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Characterization of Grass Degrading Bacteria Active on β-1,3-1,4-D-glucans from *Bacillus subtilis* GN156 Potential Use for Grass Silage-Making

Jirawan Apiraksakorn, Tonglian Buwjoom and Sunee Nitisinprasert

ABSTRACT

One hundred and sixty-one bacterial isolates were screened for (i) the stability of CM-cellulase at high temperature of 60°C as primary screening, (ii) the stability of pH and temperature of 3-7 and 30-60°C, respectively and (iii) the activities of pH and temperature range following stability study. The isolate GN156 showed high stability of CM-cellulase activity at the pH and temperature of 3.7 - 7.2 and 30 – 70°C, respectively. Based on physical and biochemical properties, this isolate was identified as *Bacillus subtilis*. The enzyme system study revealed various hydrolytic enzymes of CM-cellulase, dextrinase, cellobiase, xylanase, polygalacturonase, polymethylgalacturonase, but, β-1,3-1,4-glucanase was the most effective enzyme. Therefore, optimum pH and temperature of β-1,3-1,4-glucanase were further studied. Interestingly, its activities appeared at wide range of pH and temperature of 5.5-9 and 40-60°C, respectively. The profile of growth and enzyme production indicated that β-1,3-1,4-glucanase produced by *B. subtilis* GN156 was associated with cell growth. Induction of β-1,3-1,4-glucanase production by 1% of CM-cellulose, pectin and xylan revealed an increment of activities of 47, 41 and 11-folds, respectively. When various concentrations of CMC were taken into account, the CMC concentration of 0.8% (w/v) provided the maximum β-1,3-1,4-glucanase production.

Key words: *Bacillus subtilis*, β-1,3-1,4-glucanase, silage, hydrolytic enzyme

INTRODUCTION

Since dairy product and premium meat quality demand have been increased, livestock farming in Thailand was therefore growing rapidly. However, lacking of grass roughage in dry period is a big problem. Thus, both good quality and large amount of grass commonly used as roughage are needed. Therefore, grass storing in a form of silage has been proposed to be benefit in both preservation and nutritional improvement.

Silage is a fermented feed under anaerobic condition. During ensiling, water–soluble carbohydrates (WSC) in the crop is consumed by lactic acid bacteria to produce lactic acid and some acetic acid that causes the pH of ensiled material decreased. Consequently, spoilage microorganisms in silage are also inhibited (Henderson, 1993). The major problem of grass ensiling in Thailand is lack of sufficient amount of suitable WSC. A shortage of sugar causes poor fermentation due to lactic acid reduction, increase in pH, ammonia and also other fermentation products (Stetälä, 1988). Generally, the tropical

Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand.
* Corresponding author, e-mail: fagisnn@ku.a.c.th

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grass contains mainly cellulose, hemicellulose and other carbohydrates which can not be metabolized by lactic acid bacteria. To improve fermentability, it is necessary to breakdown carbohydrates into soluble and consumable form. Addition of enzymes to forage is becoming more common for the purpose of enhancing the WSC in the ensiling process (Stefanie *et al.*, 2000). Application of hydrolytic enzymes from fungi have been previously studied. Jaakkola (1990) found that addition of enzyme mixture of cellulase, hemicellulase and glucosidase from different fungal sources did not affect cell wall degradation. However, when more cellulase concentration was added, the degadation activity increased. In contrast, commercial hydrolytic enzymes from *Acremonium* sp. applied to various varities of grass in Thailand did not improve silage quality (Ohmomo, 1995). It is possible that the proper enzyme mixture has the potential to improve degradation activity.

Therefore, the objective of this study was to isolate the effective hydrolytic enzymes producing bacteria and to characterize the physical and chemical properties of interesting enzyme being important to grass degradation.

