MATERIALS AND METHODS

1. Microorganism

Bacillus subtilis GN156 was isolated and kept at Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Thailand.

2. Plant material

Napier grass (*Pennisetum purpureum*) was used as an inducer and to study synergistic effects, had an approximate age of 40 days and a height of 0.75 – 1 m. It had a moisture content of 60-65 % and was cut 5 inches above ground. After wilting for 2 days, the grass was chopped and milled to the size of 1 mm with CyclotecTM 1093 Mill (Foss, Denmark).

3. Growth determination

A colony of *B. subtilis* GN156 was grown in 5 ml of NB medium under aerobic conditions at 150 rpm for 18-20 h at 37°C. One percent (v/v) of innoculum was transferred into 100 ml of NB, NB with 1 % (w/v) grass (NBG) and NB with 1 % (w/v) CMC (NBC). The cultivations were continued under aerobic conditions at 150 rpm and at 37°C. The samples were taken every 2 h for 24 h to analyze cell number by viable plate count and β-1,3-1,4-glucanase activity.

4. Enzyme production

A colony of *B. subtilis* GN156 was grown in 5 ml of NB medium under aerobic conditions at 150 rpm for 18-20 h at 37°C. One percent (v/v) of innoculum was transferred into 100 ml of NB, which contained 1% (w/v) CMC, in a 250 ml flask. After 24 h of incubation, the culture was centrifuged at 4°C, 11,000 g for 15 min and the supernatant was stored at -20°C for further study.

5. Determination of enzyme activities

5.1 β-1,3-1,4-glucanase activity

β-1,3-1,4-glucanase activity was determined by the modified method of Okeke and Obi (1995) using reaction mixtures containing of 0.1 ml of sample and 0.1 ml of 1 % (w/v) barley β-glucan (Sigma) in 50 mM citrate phosphate buffer pH 5.5 at $50^{O}C$ for 20 min. The reaction was stopped by placing in boiling water for ten min. The amount of reducing sugar released was determined by the Dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of enzyme was defined as the amount of enzyme that released 1 μmol of glucose per min.

5.2 Carboxymethyl cellulase activity

Carboxymethyl cellulase (CMCase) activity was determined using the same procedure as β-1,3-1,4-glucanase activity. But 1 % (w/v) of carboxymethyl cellulose (Sigma) was used as substrate instead. One unit of CMCase was defined as mentioned above.

5.3 Dextrinase activity

Dextrinase activity was determined using the same procedure as β-1,3-1,4-glucanase activity. But 1 % (w/v) of dextrin (Sigma) was used as substrate instead. One unit of dextrinase was defined as mentioned above.

5.4 Xylanase activity

Xylanase activity was measured by the same method as β-1,3-1,4-glucanase activity, but 1% oat spelt xylan (Sigma) was used as substrate instead. One unit of enzyme was defined as the amount of xylanase that released reducing sugar equivalent to 1 μmol of xylose per min.

5.5 Pectin esterase activity

Pectin esterase activity was measured using the modification method of Huang and Mahony (1999) by titration of carboxyl groups released from pectin (Sigma) at 50°C for 20 min. 0.5 ml enzyme was mixed with 4.5 ml, 0.5% pectin in 50 mM cirtrate phosphate buffer pH 5.5. The acid released was titrated with 0.01 N NaOH. One unit of pectin esterase was defined as the amount of enzyme which released 1 μmole of carboxyl groups per min under the assay conditions.

5.6 Pectin lyase activity

Pectin lyase activitiy was determined by measuring the increase in A_{235} of 4-5 unsaturated trans-elimination products of pectin. Modification method of Huang and Mahony (1999), 0.1 ml enzyme was mixed with 0.4 ml, 0.5 % pectin in 50mM cirtrate phosphate buffer pH 5.5, incubated at $50^{O}C$ for 20 min. 4 ml, 0.01 M HCl 4 ml was added and absorbance was measured at 235 nm. One unit of pectin lyase was defined as the amount of enzyme which released unsaturated trans-elimination products showing absorbance of 0.2 at 235 nm.

5.7 Pectate lyase activitiy

Pectate lyase activitiy was determined by the same method as pectin lyase activity, except that 0.5% polygalacturonic acid (PGA) (Sigma) was used as substrate. One unit of pectate lyase was defined as the amount of enzyme which released unsaturated trans-elimination products showing absorbance of 0.2 at 235 nm.

5.8 Polygalacturonase activity

Polygalacturonase activity was measured by using two different methods.

