

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

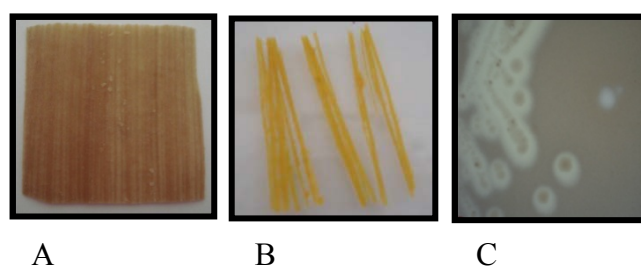


Figure 4.1 Degradation pattern on corn hull and cellulose by NKP; control corn hull (A), residues of corn hull after cultivation for 7 days (B), clear zone on cellulose agar plate (C).

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).

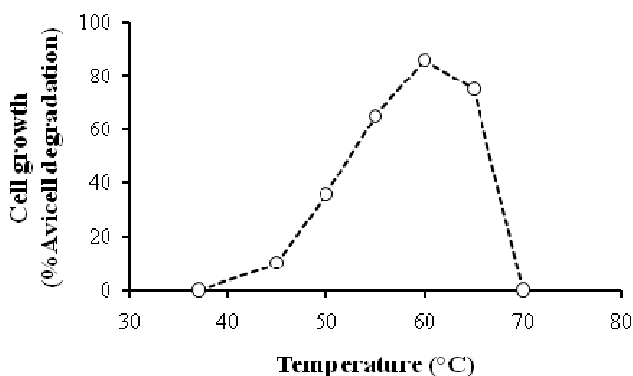


Figure 4.2 Growth of the NKP on BM media contained 1% Avicel at 37 °C to 80 °C. The growth of NKP was determined by reduction in dry weight of Avicel (%Avicel degradation).

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).

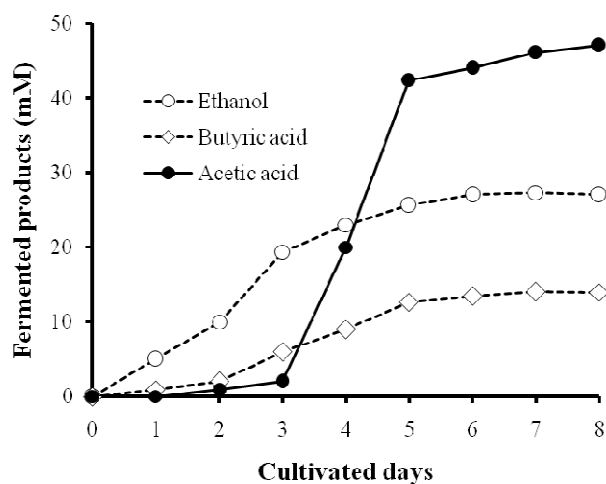


Figure 4.3 Time courses of fermented products from the NKP on Avicel cultivation at 60 °C under anaerobic condition

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.

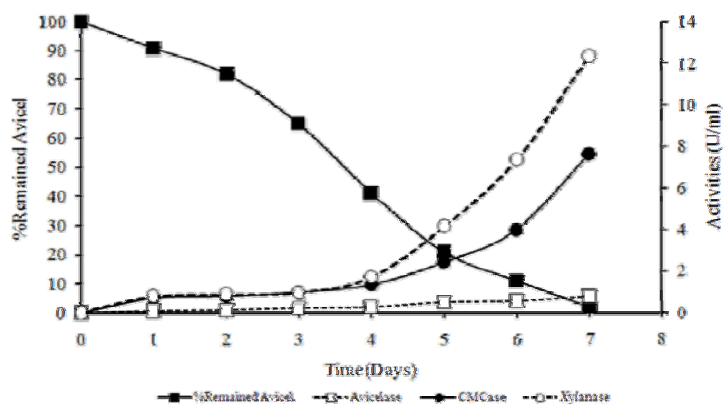


Figure 4.4 Profiles of remained Avicel and enzyme production by the NKP at pH 7.0 and 60 °C under an anaerobic condition.

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 NPK with cellulosome of *C. thermocellum* from strains JW20 and ATCC 27405.

Enzymes	Specific activities (U/ mg of protein)		
	NKP	<i>C. thermocellum</i> *	
		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 band but 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).

