Symbiotic Behavior during Co-culturing of *Clostridium thermocellum* NKP-2 and *Thermoanaerobacterium thermosaccharolyticum* NOI-1 on Corn Hull

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The symbiosis of co-culturing between *Clostridium thermocellum* NKP-2 and *Thermoanaerobacterium thermosaccharolyticum* NOI-1 is described. An efficient biomass-degrading enriched culture was isolated from soil that contained two different bacterial strains showing homology to *C. thermocellum* and *T. thermosaccharolyticum*. The enzymatic system produced from the isolated strains when cultivated individually on corn hulls demonstrated different cellulolytic and xylanolytic enzyme activities. Strain 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 primarily short- and side-chain cleaving enzymes such as cellobiohydrolase, β-glucosidase, β-xylosidase, acetyl esterase, and especially α-L-arabinofuranosidase. Enhancement of corn hull utilization, cell growth, and fermentation products (ethanol, butanol, acetic acid, butyric acid, H_2, and CO_2) was greatly increased during co-culturing compared with individual cultivation of both strains. The symbiotic behavior between both strains was one of mutualism, in which the synergistic degradation of corn hulls by co-action of cellulolytic and xylanolytic enzymes promoted hydrolysis of biomass for growth and fermented products.

Keywords: Cellulolytic-xylanolytic enzymes; Clostridium thermocellum; Co-culturing; Corn hull; Thermoanaerobacterium thermosaccharolyticum

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INTRODUCTION

Plant biomass is an important renewable energy source that is composed of polysaccharides such as cellulose and hemicelluloses (Ghosh and Ghose 2003). Corn, or maize (*Zea mays* L.), is widely cultivated in the world as food and animal feed. In Thailand, corn is one of the five main crop economies, and approximately 4.96 million tons were produced in 2012 (source: Office of Agricultural Economics, Thailand; http://www.oae.go.th). After harvesting, corn hulls and other residual materials are generated. Therefore, corn hull residues are of interest as an alternative renewable energy source. Because natural cellulose fibers are protected by a matrix of hemicellulose and lignin (Galbe and Zacchi 2007), their strong inter-chain hydrogen-bonding network and higher-order structure in plants contributes to biomass recalcitrance (Himmel *et al*. 2007).

Thus, the organization of and interactions among these polymers of the cell wall constitute a barrier to depolymerizing enzymes. Therefore, synergism of enzymatic systems plays an important role in biomass degradation. Enzymatic hydrolysis is a promising way to obtain sugars from biomass in which biodegradation of cellulose and hemicellulose materials to monomeric sugars is conducted through the concerted actions of enzymes, including cellulolytic enzymes, xylanolytic enzymes, and ligninases (Lee 1997; Lopez *et al*. 2007).

The cellulose-hydrolyzing enzymes are divided into three major groups: endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21) (Kumar *et al.* 2008). Hemicellulose has an extremely heterogeneous chemical composition and mostly consists of arabinoxylan in grasses (Mazumder and York 2010). Arabinoxylans occur as heteropolysaccharides, containing a back bone chain and different groups on the side chains such as acetyl, arabinosyl, and glucuronosyl residues (Biely 1985). Various enzymes, such as endo-1,4-β-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), α-glucuronidase (EC 3.2.1.1), α-L-arabinofuranosidase (EC 3.2.1.55), and acetyl esterase (EC 3.1.1.6), are responsible for the degradation of arabinoxylan. All these enzymes act cooperatively to convert xylan to its constituent sugar (Shallom and Shoham 2003).

Many microorganisms, such as fungi and other aerobic and anaerobic microorganisms that produce cellulosic and hemicellulosic enzymes, have been reported and characterized (Lynd *et al*. 2002; Warren 1996). Currently, efforts at finding effective enzymatic systems from various cellulolytic and/or xylanolytic microorganisms are still ongoing. The biodegradation of plant cell wall polysaccharides through the use of microbial co-cultures or complex communities has been proposed as a highly efficient approach for biotechnological applications because it avoids the problems of feedback regulation and metabolite repression posed by using only a single strain (Haruta *et al*. 2002; Soundar and Chandra 1987; Torre and Campillo 1984). Symbiotic interactions between cellulolytic and non-cellulolytic microorganisms in cellulose degradation have been reported (Pohlschroeder *et al.* 1994; Valaskova *et al*. 2009). Many reports have shown *C. thermocellum* to be a cellulose-degrading bacterium with a 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). *T. thermosaccharolyticum* is a thermophilic, anaerobic bacterium that is able to grow under the same conditions as *C. thermocellum*. *T. thermosaccharolyticum* produces primarily endocellulase-free enzymes, amylolytic enzymes, or dextranase and utilizes a variety of carbon sources, such as hexoses, pentoses, and starch (Chimtong *et al*. 2011; Ganghofner *et al*. 1998; Hoster *et al*. 2001).

