RESULTS AND DISCUSSION

1. Enzyme system of *Bacillus subtilis* GN156

To investigate *Bacillus subtilis* GN156 hydrolytic action, the activities against some of polysaccharides, and disaccharides substances were determined. The results showed the highest activity of 4.61 U/ml on barley β-glucan (Table 12), while the activities on xylan, dextrin, CMC and cellobiose were 0.56, 0.57, 0.05 and 0.05 U/ml, respectively. However, no activity was found against laminarin. Considering pectic substance degradation, it showed no activities of pectin esterase, pectin lyase and pectate lyase, only low activities of polygalacturonase against pectin and polygalacturonase against PGA of 0.15 and 0.14 U/ml were detected, respectively.

Table 12 Activity of enzyme from *Bacillus subtilis* GN156 on various substrates

Enzymes	Activity (U/ml)	Relative activity (%)
β-1,3-1,4-glucanase	4.6138	100
CMCase	0.0536	1.16
Xylanase	0.5642	12.23
Laminarinase	0	0
Dextrinase	0.5728	12.41
Cellobiase	0.0532	1.15
Pectin esterase	0	0
Pectin lyase	0	0
Pectate lyase	0	0
Polygalacturonase against pectin	0.1469	3.18
Polygalacturonase against PGA	0.1379	2.99

The cell - free culture fluid (CFC) showed high activity to β-glucan which contained both β-1,3 and β-1,4 glycosidic linkage, while low activity to β-1,4 glycosidic linkage in CMC or no activity against β-1,3 glycosidic linkage in larminarin. Thus, it could be concluded that the CFC of this strain was specific to only β-1,4 glycosidic linkages in β-glucan containing both 1,3 and 1,4 linkages, but not to the hydrolysis of β-glucan containing only 1,3 or 1,4 linkage. This characteristic was defined as the action of β-1,3-1,4-glucanase (Dixon and Webb, 1979). Hence, degradation of barley β-glucan by the enzyme of *B. subtilis* GN156 was an action of β-1,3-1,4-glucanase, but there was no synergistic effect of 1,4 and 1,3 glycolysis.

Many bacterial enzymes have shown both β-1,3-1,4-glucanase and β-1,3-glucanase activities, for example, recombinant *E. coli* JM101 (Louw *et al.*, 1993), recombinant *Streptococcus bovis* JB1 (Ekinci *et al.*, 1997), *Bacillus* sp. BE1, *Bacillus* sp. FE1, *Pseudomonas* PE1 and *Pseudomonas* PE2 (Kitamura *et al.*, 2002). But *B. subtilis* GN156 hydrolyzed only β-1,4 linkages which may leave the structure of β-1,3 linkages.

Among the hydrolytic enzymes of *B. subtilis* GN156, β-1,3-1,4-glucanase was the most effective enzyme, while both xylanase and dextrinase were only 12 % relative activity compared to β-1,3-1,4-glucanase. These results showed low of the hydrolytic action of the enzyme to β-1,4-linked xylose and α-1,4-linked glucan. Moreover, the enzyme showed low activities to both CMC and cellobiose (1 % of relative activity) which composed of β-1,4-linked glucan. It could be concluded that the hydrolytic enzymes of *B. subtilis* GN156 were very specific to mixed linkage of β-1,3-and β-1,4-glucan but low hydrolytic action to both polymer (CMC) and dimer (cellobiose) of only β-1,4-linked glucan substrates. Furthermore, polygalacturonase activities against both pectin and PGA were not significant that showed the enzyme could depolymerize both pectin and PGA only 3 % of relative activity.

