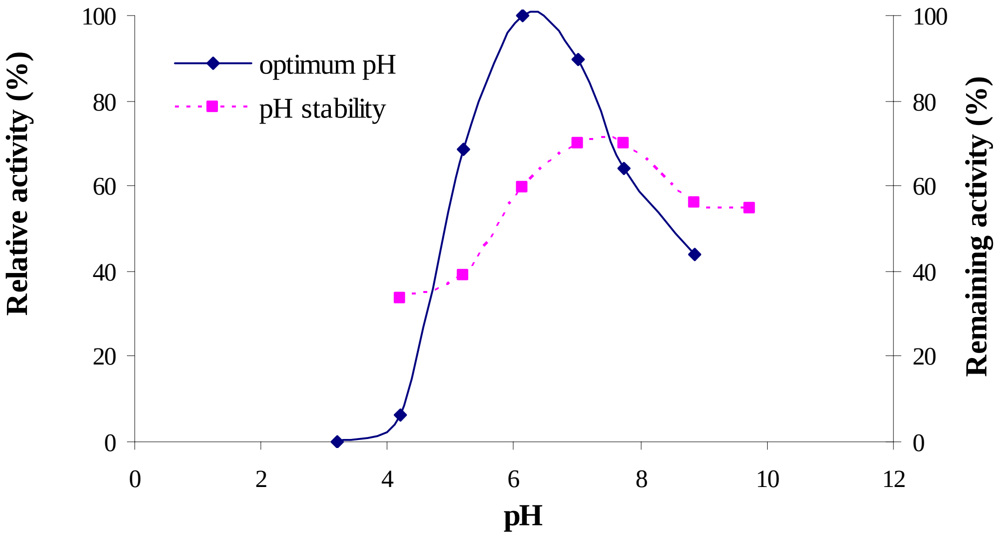

Figure 33 The pH effect on β-1,3-1,4-glucanase J1 from *B. subtilis* GN156 was carried out at 50°C for 20 min. (50 mM citrate buffer pH 3-6, 50 mM phosphate buffer pH 6-8, 50 mM glycine-NaOH buffer pH 9-10). The pH stability of the enzyme was determined at various pH values at 4°C for 2 h. Remaining activity was assayed at pH 6, 50°C for 20 min.

7.2 The effect of temperature on J1

The optimum temperature for J1 was observed by conducting the activity assays on β-glucan in citrate buffer pH 6 at various temperatures from 20-70°C. The highest activity of J1 was at 60°C as shown in Figure 34, which was the same as the optimum temperature of the crude enzyme from *B. subtilis* GN156 and also corresponded to most of the β-1,3-1,4-glucanases that usually have an optimum temperature around 45-65°C (Planas, 2000), except that thermoactive β-1,3-1,4-glucanases from recombinant *Escherichia coli* containing the β-1,3-1,4-glucanase gene from *Clostridium thermocllum* showed an optimum temperature around 80°C (Schimming *et al*., 1991).

Considering stability of J1 to various temperatures, J1 was stable at 20-50°C for 30 min with remaining activity around 50 %. It was clear that J1 was not a thermostable enzyme. Similar results were reported by Akiyama, *et al*. (1996) in that the optimum temperature for the purified β-1,3-1,4-glucanases from rice was 50°C and the activity was lost rapidly at temperature between 50°C and 60°C. These results differ from the thermostable β-1,3-1,4-glucanases from *B. brevis* gene (Louw *et al.*, 1993) and from *Clostridium thermocllum* gene (Schimming *et al*., 1991) that showed 75 % remaining at 65°C for 1 h and 65 % at 75°C for 24 h, respectively.

