

## 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 *Thermoanaerobacterium*spp., 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<sub>2</sub>CO<sub>3</sub> 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<sub>2</sub>. 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 cells 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 ×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.

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

where;             $A = \text{OD}_{400}$  of initial turbidity of the suspension  
                       $B = \text{OD}_{400}$  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 °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 min 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 min 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-xylopyranoside 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 °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).

$\beta$ -Glucosidase and  $\alpha$ -L-arabinofuranosidase activities were measured under the same condition as  $\beta$ -xylosidase activity as mentioned above except for the substrates. For the  $\beta$ -glucosidase assay, 1 mM *p*-nitrophenyl- $\beta$ -D-glucopyranoside was used as the substrate, and for the  $\alpha$ -L-arabinofuranosidase assay, 0.83 mM *p*-nitrophenyl- $\alpha$ -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  $\beta$ -D-cellobioside as the substrate. The reaction mixture consisted of 0.5 ml of 0.5 mM *p*-nitrophenyl  $\beta$ -D-cellobioside in 10 mM phosphate buffer, pH 6.0 and 0.5 ml of enzyme. The reaction mixture was incubated at 60 °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.

$\beta$ -Xylosidase,  $\beta$ -glucosidase,  $\alpha$ -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 electrophoresis, 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<sub>2</sub>CO<sub>3</sub>-NaHCO<sub>3</sub> buffer (pH 9.0 to 10.0), NaHCO<sub>3</sub>-NaOH buffer (pH 9.6 to 11.0) and Na<sub>2</sub>HPO<sub>4</sub>-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).

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

### 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 hydrolyzed 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).

Comparison 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<sub>254</sub> (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  $\alpha$ -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<sub>1</sub>-X<sub>6</sub>) and glucose (G<sub>1</sub>-G<sub>5</sub>) 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 and xylanolytic 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)

$$\text{Degree of synergy} = \frac{\text{RS}_{\text{combined enzymes}}}{\text{RS}_{\text{individual of NKP-2}} + \text{RS}_{\text{individual of NOI-1}}}$$

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.