Recently, more attention has focused on providing a promising solution for efficient and economical lignocellulose conversion to new value-added bioproducts. Most studies are on the co-cultivation of cellulolytic and non-cellulolytic microorganisms, such as the co-culturing of *C. thermocellum* JN4 and *T. thermosaccharolyticum* GD17 (Liu *et al*. 2008) or *C. thermocellum* and *C. thermopalmarium* to produced hydrogen (Geng *et al*. 2010). A co-culture of *Caldicellulosiruptor* sp. strain DIB 087C and *Thermoanaerobacter* sp. strain DIB 097X is particularly effective in the conversion of cellulose to ethanol (Svetlitchnyi *et al*. 2013).

Recently, stable coexisting biomass-degrading bacteria were isolated from the soil by our colleagues. In the interest of studying their mutualistic behavior, the identities of

the coexisting biomass-degrading bacteria, which are capable of degrading different biomass and produce biomass-degrading enzymes, were determined. To date, an anaerobic, thermophilic, and cellulolytic bacterium, *C. thermocellum* NKP-2, and a non-cellulolytic bacterium, *T. thermosaccharolyticum* NOI-1, have been successfully isolated from a consortium of bacteria. In this study, an enzymatic system was analyzed in which a symbiotic relationship was produced when co-culturing *C. thermocellum* NKP-2 and *T. thermosaccharolyticum* NOI-1 on corn hulls.

EXPERIMENTAL

Microorganisms and Medium

C. thermocellum NKP-2 and *T. thermosaccharolyticum* NOI-1 were isolated from soil at farm sites in Nakhonpathom province, Thailand. Fresh cultures were maintained by routinely transferring 10% (v/v) inoculum into fresh basal media (BM) containing (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 hydrochloride, and 0.2 mL of 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 pH of the media was adjusted to 7.0 and autoclaved at 120 ^oC for 15 min. The solid medium contained 15 g of agar per liter. The media were prepared under anaerobic conditions, as previously described (Chimtong *et al*. 2011).

Corn Hull Preparation

Corn hulls were collected from Lampang province, Thailand. They were first cut with scissors to piece size (1 cm × 1 cm) and then washed several times to remove contaminants and dried to a constant weight before use in this study. The components in corn hulls, *e.g*., 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). Ball-milled corn hulls were prepared similarly to ball-milled cellulose as previously described by Mariko *et al*. (2004), except for using corn hull as the raw material.

Screening and Isolation

The bacteria strains NKP-2 and NOI-1 were isolated. Briefly, corn hulls were used as a carbon source. The cultures were incubated at 60 °C under anaerobic conditions. The screening step was carried out several times to obtain promising candidates. The sample showing the most effective corn hull degradation was enriched. The sample was transferred into ball-milled corn hull agar plates for isolation. Then, the ball-milled corn hull agar plates were kept in anaerobic jars and incubated at 60 °C. A single colony was streaked onto a new plate. Two kinds of colony characteristics appeared on the ball-milled corn hull agar plates, even though a single colony was picked and streaked. Purification of each strain by the single colony isolation technique (on ball-milled corn hull) was not successful; thus, follow-up purification was performed using different carbon sources, where colonies were were streaked on cellulose or xylan agar plates. One of the strains (namely NOI-1) could not grow on the cellulose powder agar plate. Therefore, strain NOI-1 was isolated using the roll tube technique described earlier

(Chimtong *et al*. 2011) with xylan as a sole carbon source 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 or xylan as the respective sole carbon sources.

16S rRNA Gene Analysis

Genomic DNA was prepared using a Qiamp DNA Stool kit (Qiagen) according to the manufacturer's protocol. 16S rRNA gene analysis was performed as described previously (Chimtong *et al*. 2011). The 16S rRNA gene was amplified by PCR using the following primers: 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR was performed with standard conditions according to the manufacturer's instructions. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Germany). The determined sequence was compared with references available in the GenBank/EMBL database using the BLAST program.

