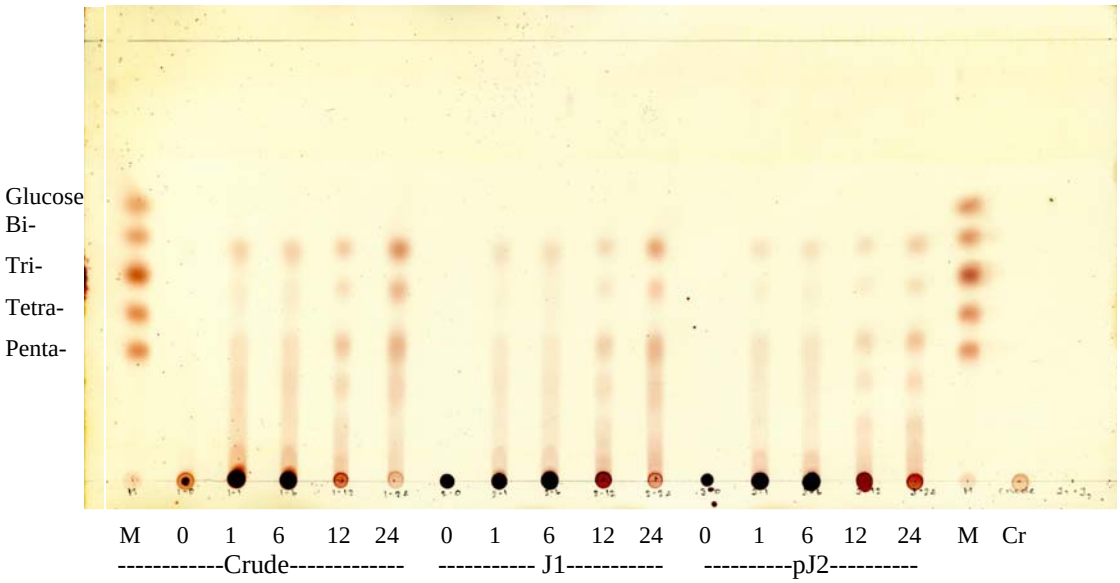

Figure 37 Thin-layer chromatography of degradation products of barley β-glucan by the β-1,3-1,4-glucanases from *B. subtilis* GN156 at various times of 0, 1, 6,12 and 24 h. M: standard oligosaccharide, C: degradation products from crude enzyme, J1: degradation products from J1, J2: degradation products from pJ2, Cr: crude enzyme.

Further quantitative determination of the oligosaccharide products from barley β-glucan hydrolysis by the crude enzyme, J1 and J2 were determined. The oligosaccharide spot intensity on TLC plates was analyzed by GeneTools program version 3.06.04 (Syngene, USA) and summarized in Table 25.

Table 25 Oligosaccharide products from hydrolysis of barley β-glucan

Enzyme	The number of oligosaccharide (% intensity)			
	A	B	C	D
Crude: 0 h	ND	ND	ND	ND
1 h	8.56	ND	ND	ND
6 h	9.32	ND	ND	ND
12 h	33.17	16.53	42.97	19.96
	(0.77)	**(0.38)**	**(1.00)**	**(0.46)**
24 h	100.00	59.32	86.44	ND
	(1.00)	**(0.59)**	**(0.86)**	ND
J1: 0 h	ND	ND	ND	ND
1 h	4.92	ND	ND	ND
6 h	6.13	ND	ND	ND
12 h	26.20	11.44	39.41	ND
	(0.66)	**(0.29)**	**(1.00)**	ND
24 h	67.97	41.72	86.95	ND
	(0.78)	**(0.48)**	**(1.00)**	ND
pJ2: 0 h	ND	ND	ND	ND
1 h	5.11	ND	0.24	ND
6 h	5.72	1.64	0.64	ND
12 h	21.43	10.36	26.54	16.28
	(0.81)	**(0.39)**	**(1.00)**	**(0.61)**
24 h	40.82	22.98	51.07	21.12
	(0.80)	**(0.45)**	**(1.00)**	**(0.41)**

ND: Not detected

( ): Ratio of oligosaccharides in the same sample

A: triose (oligosaccharides with mobilities intermediate between cellobiose – cellotriose)

B: tetraose (oligosaccharides with mobilities intermediate between cellotriose – cellotetraose)

C: pentaose (oligosaccharides with mobilities intermediate between cellotetraose – cellopentaose)

D: Oligosaccharides, which molecules were larger than cellopentaose.

