterium*spp. was used (Dotzauer et al., 2002). Results indicated that strain NKP-2 was not contaminated with the strain NOI-1 as the expected band (385 bp) were not observed on agarose gel of PCR product (Fig. 4.8). Similarly, the strain NOI-1 was tested contamination with NKP-2, but using a different set of primer (Cth-P and Cth-E) which is specific for *C. thermocellum*(Erbeznik et al., 1997). Results indicated that strain NOI-1 was not contaminated with the strain NKP-2 as the expected band (409 bp) were not observed on agarose gel of PCR product (Fig. 4.8). These results indicated that the strain NOI-1 and NKP-2 were pure culture. Thus, these strains were designed as *C. thermocellum* NKP-2 and *T. thermosaccharolyticum* NOI-1, respectively.

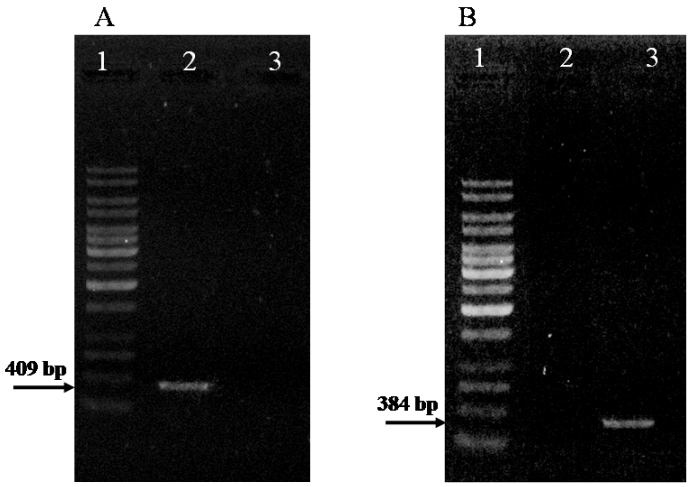


Figure 4.8 Specific PCR for *C. thermocellum* (A) and *T. thermosaccharolyticum* (B) with primers in material and method. Lane 1, 1 kbp DNA ladder; lane 2, strain NKP-2; lane 3, strain NOI-1

However, when each strain was incubated individually, corn hull utilization was lower as compared to when mixed culture was used. This is in agreement with those previously reported whereby both cellulolytic bacteria and non-cellulolytic bacteria can live symbiotically in several places in the nature (Freier et al., 1988). These bacteria in a mixed culture depend on each other for nutrient flow, carbon digestion, removal toxic metabolites or pH balance. Many reports have showed that *C. thermocellum* is a cellulose-degrading bacterium with the high rate of cellulose degradation (Demain et al., 2005; Lamed and Zeikus, 1980; Lynd, 1989; Lynd et al., 2002). Several strains of *C. thermocellum* have been described that actively utilize cellulose and cellobiose but not pentoses(Lamed and Zeikus, 1980; Ng et al., 1977). As for *T. thermosaccharolyticum*, it was a thermophilic, anaerobic bacterium that was able to grow under the same conditions of *C. thermocellum*.

Total genomic DNA of both strains grown in basal medium with cellobiose as a carbon source was extracted. Specific PCR amplifications were performed using EUB8f and U1492r primer designed to specifically amplified 16S rRNA gene of both NKP-2 and NOI-1 under condition as describe by manufacturer (Qiagen). Results of agarose gel electrophoresis of PCR product shows a single band (approximately 1,500 bp) corresponding to the expected sized of amplified DNA (Fig. 4.9). Sequence alignment of NKP-2 16S rRNA (1,432 bp) were shown in Appendix B.1. Search for sequence similarity from National Center for Biotechnology Information databases (NCBI

database) confirmed that NKP-2 shows similarity (99%) to *Clostridium thermocellum* NKP-2 (Table 4.4). Sequence alignment of NOI-1 16S rRNA (1,422 bp) were shown in Appendix B.2 Search for sequence similarity from NCBI database confirmed that NOI-1 shows highest similarity at 99% with *T. thermosaccharolyticum*, 97% with *T. aotearoense*and *T. aciditolerans*, and 96% with *T. islandicum*, *T. thermosulfurigenes*, *T. xylanolyticum*, and *T. saccharolyticum* (Table 4.5).

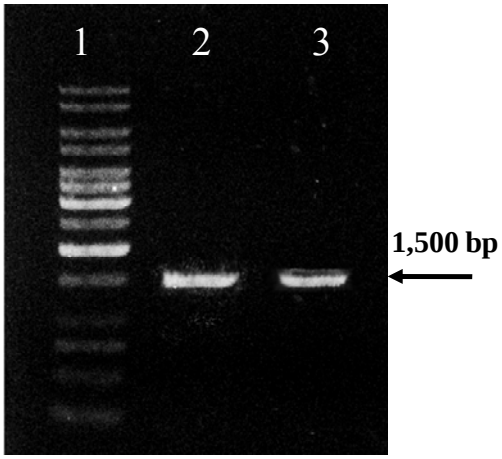


Figure 4.9 Agarose gel electrophoresis of PCR product amplification with EUB8f and U1492r primer sets. Lane 1, 1 kbp DNA ladder; lane 2-3, Amplified PCR products from total genomic DNA extraction of strain NKP-2 and strain NOI-1, respectively

Table 4.4 Homology with bacterium strain NKP-2 in a phylogenetic analysis based on the 16s rRNA sequence.

Bacterium	Homology (%Identity)
*C. thermocellum*ATCC 27405 (NR_074629.1)	99%
C. thermocellum DSM 1313 (NR_074578.1)	99%
*C. thermocellum*JCM 9323 (AB588017.1)	99%
*C. thermocellum*JN4 (EF680276.1)	99%
C. thermocellum GP1 (FN555230.1)	99%
C. thermocellum CTL-6(FJ599513.1)	99%
C. thermocellum mbf-VZ-093 (HF674392.1)	99%
Clostridium sp. CT1 (FJ808607.1)	99%
C. straminisolvens CSK1(NR_024829.1)	96%

Table 4.5 Homology with bacterium strain NOI-1 in a phylogenetic analysis based on the 16s rRNA sequence.

Bacterium	Homology (%Identity)
T.thermosaccharolyticum	99%
T. thermosulfurigenes	97%
T. xylanolyticum	97%
T. lactoethylicum	96%
T. aciditolerans	96%
T. islandicum	96%
T. saccharolyticum	96%
T. polysaccharolyticum	88%
T. zeae	88%

The phylogenetic tree based on 16S rRNA gene sequences of strain NOI-1 was aligned by using CLUSTAL W, version 1.81 and then was constructed by using the neighbour-joining method in MEGA, version 3.1 (Kumar and Singh, 2001). The confidence values of branches of the phylogenetic tree determined using bootstrap analyses (Felsenstein, 1985)based on 1,000 resampling shows that strain NOI-1 was located within the lineage of the genus *Thermoanaerobacterium* (Fig. 4.10), indicating that strain NOI-1 belongs to *T. thermosaccharolyticum*. The strain NOI-1 was deposited at MIRCEN culture collection, Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, Thailand under the accession number of TISTR1916. Therefore, the strain NKP-2 and NOI-1 were identified as *C. thermocellum* NKP-2 and *T. thermosaccharolyticumstrain* NOI-1, respectively.

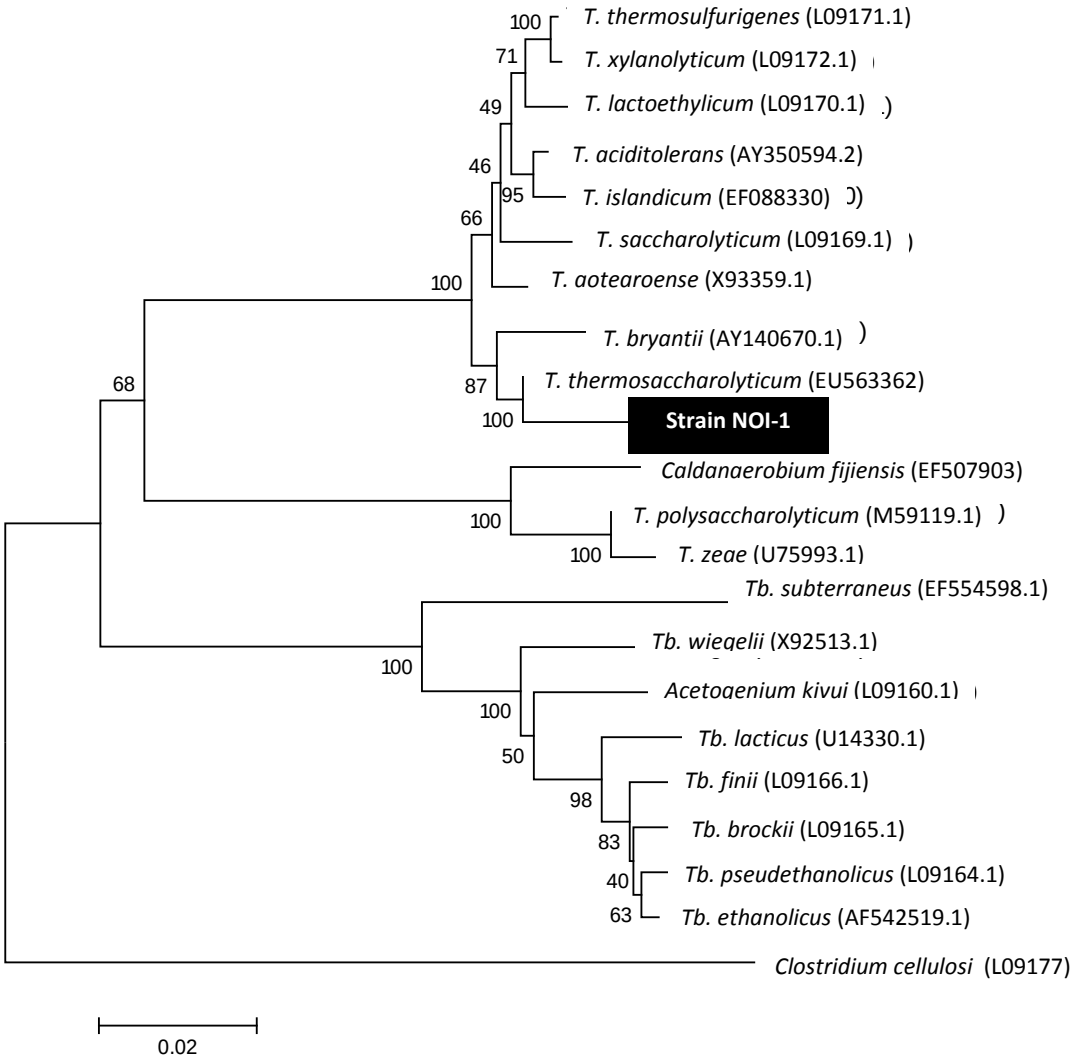


Figure 4.10 Phylogenetic tree showing the phylogenetic position of the isolated strainNOI-1 based on the 16S rRNA gene sequences of the family *Thermoanaerobacteriaceae*. The numbers at the nodes indicate the levels of bootstrap support percentages based on the neighbor-joining of 1,000 replicates. The scale bar represents 0.02 sequence difference. GenBank accession numbers are given in parentheses. The following abbreviate character codes, *T.* and *Tb.* are *Thermoanaerobacterium* and *Thermoanaerobacter*, respectively.

Many reports had showed that *C. thermocellum* is a cellulose-degrading bacterium with high rate of cellulose degradation(Demain et al., 2005; Lamed and Zeikus, 1980; Lynd et al., 1989; Lynd et al., 2002). Several strains of *C. thermocellum* have been described to actively utilize cellulose and cellobiose but not pentoses(Lamed and Zeikus, 1980; Ng et al., 1977).In addition, it has also been shown that cultures of *C.*

thermocellum are frequently associated with glycolytic, noncellulolytic and thermophilic bacteria(Freier et al., 1988; McBee, 1950)and co-cultures of *C. thermocellum* JN4 and *T. thermosaccharolyticum* GD17 have demonstrated the enhancement of bioenergy and hydrogen production(Liu et al., 2008; Saddler and Chan, 1984).Therefore, it is to our interest to know the reason behind this preference of symbiotic interaction between two bacteria in nature. Investigation of the individual and co-cultivation of *C. thermocellum*NKP-2 and *T. thermosaccharolyticum* NOI-1 will explain the synergism of enzyme production from both strains using corn hulls as a sole carbon source.