Nucleotide Sequence Accession Number

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

Enzyme Assays

Xylanase, β-xylosidase, α-L-arabinofuranosidase, β-glucosidase, and acetyl esterase activities were determined as described previously (Ratanakhanokchai *et al*. 1999). However, the xylanase assay used birchwood xylan (BWX) as the substrate. CMCase and avicelase activities were performed under the same conditions as the xylanase assay using carboxymethylcellulose and Avicel as the substrates, respectively. The reducing sugars were determined using the Somogyi-Nelson method (Nelson 1944). One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugars per minute. The cellobiohydrolase activity was determined using the method of Kohring *et al*. (1990) with *p*-nitrophenyl-β-D-cellobioside as the substrate. The β-xylosidase, α-L-arabinofuranosidase, β-glucosidase, acetyl esterase, and cellobiohydrolase activities were expressed as μmols of *p*-nitrophenol released per minute per milliliter of enzyme solution.

Cultivation

Batch cultivation of individual and co-cultures 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. All cultures had an initial pH at 7.0, with no pH control during cultivations. An inoculation volume of 14 mL (0.22 g/L of total cells) of bacteria suspension at exponential growth phase were transferred into 86 mL of BM medium for all cultivations. For the co-culture, the *C. thermocellum* NKP-2/*T. thermosaccharolyticum* NOI-1 ratio was 5:2, which was determined to be a suitable combination (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. Moreover, cultivation of individual and co-cultures without corn hulls were measured with the fermentation products as controls.

Analytical Methods

Bacterial growth analysis

Bacterial growth cultures were centrifuged at 8,000×g for 15 min. The culture supernatants at the stationary growth phase were collected to determine their enzyme activities and products. 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) polysorbate 80 (Tween 80®) at 4 °C for 30 min with occasional stirring and removal of residual corn hulls (Lamed *et al*. 1983). After centrifugation, the cell suspension was used for measuring cell growth and the remaining corn hull was quantified by a gravimetric determination after being dried at 80 °C until a constant weight was reached.

Sugar product analysis

Soluble sugars from the growth culture broth were 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 of α-diphenylamine dissolved in a solution of aniline/phosphoric acid/acetone (1.0:7.5:50.0 by volume) (Ratanakhanokchai *et al*. 1999). Series of xylose (X1-X6) and glucose (G1-G5) from Megazyme (Wicklow, Ireland) were used as the standards.

Fermentation product analysis

Fermentation products in the supernatant of grown cultures 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. Nitrogen gas was used as the carrier gas with flow rate of 3 mL/min.

Gas analysis

The biogas composition was measured using GC (model Shimadzu GC-2014 equipped with Porapak N and Porapak Q columns and a thermal conductivity detector) using argon as a carrier gas. The operational temperatures at the injection port, column oven, and detector were 80, 100, and 120 °C, respectively. One milliliter of the formed gases from the culture was injected directly into the GC column and compared with combined standard gases (10%H_2, 20% CO_2, and 60%CH_4).

RESULTS AND DISCUSSION

Corn hulls were collected from Lampang Province, Thailand and their composition was determined. Corn hulls (% dry matter) collected in this study contained cellulose, hemicellulose, lignin, and other materials at 34.15, 45.83, 14.12, and 5.90%, respectively.

Symbiotic microbial associations have evolved in environments in which biomass is degraded interdependently. In this study, a microbial consortium capable of degrading corn hulls was screened from an enrichment culture. This brings forth the requirement to isolate and characterize each of the members in the cultures, followed by the study of the roles and relationships among the members of the community. We found that the efficient