MATERIALS AND METHODS

Sample sources

The samples for screening of hydrolytic enzyme producing microorganisms obtained from various sources in North, Northeast and the Middle of Thailand were grass, silages, soil and cow feces,

Screening technique

A 10 % (w/v) sample in normal saline (0.85% NaCl) was 10-fold serially diluted and spread on NA medium containing 1% Carboxymethyl cellulose (CMC). After incubation at 37°C for 24 h, colonies with clear zone were primarily screened and later grown individually in NB medium containing 1% CMC under aerobic conditions at 150 rpm for 18-20 h at 37°C. Cell free culture containing enzyme components (CFC) was further tested for high temperature stability. Enzyme sample was incubated at 60°C for 60 min. Each of 2 ul treated enzyme or untreated one was transferred as a droplet on 1.5 % agar plate containing 1% CMC and then let it dry. The enzyme reaction was performed by incubating at 50°C for 20 min. Then, the reaction plate was rinsed with 1 M Tris –HCl pH 7.5, stained with 1% Congo red for 10 min and destained with 1 M NaCl. The stability was determined as equal clear zone appearance of temperature treated and untreated sample.

Secondary screening was investigated according to enzyme activity and stability in a wide range of pH and temperature from selected isolates of primary screening. The effects of pH on enzyme activity and stability were studied in citrate phosphate buffer pH 3-7, while the effects of temperature were from 30-60°C.

Identification of selected isolate

Morphology of 24 h cell culture on NA plate was studied by a light microscope (Olympus Co., Japan). Gram staining was followed by the method of Beisheir (1991). Spore-forming was determined by the method described by Gerhardt *et al.* (1981).

Biochemical test was performed by using API 20 E and API 50 CHB Kit, the results were analysed by APILAB Plus program version 2.1 (Biomericux Sa Co., France).

Enzyme production

The selected isolate was grown in 50 ml NB medium in 250 ml flask under aerobic conditions at 150 rpm for 18-20 h at 37°C. 1% innoculum (v/v) was transferred into 100 ml of NB, which contained 1% (w/v) inducer. After 24 h of incubation, the culture was centrifuged at 4°C, 11,000g for 15 min and the supernatant was stored at -20°C for further study.

Determination of enzyme activities

Carboxymethyl cellulase activity (CM-cellulase) was detemined by the modified method of Okeke and Obi (1995) by performing the reaction mixture of 0.1 ml of sample and 0.1 ml of 1 % (w/v) CMC (Sigma) in 50 mM citrate phosphate buffer pH 5.5 at 50°C for 20 min. The amount of reducing sugar released was determined by Dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of enzyme was defined as the amount of enzyme that released glucose equivalent to 1 μmol of glucose per min.

β-1,3-1,4-glucanase activity was measured using the same procedure as carboxymethyl cellulase activity. However, 1 % (w/v) of barley β-glucan (Sigma) was used as substrate. One unit of enzyme was defined as mentioned above

Xylanase activity was measured by the same method described by the modified method of Okeke and Obi (1995), but 1% (w/v) of oat spelt xylan (Sigma) was used as substrate. One unit of enzyme was defined as mentioned above.

Pectin methylesterase activitiy was measured using the modification method of Huang and Mahomey (1999) by titration of carboxyl groups released from pectin (Sigma) at 50°C for 20 min. Approximately 0.5 ml of enzyme sample was mixed with 4.5 ml of 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 methylesterase was defined as the amount of enzyme which released 1 mmole of carboxyl groups per min under the assay condition.

Pectin lyase activitiy was determined according to the modification method of Huang and Mahomey (1999). 0.1 ml enzyme sample was mixed with 0.4 ml of 0.5 % pectin in 50mM cirtrate phosphate buffer pH 5.5, incubated at 50°C for 20 min. 4 ml of 0.01 M HCl was added and absorbance was measured at 235 nm. One unit of pectin lyase was defined as the amount of enzyme which released 4-5 unsaturated trans-elimination products showing absorbance of 0.2 at 235 nm.

Pectate lyase activitiy was determined by the same method of pectin lyase activity determination, 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.

Polygalacturonase activity was measured by the method of pectate lyase activity assay as mensioned above, except that 1% pectin was used as a substrate. One unit of enzyme was defined as the amount of enzyme that released galactose equivalent to 1 μmol of galacturonic acid per min.

Polymethylgalacturonase activity was measured by the method of carboxymethyl cellulase activity assay mensioned elsewhere, except that 1% PGA was used as substrate. One unit of enzyme was defined as the amount of enzyme that released reducing sugar equivalent to 1 μmol of galacturonic acid per min.