9.1 Barley β-glucan degradation by crude enzyme

The results of oligosaccharide products from barley β-glucan hydrolysis by the crude enzyme from *B. subtilis* GN156 are shown as Figures 37 and 38. Oligosaccharides triose (A), tetraose (B), pentaose (C) and the oligosaccharides, whose molecules were larger than cellopentaose (D) were detected during 24h reaction time. The number of A increased to 33 and 100 % when the reaction time reached to 12 and 24 h, respectively. Mean while, higher molecule of B, C and D were detected for 16.5, 43 and 20 % at 12 h and 59, 86 % and not detected at 24 h, respectively. It was clearly shown that degradation of barley β-glucan by crude enzyme provided the highest number of cellotriose followed by cellopentaose and cellotetraose in order.

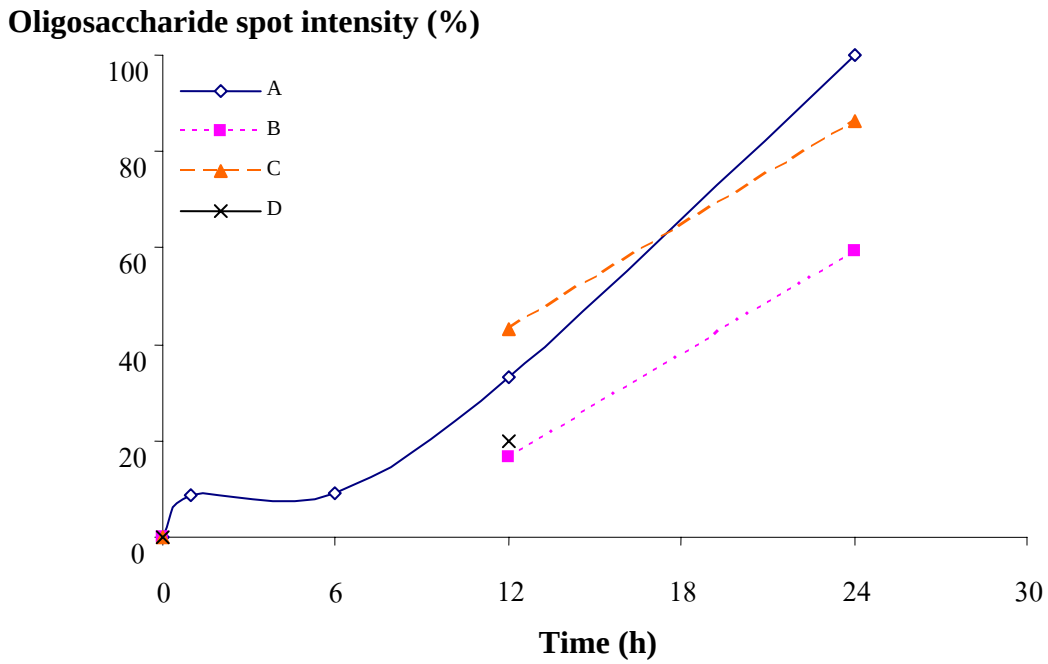

Figure 38 Oligosaccharide products from barley β-glucan hydrolysis by crude enzyme from *B. subtilis* GN156. A: trioses, B: tetraose, C: penta, D: oligosaccharides, which molecules were larger than cellopentaose.

Akiyama *et al.* (1996) reported that two major oligosaccharides with mobilities intermediate between cellobiose – cellotriose and cellotriose – cellotetraose were detected from the 6 h hydrolysis of barley β-glucan by the purified rice β-1,3-1,4-glucanase. In contrast, oligosaccharides with mobilities intermediate between cellotetraose – cellopentaose and the oligosaccharides, which are larger than cellopentaose were not reported. Comparing to this stud, these major oligosaccharides of cellotriose, cellotetraose and cellopentaose appeared after 24 h hydrolysis. It was noticed that degradation pattern of rice β-1,3-1,4-glucanases and those from *B. subtilis* GN156 were different.