4.2.2.2 Symbiotic behavior of co-culturing *C. thermocellum* NKP-2 and *T. thermosaccharolyticum* NOI-1 on corn hull

In previous study, *C. thermocellum*NKP-2 and *T. thermosaccharolyticum* NOI-1 were isolated from a mixed culture of bacteria (NKP) which have proven to be efficient in degrading corn hull. These bacteria often co-exist in their natural habitats and are difficult to be separate. Their coexistence helps in the degradation of biomass leading to a better use of biomass whilst offering a promising new way to improve the conversion efficiency of biomass. This relationship may be defines as a symbiotic mutualism relationship which is the relationship between individuals of different species in which both individuals benefit from the association to enhance their survival, growth or competency, removal of toxic metabolites, growth factors or sufficient nutrition.In this study, the enzyme production were investigated by cultivating *C. thermocellum*NKP-2 and *T. thermosaccharolyticum* NOI-1 individually and together using corn hulls as a sole carbon source. Enzyme activities were determined from culture supernatant after the culture reached the stationary growth phase (Table 4.6). Results shows that strain NKP-2 produced CMCase, avicelase and xylanase as the main enzymes while strain NOI-1 did not produce CMCase and avicelase, and produces less xylanase (2.9-fold lesser) than strain NKP-2. However, strain NOI-1 produces higher cellobiohydrolase, β-glucosidase, β-xylosidase, α-L-arabinofuranosidase, acetyl esterase than strain NKP-2 (1.3-, 1.4-, 3.4-, 27.1- and 2.4-fold, respectively). These results shows that both strain exhibits different enzyme production system in which the NKP-2 strain generally produces cellulose- and xylan-main chain cleaving enzymes, whereas NOI-1 strain produces mainly short- and side-chain cleaving enzymes. Corn hull contains cellulose, hemicellulose, lignin, and other materials. The cellulose fibrils are enclosed by a

network of hemicellulose and lignin. Therefore, complete and rapid hydrolysis of corn hulls required synergetic action of both cellulolytic and xylanolytic enzyme (Murashima et al., 2003) in which xylanolytic enzymes hydrolyzed the outer hemicellulose component of the plant cell wall first to allow the cellulolytic enzymes to hydrolyze the inner cellulose component afterwards (Shallom and Shoham, 2003). Hence, this may be the reason behind the symbiotic relationship created by these microbes in their nature habitats.

Table 4.6 Cellulolytic and xylanolytic enzyme activities of *C. thermocellum* NKP-2 and *T. thermosaccharolyticum* NOI-1 on corn hull cultivation at the stationary growth phase.

Enzymatic activities	**Total activities (U)**	
	C. thermocellum **NKP-2**	***T. thermosaccharolyticum*** **NOI-1**
CMCase	19.50	ND
Avicelase	0.24	ND
Xylanase	36.00	12.30
Cellobiohydrolase	0.42	0.56
β-Glucosidase	0.27	0.39
β-Xylosidase	0.16	0.55
α-L-Arabinofuranosidase	0.14	3.82
Acetyl esterase	0.04	0.11

ND, were not be able to detect under the assay condition

The cell growth of *C. thermocellum* NKP-2 and *T. thermosaccharolyticum* NOI-1 cultivated individually and to gather on corn hull were studied and shown in Fig. 4.11. Fermentation was carried out with same starting inoculum size, either when culturing individually or co-cultivating it. Result shows that the relationship of co-culturing both strain of bacteria on corn hulls exhibited a shorter lag phase compared to culturing individual strain of NKP-2. In addition, strain NOI-1 did not exhibit an apparent lag phase. Furthermore, co-culture generally shows a faster exponential growth rate compared to the individual culture of strain NKP-2. At the stationary growth phase, the

growth of individual culture of strain NKP-2 and strain NOI-1 were 0.14 and 0.23 g/l of the total cell, respectively, whereas the co-culturing with both strains was 0.33 g/l of the total cell. Approximately 15% and 12% of the corn hulls were utilized by the individually-culture of strain NKP-2 and strain NOI-1, respectively, whereas the corn hull utilization by co-culturing of both strains was 22% at 36 hours. By comparison, it was found that co-culturing demonstrated higher cell growth and corn hull utilization than those individually-cultured NKP-2(2.4-fold and 1.5-fold respectively) or NOI-1(1.5-fold and 1.8-fold, respectively). This observation may indicate that strain NOI-1 were probably mostly xylan degrading enzymes which hydrolyzed arabinoxylan (a major component of hemicelluloses), (Galbe and Zacchi, 2007; Mazumder and York, 2010). Although strain NKP-2 produced endo-xylanase, this strain had low debranching-enzymes, especially arabinofuranosidase. Thus, the lag phage of strain NKP-2 was longer than the co-culture and individual-culture of strain NOI-1.

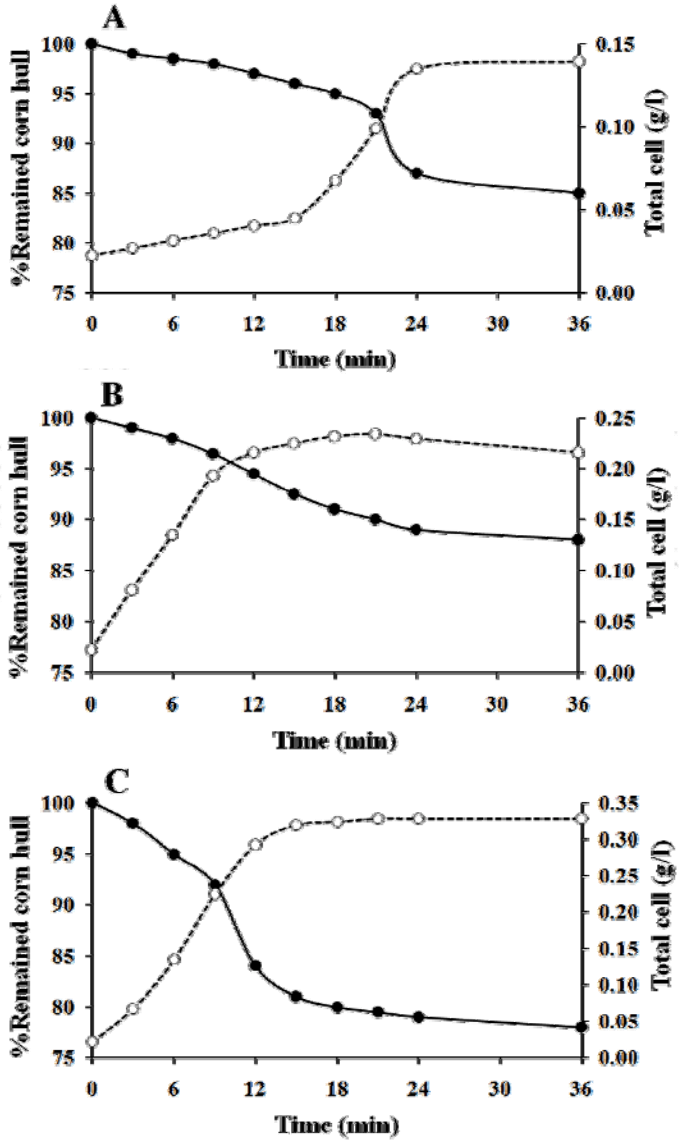


Figure 4.11 Time courses of cell growth and remaining corn hulls of (A) *C. thermocellum* NKP-2, (B) *T. thermosaccharolyticum* NOI-1 and (C) the co-culture of both strains at 60 °C and pH 7.0

Furthermore, the remaining soluble sugar (300 mg/l) in the individualculture of strain NKP-2 was probably branched-oligosaccharides because the size of the sugar did not relate to any of the standard sugars (Fig. 4.12). In addition, this oligosaccharide could be a branched-xylooligosaccharides because xylanolytic enzyme systems of the strain NKP-2 were not complete and especially had very low α-L-arabinofuranosidase activity. In contrast, this sugar was not present in the individual-cultured strain NOI-1

and the co-cultured supernatant, perhaps due to the utilization of this sugar by the bacterium in the culture.

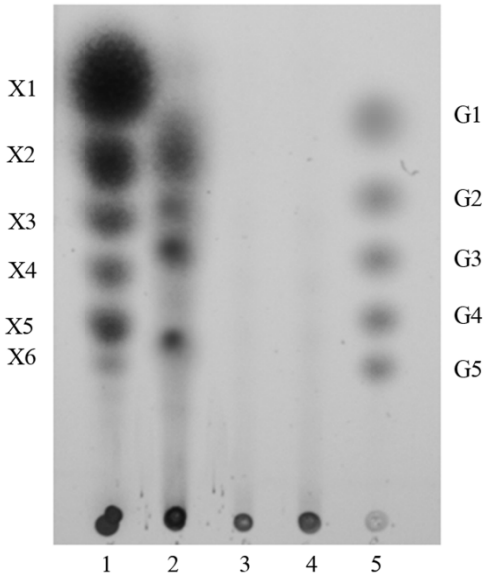


Figure4.12 Thin layer chromatography of sugars from the culture supernatant at the stationary growth phase by individual mono- and co-cultures of *C. thermocellum* NKP-2 and *T. thermosaccharolyticum* NOI-1. Lane 1: standard X1-X6, Lane 2: *C. thermocellum* NKP-2, Lane 3: *T. thermosaccharolyticum* NOI-1, Lane 4: co-culture, and Lane 5: standard G1-G5

The fermentation end products were determined by gas chromatography and results are shown in Fig. 4.13. Ethanol, butanol, acetic acid, butyric acid, hydrogen gas and carbon dioxide gas were the expected end products of the fermentation. Results show that butanol was not present after cultivation with strain NKP-2 alone. Furthermore, co-culture generally has higher ethanol (1.5-fold), acetic acid (2.2-fold), butyric acid (8.1-fold), H_2 (1.5 fold) and CO_2 (1.5-fold) than the individually-cultured strain NKP-2 and ethanol (1.5-fold), butanol (2.1-fold), acetic acid (2.1-fold), butyric acid (2.5-fold), H_2 (1.2 fold) and CO_2 (1.3-fold) than the individually-cultured strain NOI-1.

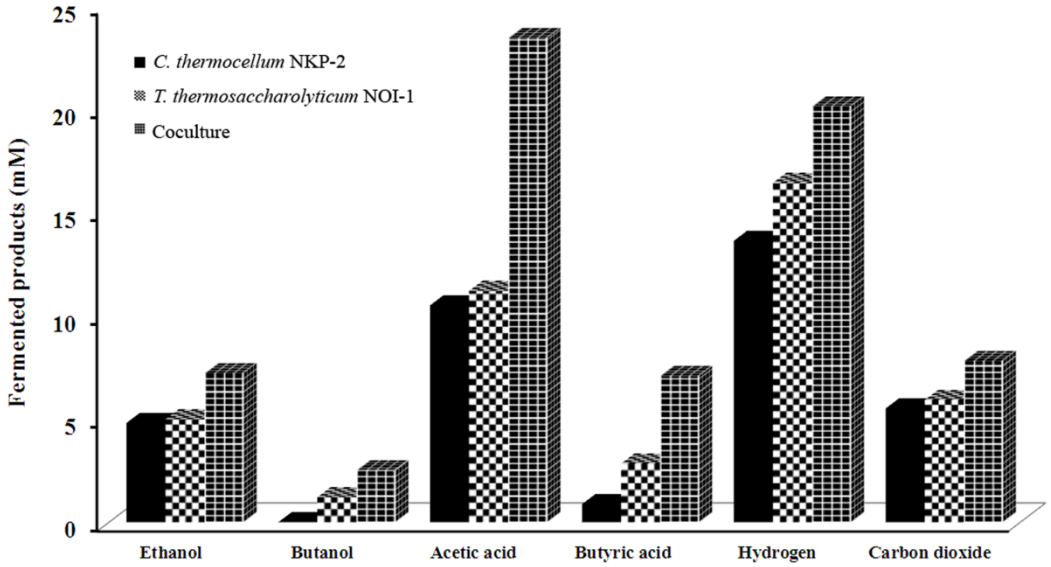


Figure 4.13 Fermentation end-products of individual-culture and co-culture of *T. thermosaccharolyticum* NOI-1 and *C. thermocellum*NKP-2 grown on corn hulls at 60 °C and pH 7.0.

The higher cell growth, carbon utilization and fermentation end products in the co-culture indicated that co-culturing of both strains improved the conversion efficiency of corn hull in term of cell growth and fermentation end products. This is in agreement with recent research in literature which shows that co-culturing bacteria can improve the degradation of biomass for increasing the yield of fermented products (Geng et al., 2010; Liu et al., 2008).Thus, it can be concluded that the mutualism relationship exist between strain NKP-2 and strain NOI-1 in corn hull fermentation, in which the cellulolytic and xylanolytic enzymes are produced from both strains. Similarly, this synergistic interaction of non-cellulolytic and cellulolytic anaerobic bacteria has also been proven (Khan and Murray, 1982; Mori, 1990; Murray, 1986).Therefore, in the subsequent study, the effect of combining each enzyme from strains NKP-2 and NOI-1 was evaluated for its efficacy in hydrolysis of biomass.