Protein assay

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

Determination of grass degradation activity

Cell-free culture fluid (CFC) containing enzyme components was filtrated with 0.2 micron filter membrane. One ml of CFC was added to the tube containing 1 g of grass powder prepared by milling and sieving (mesh number 120). The mixture was incubated for 24 h at experimental temperatures. Then the extract solution was prepared using the method of Ohmomo *et al*. (1995). In brief, 10 ml of distilled water was added into the reaction mixture, shaked at 100 rpm for 2 h and then filtrated through Whatman No.4 paper filter. To remove all residual soluble carbohydrates, the retentate was washed with 10 ml distilled water twice. All filtrates were pooled and adjusted to the

final volume of 25 ml. The complete mixed solution was further analyzed of reducing sugar content by DNS method (Miller, 1959).

RESULTS AND DISCUSSION

Screening of thermostable CM-cellulase producing bacteria

A total of 161 islolates were primarily screened from 15 samples in the areas of North, Northeast and the Middle of Thailand. Only 14 isolates of AM110, AN114, DN138, EN215, GN156, GN231, GN232, HN164, HN166, M10, MM252, ON271, RN278 and SN280 showing thermostable CM-cellulase activity were selected for further study. It seemed that all effective isolates distributed to every parts of Thailand studied.

Since grass ensiling process occurred at the wide ranges of both pH and temperature, investigation of CM-cellulase which was active at the pH and temperature of 3 – 7 and 30 - 60°C, respectively was decided for secondary screening. The results are shown in Table 1. Only 7 isolates of AN114, EN215, GN156, GN232, HN166, M10 and SN280 could produce enzymes which showed relative activity higher than 60% at temperature ranges of 30-60°C. However, only 5 isolates, AN114, GN156, HN166, M10 and SN280 were stable at the same temperature range.

To determine the effect of pH to the enzyme activity of these 5 isolates, citrate phosphate buffer pH 3, 4, 5 and 7 were used to perform enzyme reaction. However due to high pH of CFC, the pH of reaction mixture changed from pH 3, 4, 5 and 7 to 3.7, 4.5, 5.3 and 7.1, respectively. Among these 5 isolates, only the enzyme from AN114, GN156 and HN166 showed higher relative activities than 60%. However, GN156 could show higher relative activity for 70 % at 4 pH levels. Moreover, it was stable with relative activity higher than 85 % at the same pH range for 1 h. Thus, the isolate GN156 was selected for further characterization.

Considering the sources of 5 isolates exhibiting high activity at wide temperature ranges, they were isolated from different sample sources: nitrifeed, grass, corn silage and rust fungi. However, only one isolate, GN156, found produced high enzyme activity at wide pH ranges. The pH of corn silage usually decreased to 3.5 at the end of ensiling. Therefore, changes of pH condition during ensiling to extreme pH might provide an advantage to obtain an effective strain.

It was quite common to find CM-cellulase producing *B. subtilis*, however, their activities were not active at high temperature (Malburg *et al.*, 1992). *B. subtilis* GN156 growed well at low temperature of 37°C but being able to produce thermostable cellulase. Therefore, it would be a promising strain for further study.

Identification of the isolate GN156

Morphology

The colony of GN156 on NA was circular, raised, translucent and smooth. It was white and butyrous. The edge was undulate. The 24 h cell culture was Gram positive, rod shape and motility. Elliosoidal endospore was found in the center of the cell. This indicated that the isolate GN156 belonged to a group of rod, spore-forming bacteria based on Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 1986).

Biochemical test

The biochemical properties and ability to utilize various carbon sources were determined using API 20 E and API 50 CHB kit, respectively. The isolate GN156 could produce beta-galactosidase, acetoin, catalase, oxidase and gelatin hydrolysis. This strain could not use citrate as a carbon source and could not reduce nitrate neither which disagreed to *B. subtilis* type strain based on Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 1986). Therefore, utilization of various carbon sources was further

Table 1 Optimum and stability of pH and temperatures of CM-cellulase from bacterial isolates screened from various sources in Thailand.

