CHAPTER III

MATERIALS AND METHODS

Instruments and chemicals; culture media ; reagents and buffers ; primers, characteristics of isolates, **nucleotide sequences and DNA G + C contents**

Name list of all instruments and chemicals; culture media; reagents and buffers; primers, characteristics of isolates, nucleotide sequences and DNA G + C contents are shown in Appendix A, B, C and D, respectively.

Methods

1. Screening of cellulase-producing bacteria

1.1 Screening of cellulase-producing bacteria on agar plate

A total of 120 soil samples were collected from Pua and Santisuk districts, Nan province, Thailand (Table 5). Cellulase producing bacteria were isolated from the soil samples not later than 24 hours after collection by an enrichment culture method. The soil sample (0.1 g) was put into a 10 ml of Cellulose powder medium (CP) in 25x250 mm test tube and incubated on a rotary shaker at 200 rpm, 40°C for 2 days. One milliliter of the culture was transferred to fresh CP medium and incubated at the same above conditions for 2 more times. The enriched cultures or their dilutions (0.1 ml) were dropped and spreaded on the CP agar medium and incubated at 40°C for 2 days. Cellulase producing capability of the cultures was screened qualitatively by method described by Teather and Wood (1982). Their colonies grown on Carboxymethyl cellulose-basal (CMC-basal) agar medium at 40°C for 2 days were flooded with 0.1% (w/v) Congo red solution for 1 minute and then washed by 0.1

M NaCl. Colonies surrounded by clearance zone were selected as cellulase producing isolates and then they were purified by streak plate method for further study.

1.2 Quantitative cellulase producing assay

Single colony of cellulase producing isolates were inoculated into 10 ml of Carboxymethyl cellulose (CMC) medium in 25x250 cm test tube and incubated on a rotary shaker at 200 rpm, 40°C for 2 days. Three milliliters of the cultures were transferred into 30 ml of CMC medium in 250 ml Erlenmeyer flask and incubated at the same aboved conditions for 2 day. Supernatants obtained after centrifugation of the cultures at 10,000 rpm (13,300 g) , 4° C for 15 min were used as crude enzyme for cellulase activity assay.

Cellulase activity assay was done by the method described by Ghose (1987). Reaction mixture composed of 0.5 ml of 2% Carboxymethyl cellulose (CMC) in 100 mM sodium phosphate buffer pH 7.0 , and 0.5 ml of crude enzyme were incubated at 40°C for 30 min. The amount of reducing sugar released was quantified by Somogyi and Nelson method (1952) using glucose as authentic sugar. After addition of enzyme solution, the reactions were stopped immediately and used as a reaction blank. One unit of cellulase was defined as the amount of enzyme yielding 1 micromole of glucose within 1 min under the assay conditions.

2. Identification methods

2.1 Cell morphology and cultural characteristics

The colonies grown on CMC agar medium at 37°C for 1 days were examined for their cell shape and colonial appearance, spore formation, motility, and pigmentation as described by Barrow and Feltham, 1993.

2.1.1 *Gram staining* Thin smear of bacterial colony on a clean slide was fixed by passing through flame and stained with Gram's crystal violet for 30 sec, rinsed with water, followed by covering with Gram's iodine solution for 30 sec then

rinsed with water, decolorized with 95% (v/v) ethanol and washed with water. Then, the smear was counter stained with safranin for 30 sec, blot dried and examined under microscope.

2.1.2 *Flagella staining* Standard microscopic slides, precleaned by the manufacturer, were used. The slide briefly flamed and drawn a thick line by a wax pencil across its width to confine a stain to be two-thirds of the slide surface. Three drops of sterile distilled water were added to this area and gently mixed with cells. There was no visible opalescence. The suspension was smeared over the staining area and then tapped off onto a disinfectant-soaked gauze sponge, and air dried on a level surface. Staining was done by the method described Forbes (1981) using 1 ml of staining solution at ambient temperature for 1 min. The slide was washed in tap water, counterstained with Hucker modified Gram crystal violet for 1 min, washed, blotted dry, and examined under oil immersion starting near the wax line.