4.2.2.3 Combination of plant cell wall degrading enzymes involed in corn hull degradation

In the previous section of this study, bacteria strain were co-cultivated on corn hull and results indicated that co-culturing is effective in increasing cell growth, fermentation end products and corn hull degradation. In this section, the synergistic effect of enzyme

mixture on biomass (corn hull) degradation was investigated. Degradation of plant cell wall polysaccharide by combination of crude enzyme from *C. thermocellum* NKP-2 and *T. thermosaccharolyticum* NOI-1 will be an interesting study since *C. thermocellum* NKP-2 produced main-chain cleaving enzymes (xylanase,cellobiohydrolase, avicelase and CMCase) whereas *T. thermosaccharolyticum* NOI-1 produced side and/or oligos-chain cleaving enzymes (xylanase, β-xylosidase, α-L-arabinofuranosidase, β-glucosidase and cellobiohydrolase) when using Avicel and OSX as a carbon sources, respectively.The crude enzyme produce from both strains was shown in Table 4.7.Theresult shown that the strain NOI-1 could producedxylanase, β-xylosidase,α-L-arabinofuranosidase, β-glucosidase and cellobiohydrolase but could not producedavicelase and CMCase. On the other hand, *C. thermocellum* NKP-2 could producexylanase,cellobiohydrolase, avicelase and CMCase but not β-xylosidase, α-L-arabinofuranosidase and β-glucosidase under the same condition. Based on the results, combination of both enzymes will provide allrequired enzyme for efficient corn hull drgradation

Table 4.7 Comparison of cellulolytic-xylanolytic enzyme activities from *T.thermosacchrolyticum* NOI-1 and *C. thermocellum* NKP-2

Specific activity (U/mg. Protein	**Stain NOI-1**	**Strain NKP-2**
Xylanase	2.55	2.90
β-Xylosidase	2.00	-
α-L-arabinofuranosidase	2.44	-
β-Glucosidase	0.49	-
Cellobiohydrolase	0.39	0.02
Avicelase	-	0.43*
CMCase	-	0.19

-, Could not detect under the assay condition

* U;One unit of enzyme activity was defined as the amount of enzymesthat liberated 1 μmol of reducing sugars per hour

Plant cell wall contains three major groups of polymer, namely, cellulose, hemicellulose and lignin(Kuhad and Singh, 1993). In term of composition, cellulose and hemicellulose encompass 40-60% of plant cell walls with lignin made up the remainder (Doi and Kosugi, 2004). Generally, cellulose fibers are well protected by a matrix of hemicellulose and lignin and its strong inter-chain hydrogen-bonding network and higher-order structure in plants contribute to biomass recalcitrance. Thus, the synergism of enzymatic action plays an important role in plant cell wall degradation(Himmel et al., 2007). This means that the completes and rapid hydrolysis of biomass will required the combination of cellulolytic-xylanolytic enzymes (Doi and Kosugi, 2004). Enzymes produced by the twobacteria strains in isolated in this study will be useful for the complete hydrolyzed plant cell wall structure. The major structure of plant cell wall might hydrolyzed with enzyme that is produced by *C. thermocellum* NKP-2, while the enzyme that produced from the *T. thermosacchrolyticum*strain NOI-1 could be used to hydrolyzed the minor structure of plant cell wall or oligosaccharide.

The influence of reaction time and ratio on degree of enzyme efficiencywas demonstrated (Koukiekolo et al., 2005; Murashima et al., 2002). Thus, in order to determine the relationship between reaction time and enzymatic efficiency, corn hull was hydrolyzed by a mixture of crude enzyme obtain from *T. thermosaccharolyticum* NOI-1 and *C. thermocellum*NKP-2 equal amounts of xylanase0.05 U/mg of protein for 1 to 5 h at 60°C. Synergistic effect was demonstrated when the enzymes were applied together, since more substrate are converted than when each of the enzymes are used seperately. As shown in Fig. 4.14, the ratio between the synergistic activity and the sum of the individual activities (the degree of synergism) increased from 1.0-fold (1 h), 1.9-fold (3 h) and 1.8-fold (5 h), according to the length of the reaction period. These results suggested that synergistic effects between endocellulase-free xylanolytic enzymes from *T. thermosaccharolyticum* NOI-1 and cellulolytic enzymes from *C. thermocellum*NKP-2 were more effective for substrate hydrolysis.

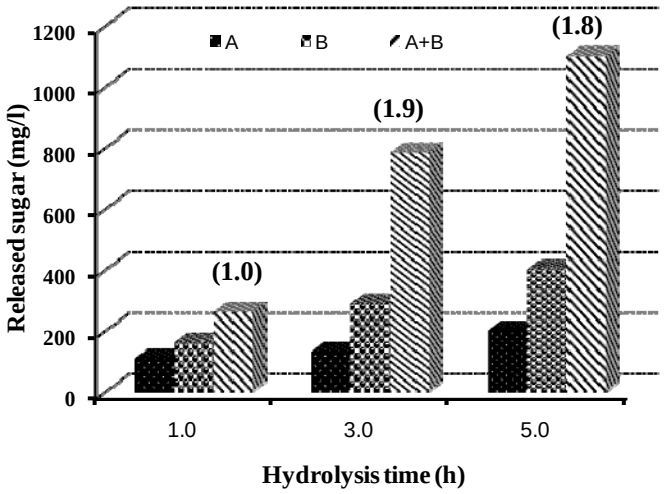


Figure 4.14 Corn hull hydrolysis time of individual and combination of crude enzyme from *T. thermosaccharolyticum* NOI-1(A) and *C. thermocellum*NKP-2 (B). Corn hull was hydrolyzed by 0.1 U/mg of protein concentration of each xylanase at 60 °C for 1, 3 and 5 h. The number in parentheses indicates degree of synergy.

Enzymatic hydrolysis of the native, crystalline cellulose is of great economic importance. It is potentially useful in biotechnological application such as treatment of cellulosic wastes and bioconversion of cellulosic substrates into solvents and fuels. Nevertheless, this approachhas its drawbacks in term of production cost as it involves a set of enzymes for the hydrolysis of heterogeneous structure substrates. Only with the assistance of multiple enzymes with different modes of action acting in a synergistic manner can this unruly substrate be effectively and completely decomposed(Koukiekolo et al., 2005). This can be done by the enzyme mixture obtain from this study, in which the major structure of plant cell wall can be hydrolyzed with cellulolytic enzymes produced by *C. thermocellum*NKP-2 whereas the minor structure of the plant cell wall can be hydrolysed by the endocellulase-free xylanolytic enzymes produced by*T. thermosaccharolyticum* NOI-1. Moreover, the enzymes from strain NOI-1 that is speculated to be mostly xylan degrading enzymes is important in hydrolyzing the xylan (as a major component of hemicelluloses) creating a way for the penetration of the NKP-2 cellulases to degrade the cellulosic component of the plant cell wall.

The initial hydrolysis of corn hull by the combination of crude enzyme from *T. thermosaccharolyticum* NOI-1 and *C. thermocellum*NKP-2 was conducted. Each of

them was adjusted to contain equal amount of xylanase activity (0.1 U/mg of protein) and both were combined in different proportions to maintain a constant sum of individual protein content. Crude enzymes from both strains were also individually tested as controls. The amount of reducing sugars released by the combination of both enzymes was significantly higher than those released by the individual enzymes, indicating a synergistically efficient effect between crude enzyme from *T. thermosaccharolyticum* NOI-1 and *C. thermocellum*NKP-2. The maximum reducing sugars were produced when the ratio of crude enzyme from *T. thermosaccharolyticum* NOI-1 and *C. thermocellum*NKP-2 was set at 2:8 (Table 4.8). These results strongly suggested that the enzymes from *T. thermosaccharolyticum* NOI-1 and *C. thermocellum*NKP-2 degraded corn hull synergistically. However, results were based on the ratio of combined crude enzyme from *T. thermosaccharolyticum* NOI-1 and *C. thermocellum*NKP-2 which contains enzyme such as avicelase, CMCase, xylanase, β-xylosidase, α-L-arabinofuranosidase, β-glucosidase and cellobiohydrolaseon the degradation of corn hull. Different biomass have different composition of lignocellulose, thus may require different ratio of these enzyme mixture. Thus further study on the effect of this enzyme combination on different biomass will be noteworthy.

Table 4.8 Relationships between ratio and synergism ofenzymes from *T. thermosaccharolyticum* NOI-1(A) and from *C. thermocellum*NKP-2 (B)on hydrolysis of corn hull.

Ratios (A:B)	Sum of reducing sugarreleased by the individualenzyme (mg/l)	Reducing sugars released by the combination of both enzymes(mg/l)	Synergism
0:10	289	289	1.0
1:9	419	764	1.8
2:8	419	1,167	2.8
3:7	419	1,032	2.5
4:6	419	823	2.0
5:5	419	783	1.9
6:4	419	656	1.6
7:3	419	630	1.5
8:2	419	399	1.0
9:1	419	239	0.6
10:0	130	130	1.0

The different lignocellulosic material, such as corn hull, corn cob, rice straw, rice husk, cassava peel, cassava pulp and sugarcane bagasses was used for the determination of synergism of the enzyme mixture from both strains. Based on preliminary experimental results about the time course of corn hull degradation (at ratio 1:1, for 3 h.), the amount of reducing sugar released was significantly increased when both crude enzyme from *T. thermosaccharolyticum* NOI-1 and *C. thermocellum*NKP-2 were present compared to when crude enzyme from *C. thermocellum*NKP-2 or crude enzyme from *T. thermosaccharolyticum* NOI-1 were used alone (Table 4.9). The highest synergy degree was observed with corn hull (1.9-fold). The relationships between structural and compositional factors reflect the complexity of lignocellulosic materials, and these variability accounts for the different degradability between different sources of lignocellulosic materials.

Table 4.9 Reducing sugars released from lignocellulosic material hydrolysis by alone and combination of endocellulase-free xylanolytic enzymes and cellulolytic enzymes combination from *T. thermosaccharolyticum* NOI-1(A) and *C. thermocellum*NKP-2 (B).

Material	**Released reducing sugar from hydrolyzed by alone or combination enzyme activities**			**Degree of synergy**
	A	**B**	**A+B**	
Corn hull	130	289	783	1.9
Rice straw	91	31	181	1.5
Cassava peel	223	83	388	1.3
Sugarcane bagasses	70	94	194	1.2
Corn cob	110	283	419	1.1
Rice husk	130	84	227	1.1
Cassava plup	302	130	456	1.1

The lignocellulose component of biomass used in this study was shown in the Table 4.10. Lignocellulosic materials is a heterogeneous complex of carbohydrate polymers (such as cellulose and hemicellulose) and lignin that made up aproximately 55–75% of carbohydrates by dry weight, with many physiochemical, structural and compositional factors hindering the enzymatic digestibility of lignocellulosic materials. The degradation of lignocellulosic materials is dependent on the action of numerous enzymes. For efficient degradation of polysaccharides, synergistic interactions between the enzymes are required for cleaving different linkagesor bond holding each polymers togather.The results suggest that the combination of crude enzyme from *C. thermocellum*NKP-2 and *T. thermosaccharolyticum* NOI-1 is effective in the degradation of lignocellulosic materials.

Table 4.10 Composition of the lignocellulosic materials

Material	Composition (% dry basis)			References
	Cellulose	Hemicellulose	Lignin	
Corn hull	16.0	72.0	12.0	(Sugawara et al., 1994)
Rice straw	35.0	25.0	12.0	(Saha, 2003)
Cassava peel	28.4	29.0	5.0	(Baah et al., 1999)
Sugarcane bagasses	40.0	24.0	25.0	(Saha, 2003)
Corn cob	45.0	35.0	15.0	(Saha, 2003)
Rice husk	35.0	25.0	20.0	(Saha, 2003)
Cassava plup	15.6	4.6	25.0	(Rattanachomsri et al., 2009)

The structure of plant cell walls gives an insight into the mechanism of synergistic action involving xylanolytic and cellulolytic enzymes. In plant cell wall, xylan chains are connected to cellulose microfibril surfaces through hydrogen bonding, generating a cross-linked structure (Carpita, 1996).This structure suggests that degradation of xylan networks between cellulose microfibrils by xylanolytic enzyme will allow cellulolytic enzyme penetrates and attached to the cellulose microfibrils, which is located within the hemicellulosic structure. In addition, further degradation of cellulose microfibrils will allows the xylanolytic enzyme to further access and degrades xylan chains enclosed within the outer polymers. This degradation model could explain the unique features of synergism between xylanolytic and cellulolytic enzymes.

In conclusion, this research has established the potential use enzyme mixture from cellulolytic bacteria coupled with non-cellulolytic bacteria to hydrolyzed lignocellulosic materials. *C. thermocellum* NKP-2 produced main-chain cleaving enzymes whereas *T. thermosaccharolyticum* NOI-1 produces side and/or oligos-chain cleaving enzymes. The synergistic hydrolysis of plant cell wall by several cellulases and xylanase are well known methods (Koukiekolo et al., 2005; Murashima et al., 2003; Olver et al., 2011; Van Dyk and Pletschke, 2012)although, to our knowledge, there have been no reports about the synergistic action between cellulolytic enzymes and xylanolytic enzyme on plant cell wall degradation. In addition, preliminary study of this research shows that cellulolytic enzymes and xylanolytic enzyme exhibits different degree of synergism between different biomass used. The synergistic effect of these enzyme

combination were the highest when used to hydrolyze corn hull, followed by rice straw, cassava peel, sugarcane bagasses, corn cob, rice husk and cassava pulp. Further study may be required to optimize the condition for degradation of different substrate. The mechanism proposed above could be a possible mechanism for the synergistic effect between cellulolytic enzymes and xylanolytic enzymes on plant cell wall degradation.