corn hull utilization candidate contained two kinds of microorganisms, *C. thermocellum* NKP-2 and *T. thermosaccharolyticum* NOI-1. Bacteria were selected that degraded corn hulls efficiently. After several rounds of enrichment and isolation on a ball-milled corn hull agar plate, it was found that the efficient candidate still contained two kinds of colonies of anaerobic bacteria based on colony morphology. After attempts to purify each strain by the single colony isolation technique (on ball-milled corn hull) failed, they were purified using different carbon sources. It was found that both strains could be separated as described in the materials and methods section. Moreover, both pure strains were then tested for contaminants using molecular techniques. Verification of the separation of the pure strain from the contaminant was performed using a modified PCR assay in which a set of primers (Thm V1; 5'-GAAGGGAGTACTACGGTAC-3', and Thm R1; 5'-TATGGTACCGTCATTTCTTT-3') specific for *Thermoanaerobacterium* spp. was used (Dotzauer *et al*. 2002). The result indicated that strain NKP-2 was not contaminated with the strain NOI-1, as the expected band (385 bp) was not observed on an agarose gel of PCR product (data not shown). Similarly, the strain NOI-1 was tested for contamination with NKP-2, but using a different set of primers (Cth-P; 5'-AACTGCAGTCGAGCGGGGATATACGGAAG-3', and Cth-E; 5'-AAGAATTCCTTCGTCCCCAATCAAAGAAG-3') specific for *C. thermocellum* (Erbeznik *et al*. 1997). The results indicated that strain NOI-1 was not contaminated with the strain NKP-2, as the expected band (409 bp) was not observed on an agarose gel of PCR products (data not shown). Therefore, the results strongly indicated that both strains were pure cultures.

The isolated strains NKP-2 and NOI-1 showed high similarities with *C. thermocellum* and *T. thermosaccharolyticum*, respectively, when they were identified using 16S rRNA gene analysis (> 99% identity). Thus, they were designated *C. thermocellum* NKP-2 and *T. thermosaccharolyticum* NOI-1. However, when each strain was incubated individually, corn hull utilization was lower than when a mixed culture was used. This is in agreement with previous reports whereby both cellulolytic bacteria and non-cellulolytic bacteria can live symbiotically in several places in nature (Freier *et al*. 1988). Therefore, they grow together during isolation. However, both strains were successfully isolated in this study using different carbon sources. As in a previous report, the *T. thermosaccharolyticum* strain NOI-1 was reported to produce a unique complex of enzymes in the form of an endocellulase-free multienzyme complex that was shown to be effective in hydrolyzing corn hull (Chimtong *et al*. 2011). Nevertheless, the enzymatic systems of both strains were not studied when cultivated on biomass. Hence, the symbiotic behavior of these strains in corn hull cultivation was studied and monitored in terms of synergistic enzymes.

In this study, the enzyme production and cell growth were investigated by cultivating *C. thermocellum* NKP-2 and *T. thermosaccharolyticum* NOI-1 individually and together using corn hulls as a sole carbon source. All cultivations were carried out with the same starting inoculum size, either when culturing individually or co-cultivating. Enzyme activities were determined from culture supernatant after the culture reached the stationary growth phase (Table 1). Results showed that strain NKP-2 produced CMCase, avicelase, and xylanase as the main enzymes, while strain NOI-1 did not produce CMCase and avicelase, and produced less xylanase (2.9-fold less) than strain NKP-2. However, strain NOI-1 produced higher amounts of cellobiohydrolase, β-glucosidase, β-xylosidase, α-L-arabinofuranosidase, and acetyl esterase than strain NKP-2 (1.3-, 1.4-, 3.4-, 27.3- and 2.8-fold, respectively). These results show that both strains contained

different enzyme production systems. The NKP-2 strain generally produced cellulose- and xylan-main chain-cleaving enzymes, whereas NOI-1 strain produced mainly short- and side-chain-cleaving enzymes. On the other hand, the result showed that the co-culturing of both strains produced more cellulolytic and xylanolytic enzymes than the individual culture. Symbiotic relationships between cellulolytic and xylanolytic microorganisms showed strong promotion of lignocellulose degradation by mixed microbial cultures.