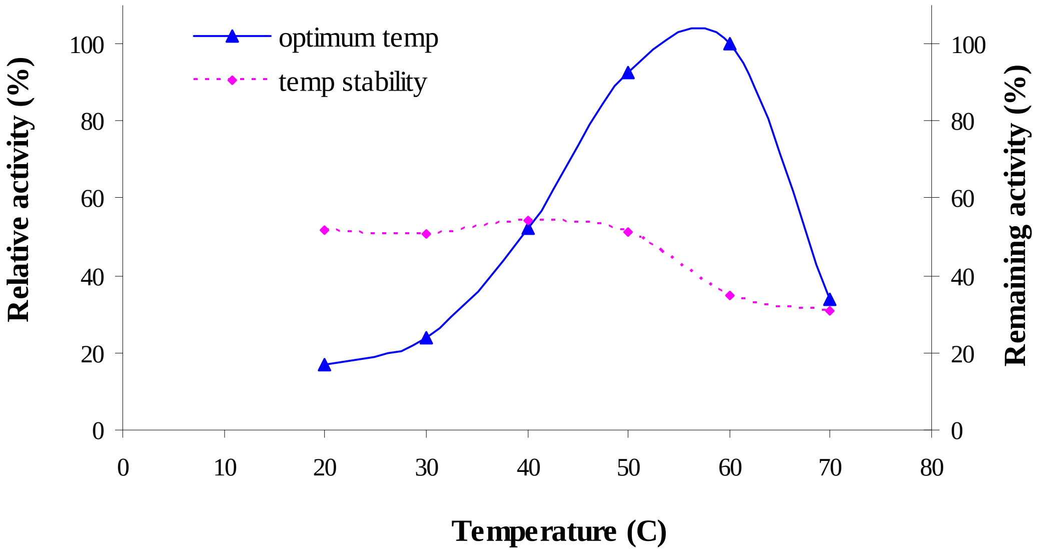

Figure 34 The effect of temperature on β-1,3-1,4-glucanase J1 from *B. subtilis* GN156 was carried out in citrate buffer pH 6. Temperature stability of the enzyme was treated at various temperatures for 30 min. The remaining activity was assay at 50°C for 20 min.

7.3 Effect of metal ion

The effect of 10 mM cation concentration were performed, J1 activity was significantly inhibited by Mn^{2+}, reduced in the presence of Cu^{2+} ions and slightly inhibited by Fe^{2+}, Ca^{2+} and Zn^{2+} ions as shown in Table 18. Moreover, its activity was not dependent on the presence of Mg^{2+}or Li^{+} in that the activity was maintained at more than 90 %. The same results of Ca^{2+} and Mg^{2+} effecting to β-1,3-1,4-glucanase activity from *Clostridium thermocellum* and expressed in *E. coli* was reported by Shimming *et al.* (1991).

Table 18 Effect of metal ions on β-1,3-1,4-glucanase J1 from *B. subtilis* GN156.

Cation	Relative activity (%)
$CuSO_4$	34.1^{d}
$CaCl_2$	87.0^{b}
$FeSO_4$	76.8^{c}
LiCl	93.2^{ab}
$MgSO_4$	93.5^{ab}
$MnSO_4$	8.7^{e}
$ZnSO_4$	87.3^{b}
Control	100^{a}

Superscript alphabet in row presents difference in statistic with alpha = 0.05

7.4 Substrate specificity

To investigate the hydrolysis action of β-1,3-1,4-glucanase J1 from *B. subtilis* GN156, the activities against various polysaccharides substances was determined. The results showed activity of J1 only on barley β-glucan (Table 19). No activity was found against CMC, xylan, laminarin and dextrin. From the definition of β-1,3-1,4-glucanases, it hydrolyses 1,4-β-D-glycosidic linkages in β-D-glucans containing 1,3 and 1,4 linkages, do not hydrolyse β-D-glucans containing only 1,3 or only 1,4 linkages (Dixon and Webb, 1979), it was clear that J1 was a β-1,3-1,4-glucanase.

The substrate specificity of β-1,3-1,4-glucanase J1 from *B. subtilis* GN156 was similar to that reported from rice (Akiyama *et al*., 1996) and *Clostridium thermocellum* (Schimming *et al*., 1991). In contrast, β-1,3-1,4-glucanase from

B. bovis indicated a high degree of specificity for barley β-glucan, but low for β-1,3-glucan laminarin (Ekinci *et al.*, 1997). In addition, the purified β-1,3-1,4-glucanase from *B. brevis* showed both β-1,3-glucanase and β-1,3-1,4-glucanase activity against laminarin and lichenan, respectively (Louw *et al.*, 1993). The distinction of these enzymes can be rather tenuous as it is based on substrate specificity for laminarin as apposed to lichenan, respectively. Since the substrate specificity of the *B. brevis* enzyme was found to be 300-fold more for lichenan than laminarin, it was therefore classified as a β-1,3-1,4-glucanase.