In this study, β-1,3-1,4-glucanase is classified in hemicellulase according to the substrate on which it acts (Bastawde, 1992, Brett and Waldron, 1996, Bayer *et al.*, 2004). Thus, the enzyme system of *B. subtilis* GN156 could be classified to 4 groups of hemicellulase (β-1,3-1,4-glucanase and xylanse), cellulase (CMCase and cellobiase), amylolytic enzyme (dextrinase) and pectinase (polygalacturonase) that show their activities corresponds to the tropical grass components ratio, which comprised 30-40 % hemicellulose, 1-5 % starch and 1-2 % pectin (Stetälä, 1989). Considering cellulose, which is one of the major component in tropical grass and found as much as 30-40 %, cellulases were low in the enzyme system of *B. subtilis* GN156. Therefore, to apply enzyme from *B. subtilis* GN156 for grass degradation, other source with high cellulase activity may be needed.

2. Growth and β-1,3-1,4-glucanase production profile

Growth of *B. subtilis* GN156 in NB medium without the other carbon sources (NB), NB with grass (NBG) and NB with CMC (NBC) were performed. The samples were taken every 2 h for 24 h to analyze cell number and β-1,3-1,4-glucanase activity. The results are shown in Figure 16. Cell growth in all treatments reached stationary phase after 4 h. However, number of viable cells from the NB treatment started to decline during 12-24 h while that from both NBG and NBC still continued during 24 h and reached 9.4×10^9 and 3.5×10^9 cfu/g at 24 h, respectively.

β-1,3-1,4-glucanase activity of 0.1-0.39 U/ml could be detected at 6 h when the growth reached stationary phase. Stulke *et al.*(1993) reported that β-1,3-1,4-glucanase is expressed when cells entered stationary stage in response to nutrient limitation. This concept was supported by the study of Tang *et al.* (2004).

The level of β-1,3-1,4-glucanase activity in the NBC cell-free culture fluid at 12, 16, 20 and 24 h were 1.78, 5.12, 5.78 and 5.96 U/ml, while the activity from NBG was 0.79, 2.91, 5.64 and 6.48 U/ml, respectively. The control exhibited only low activities of 0.66 – 0.88 U/ml. It was found that β-1,3-1,4-glucanase was induced by

both CMC and grass. It was noticed that β-1,3-1,4-glucanase production from NBC was faster than from NBG during 12-16 h. However, grass induction treatment reached maximum production of 6.48 U/ml after 24 h. Both CMC and grass induced higher β-1,3-1,4-glucanase activity than the control for 6.73 and 7.36 folds, respectively. Therefore, this would be a potential use to increase grass degradation in the beginning of the silage fermentation.

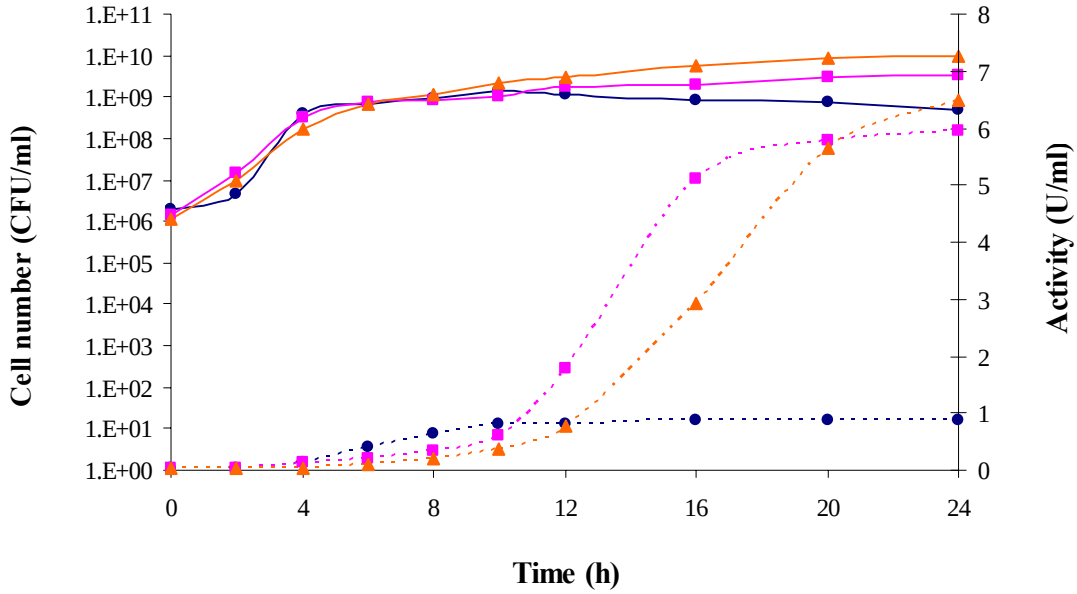

Figure 16 Effect of various inducers to growth and β-1,3-1,4-glucanase production. ♦ Control, ■ CMC, ▲ Grass, —— Cell number and ----- Activity.