Table 1. Cellulolytic and Xylanolytic Enzymes of *C. thermocellum* NKP-2, *T. thermosaccharolyticum* NOI-1 and Co-culture of Both Strains on Corn Hull Cultivation at the Stationary Growth Phase

Enzyme	Total activity (U)		
	C. thermocellum NKP-2	*T. thermosaccharolyticum* NOI-1	Co-culture
CMCase	19.50	ND	43.06
Avicelase	0.24	ND	1.33
Xylanase	36.00	12.30	54.42
Cellobiohydrolase	0.42	0.56	1.42
β-Glucosidase	0.27	0.39	1.11
β-Xylosidase	0.16	0.55	1.06
α-L-Arabinofuranosidase	0.14	3.82	4.92
Acetyl esterase	0.04	0.11	0.16

The cell growth of *C. thermocellum* NKP-2 and *T. thermosaccharolyticum* NOI-1 cultivated individually and together on corn hull was studied, and the results are shown in Fig. 1. It was found that co-culturing both strains on corn hulls gave rise to a shorter lag phase compared to culturing the individual strain of NKP-2, whereas strain NOI-1 did not exhibit an apparent lag phase. Furthermore, co-culture generally showed a faster exponential growth rate compared to the individual culture of strain NKP-2. At the stationary growth phase, the masses of the individual cultures of strains NKP-2 and NOI-1 were 0.14 and 0.22 g/L of the total cell biomass, respectively, whereas that of the co-cultured strains was 0.33 g/L of the total cell biomass. Approximately 15% and 12% of the corn hulls were utilized by the individually cultured strains NKP-2 and NOI-1, respectively, whereas the corn hull utilization by co-culturing both strains was 22% at 36 h. Afterward, the remaining corn hulls of cultivations were analyzed for cellulose, hemicellulose, and lignin contents. The component of remaining corn hull revealed that approximately 8.9 and 13.1% of cellulose and hemicellulose were utilized by co-culturing both strains when compared with original corn hull. Individual culture of the strain NKP-2 utilized approximately 7.2 and 7.8% of cellulose and hemicellulose, respectively, whereas individual culture of the strain NOI-1 utilized only hemicellulose (approximately 12.0%). The results indicated that the corn hull degradation corresponds

to enzyme production from each strain, while lignin content did not change in individual and co-cultures. Moreover, *C. thermocellum* and *T. thermosaccharolyticum* are commonly cellulolytic and/or xylanolytic producing bacteria.