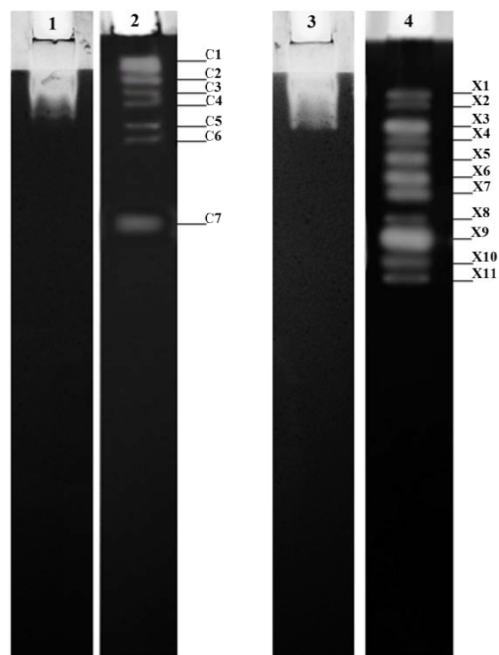


Figure 4.5 Zymogram analysis for CMCase and xylanase activities in culture supernatants of day 7 culture produced by the NKP at pH 7 and 60 °C under anaerobic condition. Lane 1; native zymograms for CMCase activity, lane 2; SDS zymogram for CMCase activities, lane 3; native zymograms for xylanase activity, 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. This allow 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.

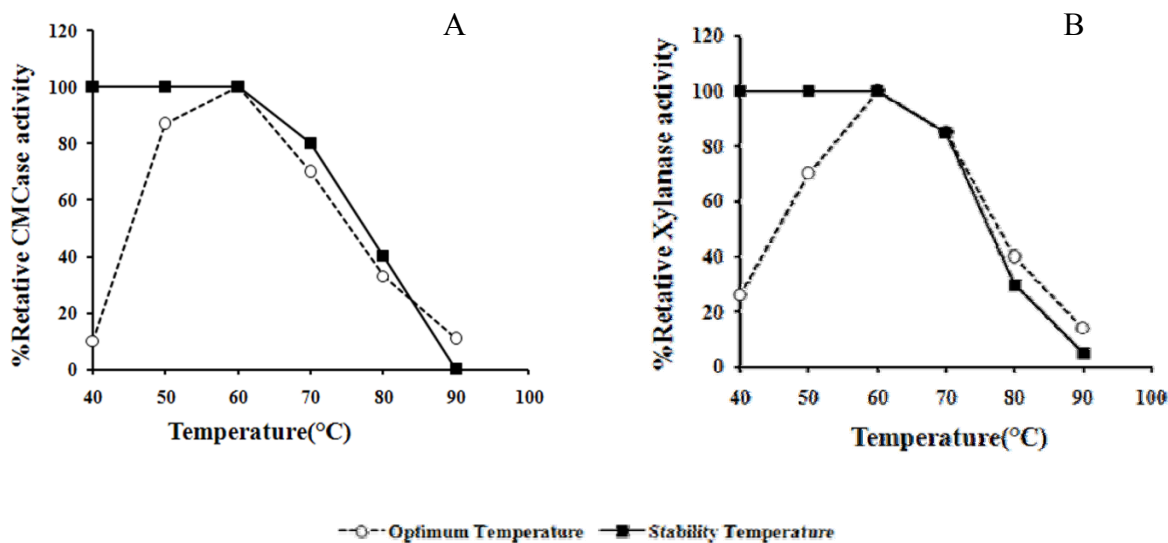


Figure 4.6 Effects of temperature on activity and stability of CMCCase (A) and xylanase (B) of crude extracellular enzymes from the NKP.

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 NKP 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).

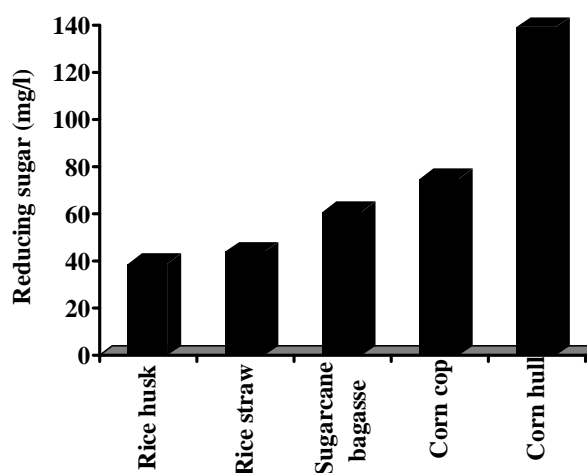


Figure 4.7 Reducing sugar content of biomass after hydrolysis with crude extracellular enzymes from the NKP for 1 hour (0.6 U of CMCase, 1.0 U of xylanase).