Furthermore, the four degradation products of cellotriose, cellotetraose, cellopentaose and the oligosaccharides, which are larger than cellopentaose from β-glucan hydrolysis by β-1,3-1,4-glucanases of *B. subtilis* GN156 correspond to those reported by Olsen *et al.* (1991). The degradation products of barley β-glucan hydrolysis by parental and hybrid β-1,3-1,4-glucanases from *B. meacerans* and *B. amyloliquefaciens* were oligosaccharide trimers, tetramers, pentamers and higher molecules of hexamers and heptamers. In contrast, traces of dimers were also reported by Olsen *et al.* (1991), but they could not be detected from the degradation product of *B. subtilis* GN156.

9.2 Barley β-glucan degradation by J1

The oligosaccharide products from barley β-glucan hydrolysis by purified J1 β-1,3-1,4-glucanase from *B. subtilis* GN156 are shown as Figures 37 and 39. The degradation patterns by J1 were quite similar to those of the crude enzyme. It was found that oligosaccharides with mobilities intermediate between cellobiose – cellotriose, which were considered to be triose, were the major products and increased with time, while other saccharides could be detect after 12 h of hydrolysis.

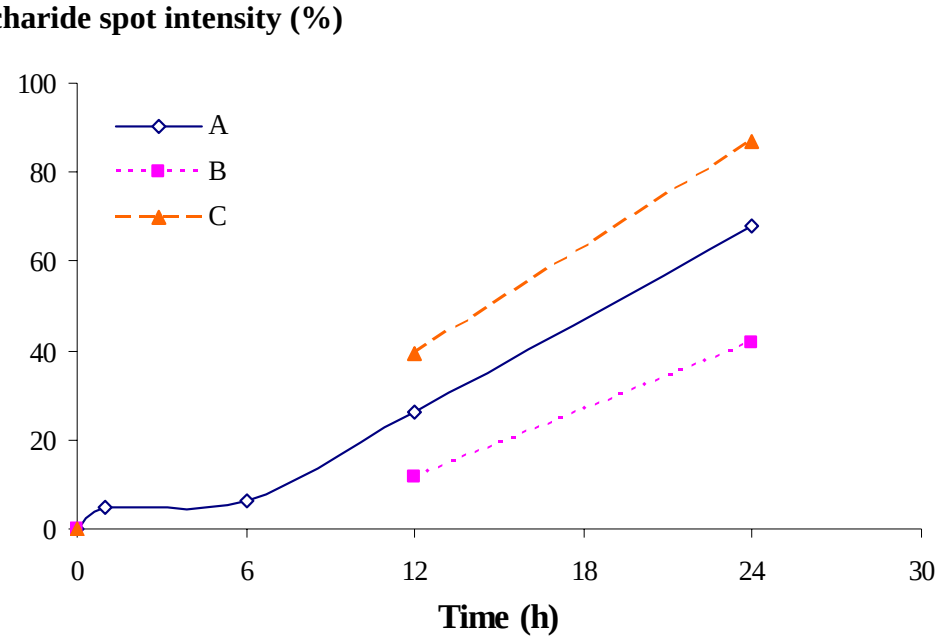

Figure 39 Oligosaccharide products from barley β-glucan hydrolysis by β-1,3-1,4 glucanase J1 from *B. subtilis* GN156. A: trioses, B: tetraose, C: penta, D: oligosaccharides, which molecules were larger than cellopentaose.

9.3 Barley β-glucan degradation by pJ2

The oligosaccharide products from barley β-glucan hydrolysis by β-1,3-1,4-glucanase J2 from *B. subtilis* GN156 are shown in Figures 37 and 40. The major product was considered to be triose and increased with time, while other saccharides of tetraose, pentaose and oligosaccharides, whose molecules were larger than cellopentaose were also detect after 12 h of hydrolysis as well. Considering, the degradation patterns by pJ2 were different from those of the crude enzyme and J1. Especially the oligosaccharides, whose molecules were larger than cellopentaose, were higher than those from crude enzyme and J1 treatment for 16 % and 21 % at 12 h and 24 h, respectively. In addition, the smaller oligosaccharides from pJ2 treatment seemed to be lower than those from crude enzyme and J1.