2.2 Physiological and biochemical characteristics

2.2.1 *Oxidase test* A few drops of 1% tetramethyl-p-phenylenediamine were dropped on sterile filter paper disc. The culture tested was then smeared across the moist paper disc with sterile loop. The appearance of dark-purple colour on paper within 30 sec denoted a positive reaction.

2.2.2 *Catalase test* Cells were transferred onto slide, and immediately covered by 3% (v/v) hydrogen peroxide. The evolution of gas bubbles indicated a positive test.

2.2.3 *Growth at different temperature* Cells were inoculated on the CMC agar medium and incubated at 10°C, 15°C, 20°C, 37°C, 45°C, 50°C, 55°C and 60°C. Growth examination was performed after 5 days.

2.2.4 *Growth at different pH* Cells were inoculated into C broth which pH adjusted to 5, 6, 8, 9 and incubated at 37°C for 5 days.

2.2.5 *Growth in different NaCl concentration* Cells were inoculated on the C agar medium containing 3 and 5% NaCl and incubated at 37°C for 5 days, then the growth was observed.

2.2.6 *L-Arginine hydrolysis* Cells were inoculated onto arginine agar slant and incubated at 37°C for 5 days. Colour change of the indicator to red indicated a positive test.

2.2.7 *Aesculin hydrolysis* Cells were inoculated into aesculin broth and incubated at 37°C for 5 days. Black colour formation indicated a positive test.

2.2.8 *Casein hydrolysis* Cells were inoculated on the C agar medium containing 1% (w/v) skim milk and incubated at 37°C for 5 days. Clear zone surrounded colony indicated tyrosine hydrolysis

2.2.9 *Gelatin hydrolysis* Cells were inoculated onto gelatin medium and incubated at 37°C for 5 days then flooded the surface with 5-10 ml of 30% (v/v) trichloroacetic acid. Clear zone surrounded colony indicated the hydrolysis.

2.2.10 *Methyl red and Voges-Proskauer* Cells were inoculated into MR-VP broth and incubated at 37°C for 5 days. The culture broth was mixed with methyl red reagent, red colour indicated a MR positive test. After adding with 5% α-naphthol solution and 40% KOH solution, strong red colour indicated a VP positive test.

2.2.11 *Starch hydrolysis* Cells were inoculated onto 10% starch agar medium and incubated at 37°C for 5 days, then flooded with Lugol's iodine solution . Clear colourless zone surrounded colony indicated starch hydrolysis.

2.2.12 *Tyrosine hydrolysis* Cells were inoculated onto the C agar medium containing 0.5% (w/v) tyrosine and incubated at 37°C for 5 days. Clear zone surrounded colony indicated tyrosine hydrolysis.

2.2.13 *Deoxyribonuclease (DNase) activity* Cells were inoculated on DNase test agar and incubated at 37°C for 5 days then flooded with 1 N HCl. Clear zone surrounded colony indicated a positive test.

2.2.14 *Indole test* Cells were inoculated into tryptone broth and incubated at 37°C for 5 days. The culture broth was mixed with Kovac's reagent . Red colour indicated a positive test.

2.2.15 *Nitrate reduction* Cells were inoculated into nitrate broth and incubated at 37°C for 5 days, then one drop each of Solution A and Solution B of nitrate reduction test reagents were added. Red colour developed within 5 minutes indicated a positive test.

2.2.16 *Simmon Citrate test* Cells were inoculated into citrate agar slant and incubated at 37 °C for 5 days. Blue colour formation indicated a positive test.

2.2.17 *Triple Sugar Iron agar (TSI)* Cells were inoculated into TSI agar slant and incubated at 37 °C for 5 days. Black colour formation indicated a positive test.

2.2.18 *Anaerobic growth* Cells were inoculated on the C agar medium and incubated at 37°C for 5 days in an anaerobic jar, then the growth was observed.

2.2.19 *Dihydroxyacetone from glycerol* Cell were inoculated on the C agar medium contain 1% glycerol and incubated at 37°C for 5 days, then flooded with Fehling's solution . Yellow colonial appearance indicated a positive test.