Source	Isolate	Relative activity of CM-cellulase (%)															
		Temperature								pH							
		Optimum				Stability				Optimum				Stability			
		30	40	50	60	30	40	50	60	3.7	4.5	5.3	7.1	3.7	4.5	5.3	7.1
1	AM110	57.3	74.9	100	100	93.6	91.2	87.7	64.9	39.7	77.3	100	97.2	78.6	100	100	97.1
	AN114	65.9	94.3	100	89.8	102.3	96.6	72.3	60.2	69.9	75.9	95.2	100	72.4	115.9	105.1	95.2
	DN138	100	81.0	0	0	84.5	81.0	0	0	95.7	100	0	0	95.5	95.7	0	0
2	EN215	87.5	81.0	100	81.0	0	0	0	81.0	0	100	100	100	0	0	114.3	0
3	GN156	62.4	83.5	100	68.2	72.9	70.6	80.0	60.0	73.0	88.9	100	85.7	91.3	92.9	92.1	114.8
	GN231	56.3	75.0	100	97.3	87.2	87.6	85.1	46.2	14.6	66.9	100	91.04	92.3	102.9	94.9	98.8
	GN232	83.9	72.6	100	75.8	0	0	75.8	0	91.3	100	91.3	100	109.5	91.3	119.0	91.3
4	HN164	51.5	73.8	100	94.9	92.2	90.8	89.9	58.9	51.53	73.8	100	94.9	96.3	96.4	91.0	101.1
	HN166	82.3	100	96.8	75.8	79.0	90.3	85.5	82.3	68.7	100	92.5	80.6	113.0	80.6	96.8	100
5	M10	96.2	88.7	100	92.5	96.2	96.2	96.2	88.7	0	0	100	95.5	0	0	109.1	0
	MM252	54.9	76.5	100	99.1	90.6	92.5	93.4	52.9	11.6	60.2	100	87.9	104.6	100.7	101.1	107.2
6	ON271	57.1	71.4	100	96.4	82.1	81.3	81.7	52.7	26.5	71.3	100	90.1	87.5	106.2	102.2	109.0
7	RN278	0	0	100	92.5	88.7	92.5	88.7	84.2	0	100	100	100	0	100	100	100
	SN280	70.8	78.1	71.9	100	104.2	97.9	102.1	88.54	50.6	85.5	100	87.9	114.3	102.8	87.9	97.3

+ Possitive result - Negative result

1: Nitrifeed, Nakornrachasima Province
2: Corn silage, Rachaburi Province
3: Corn silage, Nakornrachasima Province
4: Grass, Rachaburi Province
5: Chaing Mai Province
6: Grass, Nakornpratom Province
7: Rust fungi

carried out and the result showed that the isolate GN156 produced acid from glycerol, L-arabinose, ribose, D-xylose, glucose, fructose, mannose, inositol, manitol, sorbitol, α-methyl-D-glucoside, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, melibiose, sacchrose, trehaose, raffinose, starch, glycogen and gentiobiose. The identification made by API data base correlation indicated 96.3 % similarity to *Bacillus subtilis*.

Grass degradation at different temperatures

The cell-free culture fluid (CFC) of GN156 exhibited grass degradation activities at various temperatures as shown in Figure 1. The activities increased 14, 7 and 6-folds at 37°C, 50° C and 60°C, respectively comparing to non-enzyme added control. The maximum activity occurred at 50°C that would be an appropriate condition for its enzymatic reaction.

Enzyme system of *Bacillus subtilis* GN156

The CFC from isolate GN156 showed clearly the increment of grass degradation. To investigate its hydrolysis action, the activities against some of polysaccharides, and disaccharides substances were performed. The results showed the highest activity of 4.61 U/ml on barley β-glucan (Table 2), 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 against laminarin was found. Considering pectic substance degradation, it showed no activities of pectin methylesterase, pectin lyase and pectate lyase, but, only low activities of polygalacturonase and polymethylgalacturonase were detected at the concentrations of 0.15 and 0.14 U/ml, respectively. The tropical grass components compose of about 30-40% cellulose, 30-40% hemicellulose, 1-5% starch and 1-2% pectin (Statälä, 1989). Various activities appearance of CM-cellulase, cellobiase, β-glucanase, xylanase, dextrinase and pectinase supported grass degradation very well.