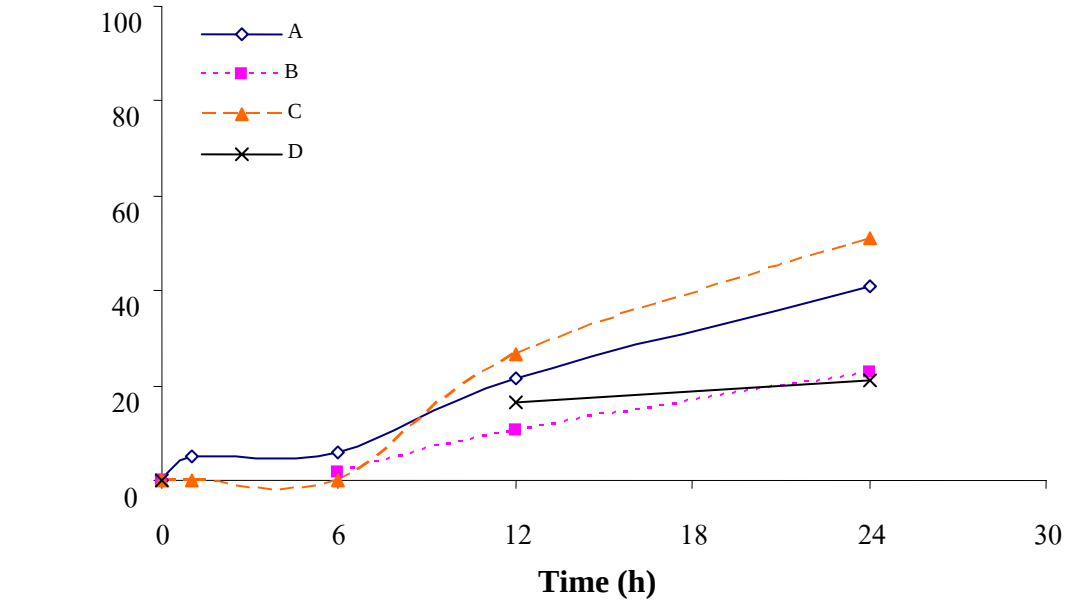

Figure 40 Oligosaccharide products from barley β-glucan hydrolysis by β-1,3-1,4-glucanase pJ2 from *B. subtilis* GN156. A: trioses, B: tetraose, C: penta, D: oligosaccharides, which molecules were larger than cellopentaose.

Considering the degradation products of barley β-glucan hydrolysis by β-1,3-1,4-glucanases from *B. subtilis* GN156 for 24 h, base on the data from Table 25 percentage of each oligosaccharide was calculated as follow:

$$\text{Oligosaccharide (\%)} = \frac{\text{Intensity of each oligosaccharide (A, B, C or D)}}{\text{Total intensity of all oligosaccharides (A + B + C +D)}} \times 100$$

For example; degradation product D from crude enzyme

$$D = \frac{86.44}{100 + 59.32 + 86.44} \times 100 = 35.17\ \%$$

Percentage of degradation products of barley β-glucan hydrolysis was summarized in Table 26.

Table 26 Percentage of degradation products of barley β-glucan hydrolysis by β-1,3-1,4-glucanases from *B. subtilis* GN156 for 24 h.

Enzyme	Oligosaccharide product			
	A	B	C	D
Crude	40.69	24.14	35.17	ND
J1	34.57	21.21	44.22	ND
pJ2	30.02	16.90	37.55	15.53

ND: Not detected

In conclusion, the degradation products of barley β-glucan hydrolysis were approximately 35 – 44 % pentaoses (oligosaccharides with mobilities intermediate between cellotetraose – cellopentaose), 30 – 40 % trioses (oligosaccharides with mobilities intermediate between cellobiose – cellotriose), 17 – 24 % tetraose (oligosaccharides with mobilities intermediate between cellotriose – cellotetraose) and 15 % oligosaccharides, larger than cellopentaose. Results from the degradation of barley β-glucan by parental and hybrid β-1,3-1,4-glucanase from *B. macerans* and *B. amyloliquefaciens* showed that the major products were oligosaccharide trimers and tetramers (approximately 50 – 60 % and 30 %, respectively), but were 4 – 6 % oligosaccharide pentamers and less than 1 % dimers (Olsen *et al.*, 1991). In addition, the major products from lichenan hydrolysis by the β-1,3-1,4-glucanase from the *Bacteriodes succinogenes* gene were trisaccharide (82%) and pentasaccharide (9.5%), while the hydrolysis of oat β-glucan yielded trisaccharide (63.5%) and tetrasaccharide (29.6%) (Elfle *et al.*,1988).

10. Synergistic effect

10.1 β-glucan degradation

The effect of the crude enzyme, J1 and pJ2 from *B. subtilis* GN156 to barley β-glucan or grass powder was investigated. Equal activities (0.036 U) of the enzymes were added separately or in combination to the reaction mixture. Samples were collected at 1, 6, 12 and 24 h. Results are shown in Table 27.