In addition, lichenan is a β-1,3-1,4-glucan containing substrate which is used for the assay of β-1,3-1,4-glucanase activity which was reported in previous studies (Schimming *et al.*, 1991; Louw *et. al.*, 1993, Akiyama *et al.*, 1996; Ekinci *et al.*, 1997). Since their different sources of β-1,3-1,4-glucan substrates, there are variations in the proportion of both types of linkage (25 – 30 % β-1,3 linkages) and in the length of the mixed-liked segment (Planas, 2000). Therefore, the recombinant β-1,3-1,4-glucanase from *Clostridium thermocellum* gene (Schimming *et al*., 1991), the recombinant β-1,3-1,4-glucanase from *Streptococcus bovis* JB1 gene (Ekinci *et al.*, 1997) and the purified rice β-1,3-1,4-glucanase (Akiyama *et al.*, 1996) showed different of activities against barley β-glucan and lichenan.

Table 19 Substrate specificity of the β-1,3-1,4-glucanase J1 from *B. subtilis* GN156 toward various substrates

Substrate	Relative activity (%)
Barley β-glucan	100
CMC	0
Xylan	0
Laminarin	0
Dextrin	0

7.5 Enzyme kinetics

The Michealis-Menten constant (K_m), maximum velocity (V_{max}) and the catalytic rate constant (k_{cat}) are unique characters of each enzyme investigated for β-1,3-1,4-glucanase J1 from *B. subtilis* GN156. Results are summarized inTable 20.

Table 20 Characteristic constants of J β-1,3-1,4-glucanase J1 from *B. subtilis* GN156

Characteristic constant	Value	
Michealis-Menten constant (K_m)	1.53	mg/ml
Maximum velocity (V_{max})	8,511	µU/ml.min
Catalytic rate constant (k_{cat})	0.074	sec^{-1}

The K_m, V_{max} and k_{cat} values of β-1,3-1,4-glucanase J1 from *B. subtilis* GN156 were 1.53 mg/ml, 8,511 µU/ml.min and 0.074 Sec^{-1}, respectively. The K_m value corresponds to the K_m values of β-1,3-1,4-glucanase for other *Bacillus sp.* of 1.2–1.5 mg/ml for barley β-glucan (Planas, 2000), but differs from the K_m value of a commercial enzyme from *B. subtilis* (0.3 mg/ml Megazyme, USA). Compare to the β-1,3-1,4-glucanase from recombinant *Streptococcus bovis* JB1, which showes the K_m values of 2.78 mg/ml and the V_{max} of 338.3 µU/ml.min (Ekinci *et al.*, 1997), J1 is significant higher effective to β-glucan.

8. Characterization of pJ2

Since pJ2 consisted of proteins J1 and J2, characterization of pJ2 might show mix properties derived from both proteins that might be similar or different from the properties of J1.

8.1 pH effect on pJ2

The optimum pH of pJ2 β-1,3-1,4-glucanase was determined by conducting the activity assays on β-glucan at 50° C at various pH values of 3-10. The highest activity was shown at 6.5 (Figure 35) which was closed to the optimum pH of 6 found for J1. Furthermore, pJ2 was stable in the pH range from 6 - 9 at 4°C for 2 h (Figure 35) with remaining activity of 50 %.

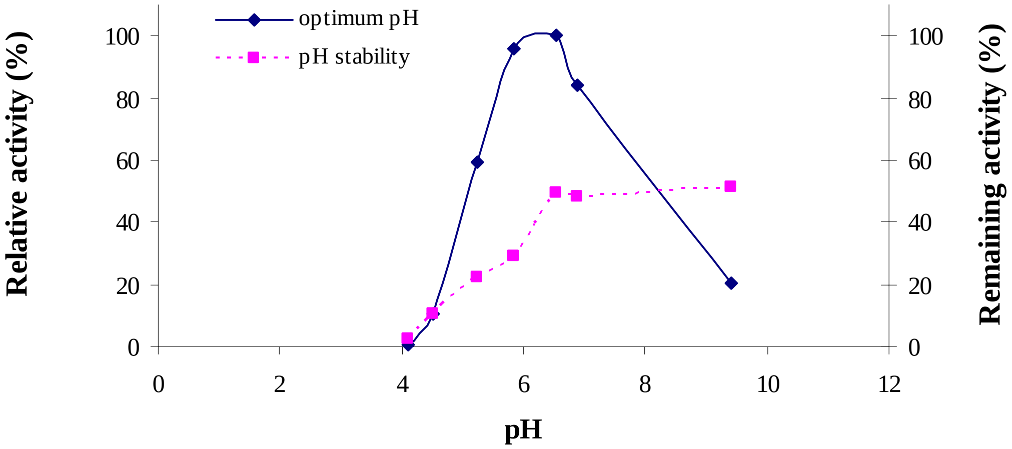

Figure 35 The pH effect on β-1,3-1,4-glucanase pJ2 from *B. subtilis* GN156 was carried out at 50°C for 20 min. (citrate buffer pH 3-6, phosphate buffer pH 6-8, glycine-NaOH buffer pH 9-10). pH stability of the enzyme was treated at various pH at 4°C for 2 h. The remaining activity was assayed at standard condition.