The CFC showed high activity to β-glucan having 1,3 and 1,4 glycosidic linkage, while it did low activity to β-1,4 glycosidic linkage alone of CMC or no activity against β-1,3 glycosidic linkage alone of larminarin.. Therefore, it could be concluded that the CFC of this strain was specific to only β-1,4 glycosidic linkages and preferred to the structure of 1,3 and 1,4 linkages which was defined as action of β-1,3-1,4-glucanase (Dixon and Webb, 1979). Hence, degradation of barley β-glucan was an action of β-1,3-1,4-glucanase, but there was no synergistic action of 1,4 and 1,3 glycolysis.

Many bacterial enzymes showed both of β-1,3-1,4-glucanase and β-1,3-glucanase activities

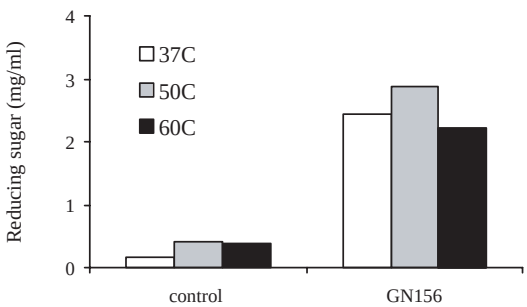

Figure 1 Reducing sugars released from grass hydrolysis at different temperatures.

Table 2 Activities of enzyme from isolate GN156 on various substrate.

Substrate	Activity (U/ml)
Barley β-1,3-1,4-glucan	4.61
CMC	0.05
Xylan	0.56
Laminarin	0
Dextrin	0.57
Cellobiose	0.05
Pectin methylesterase	0
Pectin lyase	0
Pectate lyase	0
Polymethylgalacturonase (PMG)	0.15
Polygalacturonase (PG)	0.14

for example recombinant *E. coli* JM101 (Louw and Reid, 1993), recombinant *Streptococcus bovis* JB1 (Ekinci *et al.*, 1997), *Bacillus* sp. BE1, *Bacillus* sp. FE1, *Pseudomonas* PE1 and *Pseudomonas* PE2 (Kitamura *et al.*, 2002). *B. subtilis* GN156 hydrolyzed only β-1,4 linkages which may left the structure of β-1,3 linkage behind. This structure was not altered by the acid used for extraction (Muller *et al.*, 1997). The oligosaccharides obtained might be useful for further prebiotic application in future study.

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

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

The effect of pH on β-1,3-1,4-glucanase

The pH optima of β-1,3-1,4-glucanase was determined by conducting the activity assays on β-glucan at 50°C in various pH values of 3-12. The results are shown in Figure 2. The activity curve showed optimal pH of 7 with the secondary inflection in the pH range of 8-9. Moscatelli *et al.* (1961) found similar results of 2 inflections in curve from *B. subtilis* at pH 6.5-6.6 and 7.1-7.3. In addition, higher optimal pH was shown by crude enzymes of *B. brevis* (Louw and Reid, 1993), of recombinant *Escherichia coli* and of *Clostridium thermocellum* (Schimming *et al*., 1991) at pH 8-10. β-glucanase from *B. subtilis* GN156 similar to other *Bacillus* sp. strains showed pH optimal at neutral to alkaline pH with secondary inflection shifting to alkaline pH. β-1,3-1,4-glucanase was completely inhibited at extreme pH of 3 and 12.

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

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

The optimum temperature for β-1,3-1,4-glucanase was observed by incubating at temperature of 20-90°C for 20 min. The temperature optima was 60°C as shown in Figure 3. On either side of this optimum temperature, the activity declined sharply. Considering its stability at 1 h, β-1,3-1,4-glucanase was stable at 20-50°C,