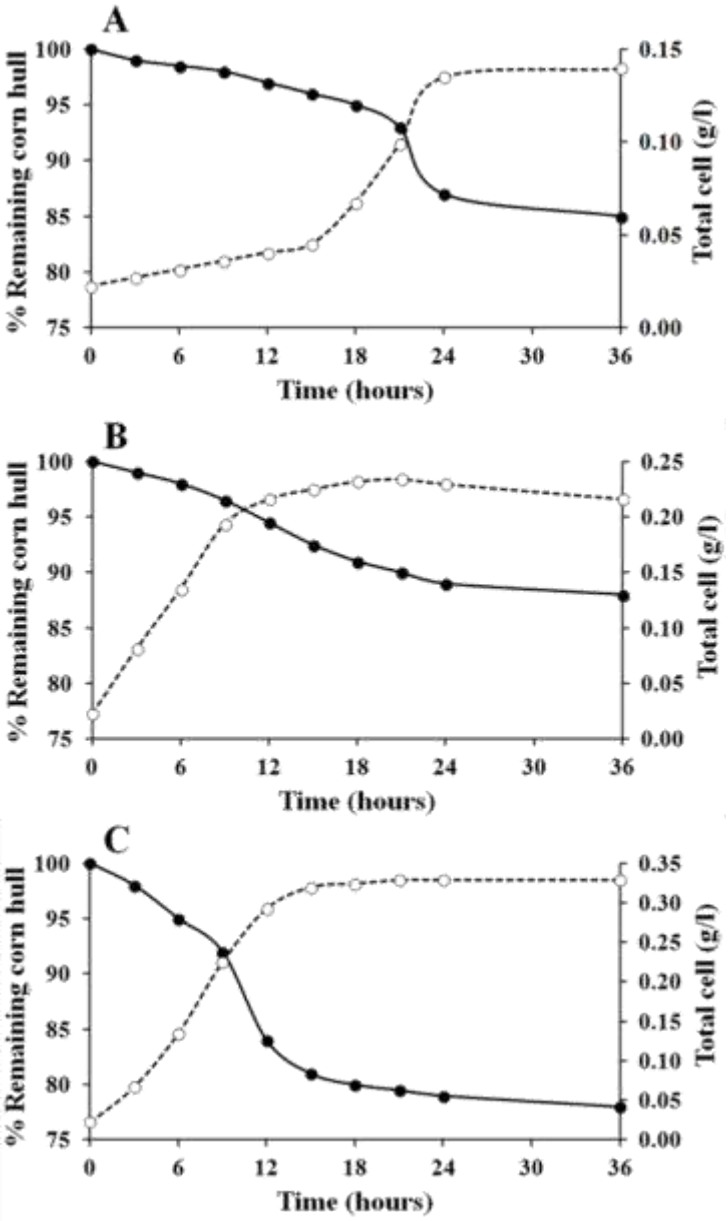

Fig. 1. Time courses of cell growth (○) and remaining corn hulls (●) of *C. thermocellum* NKP-2 (A), *T. thermosaccharolyticum* NOI-1 (B), and the co-culture of both strains (C) at 60 °C and initial pH 7.0

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 requires synergetic action of both cellulolytic and xylanolytic enzymes (Murashima *et al*. 2003), in which xylanolytic enzymes hydrolyze the outer component, arabinoxylan, a major component of hemicellulose in grasses, and the cellulolytic enzymes hydrolyze the inner cellulose component afterwards

(Galbe and Zacchi 2007; Mazumder and York 2010; Shallom and Shoham 2003). This may be the reason for the symbiotic relationship created by these microbes in their natural habitats.

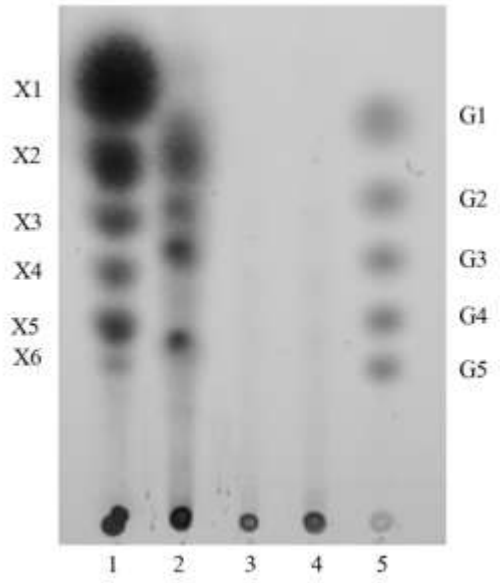

Fig. 2. Thin layer chromatography of sugars from the culture supernatant at the stationary growth phase by individual mono- and co-cultures of *C. thermocellum* NKP-2 and *T. thermosaccharolyticum* NOI-1. Lane 1: standard X1-X6, Lane 2: *C. thermocellum* NKP-2, Lane 3: *T. thermosaccharolyticum* NOI-1,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

Although strain NKP-2 produced endo-xylanase, this strain had a low amount of debranching enzymes, especially arabinofuranosidase (Table 1). Thus, the hydrolysis of arabinoxylan of corn hulls by the enzymatic system of strain NKP-2 was not complete, and the hydrolysis products could not be utilized by the microorganism. The lag phase of strain NKP-2 was longer than that of the co-culture and the individual culture of strain NOI-1. The remaining soluble sugar (300 mg/L) in the individual culture of strain NKP-2 was analyzed. The size of the sugars did not relate to any of the standard series of xylo- and gluco-oligosaccharides (Fig. 2, lane 2), indicating that these sugars could be branched-xylooligosaccharides. In contrast, these sugars were not present in the individual culture of strain NOI-1 and the co-cultured supernatants (Fig. 2, lanes 3 and 4), perhaps due to the utilization of all sugars by those bacteria in the culture.

Figure 3 shows the fermentation products at the stationary growth phase. Ethanol, butanol, acetic acid, butyric acid, hydrogen gas, and carbon dioxide gas were the expected end products of the fermentation. Results showed that butanol was not present after cultivation with strain NKP-2 alone. Furthermore, co-culturing produced higher ethanol (1.5-fold), acetic acid (2.2-fold), butyric acid (7.2-fold), H_2 (1.4-fold), and CO_2 (1.5-fold) contents than the individually cultured strain NKP-2. The amounts of ethanol (1.5-fold), butanol (2.1-fold), acetic acid (2.1-fold), butyric acid (2.4-fold), H_2 (1.2-fold), and CO_2 (1.3-fold) were also increased over the individually cultured strain NOI-1. During the cultivation of individual culture strain NKP-2 and NOI-1 there was progressive decrease in pH from 7.0 to 6.5, whereas co-culturing decreased pH from 7.0 to 6.2 after 36 h. Furthermore, both strains were cultivated in the media without corn hulls as the sole carbon source. The result showed that both strains could not grow well

(minimal growth) when cultured only in yeast extract. Thus, the metabolite products could not be detected under this cultivation.