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 NKP and 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 (Erbeznic 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, which was efficient in degrading corn hull symbiotically. Subsequently, both strains were isolated by their biochemical properties. The strain NKP-2 was able to grow 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-cellulolytic 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.

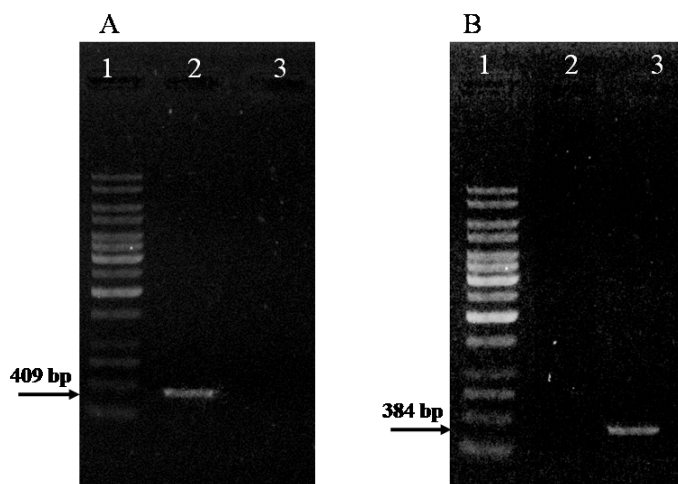


Figure 4.8 Specific PCR for *C. thermocellum* (A) and *T. thermosaccharolyticum* (B) with primers in material and method. Lane 1, 1 kbp DNA ladder; lane 2, strain NKP-2; lane 3, strain NOI-1

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).

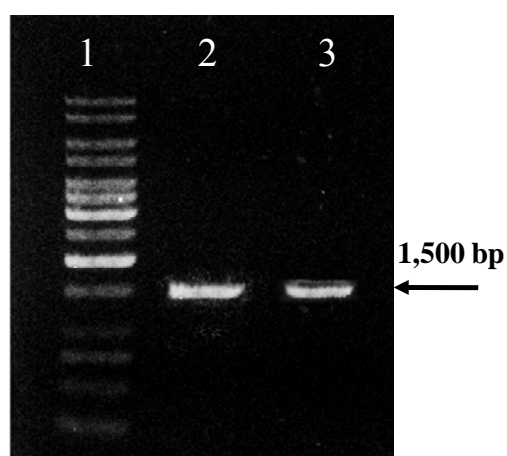


Figure 4.9 Agarose gel electrophoresis of PCR product amplification with EUB8f and U1492r primer sets. Lane 1, 1 kbp DNA ladder; lane 2-3, Amplified PCR products from total genomic DNA extraction of strain NKP-2 and strain NOI-1, respectively

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

Bacterium	Homology (%Identity)
<i>C. thermocellum</i> ATCC 27405 (NR_074629.1)	99%
<i>C. thermocellum</i> DSM 1313 (NR_074578.1)	99%
<i>C. thermocellum</i> JCM 9323 (AB588017.1)	99%
<i>C. thermocellum</i> JN4 (EF680276.1)	99%
<i>C. thermocellum</i> GP1 (FN555230.1)	99%
<i>C. thermocellum</i> CTL-6(FJ599513.1)	99%
<i>C. thermocellum</i> mbf-VZ-093 (HF674392.1)	99%
<i>Clostridium</i> sp. CT1 (FJ808607.1)	99%
<i>C. straminisolvens</i> 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)
<i>T. thermosaccharolyticum</i>	99%
<i>T. thermosulfurigenes</i>	97%
<i>T. xylanolyticum</i>	97%
<i>T. lactoethylicum</i>	96%
<i>T. aciditolerans</i>	96%
<i>T. islandicum</i>	96%
<i>T. saccharolyticum</i>	96%
<i>T. polysaccharolyticum</i>	88%
<i>T. zeae</i>	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. thermosaccharolyticum* strain NOI-1, respectively.