3. Effect of inducers to β-1,4-1,3-glucanase production

Polysaccharides of CMC, pectin and xylan introduced individually to nutrient broth were studied for the induction of β-1,3-1,4-glucanase. The results are shown in Figure 17. CMC and pectin supplements induced high β-1,3-1,4-glucanase activity of 8.5 and 7.6 U/ml which were 47 and 41 folds higher than the control, respectively while xylan was also induced the production, though only increased 10.8-fold. El-Helow and El-Ahawany (1999) have studied 5 inducers, galactomannan, lichenan,

pectin, starch and xylan, the results showed that supplementation with pectin enhanced maximum β-1,3-1,4-glucanase production as well. Regarding to these results, not only pectin but also CMC was the effective inducer for the β-1,3-1,4-glucanase production.

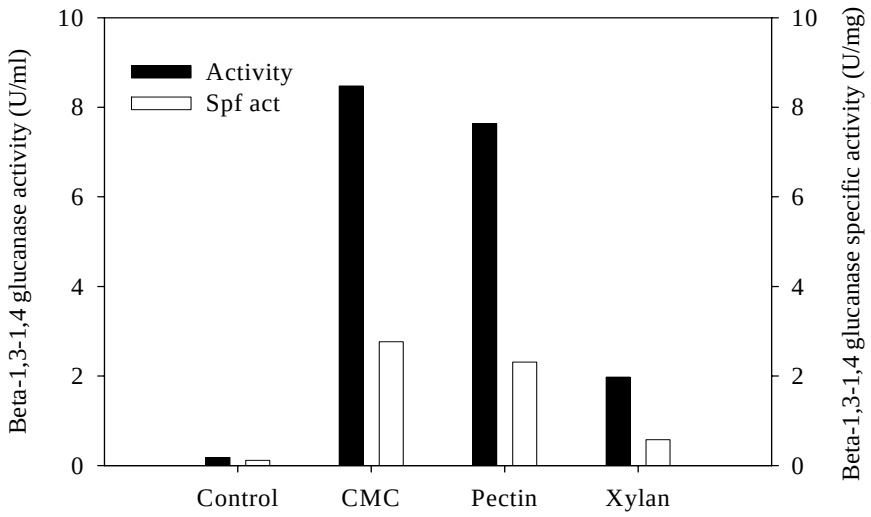

Figure 17 Effect of inducers on the β-1,4-1,3-glucanase production by *B. subtilis* GN156.

To investigate the effect of CMC concentration on β-1,3-1,4-glucanase production, the amount of CMC was varied from 0-1 g in 100 ml nutrient broth. The results showed that the activity of 2.87, 4.54, 5.19 and 5.82 U/ml increased with increasing CMC concentrations 0.2, 0.4, 0.6 and 0.8 %, respectively (Figure 18). However, the activity was lowered when the CMC concentration was 1 % but the specific activities were comparable to that with 0.8 % CMC. It was clearly shown that β-1,3-1,4-glucanase production by *B. subtilis* GN156 depended on the amount of CMC. The concentration of 0.8 % (w/v) CMC provided the highest activity.