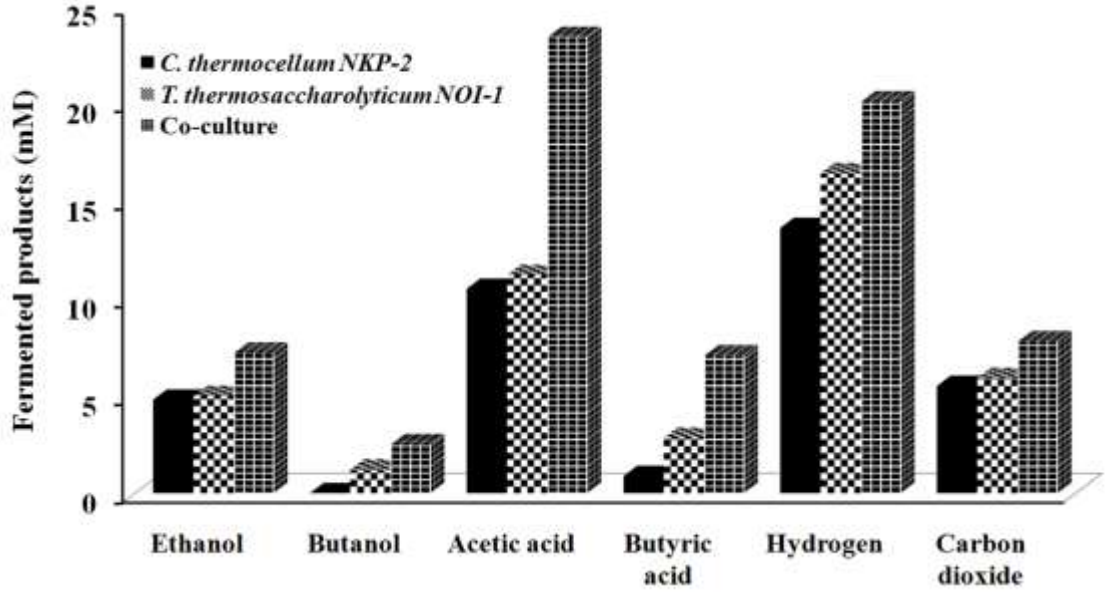

Fig. 3. Fermentation products of individual cultures and co-culture of *T. thermosaccharolyticum* NOI-1 and *C. thermocellum* NKP-2 grown on corn hulls at 60 °C and initial pH 7.0

The higher cell growth, carbon utilization, and fermentation products in the co-culture indicated that co-culturing of both strains improved the conversion efficiency of corn hulls in terms of cell growth and fermentation products. This is in agreement with recent research that shows that co-culturing bacteria can improve the degradation of biomass to increase the yield of fermented products and bioenergy (hydrogen) (Geng *et al*. 2010; Liu *et al*. 2008). Thus, it can be presumed that a mutualistic relationship exists between strain NKP-2 and strain NOI-1 in corn hull fermentation, in which completely cellulolytic and xylanolytic enzyme systems are co-produced from both strains.

CONCLUSIONS

1. Coexisting biomass-degrading bacteria with the ability to efficiently degrade corn hulls were enriched. Reports in the literature generally indicate that coexisting cellulolytic and non-cellulolytic bacteria are difficult to separate. However, two bacteria strains, NKP-2 and NOI-1, were successfully isolated from a consortium of bacteria using different carbon sources.
2. This is the first report of co-culturing of *C. thermocellum* and *T. thermosaccharolyticum* on corn hulls in terms of an enzymatic symbiosis. It can be concluded that mutualism exists between strain NKP-2 and strain NOI-1 in corn hull fermentation, in which the synergistic cellulolytic and xylanolytic enzymes are co-produced from both strains. The symbiotic mechanism between strains NKP-2 and NOI-1 was studied by comparing the enzymatic system when it was used separately or combined to promote hydrolysis of biomass.

3. Results of these studies demonstrated that the co-culture of strain NKP-2 and strain NOI-1 can be applied to the biodegradation of biomass to improve the production of value-added products.

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