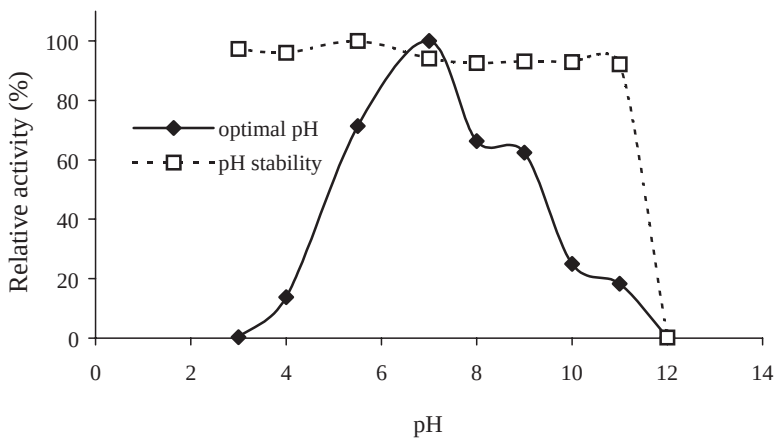

Figure 2 The pH effect on β-1,3-1,4-glucanase activity from isolate GN156 was carried out at 50°C for 20 min. (citrate phosphate buffer pH 3-5.5, phosphate buffer pH 7-9, glycine-NaOH buffer pH 10-12). pH stability of the enzyme was treated at various pH at 4°C for 24 h. The remaining activity was performed by standard condition at pH 5.5, 50°C for 20 min.

while the enzyme was inhibited at higher temperature of 60°C and completely inhibited at 70-90°C. Moscatelli *et al.* (1961) found that β-1,3-1,4-glucanase from *B. subtilis* had optimum temperatures at 50-60°C but it showed significant loss of activity in 5 min at 60°C. However, Louw and Reid (1993) reported a thermostable β-1,3-1,4-glucanase from *B. brevis* showing optimum temperatures at 65-70°C, while crude enzyme from recombinant *Escherichia coli* containing β-1,3-1,4-glucanase gene from *Clostridium thermocellum* showed the highest activity at 80° C (Schimming *et al*., 1991). It clearly concluded that β-1,3-1,4-glucanase from GN156 was not thermostable.

Effect of inducers on β-1,3-1,4-glucanase production

Polysaccharides of CMC, pectin and xylan introduced individually to nutrient broth

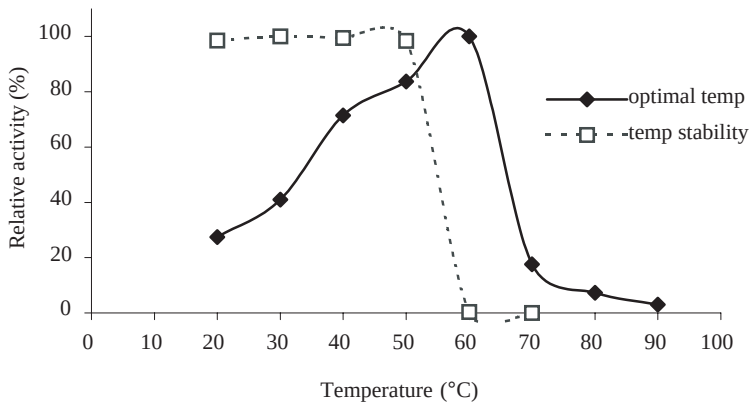

Figure 3 Effects of temperature on β-1,3-1,4-glucanase activity from isolate GN156 carried out in citrate phosphate buffer pH 5.5, temperature stability of the enzyme treated at various temperature for 1 h, the remaining activity performed by standard condition at 50°C for 20 min.

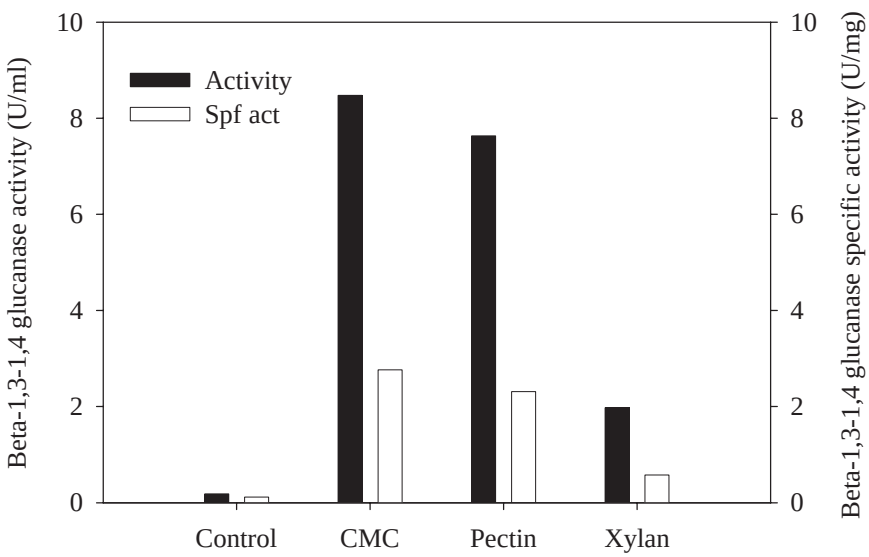

Figure 4 Effects of inducers on β-1,3-1,4-glucanase production.