Table 27 Reducing sugar released from β-glucan degradation by enzymes at various time

Enzyme	Reducing sugar (mg/ml)			
	Incubation time (h)			
	1 h	6 h	12 h	24 h
C	0.7133 [a]	1.8710 [a]	2.1788 [a]	2.5824 [a]
J1	0.5409 [c]	1.5551 [b]	1.7400 [b]	2.4420 [a]
pJ2	0.3836 [d]	1.4103 [b]	1.7149 [b]	2.0960 [b]
C + J1	0.6769 [ab]	1.9349 [a]	2.1938 [a]	2.6927 [a]
C + pJ2	0.6161 [b]	1.7751 [a]	2.0183 [a]	2.5749 [a]

Superscript alphabet in row presents difference in statistic with alpha = 0.05
C: Crude enzyme, J1: the purified J1, pJ2: the partial purified J2

The highest degradation occurred with the crude enzyme, while degradation by pJ2 was lowest (Figure 41). Even though the activity of each enzyme was equal, the reducing sugar released by each β-glucan hydrolysis treatment was different. This might be due to variation in stability of the enzymes, as proposed in the

previous study that the crude enzyme from *B. subtilis* GN156 was thermostable, while J1 and pJ2 less stable to temperature than the crude enzyme. Purity of the enzyme might therefore caurse differences in hydrolysis.

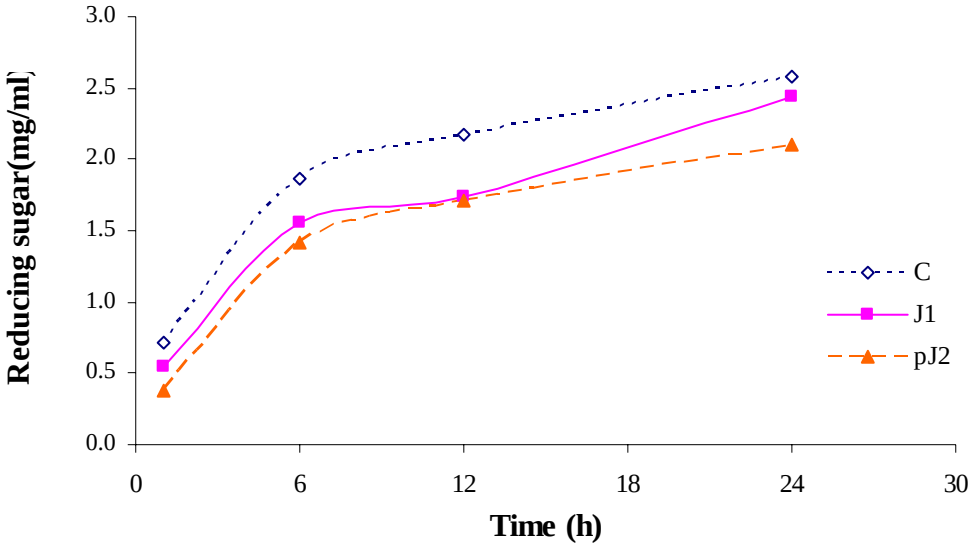

Figure 41 Barley β-glucan degradation at various times by C: Crude enzyme, J1: the purified J1, pJ2: the partial purified J2.

β-glucan degradation by either crude enzyme (C) alone or combination of C + J1 or C + pJ2 was not significant different (Figure 42 and 43). This showed that addition of either J1 or pJ2 provided the same reaction rate to crude enzyme.