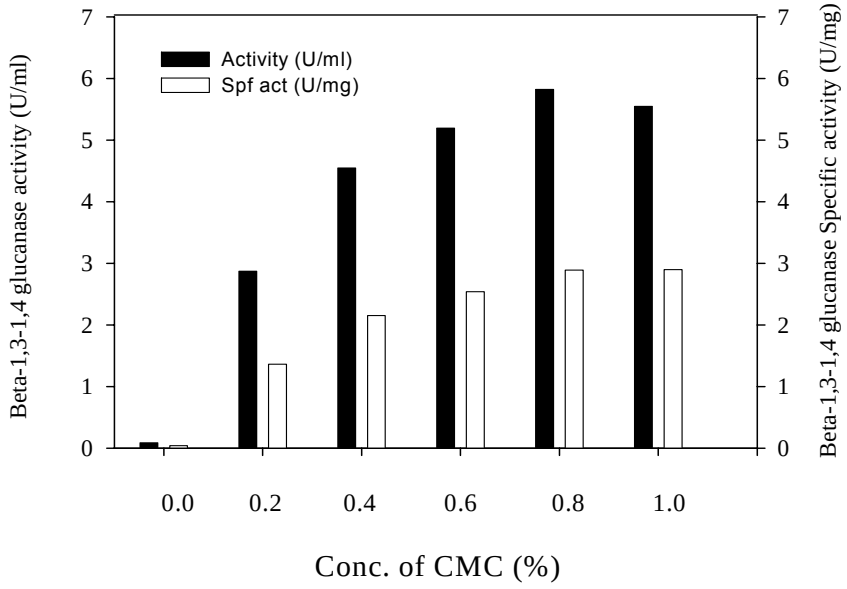

Figure 18 Effect of CMC concentration on β-1,3-1,4-glucanase production by *B. subtilis* GN156.

4. Characterization of β-1,3-1,4-glucanase

The high β-1,3-1,4-glucanase activity played a role in β-1,3-1,4-glucan hydrolysis which is a major hemicellulose in grass (Brett and Waldron, 1996). Therefore, its physical and chemical properties of pH and temperature were more interesting to study.

4.1 pH effect on β-1,3-1,4-glucanase

The pH optima of the crude β-1,3-1,4-glucanase was determined by conducting the activity assays on β-glucan at 50° C at various pH values of 3-12. The results are shown in Figure 19. The activity curve showed an optimal pH of 7 with a secondary inflection in the pH range of 8-9. Moscatelli *et al*. (1961) has found similar results of 2 inflections in the curve from *B. subtilis* at pH 6.5-6.6 and 7.1-7.3. Higher optimal pH of 8-10 was observed in crude enzymes of *Bacillus brevis* (Louw *et al.*, 1993), of recombinant *Escherichia coli* and of *Clostridium*

thermocellum (Schimming *et al*., 1991). It seems that β-glucanase from *Bacillus* sp. strains including *B. subtilis* GN156 showed optimal pH at neutral to alkaline pH with secondary inflection shifting to alkaline pH. No activity could be detected at the extreme pH values of 3 and 12.

Considering pH stability, it was found that β-1,3-1,4-glucanase was stable at various pH values from 3-11 at 4°C for 24 h. However, at a high pH of 12, β-1,3-1,4-glucanase activity was completely denatured.