were studied for β-1,3-1,4-glucanase induction. The results are shown in Figure 4. CMC and pectin supplement induced high β-1,3-1,4-glucanase activity of 8.5 and 7.6 U/ml which were higher 47 and 41 folds than the control, respectively, while xylan activity was only 10.8 folds. El-Helow and El-Ahawany (1999) studied 5 inducers, galactomannan, lichenan, pectin, starch and xylan, supplementation with pectin enhanced maximum β-1,3-1,4-glucanase production. These results supported high production of β-1,3-1,4-glucanase from the cultivation of strainGN156 having pectin as an inducer. Therefore, both pectin and CMC were effective inducers for β-1,3-1,4-glucanase production.

To investigate the effect of CMC level on β-1,3-1,4-glucanase production, various CMC concentrations of 0, 0.2, 0.4, 0.6, 0.8 and 1% of nutrient broth (w/v) were studied. The results are shown in Figure 5. The increments of CMC concentration of 0.2, 0.4, 0.6 0.8 and 1 % induced enzyme activities of 2.87, 4.54, 5.19 5.82 and 5.55 U/ml respectively. At the concentration ranges of 0-0.8%, higher CMC concentration induced higher enzyme activity. However, enzyme activity started to decrease when high concentration of 1% was used. It was clearly shown that the induction of 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.

Effect of grass on the growth and β-1,3-1,4-glucanase production

The growth and enzyme production of *Bacillus subtilis* GN156 in NB medium alone (NB) and NB with other carbon sources of grass (NBG) and CMC (NBC) were perfomed. The samples were taken every 2 h for 24 h to analyse cell number and β-1,3-1,4-glucanase activity. The results are shown in Figure 6. Cell growth on NBG was not different from the NBC and NB during 10 h. They all reached stationary phase after 4 h. However, cell growth from the NB treatment started to decline during 12-24 h while the ones from both NBG and NBC still grew during 24 h and reached 9.4×10^9 and 3.5×10^9 cfu/g at 24 h, respectively.

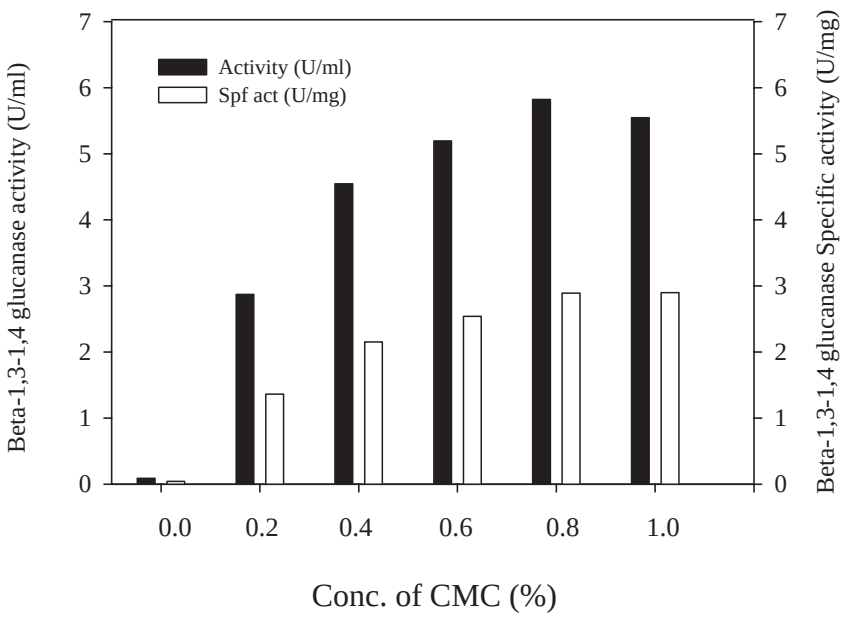

Figure 5 Effects of CMC concentration on β-1,3-1,4-glucanase production by*Bacillus subtilis* GN156.