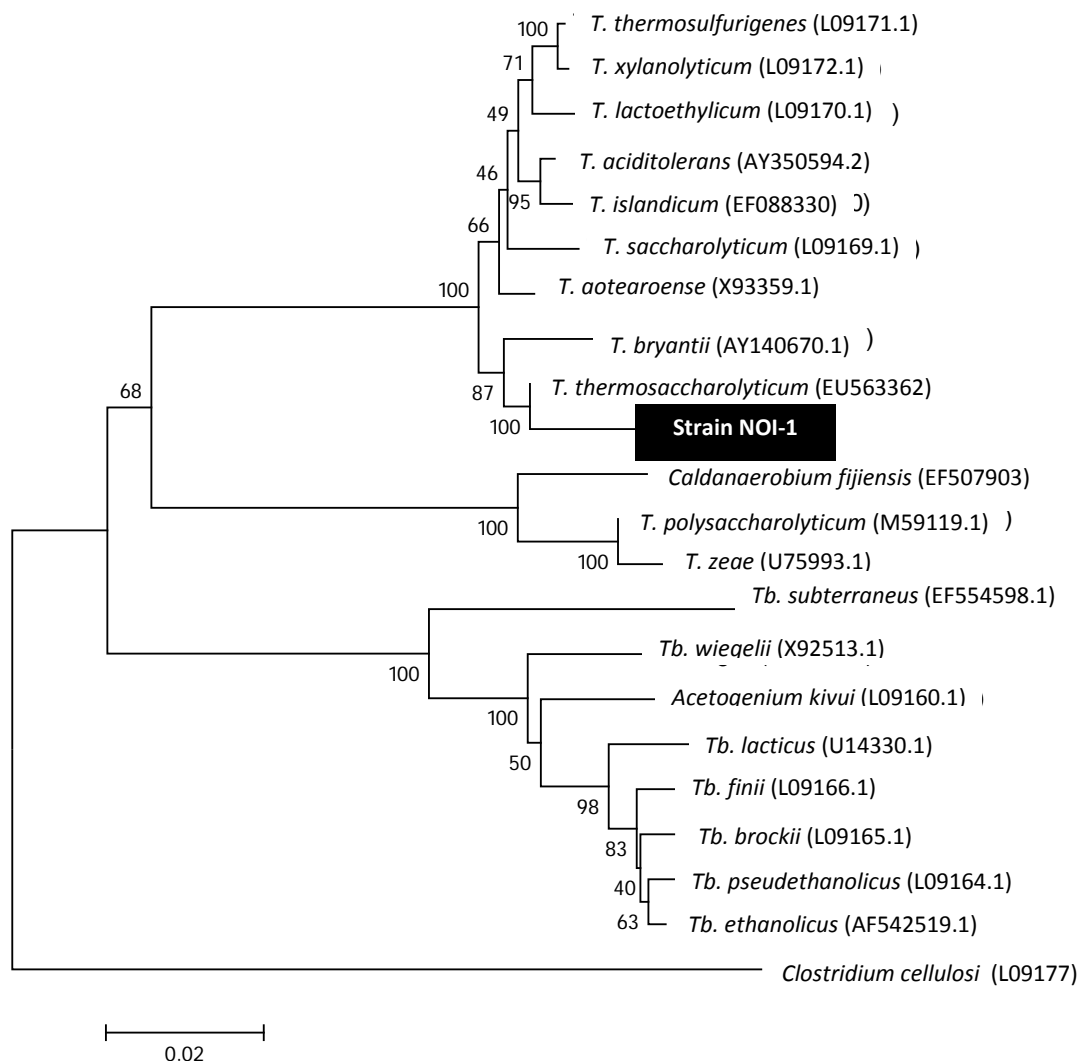


Figure 4.10 Phylogenetic tree showing the phylogenetic position of the isolated strain NOI-1 based on the 16S rRNA gene sequences of the family *Thermoanaerobacteriaceae*. The numbers at the nodes indicate the levels of bootstrap support percentages based on the neighbor-joining of 1,000 replicates. 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 defined 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)	
	<i>C. thermocellum</i>	<i>T. thermosaccharolyticum</i>
	NKP-2	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 phase of strain NKP-2 was longer than the co-culture and individual-culture of strain NOI-1.