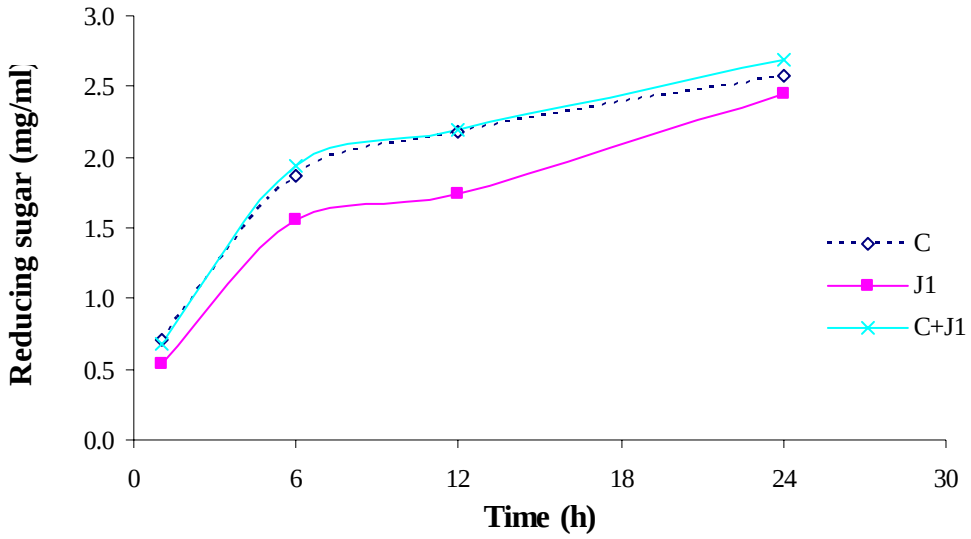

Figure 42 Barley β-glucan degradation at various times by C: Crude enzyme, J1: the purified J1 and the combination of C + J1

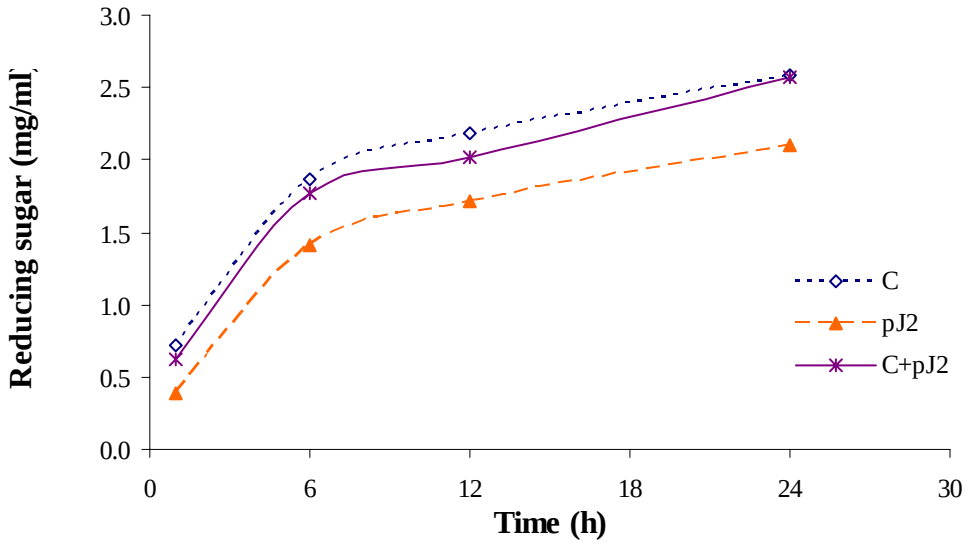

Figure 43 Barley β-glucan degradation at various times by C: Crude enzyme, pJ2: the partial purified J2 and the combination of C + pJ2.

10.2 Grass degradation

These studies were conducted the same as β-glucan degradation. An equal activity of 0.036 U of the enzymes were added separately or in combination to grass powder. Degradation products were collected at 1, 6, 24 and 48 h as shown in Table 28.

Table 28 Reducing sugar released from grass degradation by enzymes at various time

Enzyme	Reducing sugar (mg/ml)			
	Incubation time (h)			
	1	6	24	48
C	0	0.0244 [a]	0.0476 [a]	0.0633 [a]
J1	0	0.0351 [a]	0.0517 [a]	0.0602 [ab]
pJ2	0	0.0207 [a]	0.0445 [a]	0.0552 [ab]
C + J1	0	0.0119 [a]	0.0338 [a]	0.0464 [b]
C + pJ2	0	0.0169 [a]	0.0379 [a]	0.0536 [ab]

Superscript alphabet in row presents difference in statistic with alpha = 0.05
C: Crude enzyme, J1: the purified J1, pJ2: the partial purified J2

At 1 h incubation time, no reducing sugar was detected from all treatments. Reducing sugar released from grass degradation during 6 and 24 h from all treatment were not significantly different, while the reducing sugar released from 24 h hydrolysis of 0.0633, 0.0602, 0.0552, 0.0464 and 0.0536 mg/ml were detected from the treatment of C, J1, pJ2, C + J1 and C + pJ2, respectively.