2.2.20 *Urease activity* Cells were inoculated onto the C agar slant medium containing urea 2% (w/v). Colour change of the indicator to pink indicated a positive test.

2.2.21 *Acid from carbohydrates* The acid from carbon sources were performed in C broth containing 22 different kinds of carbon sources, 0.5% (w/v), including L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, glycerol, inulin, lactose, *myo*-inositol, maltose, D-mannitol, mannose, melibiose, melezitose, raffinose, rhammose, D-ribose, salicin, sucrose, D-sorbitol, D-trehalose, D-xylose. The media were adjusted to pH 7.2 and phenol red 0.2% solution (w/v) was

added as an indicator. Cells were inoculated into C broth containing each kind of carbon sources and incubated at 37°C for 5 days. The colour of culture broth changed from red to yellow indicated a positive result.

2.3 Chemotaxonomy

2.3.1 *Cell wall analysis* (Komagata and Suzuki, 1987)

Whole-cell hydrolysis ; approximate 3 mg of dried cells were hydrolysed with 1 ml of 6 N HCI in a screw-capped tube at 100°C for 18 hours. After cooling, the hydrolysate was filtered and dried to dryness by a rotary evaporator. The dried material was dissolved in 1 ml of water and repeat drying. The residue was redissolved in 0.3 ml of water and analysed by thin-layer chromatography (TLC). Each samples was applied as 3 µl on the base line of a plastic cellulose TLC plate (Merck No. 5577, E. Merck, Darmstadt, FRG). One µl of 0.01 M DL-diaminopimelic acid (DAP) was applied as reference. TLC was developed with methanol-water-6N HCI-pyridine (80:17.5:1.5:10, v/v) system which last 3 hours or more, then visualized by spraying with 0.2% (w/v) ninhydrin in water-saturated n-butanol followed by heating at 100°C for 5 min. DAP isomers appeared as dark-green spots and the developed spot gradually disappeared in a few hours.

2.3.2 *Quinone analysis* (Komagata and Suzuki, 1987)

Dried cells (100-500 mg) were extracted with chloroform:MeOH (2:1) for a few hours. The suspension was then filtered and dried under rotary evaporator. The dried sample was dissolved in a small amount of acetone and applied onto a silica gel TLC (Merck no.1.05744). The applied TLC was then developed with petroleum ether-diethyl ether system (85: 15, v/v) and the band of menaquinone was detected by using a UV lamp (254 nm). The menaquinone band was scraped and dissolved in HPLC-grade methanol. The suspension was filtered and dried under N_2 gas. The menaquinone sample was analyzed by HPLC employing methanol-isopropanal (4:1) with the µ-BondapakC_{18} column (Water Associates, Milford, Mass., USA).

2.3.3 *DNA base composition* DNA was isolated by the method described by Saito and Miura (1963). Briefly, log phase cells grown in the complex agar medium at 37°C for 1 day were harvested by scraping and suspended in 10 ml of saline-EDTA buffer pH 8.0 . Bacterial cell lysis was induced by 20 mg/ml lysozyme in 0.1 M Tris buffer pH 9.0 containing 10% (w/v) sodium dodecyl sulfate (SDS) at 55°C for 10 min. After cell lysis, the suspension became turbid to opalescent and viscous. Protein was denatured by extracting with 4 ml of phenol-chloroform (1:1 v/v) for 30 sec, and then centrifuged at room temperature, 12,000 rpm (9,200 g) for 10 min. DNA was precipitated by adding cold 95% (v/v) ethanol into supernatant. The DNA precipitate was spooled with a grass-rod, rinsed with 70% then 95% (v/v) ethanol, air dried, then dissolved in 5 ml of 0.1 x SSC. RNase A solution (0.3 ml) was added into the DNA solution, incubated at 37°C for 20 min, then extracted by 2 ml of phenol-chloroform. After centrifugation at room temperature, 12,000 rpm (9,200 g) for 10 min, the upper layer was transferred to new tube. The DNA was precipitated by adding cold 95% (v/v) ethanol and spooled with a grass-rod then rinsed with 70% then 95% (v/v) ethanol. The DNA precipitate was air dried and dissolved in 5 ml of 0.1 x SSC. The purity and quality of DNA were determined from the ratio of an absorbance at 260 and 280 nm (A_{260}/A_{280}) as described by Marmur and Doty (1962).