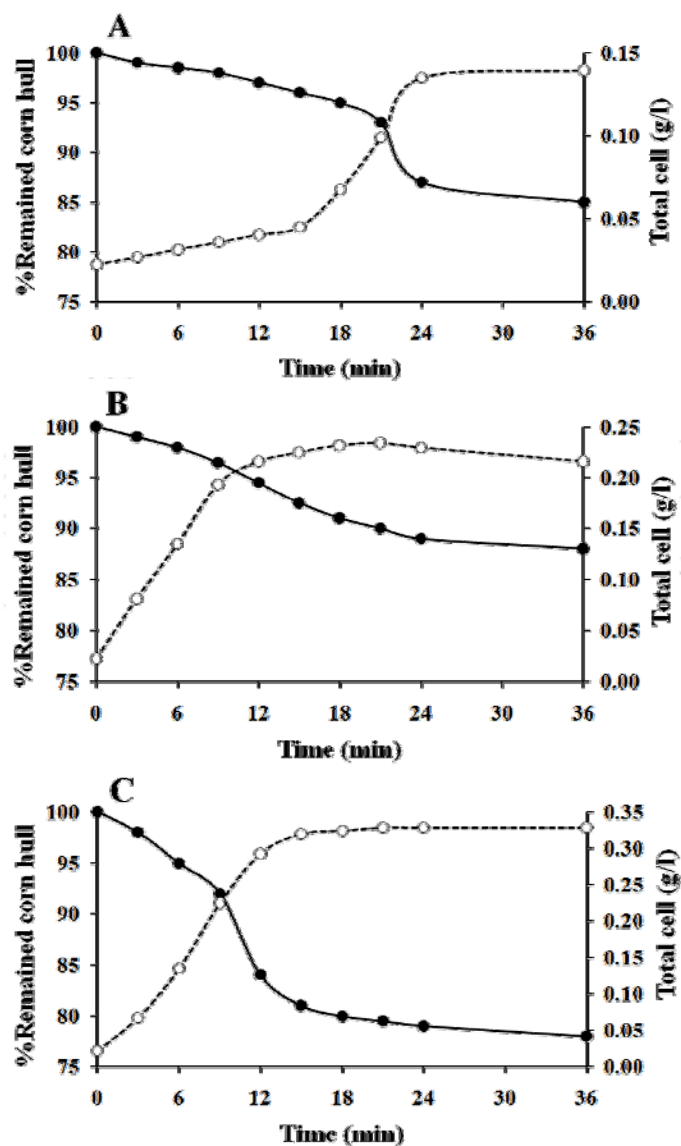


Figure 4.11 Time courses of cell growth and remaining corn hulls of (A) *C. thermocellum* NKP-2, (B) *T. thermosaccharolyticum* NOI-1 and (C) the co-culture of both strains at 60 °C and pH 7.0

Furthermore, the remaining soluble sugar (300 mg/l) in the individual culture 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.

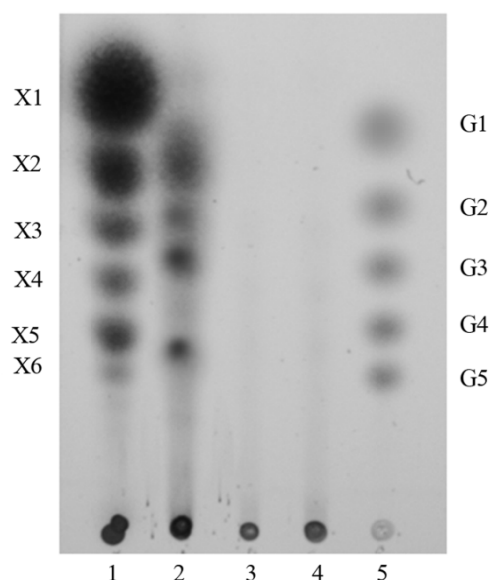


Figure 4.12 Thin layer chromatography of sugars from the culture supernatant at the stationary growth phase by individual mono- and co-cultures of *C. thermocellum* NKP-2 and *T. thermosaccharolyticum* NOI-1. Lane 1: standard X1-X6, Lane 2: *C. thermocellum* NKP-2, Lane 3: *T. thermosaccharolyticum* 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), H₂ (1.5 fold) and CO₂ (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), H₂ (1.2 fold) and CO₂ (1.3-fold) than the individually-cultured strain NOI-1.