CHAPTER 5
STUDY ON ENDOCELLULASE-FREE MULTIENZYME COMPLEX LIKE XYLANOSOME

5.1 Introduction

The strain NOI-1 was isolated from a biomass degrading community, NKP and was identified as *T. thermosaccharolyticum.* This bacterium could not grow on cellulose as sole a carbon source but can grow on xylan very well. This strain could produce endocellulase-free-xylanolytic enzyme. Xylan-degrading enzyme have attracted much attention because of their applications in industrial processes such as pre-bleaching of paper pulps, xylooligosaccharide production, modification of cereal-based food stuffs, improving the digestibility of animal feed stocks, and bioconversion of lignocellulosic materials into fermentable products (Beg et al., 2001). In these applications, it is important for the enzyme preparation to be cellulase-free, in order to retain the integrity of the pulps or fibers for cellulose pulps preparation and textile fibers liberation (De Oliveira da Silva and Carmona, 2008). Aerobic and anaerobic mesophilic microorganisms are known to produce xylan-degrading enzymes, however, most studies have focused on aerobic bacteria and fungi (Coughlan and Hazlewood, 1993). However, most industrial applications were carried out at high temperature, thus, thermostable enzymes will be more preferable (Maheshwari and Kamalam, 1985). Recently, thermophilic microorganisms have been recognized for production of thermostable enzymes, with few reports on the production of endocellulase-free thermostable xylanases (Maalej et al., 2009). Multiple forms of cellulases and xylanases are organized into such complexes which are dedicated to hydrolyze lignocellulosic substances effectively because of their ability to bind to insoluble cellulose and/or xylan via cellulose-binding and xylan-binding modules, respectively (Bayer et al., 2004). Thus, the arrangement of cell wall-degrading enzymes into a multienzyme complex provides advantages over single enzyme systems (Shoham et al., 1999). *T. thermosacharolyticum* are thermophilic anaerobic bacteria that are of considerable interest for hydrogen production and thermostable amylolytic enzymes production (Ganghofner et al., 1998; O-Thong et al., 2008). Furthermore, the enzymatic system of *Thermoanaerobacterium* has not been reported in term of their multienzyme complexes.

Therefore, in this study, *T. thermosaccharolyticum* strain NOI-1 and its enzyme was characterized.

5.2 Results and Discussion

5.2.1 Characterization of bacterium, the strain NOI-1

The strain NOI-1 was identified by sequence similarity with the sequences deposited in NCBI databases. Results show that strain NOI-1 shows high similarity (99%) with *T. thermosaccharolyticum* and will be refered as *T. thermosaccharolyticum* NOI-1 here after. The morphology of strain NOI-1 cell was observed by scanning electron microscopy (Fig. 5.1). Results indicates that *T. thermosaccharolyticum* NOI-1 is a rod-shaped (0.2-0.5 µm in width and 2.0-5.0 µm in length), Gram-positive, strictly anaerobic, and endospore-forming bacteria. The adhesions of the cells of strain NOI-1 to insoluble materials was investigated by growing strain NOI-1 in BM medium containing OSX. The result showed that the strain NOI-1 cells could adhere to both insoluble xylan and Avicel at 71.2% and 63.0%, respectively. SEM observation of cell adhesion to insoluble xylan was shown in Fig. 5.1.

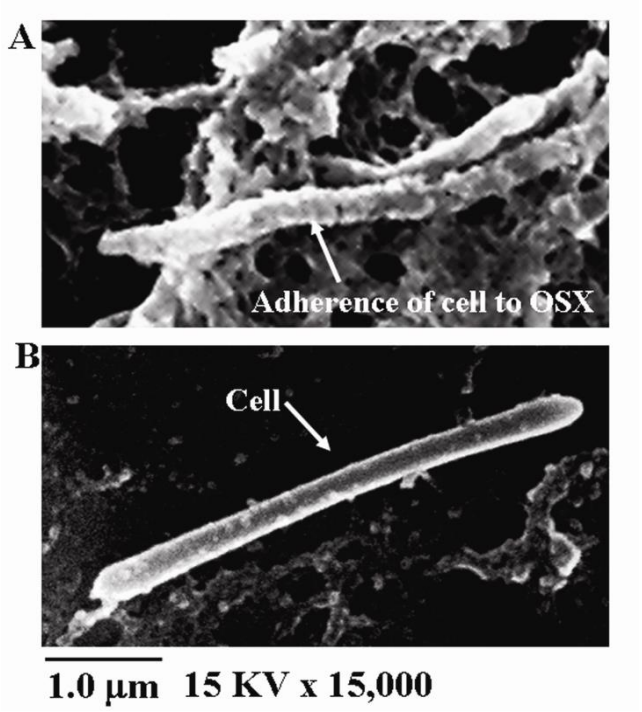


Figure 5.1 SEM of morphology and cell surface structure of *T. thermosaccharolyticum* strain NOI-1 harvested at (A) the exponential growth phase showing the adhesion of cell to OSX and (B) early declining growth phase showing no adhesion of cell to OSX.

SEM observation of cell surfaces of cell harvested during the exponential growth phase (Fig 5.1A) shows that the surfaces of cells were adhered to xylan, which was similar those observe by the multienzyme complex-bound cell surface such as *C. thermocellum* (Bayer and Lamed, 1986), *Fibrobacter succinogens* (Morris, 1988), *Ruminococcus albus* (Rincon et al., 2003), *Paenibacillus curdlanolyticus* (Pason et al., 2006) and *Tepidimicrobium xylanilyticum* (Phitsuwan et al., 2010) to insoluble substances. In contrast, no adhesion of xylan to cell surface was observed on cells harvested at the declining growth phase (Fig. 5.1B). The releasing of multienzyme complex from the cell surface into the culture medium is probably due to the presenceof less amount of xylan, the cells deteriorated and occurrence of some hydrolysis products and/or metabolic products during cultivation (Waeonukul et al., 2009). The strain NOI-1 cell was found to adhere to insoluble xylan and Avicel as the same as multienzyme complex-producing strain cells (Pason et al., 2006). These results suggested that the strain NOI-1 has an essential component responsible for anchoring the cellulose and xylan present on the cell surfaces.

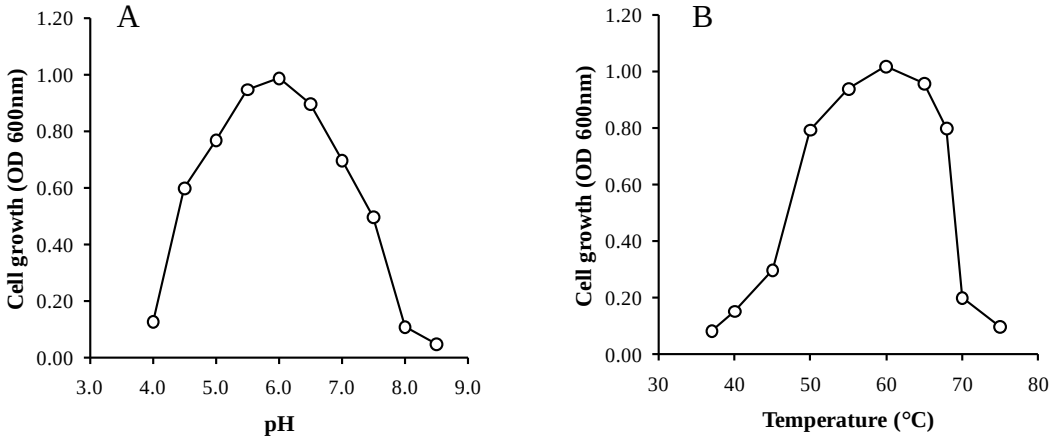


Figure 5.2 Effect of pH (A) and temperature (B) on cell growth using OSX as sole a carbon source.

The effect of temperature and pH on cell growth of the *T. thermosaccharolyticum* NOI-1 was studied. Results indicate that it was able to grow at various temperatures, ranging from 50°C to 68°C and at pHs, ranging from 4.5 to 7.5. Temperature and pH optima of this were observed at 60°C and 6.0, respectively (Fig. 5.2), similar to several other report for the genus *Thermoanaerobacterium* such as *T. thermosaccharolyticum* strain FH1 (Hoster et al., 2001), *T. saccharolyticum* (Lee et al., 1993) and *T. xylanolyticum* (Zhang et al., 2003). Carbon utilization of strain NOI-1 was shown in Table 5.1. Results indicates that the strain NOI-1 can utilized a variety of the carbon sources such as L-arabinose, D-fructose, D-galactose, D-glucose, D-mannose, D-xylose, cellobiose, lactose, maltose, sucrose, trehalose, raffinose, starch, xylan and manitol. However, it could not utilize D-rhamnose, dextran, pectin, cellulose powder and glycerol. According to the carbon source utilization, the strain NOI-1 were found to be different when compared with other strains of *T. thermosaccharolyticum* such as DSM571 (Lee et al., 1993) , FH1 (Hoster et al., 2001) and PSU-2 (O-Thong et al., 2008). The strain PSU-2 can utilize D-rhamnose but the strains NOI-1 and DSM571 can not utilize. For dextran utilization, the strains FH1 and PSU-2 can utilize but the strains DSM571 and NOI-1 not utilize. Furthermore, the strain NOI-1 can utilize manitol but not for the other strains. Thus, it can be concluded on the basis of the 16S rRNA gene analysis and the carbon sources utilization that the strain NOI-1 represents a new strain within the species of *T. thermosaccharolyticum.*

Table 5.1 Utilization of carbon sources for the strain NOI-1 compared with other strains of *T. thermosaccharolyticum*, DSM571 (Lee et al., 1993), FH1 (Hoster et al., 2001), and PSU-2 (O-Thong et al., 2008).

Carbon sources	**DSM571**	**FH1**	**PSU-2**	**NOI-1**
L-Arabinose	+	+	+	+
D-Fructose	+	+	+	+
D-Galactose	+	ND	+	+
D-Glucose	+	+	+	+
D-Mannose	+	ND	+	+
D-Rhamnose	-	ND	+	-
D-Xylose	+	+	+	+
Cellobiose	+	+	+	+
Lactose	+	+	+	+
Maltose	+	+	+	+
Sucrose	+	+	+	+
Trehalose	+	ND	+	+
Raffinose	ND	+	+	+
Dextran	-	+	+	-
Pectin	-	-	-	-
Starch	+	+	+	+
Xylan	+	+	+	+
Cellulose	ND	ND	+	-
Manitol	-	-	-	+
Glycerol	-	-	-	-

+, growth; -, no growth; ND, not determined

The fermentation products were determined at stationary phase (Fig. 5.3). Ethanol, acetic acid and butyric acid were produced as main fermentation products when strain NOI-1 was grown on OSX at 60 °C under anaerobic condition. The result showed that ethanol (58.34 % of total products) was the major product which was converted from OSX, which were similar to the fermentation product obtain through fermentation by other *Thermoanaerobacterium* species such as *T. polysaccharolyticum* and *T. zeae*

(Cann et al., 2001). This shows that ethanol was the major fermentation product, indicating that the strain NOI-1 could convert xylan to ethanol directly.

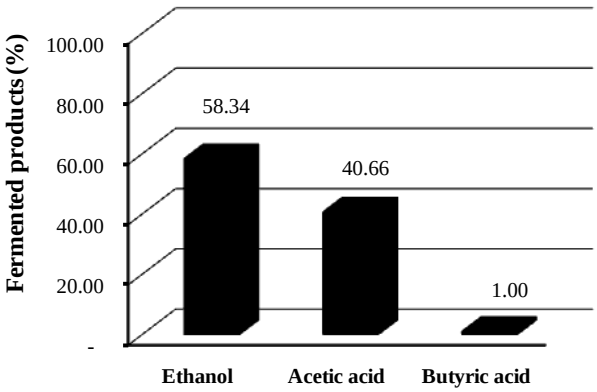


Figure 5.3 Fermented products in culture medium of OSX-grown cells at stationary phase (3 days)

5.2.2 Characterization of crude enzymes from *T. thermosaccharolyticum* strain NOI-1

When *T. thermosaccharolyticum* strain NOI-1 was grown in BM medium containing OSX as a carbon source, it produces endocellulase-free xylanolytic enzymes under anaerobic and thermophilic conditions.Time courses for production of xylanase in pellet and culture supernatant are shown in Fig.5.4. In the pellet, the highest xylanase activity was observed at the exponential growth phase (1 day), and then rapidly decreased, while xylanase activity in the culture supernatant rapidly increased at the exponential growth phase and slightly increases thereafter (2-3 days) and remained nearly constant at the stationary growth phase (3 days).