8.2 The effect of temperature on pJ2

The highest activity of pJ2 was occured at 60°C (Figure 34) which was the same for J1. Considering the activity curves, pJ2 showed a similar pattern of activity as the crude enzyme (Figure 36 and 5), which had a secondary inflection at 40°C, unlike the activity curve of J1 which had only one peak of activity without a secondary inflection (Figure 34). It seems that the HMW exhibited optimum temperature at 40°C. pH optimum and stability of J1 and pJ2 were similar but they were different in thermostability. Purified J1 was stable at 20-50°C for 30 min with remaining activity around 50 % (as dertermined previously), but pJ2 was more thermally unstable, the remaining activity was decreased to 20 % at 20-40°C and was completely destroyed at 50-70°C.

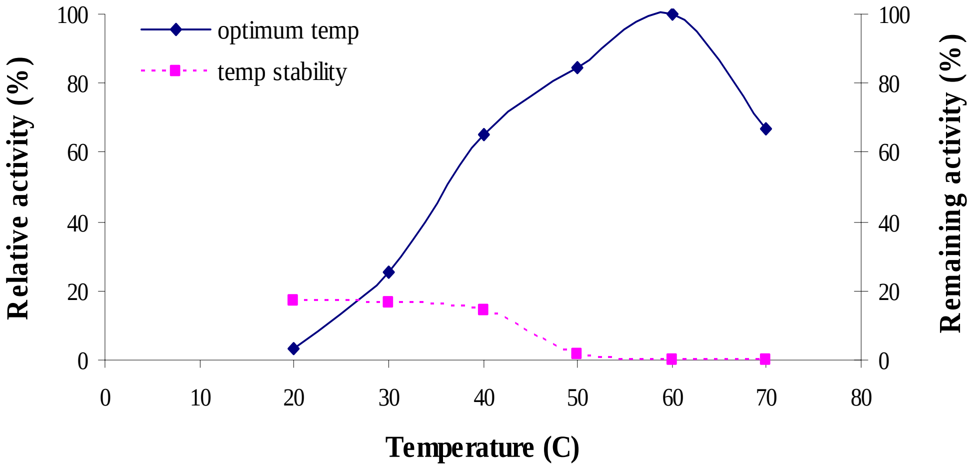

Figure 36 The effect of temperature on β-1,3-1,4-glucanase pJ2 from *B. subtilis* GN156 was carried out in citrate buffer pH 6. Temperature stability of the enzyme was treated at various temperatures for 30 min. The remaining activity was assay at 50°C for 20 min.

8.3 Effect of metal ions

At the individual divalent cation concentration of 10 mM, the presence of each cation showed significantly different affects on β-1,3-1,4-glucanase pJ2 activity. It was clear that pJ2 activity was completely inhibited by Mn^{2+} ions, reduced in the presence of Cu^{2+} ions and slightly inhibited by Ca^{2+}, Mg^{2+} and Zn^{2+} as shown in Table 21. While, its activity was not significantly dependent on the presence of Li^{+} and Fe^{2+} maintained activity of 99 % and 93 %, respectively.

Table 21 Effect of metal ions on β-1,3-1,4-glucanase pJ2 from *B. subtilis* GN156.

Cation	Relative activity (%)
$CuSO_4$	34.5^{E}
$CaCl_2$	88.5^{BC}
$FeSO_4$	92.9^{ABC}
LiCl	98.7^{A}
$MgSO_4$	87.6^{C}
$MnSO_4$	0^{F}
$ZnSO_4$	72.1^{D}
Control	100^{A}

Superscript alphabet in row presents difference in statistic with alpha = 0.05

8.4 Substrate specificity

To investigate the hydrolysis action of pJ2 β-1,3-1,4-glucanases from *B. subtilis* GN156, the activities against various polysaccharides substances were determined. The results showed activity of pJ2 only on barley β-glucan (Table 22). No activity was found against CMC, xylan, laminarin and dextrin, and chitin. It was therefore clear that pJ2 was a β-1,3-1,4-glucanase, therefore similar to J1 as described previously.

