CHAPTER III

MATERIALS AND METHODS

Instruments and chemicals; culture media; reagents and buffers; physiological and biochemical characteristics of isolates, primers, 16S rRNA gene sequences and DNA G + C contents

Name list of all instruments and chemicals; culture media; reagents and buffers; physiological and biochemical characteristics of isolates, primers, 16S rRNA gene sequences and DNA G + C contents are shown in Appendix A, B, C and D, respectively.

Methods

3.1 Screening of cellulase and xylanase producing bacteria

3.1.1 Isolation of cellulase producing bacteria

Cellulase producing bacteria were isolated from 40 soil samples collected from Tadan district, Nakhon Nayok province; Nasuan district, Suratthani province; Hadyai district, Songkla province; Puka district, Nan province and 10 biofertilizers, not later than 24 hours after collection by an enrichment culture method. The sample (0.1 g) was put into 10 ml of cellulose powder medium (CPY) 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 CPY medium and incubated at the same above conditions for 2 more times. The enriched cultures or their dilutions (0.1 ml) were spreaded on CPY 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). The 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 clear zone were selected as cellulase producing isolates and then they were purified by streak plate method. Colony diameter and clear zone diameter of the purified cultures were measured. Hydrolysis capacity (HC) value was calculated from clear zone diameter divided by colony diameter.

3.1.2 Quantitative cellulase producing assay

Single colony of the 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 above 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 method described by Ghose (1987). Reaction mixture composed of 0.5 ml of 2% (w/v) carboxymethyl cellulose (CMC) for endoglucanase activity (CMCase) or 0.4% (w/v) of salicin for ß-glucosidase activity in 100 mM sodium phosphate buffer pH 7.0 , and 0.5 ml of the crude enzyme were incubated at 40°C for 30 min. The amount of reducing sugar released was quantified by Somogyi and Nelson method (Nelson, 1944; Somogyi, 1952) using glucose as authentic sugar. The reaction stopped immediately after addition of enzyme solution was used as a reaction blank. One unit of cellulase was defined as an amount of the enzyme yielding 1 micromole of glucose equivalent within 1 min under the assay conditions.

3.1.3 Isolation of xylanase producing bacteria

Xylanase producing bacteria were isolated from 45 soil samples collected from Nangrong and Tadan district, Nakhon Nayok province and Puka district, Nan province by the same method as described in 3.1.1, except xylan medium (XPY) was used instead of CPY and CMC-basal medium.

3.1.4 Quantitative xylanase producing assay

Xylanase production of the bacteria isolated was quantitatively determined by the same method as described in 3.1.2, except the bacteria were grown in xylan medium instead of CMC medium and oat splet xylan was used as substrate for enzyme activity assay instead of carboxymethyl cellulose. One unit of xylanase was defined as an amount of the enzyme yielding 1 micromole of xylose equivalent within 1 min under the assay conditions.

3.2 Identification methods

3.2.1 Cell morphology and cultural characteristics

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

3.2.1.1 *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.

3.2.1.2 *Flagella staining* Standard microscopic slide precleaned by the manufacturer was used. The slide was 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 dropped to this area and gently mixed with cells. 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. There was no visible opalescence. Staining method described by Forbes (1981) was performed using 1 ml of staining solution at ambient temperature for 1 min. Then the slide was washed in tap water, counterstained with Hucker modified Gram crystal violet for 1 min, washed, blot dried, and examined under oil immersion starting from near to the wax line.

3.2.2 Physiological and biochemical characteristics

3.2.2.1 *Oxidase test* A few drops of 1% (w/v) N,N,N',N'-Tetramethyl-1,4-phenylenediamine dihydrochloride 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 color on paper within 30 sec indicated a positive reaction.

3.2.2.2 *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.

3.2.2.3 *Growth at different temperature* Cells were inoculated on the CMC or xylan 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.

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

3.2.2.5 *Growth in different NaCl concentration* Cells were inoculated on the PY agar medium containing 3 and 5% (w/v) NaCl and incubated at 37 °C for 5 days, then the growth was examined.

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

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

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

3.2.2.9 *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.

3.2.2.10 *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 color developed indicated a MR positive test. After adding with 5% (v/v) α-naphthol solution and 40% (v/v) KOH solution, strong red color developed indicated a VP positive test.