At the beginning of cultivation, xylanase activity could be detected in the pellet more than culture supernatant. At the stationary growth phase, as the enzyme was released from the pellet into culture supernatant, most of the enzyme was found in culture supernatant. The result was similar to those of bacteria which produced multienzyme complex around the cell surfaces and adhere to these substrates and secreted to culture supernatant at the late stationary growth phase (Doi et al., 2003; Pason et al., 2006; Van Dyk et al., 2009).

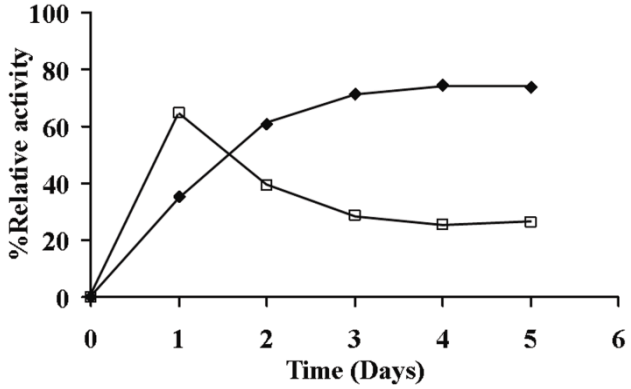


Figure 5.4 Time courses of xylanase production of the strain NOI-1 in pellet (□) and culture supernatant (■).

After the bacterium was cultivated on xylan, at the stationary growth phase (3 days), enzyme activities were determined in the culture supernatant (extracellular protein) and pellet-bound protein. Enzyme activities of the extracellular and pellet-bound protein are shown in Table 5.2. The result showed that xylanase, β-xylosidase, α-L-arabinofuranosidase, acetyl esterase, β-glucosidase and cellobiohydrolase were detected in both fractions whereas avicelase and CMCase were not detected in both fractions. Additionally, the hydrolytic activities on other polysaccharides such as mannan and starch were detected in the extracellular fraction, but only starch hydrolysis were detected in the pellet bound fraction. Thermophilic xylanases have been reported from *Thermotoga maritime*, *Clostridium thermohydrosulfuricu* (Sonne-Hansen et al., 1993), *Clostridium stercorarium* (Sakka et al., 1994), *Cellulomonas fimi* (Nikolova et al., 1997) etc., but there are only a few reports on endocellulase-free thermostable xylanases from bacteria and fungi such as *Thermomyces lanuginosus* (Gomes et al., 1993), *Saccharomonospora viridis* (Roberts et al., 1990), *Bacillus* sp. (Dey et al., 1992) and *Chainia* sp. (Bandivadekar and Deshpande, 1994). The strain NOI-1 exhibited significant amounts of xylanolytic enzymes production at 60°C under anaerobic conditions and this enzyme was endocellulase-free xylanolytic enzymes. The endocellulase-free xylanolytic enzymes produced from this strain may potentially be applied in enzymatic hydrolysis of xylan of lignocellulosic materials, kraft pulps for paper industries, textile processes and the food and feed industries (Gomes, 1994). Furthermore, the strain NOI-1 exhibited significant amounts of xylanolytic enzymes

production at 60°C under anaerobic conditions with lack of CMCase and avicelase activities. However, most of enzyme activities were detected in the extracellular fraction at strationary phase. Thus, the extracellular protein was used for further study.

Table 5.2 Enzymatic activities of culture supernatant (extracellular protein) and pellet-bound protein from *T. thermosacharolyticum* strain NOI-1

Enzymes	**Specific activities (U/mg Protein)**	
	Extracellular	**Pellet-bound**
Xylanase	2.55	0.69
β-Xylosidase	2.00	0.67
α-L-arabinofuranosidase	2.44	2.17
Acetyl esterase	0.01	0.01
β-Glucosidase	0.49	0.41
Cellobiohydrolase	0.39	0.36
Avicelase	ND	ND
CMCase	ND	ND
Amylase	0.90	0.5
Mannanase	0.21	ND

ND, were not be able to detect under the assay condition

Binding ability of crude enzyme from extracellular protein obtained at stationary phase was explained. Since the strain NOI-1 cells could adhere to insoluble polysaccharides, which were similar to those of the multienzyme complex-bound cells, the ability of the crude enzyme to bind to insoluble polysaccharides was investigated. The results showed that crude enzyme was able to bind to OSX and Avicel at 40.7% and 35.2%, respectively, and exhibited stronger affinity to OSX than to Avicel (Fig. 5.5). The binding ability of enzymes to insoluble substrates plays an important role in the efficiency of enzymatic hydrolysis of the insoluble substrates (Bayer et al., 2004). Many investigators have reported that the multienzyme complex contained catalytic and non-catalytic domains such as cellulose-binding domains (CBDs) and/or xylan-binding domains (XBDs) (Kulkarni et al., 1999). This may indicate that the multienzyme complex produced by the strain NOI-1 might have XBD and CBD.

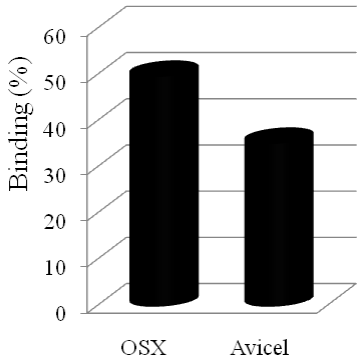


Figure 5.5 Binding abilities of crude enzyme from the strain NOI-1at stationary phase to insoluble polysaccharide

The extracellular and pellet-bound proteins were analyzed by Native-zymograms, and SDS-zymograms, using soluble xylan as substrate to determine the activity of xylanase subunits. Native-zymograms showed one band of high molecular mass (Fig 5.6, 1A and 1B) for both pellet bound and extracellular proteins whereas SDS-zymograms, showed 6 subunits of xylanase activity in the pellet bound protein (Fig 5.6, 2A) and 9 subunits of xylanase activity in extracellularprotein(Fig 5.6, 2B). Among them, 6 proteins with molecular-mass of 115, 100, 91, 84, 74 and 59 kDa showed xylanase activity in both the pellet bound and extracellular proteins. These results indicated that many proteins possessing xylanase activities were assembled into a multienzyme complex (xylanosome) in the extracellular and pellet bound fraction. At stationary growth phase of the strain NOI-1, xylanase subunit in extracellular protein exhibits higher activity than those of pellet bound protein which matched the result to determination of xylanase activity so showed higher xylanase activity in extracellular protein than pellet bound protein (Table 5.2). Thus, extracellular protein was used future study.

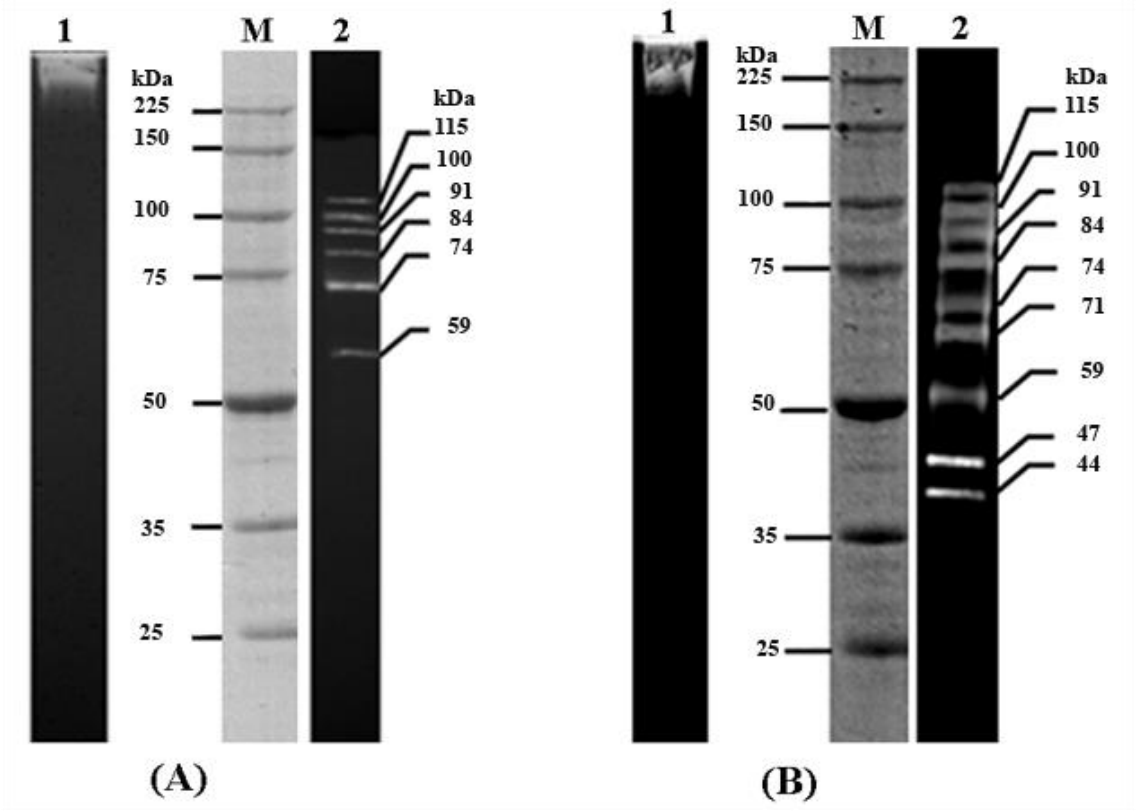


Figure 5.6 Patterns of xylanase activities by zymogarm gel electrophoresis from (A) pellet-bound protein and (B) extracellular protein. Lane 1, Native-zymogram; Lane 2, zymogram analysis of xylanase activity; M, Molecular mass in kDa

Crude enzyme from extracellular protein obtained from stationary growth phase of the strain NOI-1 was studied for the effect of pH and temperature on xylanase activity and stability. Xylanase activity was tested from pH 4.0 to 12.0 (Fig. 5.7A) and the optimum pH was found to be 6.0. However, a relatively broad pH plateau was observed between pH 4.0 to 10.0, exhibiting a relative activity close to the optimal value. The pH stability of the xylanase of the strain NOI-1 was assayed from pH 4.0 to12.0 (Fig. 5.7A). Results concluded that it has high stability (above 80%) in the pH range of 5.0 to 10.0, and relatively low stability (below 80%) below pH 4.0 and above pH 11.0. This means that this xylanase exhibits a broad pH stability, in agreement with the general observation of *Bacillus* spp. and other bacteria xylanases which are also active over a broad pH range (Kulkarni et al., 1999).

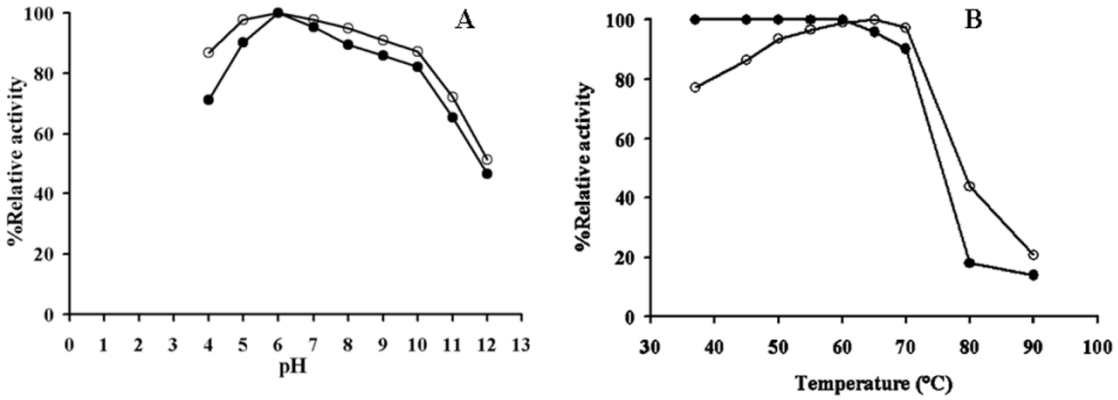


Figure 5.7 Effect of pH on activity (○) and stability (●) of xylanase (A) and effect of temperature on activity (○) and stability (●) of xylanase (B)

In order to determine the effect of temperature on enzyme stability and optimum temperature for this enzyme, enzymatic assay was performed at the temperature range of 37-90 °C. Xylanase from the strain NOI-1 exhibited its optimum activity at 60 °C, and was stable up to 70 °C for 60 min (Fig. 5.7B). Optimal condition (pH and temperature) as well as the stability of enzyme represent an important properties for industrial enzymes (Gattinger et al., 1990). Xylanases with high thermostability and pH are required in the pulp and paper industry (Karlsson et al., 1998). Since the xylanase produces the strain NOI-1 could be active in the broad ranges of pH and temperature, it would be suitable for application in paper industry.