DNA base composition was analyzed by the method described by Tamaoka and Komagata (1984), DNA was hydrolysed to nucleosides by nuclease P1 (EC 3.1.3.30) and alkaline phosphatase (EC 3.1.3.1). DNA sample (0.5-1.0 g of DNA/litre of distilled water ; OD_{260} = 10-20) was boiled in boiling water bath for 5 min and immediately cooled in ice water. Ten microliter of the DNA was mixed with 10 µl of nuclease P1 solution, incubated at 50°C for 1 hour, and then 10 µl of alkaline phosphatase solution was added and keep at 37°C for 1 hour. DNA base composition of DNA hydrolysate was analyzed by HPLC using conditions as shown in Table 4

Table 4 HPLC conditions for DNA base composition analysis

Detector wave length	270 nm
Column	Nakarai Cosmosil packed column $5C_{18}$ (150x4.6 mm)
Column temperature	Room temperature
Eluent	0.2 M $NH_4H_2PO_4$: acetonitrile (20:1, v/v)
Flow rate	1 ml/min
Sample	5-10 µl

2.4 16S rDNA sequence and phylogenetic analysis

The 16S rRNA gene was PCR amplified using 9F (5'GAGTTTGATCCTGGCTCAG'3, *Escherichia coli* numbering) as forward primer, and 1541R (5'AAGGAGGTGATCCAGCC'3) as reverse primer. Sequence of the amplified product was analyzed by an automated DNA sequencer, ABI PRISM 377 Genetic analyzer (Applied Biosystems) using the following primers : 339F (5'CTCCTACGGGAGGCAGCAG'3),785F (5'GGATTAGATACCCTGGTAGTC'3), 1099F (5'GCAACGAGCGCAACCC'3), 357R (5'CTGCTGCCTCCCGTAG'3) and 802R (5'TACCAGGGTATCTAATCC'3) or primers : 8-27f (5'-AGAGTTTGATC (A/C)TGGCTCAG-3'), 530f (5'-GTGCCAGC(A/C)GCCGCGG-3'), 1114f (5'-GCAACGAGCGCAACCC-3'), 1392r (5'-ACGGGCGGTGTGT(A/G)C-3').

The DNA sequence was multiply aligned by CLUSTAL X program (version 1.83; Thompson *et al.*, 1994), then the alignment was manually verified and edited prior to construction of a phylogenetic tree. The phylogenetic tree was constructed by neighbour-joining method (Saitou and Nei, 1987) in MEGA program version 2.1 (Kumar *et al.*, 2001). The confidence values of branches of the phylogenetic tree were determined by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. The values for sequence similarity among the closest strains were calculated manually after

pairwise alignments obtained using the CLUSTAL X version 1.83. Gap and ambiguous nucleotides were eliminated from the calculations.

3. Effect of pH and temperature on cellulase production

The effect of pH and temperature on cellulase production of the 2 most highest cellulase producing strains, P3-1 and P4-6; and representative strains of high HC value isolates; P2-1, P2-3, P7-7 were examined by cultivating in CMC medium at various pH, or various temperature with shaking (200 rpm) for 2 days. After centrifugation at 10,000 rpm (13,300 g), 4° C for 15 min, the supernatants were analysed for cellulase activity.

4. Effect of pH and temperature on cellulase activity

The effect of pH and temperature on cellulase activity of the 2 most highest cellulase producing strains, P3-1 and P4-6 were examined by culturing in CMC medium at pH 7, 40° C with shaking (200 rpm) for 2 days. After centrifugation at 10,000 rpm (13,300 g), 4° C for 15 min, the supernatants were analysed for cellulase activity (various pH, or various temperature).