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

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

3.2.2.13 *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.

3.2.2.14 *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 color developed indicated a positive test.

3.2.2.15 *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 color developed within 5 minutes indicated a positive test.

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

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

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

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

3.2.2.20 *Acid from carbohydrates* The acid from carbon sources were performed in PY broth containing each of 22 different kinds of carbon sources tested at 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 0.2% (w/v) phenol red was added as an indicator. Cells were inoculated into PY broth containing each kind of carbon sources and incubated at 37°C for 5 days. The color of culture broth changed from orange to yellow indicated a positive result.

3.2.3 Chemotaxonomic characterization

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

Dried cells approximate 3 mg were hydrolysed with 6 N HCl (1 ml) 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 distilled water (1 ml) and repeated drying. The dry residue was redissolved in distilled water (0.3 ml) and analysed by thin-layer chromatography (TLC). Each samples (3 µl) was applied on the base line of a plastic cellulose TLC plate (Merck No. 5577, E. Merck, Darmstadt, FRG). One microliter of 0.01 M DL-diaminopimelic acid (DAP) was applied as reference. TLC was developed with methanol-water-6N HCl-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.

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

Dried cells (100-500 mg) were extracted with chloroform: MeOH (2:1) for a few hours, 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, E. Merck, Darmstadt, FRG). The applied TLC was then developed with petroleum ether-diethyl ether system (85: 15, v/v) and menaquinone band was visualized by UV (254 nm) radiation. 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).

3.2.4 DNA base composition

DNA was isolated by the method described by Saito and Miura (1963). Log phase cells grown on CPY or XPY 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 addition of lysozyme at final concentration of 20 mg/ml in 0.1 M Tris buffer pH 9.0 containing 10% (w/v) sodium dodecyl sulfate (SDS) and incubation at 55°C for 10 min. After cell lysis, the cell 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 glass 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 glass 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 quantity 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) which the 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 solution 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 kept at 37°C for 1 hour. DNA base composition of DNA hydrolysate was analyzed by HPLC using conditions as shown in Table 3.1

Table 3.1 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

3.2.5 16S rRNA gene 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, USA) using the following primers : 357R (5'CTGCTGCCTCCCGTAG'3), 802R (5'TACCAGGGTATCTAATCC'3), 1115R (5'AGGGTTGCGCTCGTTG'3) and 1541R (5'AAGGAGGTGATCCAGCC'3) The DNA sequence obtained was multiply aligned by CLUSTAL X program (version 1.83; Thompson *et al.*, 1997), then the alignment was manually verified and edited prior to construction of a phylogenetic tree. The phylogenetic tree was constructed by neighbor-joining method (Saitou and Nei, 1987) in MEGA program version 4.0 (Tamura *et al.*, 2007). The confidence value 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 MEGA program version 4.0 (Tamura *et al.*, 2007) Gap and ambiguous nucleotides were eliminated from the calculations.

3.3 Effect of pH and temperature on enzyme activity

Cellulase :

Highest endoglucanase and ß-glucosidase producing strains, highest cellulase hydrolysis capacity strain and strain produced clear zone on cellulose powder medium were chosen for this study.

Xylanase :

Highest xylanase producing strains from different phenotypic groups and highest xylan hydrolysis capacity strain were chosen for this study.

An effect of pH and temperature on cellulase and xylanase activities of the chosen strains were examined by culturing in CMC or xylan medium at 200 rpm, 40° C, pH 7 for 2 days. After centrifugation at 10,000 rpm (13,300 g), 4° C for 15 min, the supernatants were analysed for cellulase and xylanase activity at various pH and temperatures.

3.4 Filter paper degradation

The above chosen cellulase producing bacteria were orthogonal mixed and cultured in the modified CMC-basal medium which 1 x 6 cm strip of filter paper (Whatman No.1) was used as carbon source instead of carboxymethyl cellulose. The mixed culture was incubated with shaking (200 rpm) at 40°C. After 1 month of cultivation, filter paper was gravimetrically determined (Updegraff, 1969), reducing sugar liberated, endoglucanase and ß-glucosidase activity in culture filtrate were analysed.