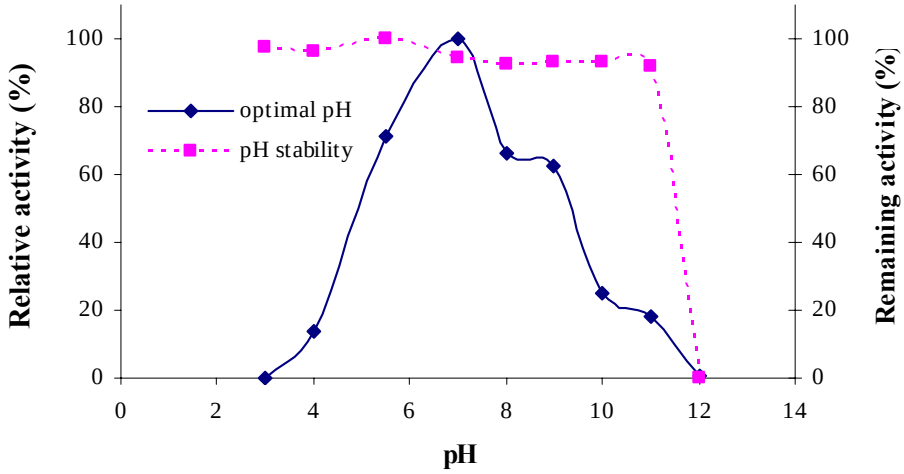

Figure 19 The pH effect on β-1,3-1,4-glucanase activity from crude enzyme of *B. subtilis* GN156 was carried out at 50°C for 20 min. (50 mM citrate phosphate buffer pH 3-5.5, 50 mM phosphate buffer pH 7-9, 50 mM glycine-NaOH buffer pH 10-12). The pH stability of the enzyme was treated at various pH at 4°C for 24 h. Remaining activity was performed at pH 6, 50°C for 20 min.

4.2 The effect of temperature on β-1,3-1,4-glucanase

The optimum temperature for the crude β-1,3-1,4-glucanase was observed by incubating at temperatures of 20-90° C. The optimum temperature was 60°C as shown in Figure 20. On either side of this optimum temperature, the activity declined

sharply only 27 % and 3 % of activity remaining at 20°C and 50°C, respectively. Considering its stability at 1 h, β-1,3-1,4-glucanase was stable at 20-50°C, while the enzyme was inhibited at higher temperature of 60°C and almost completely denatured at 70-90° C. Moscatelli *et al.* (1961) found that β-1,3-1,4-glucanase from *B. subtilis* had an optimum temperature at 50-60°C but it showed a significant loss of activity in 5 min at 60°C. However, Louw *et al.* (1993) reported a thermostable β-1,3-1,4-glucanase from *Bacillus brevis* with an optimum temperature at 65-70°C, while the crude enzyme from recombinant *Escherichia coli* containing the β-1,3-1,4-glucanase gene from *Clostridium thermocellum* showed the highest activity at 80°C (Schimming *et al*., 1991). It is clearly concluded that β-1,3-1,4-glucanase from *B. subtilis* GN156 is not thermostable.

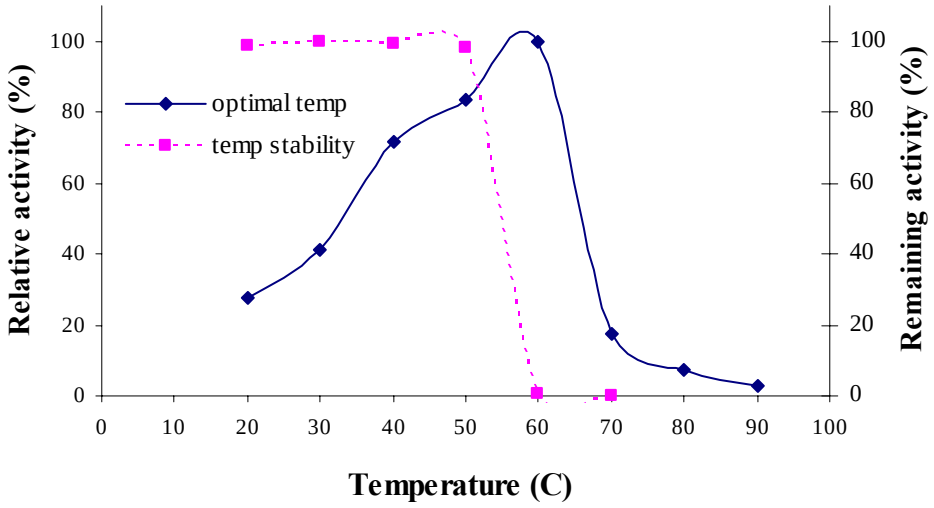

Figure 20 The effect of temperature on β-1,3-1,4-glucanase activity from crude enzyme of *B. subtilis* GN156 was determining in 50 mM citrate phosphate buffer pH 5.5. Temperature stability of the enzyme was treated at various temperatures for 1 h. The remaining activity was performed at pH 6, 50°C for 20 min.

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