0.1-0.39 U/ml of β-1,3-1,4-glucanase activity could be detected when the growth reached stationary phase at 6 h. Stulke *et al.*(1993) reported that β-1,3-1,4-glucanase is expressed when cells enter stationary stage in response to nutrient limitation. This aspect was supported by the study of Tang *et al.*(2004) which proposed that β-glucanase produced by *B. subtilis* ZJF-1A5 was associated partially with cell growth.

The induction of β-1,3-1,4-glucanase activity by CMC exhibited 1.78, 5.12, 5.78 and 5.96 U/ml while the one by grass were 0.79, 2.91, 5.64 and 6.48 U/ml after 12, 16, 20 and 24 h, respectively. Interestingly, the control exhibited only low activities of 0.66 – 0.88 U/ml. It was noticed that β-1,3-1,4-glucanase production from NBC was higher than NBG during 16 h. However, grass induction treatment reached maximum production of 6.48 U/ml after 24 h. Both CMC and grass induced the higher β-1,3-1,4-glucanase activity 6.73 and 7.36 folds. than the control Therefore, this kind of induction would increase grass degradation, especially, when silage fermentation started during 24 h.

CONCLUSION

To solve the problem of lacking of sufficient amount of WSC in grass ensiling process, addition of effective hydrolytic enzymes to structural carbohydrates was proposed. GN156 was screened for the stability of CM-cellulase at high temperature and the activity at wide ranges of pH and temperature. It was identified as *B. subtilis*. The activities of grass degradation clearly increased at various temperatures of 37, 50 and 60° C. Study on the enzymatic system of *B. subtilis* GN156, exhibited the activities of β-1,3-1,4-glucanase, CMCase, xylanase, dextrinase, cellobiase, polymethylgalacturonase and polygalacturonase. Among these enzymes, β-1,3-1,4-glucanase provided the highest activity with an optimal pH and temperature of 7 and 60°C and stability of 3-11 and 20-50°C, respectively. Both CMC and grass used as inducers could enhance β-1,3-1,4-glucanase production 6.73 and 7.36 folds comparing to the control. The stability of wide ranges of both pH and temperature would be an advantage for natural ensiling process.

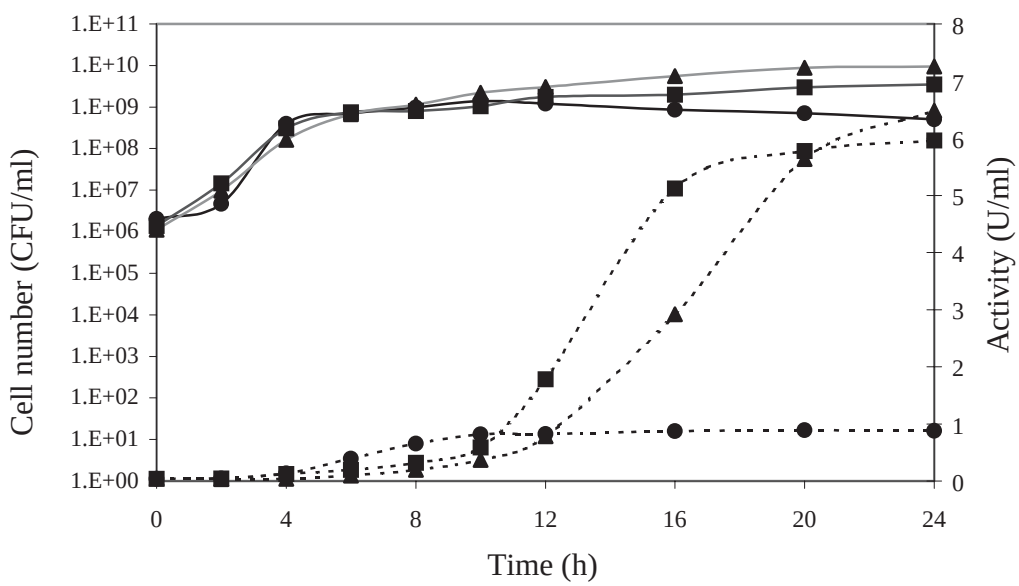

Figure 6 Effect of various inducers on growth and -1,3-1,4-glucanase production.
● Control, ■ CMC, ▲ Grass, —Cell number and ---- Activiy.

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