Xylan-hydrolysis efficiency of endocellulase-free xylanolytic enzyme from the strain NOI-1 was explained. The crude enzyme from *T. thermosaccharolyticum* NOI-1 was incubated with 1% (dry weight) xylans (OSX, BWX and LWX) and the amount of reducing sugar released was determined at 0, 5, 10, 15, 20, 45 and 60 min. Results shows that hydrolysis of xylan increase rapidly during the first 10 min of fermentation as indicated by the rapid increase in reducing sugar amount in the fermentation medium (Fig. 5.8). In addition, the crude enzyme from *T. thermosaccharolyticum* NOI-1 was able to hydrolyze LWX better BWX and OSX. These results indicated that this crude enzyme is more efficient in degradation of hard wood (LWX and BWX) than soft wood (OSX) because softwood xylans are shorter and less branched (degree of polymerization

between 70 and 130) than hardwood xylans. Furthermore, the content, position, and structure of xylan are one of the factors affecting the hydrolysis resistance of biomass (Liab et al., 2000).

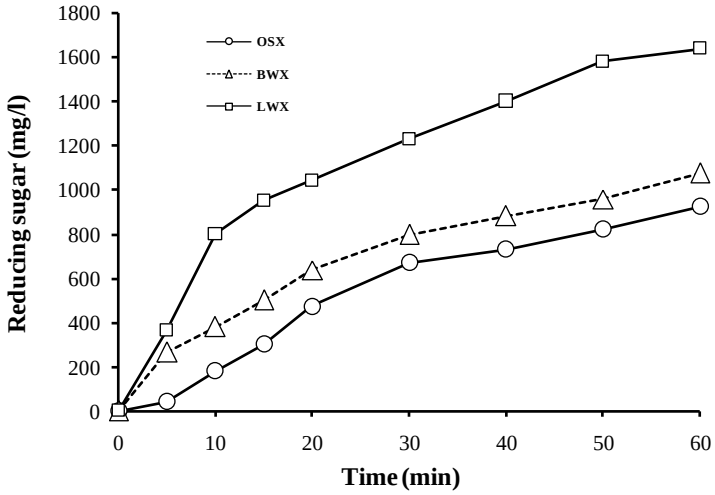


Figure 5.8 Time course of hydrolysis of pure xylans (OSX, BWX and LWX) by crude enzyme (1.0 U xylanase) from *T. thermosaccharolyticum* NOI-1(A); OSX, (o); LWX, (□); BWX, (Δ)

The hydrolysis of various xylan substrates by crude enzyme from *T. thermosaccharolyticum* NOI-1 was examined by analysis of products using TLC (Fig. 5.5B). The hydrolysis products of OSX, BWX and LWX were xylose and a series of XOs larger than xylobiose. The results showed that xylanolytic enzymes from the strain NOI-1 may be used in producing XOs. XOs showed a remarkable potential for utilization in many fields, including pharmaceuticals, feed formulations, and agricultural application (Vazquez et al., 2001).

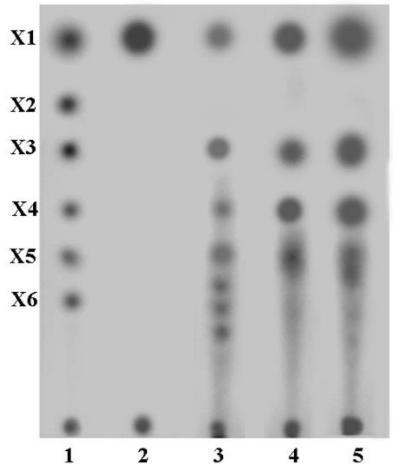


Figure 5.9 Thin layer chromatography of the hydrolysis products of xylan by crudexylanase (1.0 U) for 60 min. Lane 1, standard xylooligosaccharides (X1: xylose, X2: xylobiose, X3: xylotriose, X4: xylotetraose, X5: xylopentaose and X6: xylohexaose); Lane 2, xylose; Lane 3-5, the hydrolysis products from OSX, BWX and LWX, respectively.

5.2.3 Isolation, purification and characterization of endocellulase-free multienzyme complex from *T. thermosaccharolyticum* strain NOI-1

In previously studied, a new thermophilic anaerobic endocellulase-free xylanolytic enzymes producing bacterium, *T. thermosaccharolyticum* strain NOI-1 was isolated. The properties of cell and protein binding ability, and subunits of xylanase in extracellular protein were determined and explained. Results indicates that this isolated enzyme possess a multienzyme complex properties. Thus, it is to our advantage to isolate, purify and characterize this multienzyme complex from crude enzyme in the extracellular protein fraction that produced by *T. thermosaccharolyticum* strain NOI-1.

Isolation and purification of this multienzyme complex from crude enzyme in extracellular protein was performed by using affinity purification on cellulose (Avicel) and gel filtration chromatography. Although, crude enzyme from the strain NOI-1 had ability to bind to OSX more than Avicel, Avicel was selected to purify the multienzyme complex for affinity column because Avicel gave a flow rate higher than xylan. The cellulose-binding proteins (mainly multienzyme complex) were collected, which was then applied onto a Sephacryl S-300 column. Only one peak was observed and the molecular mass was estimated to be 1,200 kDa (Fig. 5.10).This purified, high molecular

mass protein shows to exhibits high xylanase activity. Therefore, the property of this protein (namely isolated multienzyme complex) was characterized.

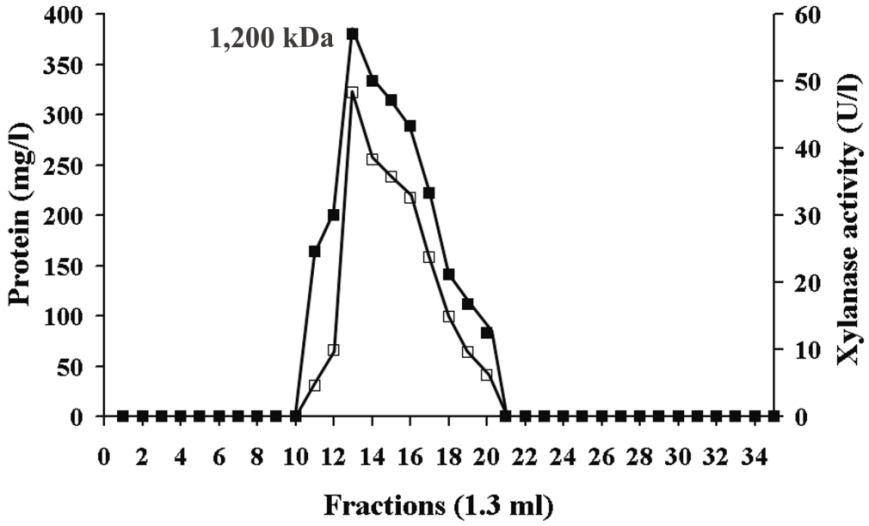


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Figure 5.10 Gel filtration chromatography on Sephacryl S-300 column of the isolatedmultienzyme complex of thestrain NOI-1, Symbols: ■, protein (mg/l); □, xylanase activity (U/l).

The isolated multienzyme complex from the strain NOI-1 was determined enzymatic activities and then compared with crude enzyme from NOI-1 and *Bacillus firmus* K-1 (Table 5.3). Generally, the isolated enzyme displayed an endocellulase-free multienzyme complex property. Compared to the crude enzyme which consist of 8 enzymatic activities, isolated the multienzyme complex only shows to have 5 enzymatic activities, with no acetyl esterase, amylase and mannanase activity detected.

Table 5.3 Enzymatic activities of crude enzyme and isolated multienzyme complex from the strain NOI-1 and crude enzyme from *Bacillus firmus* K-1

Enzymes	Specific Activity (U/mg Protein)		
	Crude enzymeof the strain NOI-1	**Isolated multienzyme complexof the strain NOI-1**	***Crude enzymeof the strain K-1**
Xylanase	2.55	9.07	4.80
β-Xylosidase	2.00	9.35	0.21
α-L-Arabinofuranosidase	2.44	11.26	0.15
Acetyl esterase	0.01	-	0.24
β-Glucosidase	0.49	0.24	-
Cellobiohydrolase	0.39	0.05	ND
Avicelase	-	-	ND
CMCase	-	-	-
Amylase	0.90	-	ND
Mannanase	0.21	-	ND

-, not able to detect under the assay condition

ND, not detected

*, (Ratanakhanokchai et al., 1999)

The hydrolysis efficiency of pure xylan (OSX) and biomass (corn hull) with three sources of xylanolytic enzyme was determined and compared. The three sources of enzyme used are: (i) isolated endocellulase-free multienzyme complex of strain NOI-1, (ii) crude enzyme of strain NOI-1, and (iii) endocellulase-free xylanolytic enzyme of *B. firmus* stain K-1 (non multienzyme complex) (Ratanakhanokchai et al., 1999). Results of the hydrolysis efficiency based on the amount of reducing sugar released were shown in Fig. 5.11. It can be seen that the isolated multienzyme complex and the crude enzyme of the strain NOI-1 hydrolyzed OSX more efficiently than enzymes from the strain K-1. In addition, the isolated multienzyme complex and crude enzyme of strain NOI-1 shows a rapid hydrolysis of OSX within the first 5 min of incubation, which slightly increased and then remained nearly constant thereafter. Contrariwise, the amount of reducing sugars released by incubation with enzymes of the strain K-1 showed slight increased along the 60 min of incubation and remained relatively constant thereafter. These might be due to the stability of the enzymes and/or end products inhibition (Bachmann and

McCarthy, 1991). Additionally, the enzyme hydrolyzed not only pure xylan but also xylan of agricultural residue such as corn hull even though without any pretreatment. The result showed that the amount of reducing sugars released from corn hull after enzyme treatment with crude enzyme, isolated multienzyme complex from the strain NOI-1, and the crude enzyme from the strainK-1 were 675.2, 1,475.1, and 250.2 μg/U enzyme, respectively. This means that the multienzyme complex of the strain NOI-1 hydrolyzed xylans more efficiently than the other two enzyme investigated.This might be due to the presence of XBD, CBD, as well as the enzymes exist as a complex of enzyme that acted synergistically during the hydrolysis of xylans.

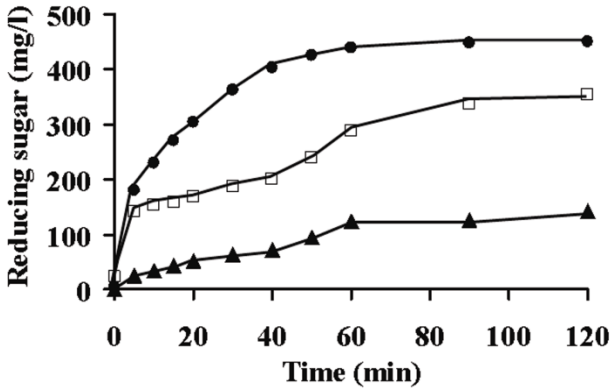

Figure 5.11 Time courses of the hydrolysis of OSX by crude enzyme (□) and the isolated multienzyme complex (●) from the strain NOI-1 and crude enzyme from *B. firmus* K-1 (▲)

The effect of pH and temperature on enzyme activity and stability (isolated multienzyme complex) was determined. The effect of pH on xylanase activity and stability of the isolated multienzyme complex from the strain NOI-1 was studied in the range of pH 4.0 to 12.0 (Fig. 5.12 A). Optimum pH was found to be 6.0 with a broad pH range between 4.0 and 10.0, exhibiting more than 85% relative activity. The pH stability of the xylanase showed high stability (above 80%) over a wide range from pH 5.0 to 10.0. Thus, isolated multienzyme complex from the strain NOI-1 could be active and stable in broad ranges of pH. The isolated multienzyme complex from the strain NOI-1 shows maximum enzyme activity at 60°C and a marked decrease in enzyme activity above 70°C for 60 min (Fig. 5.12 B). Therefore, crude enzyme from the strain NOI-1

can be applied in various industries such as pulp and paper,food/feed and enzymatic hydrolysis of xylan in lignocellulosic materials (De Oliveira da Silva and Carmona, 2008).