5.8.1 Polygalacturonase activity against pectin was measured the same method as described of pectate lyase activity assay, but 1% pectin was used as a

substrate instead. One unit of polygalacturonase activity was measured and defined as the amount of enzyme that released galactose equivalent to 1 μmol of galacturonic acid per min.

5.8.2 Polygalacturonase activity against PGA was measured by the same method as described of β-1,3-1,4-glucanase activity assay, but 1% PGA was used as substrate instead. One unit of polygalacturonase activity was defined as the amount of enzyme that releases reducing sugar equivalent to 1 μmol of galacturonic acid per min.

6. Protein determination

Protein concentration was determined by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

7. Enzyme purification

7.1 pervaporation

The concentrated enzyme was prepared by slow pervaporation at 37°C or 55°C with modified method of Chenoweth Laboratory, University of Massachusetts, Amherst, USA. One hundred ml cell free supernatant in dialysis tube was hanged overnight until the volume reduced to 12.5 ml.

7.2 Gel filtration chromatography

Sephacryl S-200 (2 x 37.5 cm) was equilibrated with 50 mM citrate phosphate buffer pH 5.5, with flow rate 14.6 ml /h, calibrated with blue dextran and a standard protein calibration kit for gel filtration (Amersham Bioscience, Sweden). To purify β-1,3-1,4-glucanase from *B. subtilis* GN156, the 8-fold concentrated enzyme from pervaporation was loaded onto the Sephacryl S-200 column and 6.68 ml

fractions were collected and assayed for β-1,3-1,4-glucanase activity as described in 5.1. Protein concentration was followed by absorbance measurements at 280 nm.

The other resin Sepharose 6B (2.5 x 32 cm) was used and the purification was performed as described above, except for a different flow rate of 25 ml/h.

7.3 Affinity chromatography

Purification of β-1,3-1,4-glucanase using affinity chromatography was performed by coupling Epoxy – activated Sepharose 6B (Bio-Rad, USA) with barley β-glucan according to instructions of the manufacturer (Bio-Rad, USA) and equilibrated with 5 mM citrate buffer pH 3.0 with flow rate of 4 ml/min. Fifty ml of cell – free culture supernatant, which was dialyzed against 5 mM citrate pH 3.0 overnight, was loaded to β-glucan coupled Epoxy – activated Sepharose (2.5 x 10 cm) and unbound protein was washed with two beds volumes of buffer. Fractions were collected and eluted with a linear gradient of 0 – 0.25 M sodium chloride in 5 mM citrate buffer. Active fractions were pooled and dialyzed against 5 mM citrate buffer pH 6.0.

The β-1,3-1,4-glucanase activity was assayed as described in 5.1. Protein concentration was followed by absorbance measurements at 280 nm and the method of Lowry *et al*. (1951) used with bovine serum albumin as a standard.

8. Molecular weight determination by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE)

Molecular weight of the purified enzyme was determined by SDS – PAGE according to Laemnli (1970) using 10 % acrylamide in separating gel (as described in Appendix A) and stained with silver stain plus kit (Bio-Rad, USA). The pre-stained marker (Bio-Rad, USA) used were: myosin (195,755), β-galcctosidase (107,181), bovine serum albumin (59,299), ovalbumin (41,220), carbonic anhydrase (27,578), soybean trypsin inhibitor (20,514), lysozyme (15,189) amd aprotinin (6,458).

9. Native - polyacrylamide gel electrophoresis (Native – PAGE)

The native purified enzyme was determined by 7.5 % acrylamide in separating gel (as described in Appendix A) and stained with a silver stain plus kit (Bio-Rad, USA). The high molecular weight native marker kit (Amersham Bioscience, Sweden) used consisted of thyroglobulin (669,000), ferritin (440,000), catalase (232,000), lactate dehydrogenase (140,000) and albumin (66,000).

10. Isoelectric point

Isoelectricfocusing was performed on Ampholine^{TM} PAGplate pH 3.5-9.5 (Amersham Biosciences, Sweden) according to instructions of the manufacturer. The broad range pI calibration kits (Amersham Bioscienve, Sweden) used were: amyloglucosidase (pI 3.50), methyl red dye (pI 3.75), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.20), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.55), horse myoglobin - acetic band (pI 6.85), horse myoglobin - basic band (7.35), lentil lectin - acidic band (pI 8.15), lentil lectin - middle band (pI 8.45) lentil - basic band (pI 8.65) and trypsinogen (pI 9.30).