Table 22 Substrate specificity of β-1,3-1,4-glucanases pJ2 from *B. subtilis* GN156 toward various substrates

Substrate	Relative activity (%)
Barley β-glucan	100
CMC	0
Xylan	0
Laminarin	0
Dextrin	0

8.5 Enzyme kinetics

The Michealis-Menten constant (K_m), maximum velocity (V_{max}) and catalytic rate constant (k_{cat}) which are unique characters of an individaul enzyme, pJ2 β-1,3-1,4-glucanase from *B. subtilis* GN156 were investigated and the results summarized in Table 23.

Table 23 Characteristic constant of β-1,3-1,4-glucanase pJ2 from *B. subtilis* GN156

Characteristic constant	Value	
Michealis-Menten constant (K_m)	4.36	mg/ml
Maximum velocity (V_{max})	7,397	µU/ml.min
Catalytic rate constant (k_{cat})	0.091	sec^{-1}

The K_m, V_{max} and k_{cat} values of β-1,3-1,4-glucanase pJ2 from *B. subtilis* GN156 were 4.36 mg/ml, 7,397 µU/ml.min and 0,091 sec^{-1}, respectively. Compare to those of J1, pJ2 seemed to low effective to barley β-glucan than J1.

The study of J1 and pJ2 properties were similar with respect to their optimum pH, pH stability, both Mn^{2+} and Cu^{2+} ion effect and substrate specificity, but significantly different in optimum temperature, temperature stability and kinetics character (Table 24) indicated that the two β-1,3-1,4-glucanases from *B. subtilis* GN156, J1 and pJ2 were different type of β-1,3-1,4-glucanases.

Table 24 Chararacteristic properties comparison of J1 and pJ2 from *B. subtilis* GN156

Properties	β-1,3-1,4-glucanase	
	J1	pJ2
Optimum pH	pH 6.0	pH 6.5
pH stability	pH 6-9 for 2 h	pH 6-9 for 2 h
Optimum temperature	60°C	40°C, 60°C
Temperature stability	stable at 20-50°C for 30 min	Unstable
Metal ions effect:		
Activity inhibition by	Mn^{2+}	Mn^{2+}
Activity reduction by	Cu^{2+}	Cu^{2+}
Activity slightly inhibition by	Fe^{2+}, Ca^{2+} and Zn^{2+}	Ca^{2+}, Mg^{2+} and Zn^{2+}
Substrate specificity	β-glucan	β-glucan
Michealis-Menten constant (K_m)	1.53 mg/ml	4.36 mg/ml
Maximum velocity (V_{max})	8,511 U/ml.min	7,397 µU/ml.min
Catalytic rate constant (k_{cat})	0.074 sec^{-1}	0.091 sec^{-1}

9. Degradation pattern

To determine the degradation products from the action of the crude enzyme, J1 and pJ2 on barley β-glucan, each reaction was performed and followed at 0, 1, 6, 12 and 24 h. The degradation products were analyzed by thin layer chromatography as shown in Figure 37.

Considering the product mobilities compared to those of the standard saccharides of cellobiose, cellotriose, cellotetraose and cellopentaose, the product mobilities were intermediate between those of the standards. It might be possible that the standards were β-D-glucosyl residues linked through β-1,4-glycosidic bonds. While the action of β-1,3-1,4-glucanase cleaves β-1,4-linkages adjacent to a 1,3-likages, the oligosaccharides thus obtained are the 1,4-linked building blocks of β-glucan with 1,3-linked end group (Johansson *et al.*, 2005). The 1,3-linked at the reducing end of the products might cause different mobilities when compared to the standards. Corresponding, a mixture of β-1,3 and β-1,4-linkages product of β-glucan hydrolysis by β-glucanase from *B. halodulans* C-125 showed mobility intermediate between laminaritriose (standard sugar for β-1,3-linkages) and cellotriose (standard sugar for β-1,4-linkages), while standard sugars for β-1,3-linkages (laminaribiose and laminaritriose) migrated ahead of standard sugars for β-1,4-linkages (cellobiose and cellotetraose). Therefore, this product was considered to be 3-O-β-D-cellobiosil-D-glucose (Akita *et al.*, 2005). In addition, the major products from β-glucan hydrolysis by β-1,3-1,4-glucanase from *Orpinomyces sp.* Strain PC-2 were triose and tetraose according to ahead migration of standards cellotriose and cellotetraose, respectively (Chen, *et al.*, 1997). Thus, the intermediate mobilities of β-glucan hydrolysis product by β-1,3-1,4-glucanase from *B. subtilis* GN156 compared to those of the standard saccharides, would be triose, tetraose, pentraose and oligosaccharides, whose molecules were larger than cellopentaose.