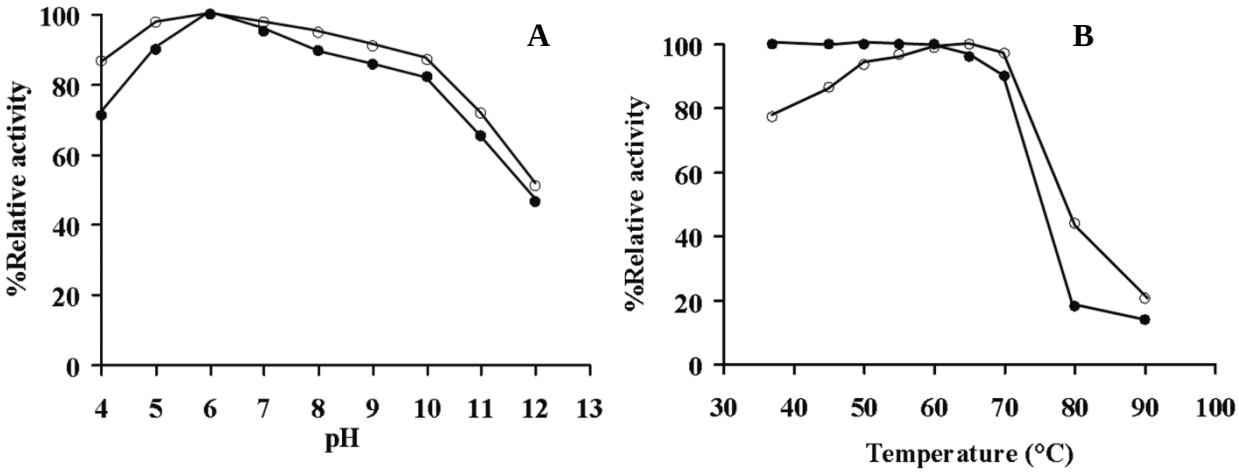

Figure 5.12 Effect of pH (A) and temperature (B) on the activity (○) and stability (●) of the isolated multienzyme complex (xylanase)

Pattern of the components of isolated multienzyme complex from the strain NOI-1 was investigated by gel-electrophoresis and zymograms and compared with those of crude enzyme. The crude enzyme exhibited 3 proteins bands on native-PAGE (Fig. 5. 13, lane 1), whereas the isolated multienzyme complex showed a single band on both native-PAGE (Fig. 5.13, lane 2) and native zymogram for xylanase activity (Fig. 5.13, lane 3), but no CMCase activity on the CMC-zymogram (Fig. 5.13, lane 4). At least 21 bands were observed on the SDS-PAGE of the isolated multienzyme complex (Fig. 5.13, lane 5) with a molecular mass range of 21 to 150 kDa. SDS-zymogram revealed 5 proteins bands (82, 104, 120, 136 and 150 kDa) having xylanase activity (Fig. 5.13, lane 6) but no CMCase activity on the CMC-zymogram (Fig. 5.13, lane 7). Moreover, the isolated multienzyme complex contained activities of β-xylosidase, α-L-arabinofuranosidase, β-glucosidase, and cellobiohydrolase (Table 5.3). Thus, the multienzyme complex of the strain NOI-1 contains xylanases associated with these enzymes. The multienzyme complex with lack of CMCase and avicelase activities has not been reported before

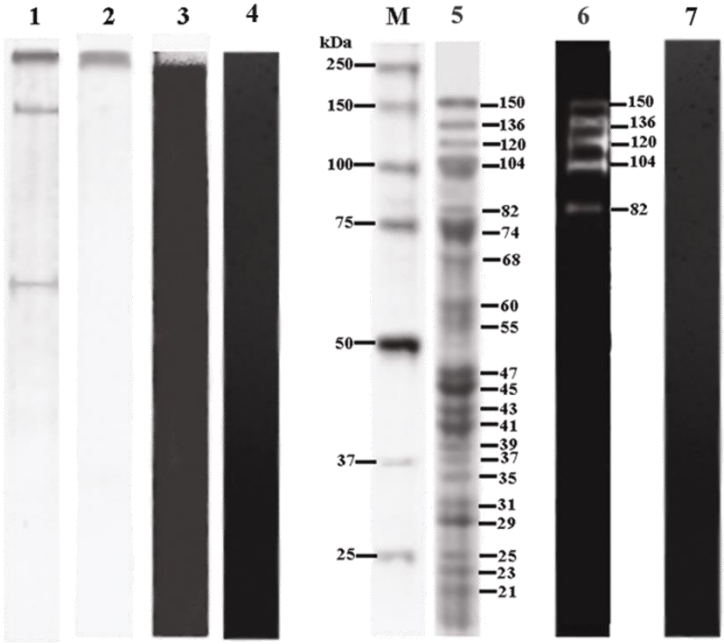

Figure 5.13 Patterns of proteins and xylanase and CMCase activities by gel electrophoresis. Lane 1, native-PAGE of crude enzyme; lanes 2-7, native–PAGE, native zymogram for xylanase and CMCase activities, SDS–PAGE, SDS-zymograms for xylanase and CMCase activities of the isolated multienzyme complex of the strain NOI-1, respectively. Molecular mass in kilodaltons is indicated on lane M.

In conclusion, the strain NOI-1 was a thermophilic, anaerobic bacterium, identified as *T. thermosaccharolyticum* strain NOI-1. The endocellulase-free multienzyme complex of this bacterium showed a molecular mass of 1,200 kDa and consists of xylanases, β-xylosidase, α-L-arabinofuranosidase, β-glucosidase and cellobiohydrolase. Moreover, it was found to hydrolyze xylan and corn hull efficiently. This is the first report of an endocellulase-free multienzyme complex produced by *Thermoanaerobacterium* species. This enzyme indicated the possible application of such enzymatic approach in some industrial processes, which require enzyme to retain its activity at high temperature, wide range of pH and with lack of endocellulase activity. Multienzyme complex, cellulosome was initially identified and described in an anaerobic, thermophilic, cellulolytic bacterium, *C. thermocellum*. The major enzyme in the cellulosome was cellulase (Lamed et al., 1983). On the other hand, xylanosomes, having a structure analogous to the cellulosomes, are discrete, multifunctional, and multienzyme

complexes that can be found in several microorganisms. These complexes play an important role in the degradation of hemicellulases (Sunna and Antranikian, 1997). Thus, the endocellulase-free multienzyme complex produced from *T. thermosaccharolyticum* strain NOI-1 like xylanosome.

CHAPTER 6
CONCLUSIONS AND SUGGESTIONS

The coexistence of two biomass degrading bacterial (NKP) with the ability to degrade corn hull efficiently was enriched. The NKP was an efficient producer of cellulolytic-xylanolytic enzymes such as avicelase, CMCase, cellobiohydrolase, β-glucosidase, xylanase, β-xylosidase, α-L-arabinofuranosidase and acetyl esterase. Characterization of the crude enzyme shows that this multienzyme complex composed of at least 7 CMCases and 11 xylanases that they are all thermostable enzymes. These data highlighted the potential of NKP as a source for cellulolytic-xylanolytic enzymes, providing insight for further studies of the use of these microorganisms for production of enzymes required for biomass conversion at high temperature. Two bacteria strain, NKP-2 and NOI-1 were isolated from NKP were characterize. Strain NKP-2 was found to be a cellulolytic bacterium which was able to grow on xylan as a carbon source, but at a slower pace (2-5 weeks) whereas the strain NOI-1 was a non-cellulolytic bacterium which could not grow on cellulose as a carbon source. Both strain were identified by sequencing their 16s rRNA, and concluded that the strain NKP-2 and NOI-1 showed the highest similarity *C. thermocellum* and *T. thermosaccharolyticum*, respectively and are denoted as *C. thermocellum* NKP-2 and *T. thermosaccharolyticum* NOI-1. Validation of purity each colony were performed by using specific primer for each strain (Cth-P and Cth-E primer for *C. thermocellum* and Thm V1 and Thm R1 primer for *T. thermosaccharolyticum*). Reports in the literature generally indicates that coexistence of cellullolytic and non-cellulolytic (Pohlschroeder et al., 1994; Valaskova et al., 2009) are difficult to be separated. Thus, the isolation of single colony could carry out several times in order to obtain a pure culture.

In addition, we investigated the study of effect of individually-cultured and co-culturing both *C. thermocellum* NKP-2 and *T. thermosaccharolyticum* NOI-1 to explain the synergism of enzymatic systems that were produced from both strains using corn hulls as a sole carbon source. This is the first report of co-culturing of *C. thermocellum* and *T. thermosaccharolyticum* on corn hulls in terms of enzymatic symbiosis. Thus, it can be conclude that mutualism exist between strain NKP-2 and strain NOI-1 in corn hull fermentation, in which the synergistic cellulolytic and xylanolytic enzymes are

produced from both strains. Several reports have described this interesting relationship of non-cellulolytic and cellulolytic anaerobic bacteria (Khan and Murray, 1982; Mori, 1990; Murray, 1986). Therefore, the symbiotic mechanism between strains NKP-2 and NOI-1 was studied by comparing the enzyme system when it was used separately and combined to promote hydrolysis of biomass. Xylanolytic enzymes can hydrolyze arabinoxylan which is the outer structure of biomass which will expose the cellulose to be hydrolyzed by cellulolytic enzymes. Results of these studies demonstrated that the co-culture of strain NKP-2 and strain NOI-1 can be applied in the biodegradation of biomass to a value added products.

The combination of the multienzyme complex from *C. thermocellum* NKP-2 and the endocellulase-free xylanolytic enzymes from *T. thermosaccharolyticum* NOI-1 for hydrolysis of plant cell wall polysaccharides was studied. *C. thermocellum* NKP-2 produced main-chain cleavaging enzymes whereas *T. thermosaccharolyticum* NOI-1 produced side and/or oligos-chain cleavaging enzymes. The synergistic hydrolysis of plant cell wall by several cellulases and xylanase are well a known phenomenon (Koukiekolo et al., 2005; Murashima et al., 2003; Olver et al., 2011; Van Dyk and Pletschke, 2012), although to our knowledge, there is no reports about the synergism between cellulolytic enzymes and endocellulase-free xylanolytic enzyme on plant cell wall degradation. Moreover, the synergism of cellulolytic enzymes and endocellulase-free xylanolytic enzymes for biomass hydrolysis differs between different types of biomass. In this study, the isolated enzyme were best used for the hydrolysis of corn hull hydrolysis, followed by rice straw, cassava peel, sugarcane bagasses, corn cob, rice husk and cassava pulp, respectively.

However, a multicomponent enzyme complex, the cellulosome, was eventually identified and described in the anaerobic, thermophilic, cellulolytic bacterium, *Clostridium thermocellum* (Lamed et al., 1983). To our understanding, the multienzyme cellulosome complex from this bacterium was subsequently augmented by additional discoveries of cellulosomes, first in other clostridial species. Therefore, it is to our interest to study the multienzyme complex from *T. thermosaccharolyticum* NOI-1.

T. thermosaccharolyticum NOI-1, a new thermophilic, anaerobic, endocellulase-free xylanolytic enzymes producing bacterium, was initially isolated from soil in Thailand. The optimal temperature and pH for growth were found to be at 60°C and pH 6.0. This strain has its potential in converting xylan to ethanol and acids. During growth on OSX, it produces endocellulase-free xylanolytic enzymes containing xylanase, β-xylosidase, α-L-arabinofuranosidase, acetyl esterase, cellobiohydrolase and β-glucosidase with no production of avicelase and CMCase. The crude enzyme is active over a broad ranges of pH and temperature, with optimum temperature of 60°C and pH 6.0. These enzymes produced were able to hydrolyzed xylan material to XOs, as well as ethanol and acetic acid.

Further studies of the multienzyme complex produced by *T. thermosaccharolyticum* strain NOI-1 shows that it consistes endocellulase-free multienzyme complex of 1,200 kDa, with xylanases, β-xylosidase, α-L-arabinofuranosidase, β-glucosidase and cellobiohydrolase activity. This enzyme shows similarity to xylanosome that was found to hydrolyze xylan and corn hull efficiently. This is the first report of an endocellulase-free multienzyme complex produced by *Thermoanaerobacterium* species. This enzyme demonstrated a potential application in some industrial processes, which require enzyme activity in to be stable at high temperature and wide range of pH, with minute or no endocellulase activity.

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Appendix A

```
   1  gccactgcgg cgcctaccat gcaagtcgag cgaagggata ctacggtacg aacttagtcg
  61  gcggacgggt gagtaacgcg tggacaatct acccctgtaga ctgggataac acctcgaaag
 121  gggtgctaat accggataat gtcaagggca tccttttcga agaaaggagg gaatcctata
 181  gtggattccc ctccctttac tattggtgag ggtaacgccc accaggcgac tatgggtacc
 241  cggcctgaga gggtgaacgg ccacctgaac tgaacacggt ccaactccta cgggaggcag
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 421  acgtgccaca ccgcggtaat actagggggc gagcttgtcc gattactgtc agcagcgcgg
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 541  gatgtgaaaa acctggtcaa ccgagggtat gcatctgaaa ctaaatagct gagtcaggag
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 661  gcgaaagcgg ctctctggac ttgaactgac gctgaggtgc gaaagcgtgg ggagcaaaca
 721  ggattagata ccctggtagt ccacgccgta aacgatggat actaggtgtg ggttagtata
 781  atccgtgccg gagttaacgc aataagtatc ccgcctgggg agtacggccg caaggttgaa
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 901  acgcgaagaa ccttaccagg gcttgacatc cacagaatcg agtagaaata cttgagtgcc
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1081  actctaccga gactgccgtg gataacacgg aggaaggcgg ggatgacgtc aaatcatcat
1141  gccctttatg ccctgggcta cacacgtgct acaatggcct gaacagaggg cagcgaagga
1201  gcgatccgga gcgaatccca gaaaacaggt cccagttcag attgcaggct gcaacccgcc
1261  tgcatgaaga cggagrrgct agtaatcgcg gatcagcatg ccgcggtgaa tacgttcccg
1321  ggccttgtac acaccgcccg tcacaccacg agagtttaca acacccgaag tcagtgacct
1381  aaccgaaagg gaggaggccg aaggtggaaa atg
```