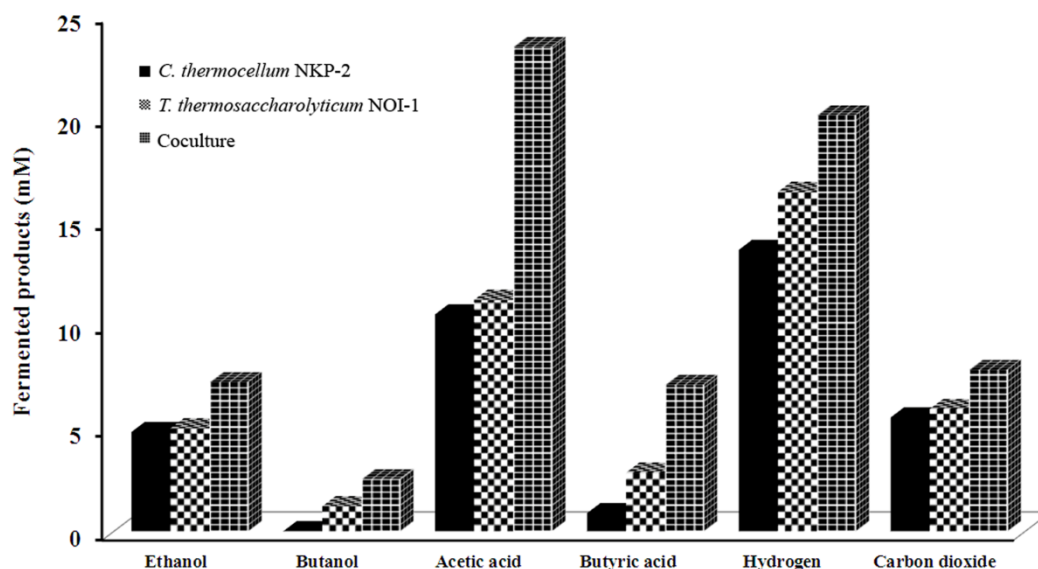


Figure 4.13 Fermentation end-products of individual-culture and co-culture of *T. thermosaccharolyticum* NOI-1 and *C. thermocellum* NKP-2 grown on corn hulls at 60 °C and pH 7.0.

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 oligo-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. The result shown that the strain NOI-1 could produced xylanase, β -xylosidase, α -L-arabinofuranosidase, β -glucosidase and cellobiohydrolase but could not produced avicelase and CMCase. On the other hand, *C. thermocellum* NKP-2 could produce xylanase, 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 all required enzyme for efficient corn hull degradation

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 enzyme that liberated 1 μ mol 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 complete and rapid hydrolysis of biomass will required the combination of cellulolytic-xylanolytic enzymes (Doi and Kosugi, 2004). Enzymes produced by the two bacteria strains 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. thermosaccharolyticum* 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 efficiency was 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 obtained from *T. thermosaccharolyticum* NOI-1 and *C. thermocellum* NKP-2 equal amounts of xylanase 0.05 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 separately. 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.

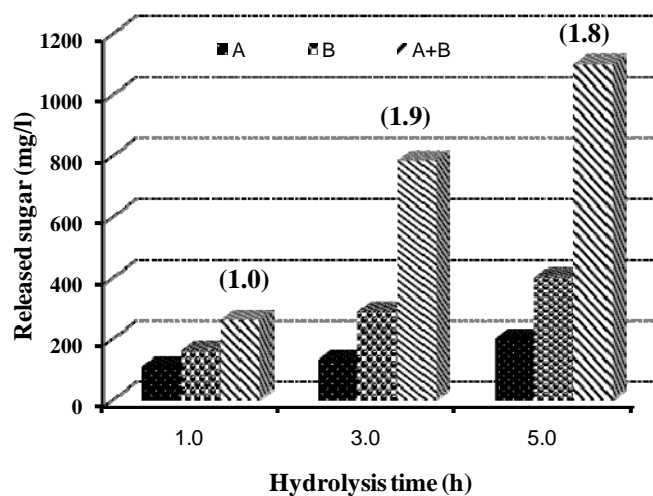


Figure 4.14 Corn hull hydrolysis time of individual and combination of crude enzyme from *T. thermosaccharolyticum* NOI-1(A) and *C. thermocellum*NKP-2 (B). Corn hull was hydrolyzed by 0.1 U/mg of protein concentration of each xylanase at 60 °C for 1, 3 and 5 h. The number in parentheses indicates degree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

Ratios (A:B)	Sum of reducing sugar released by the individual enzyme (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 approximately 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 linkages or bond holding each polymers together. 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.