11. Optimum pH and pH stability

The optimum pH of the enzyme activity was determined with modification of reaction mixture containing 100 µl of the enzyme solution and 100 µl of 1 % (w/v) β-glucan in various pH buffers in the range of 3.0 – 9.0, the reactions were incubated at 50°C for 20 min.

The effect of pH on crude enzyme stability was assayed by measuring the remaining activity after incubating the enzyme solution in the buffers that had their pH range of 3.0 – 9.0 for 24 h at 4°C. After that the enzyme pH was brought back to pH 6. The enzyme activity was assay at pH 6 and 50°C for 20 min.

The effect of pH on the purified enzymes stability were followed by above method but the enzyme solutions in the buffers that had their pH range of pH 3.0 – 9.0 were incubate at 4°C for 2 h instead.

12. Optimum temperature and temperature stability

The optimum temperature of the enzyme activity was determined by measuring its activity at various temperatures for 20 min.

The effect of temperature on enzyme stability was determined by measuring the remaining activity after incubating the enzyme in optimum pH buffer at various temperatures for 30 min. Reaction of the enzyme was performed at 50°C for 20 min.

13. Determination of K_m and V_{max}

These reactions were performed with various concentration of substrate from 0.1-1 % (w/v) under the optimum conditions and followed their activities every 5 min for 0-30 min. K_m and V_{max} were determined from Lineweaver – Burk plots.

14. Effect of metal ions

The effect of various metal ion especially cations, Cu^{2+}, Ca^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and Li^{+} on the enzyme activity was determined by measuring the activity of the enzyme in the presence of 10 mM of each cation in the reaction mixture using 50 mM citrate buffer pH 6.0 under the optimum conditions.

15. Substrate specificity

The activity of β-1,3-1,4-glucanase ons various substrates 1% (w/w): barley β-glucan, CMC, xylan, laminarin, dextrin and chitin was determined as described in 5.1 under the optimum conditions.

16. Synergistic effect of enzymes on substrate degradation

16.1 Barley β-glucan degradation

The synergistic effect of β-1,3-1,4-glucanases from *B. subtilis* GN 156 to barley β-glucan degradation was investigated by the mixture reaction of 0.6 ml, 0.06 U/ml of different enzyme sources in the ratio as shown in Table 10 and 0.6 ml of 1 % β-glucan in 50 mM citrate buffer pH 6.0. The experiment was performed in triplicate and the reaction mixtures were incubated at 50°C, samples were collected at 0, 1, 6, 12 and 24 h and the reaction mixture was stopped in boiling water for 10 min. The amount of reducing sugar released was determined by the dinitrosalicylic acid (DNS) method (Miller, 1959).

Table 11 Ratio of the enzyme combination added to substrates in synergism study.

Enzyme	Volume of enzyme added (ml)		
	C	J1	J2
C	0.6	-	-
J1	0.6	-	-
pJ2	0.6	-	-
C + J1	0.3	0.3	-
C + pJ2	0.3	-	0.3

C: crude enzyme, J1: the purified J1, pJ2: the partial purified J2

16.2 Grass degradation

Synergistic effect of β-1,3-1,4-glucanases from *B. subtilis* GN 156 to grass degradation was investigated by the same method of β-1,3-1,4-glucanase activity assay, except that 1% grass powder used as a substrate, as described in 2. Samples were collected at 0, 1, 6, 24 and 48 h, centrifuged at 4°C, 11,000 g for 15 min and the reaction were stopped in boiling water for 10 min. The amount of reducing sugar released was determined by DNS method (Miller, 1959).

17. Thin-layer chromatography (TLC) analysis

The action pattern of β-1,3-1,4-glucanases to barley β-glucan hydrolysis was determined by the modified method of Akiyama, *et al.* (1996) by thin-layer chromatography (TLC) using glucose and cellooligosaccharides C_2 - C_5 (Sigma) as standards. Two μl of the degradation products, containing 2 μg/μl reducing sugar, were applied on Kieselgel 60 (Merck) and developed for 90 min in mixture solvent of butanol : isopropanol : ethanol : deionized water in the ratio of 2 : 3 : 3 : 2, respectively. The brown spots of sugars were developed by dipping in 0.2 % (w/v) orcinol in 10 % (v/v) sulfuric in ethanol and further placing in 100°C for 15 min.

18. Place

All of experiments were carried out in the Department of Biotechnology, Faculty of Agro – Industry, Kasetsart University, Thailand and the Department of Food Science, Chenoweth Laboratory, University of Massachusetts, Amherst, USA.

19. Duration

The experiments were carried out from March 2001 to February 2006.