Figure A.1 The 16S rDNA sequence of *Thermoanaerobacterium thermosaccharolyticum* strain NOI-1

```
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  61 atatcctagc ggcggacggg tgagtaacgc gtgggtaacc tacctcatac agggggataa
 121 cacagggaaa cctgtgctaa taccgcataa tataacgggg cggcatcgtc ctgttatcaa
 181 aggagaaatc cggtatgaga tgggcccgcg tccgattagc tggttggtga ggtaacggct
 241 caccaaggcg acgatcggta gccgaactga gaggttggtc ggccacattg ggactgagac
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 541 atgcaagtca gatgtgaaat tccggggctt aaccccggcg ctgcatctga aactgtatct
 601 cttgagtgct ggagaggaaa gcggaattcc tagtgtagcg gtgaaatgcg tagatattag
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 721 gtggggagca aacaggatta gataccctgg tagtccacgc cgtaaacgat ggatactagg
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 841 agtacggccg caaggttgaa actcaaagga attgacgggg gcccgcacaa gcagtggagt
 901 atgtggttta attcgaagca acgcgaagaa ccttaccagg gcttgacatc cctctgacag
 961 ctctagagat agggcttccc ttcggggcag aggagacagg tggtgcatgg ttgtcgtcag
1021 ctcgtgtcgt gagatgttgg gttaagtccc gcaacgagcg caacccttgt cgttagttgc
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1141 acgacgtcaa atcatcatgc cccttatgtc ctgggctaca cacgtactac aatggctgct
1201 acaaagggaa gcgataccgc gaggtggagc aaatccccaa aagcagtccc agttcggatt
1261 gcaggctgaa actcgcctgc atgaagtcgg aattgctagt aatggcaggt cagcatactg
1321 ccgtgaatac gttcccgggc cttgtacaca ccgcccgtca caccatgaga gtctgcaaca
1381 cccgaagtca gtagtctagc cgcagaggag agcgcgccga aggtggggca ag
```

Figure A.2 The 16S rDNA sequence of *Clostridium thermocellum* strain NKP-2

Appendix B

Determination of protein by Lowry method (Lowry, 1951)

1. A sample 0.2 ml and add 1.0 ml of reagent C.
2. Mix and stand for 10 min.
3. Add 0.1 ml of Folin-ciocalteu reagent.
4. Mix and stand for 30 min.
5. Measure absorbance at 660 nm against a blank of 0.2 ml of distilleded water instead of sample processed through steps 1-4.
6. Bovine serum albumin solution was prepared to final concentration at 50, 100, 150, 200, 300 and 400 mg/ml for standard curve.

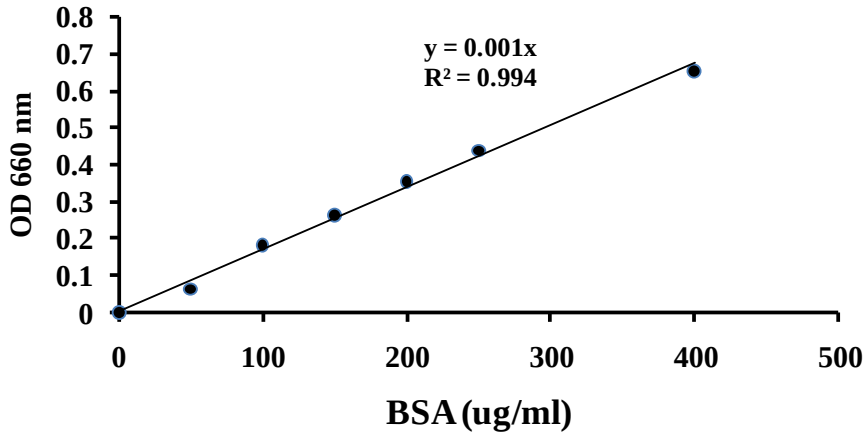

Figure B.1 Standard curve of bovine serium albumin (BSA) solution

Determination of reducing sugars by Somogyi-Nelson method (Nelson, 1944)

1. Take 0.6 ml sample and add 1 ml of Somogyi reagent.
2. Reaction mixture tube was boiled for 15 min.
3. Stand until cool, add 1 ml of Nelson reagent and mix well.
4. Add 2 ml of distilled water, mix and centrifuge at 10,000 rpm for 5 min.
5. Measure absorbance at 520 nm of supernatant against a blank of 0.6 ml of distilled water of instead sample processed through steps 1-4.

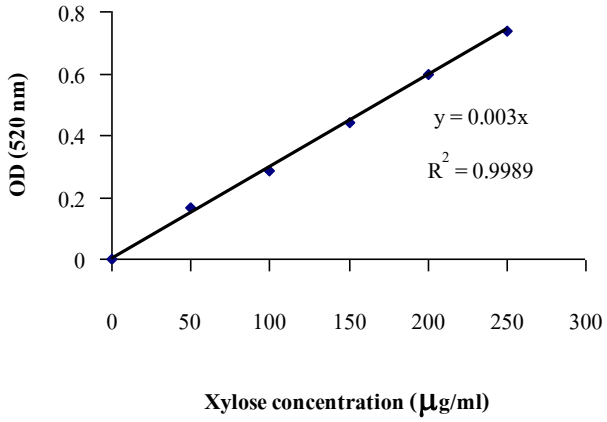

Figure B.2 Standard curve of xylose solution

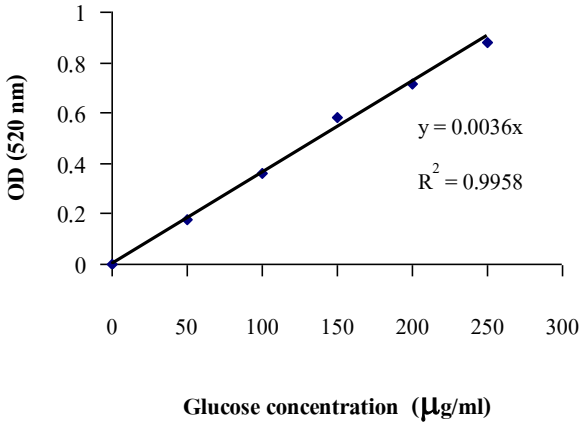

Figure B.3 Standard curve of glucose solution

Preparation of *p*-nitrophenol standard curve

1. The *p*-nitrophenol solution was prepared to final concentration at 5, 10, 15, 20, 25 and 50 μg/ml for standard curve.
2. Each concentration of *p*-nitrophenol solution (1.1 ml) was added into test tube and stopped reaction with 2 ml of 0.4 M Na_2CO_3.
3. Measured absorbance at 405 nm against a blank of 1.1 ml of distilleded water instead of sample processed through steps 1-2.

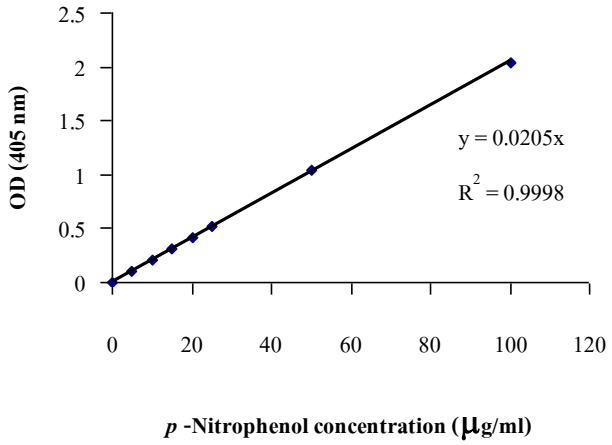

Figure B.4 Standard curve of *p*-nitrophenol solution

Electrophoresis method (Laemmli, 1970)

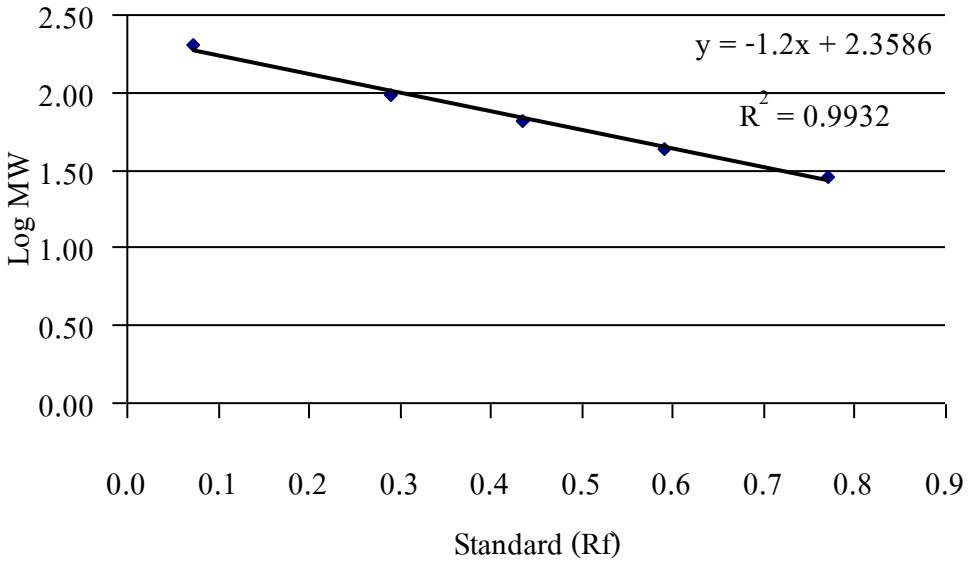

Figure B.5 Standard protein pattern of 10% SDS-PAGE

Zymogram (Ratanakhanokchai, 1999)

1-4. Prepare gel like SDS-PAGE method but add 0.2% substrate (soluble xylan or CMC) in separating gel.

5. Applied samples which want to know their purity and find theirs molecular. After boiled with sample buffer at 100 °C, 4 min and cooled then fill them in the gap above stacking gel while applied standard protein at the same time.

6-9 Do as SDS-PAGE method.

10. Wash SDS out of gel with 25% isopropanol 1-2 times.

11. Continue washing with sodium phosphate buffer 4 times, 30 min at 4 °C

12. Submerge gel in sodium phosphate buffer and incubated 50 °C, 1 hr.

13. Adsorb color by submerge gel in congo red, 15 min.

14. Wash excess color with 1.0 M NaCl.

15. Stop reaction by add acetic acid.

Sephacryl S-300 Gel filtration

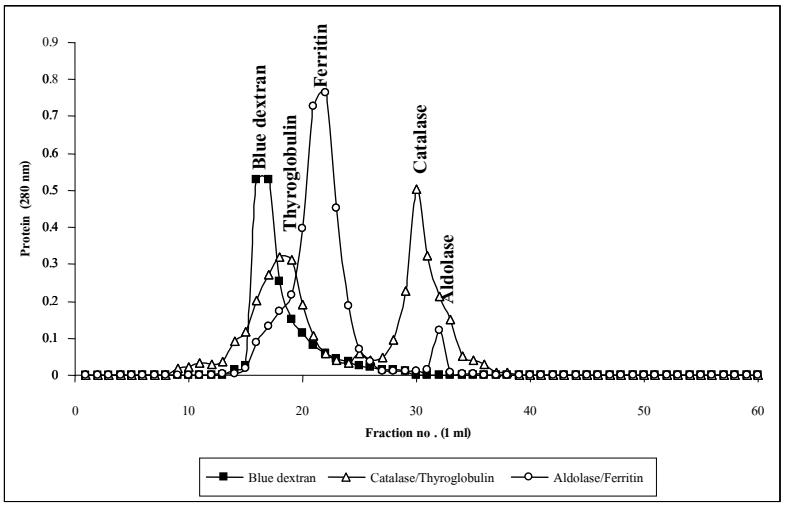

Figure B.6 Standard protein pattern of gel filtration (Sephacryl S-300); 0.67×50.0 cm^2, flow rate 0.3 ml/min., eluted with 50 mM phospate buffer pH 6.0

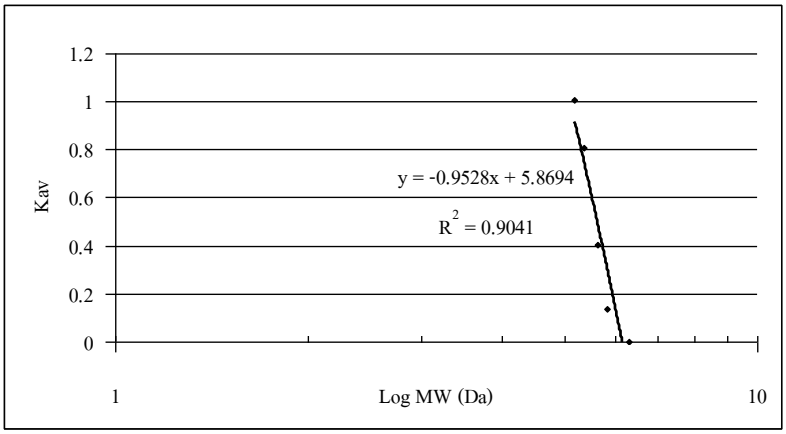

Figure B.7 Standard curve of standard protein for gel filtration (Sephacryl S-300); 0.67×50.0 cm^2, flow rate 0.3 ml/min., eluted with 50 mM phospate buffer pH 6.0

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