Grass degradation by separate C, J1 or pJ2 as shown in Figure 44 were not significantly different, while the combination of C + J1 (Figure 45) and C + pJ2 (Figure 46) showed lower degradation than those from separate C, J1 or pJ2.

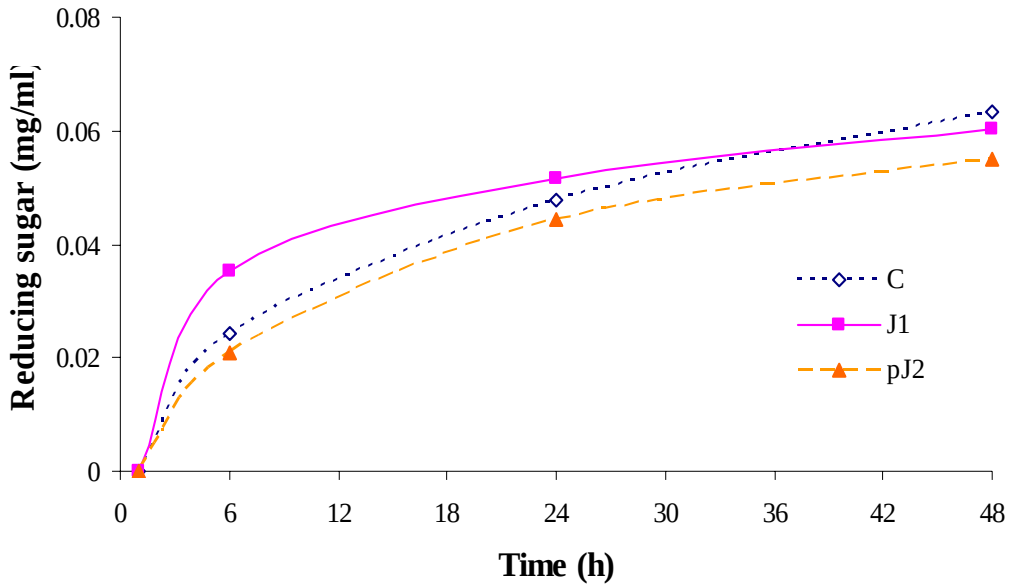

Figure 44 Grass powder degradation at various times by C: Crude enzyme, J1: the purified J1, pJ2: the partial purified J2.

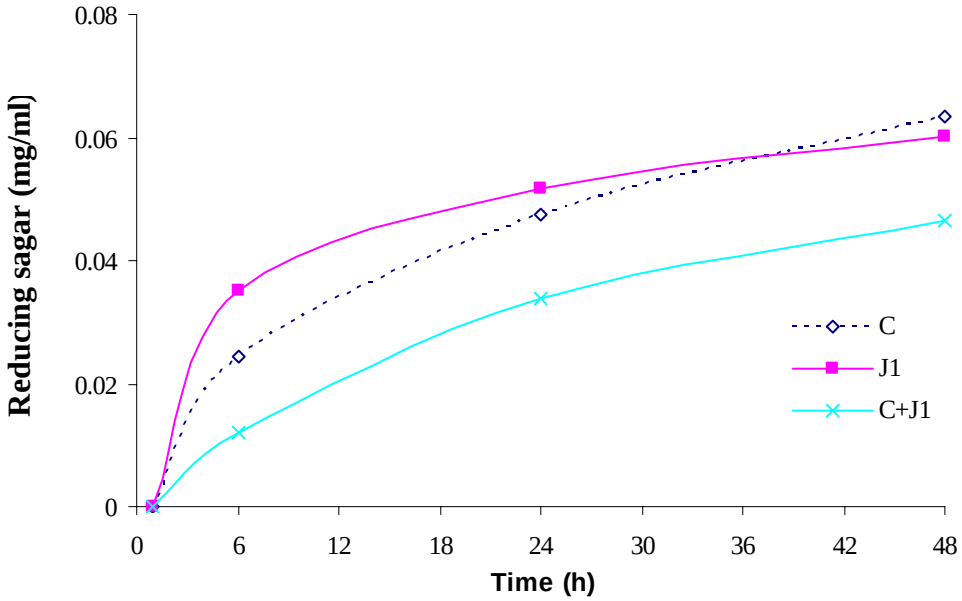

Figure 45 Grass powder degradation at various times by C: Crude enzyme, J1: the purified J1 and the combination of C + J1.

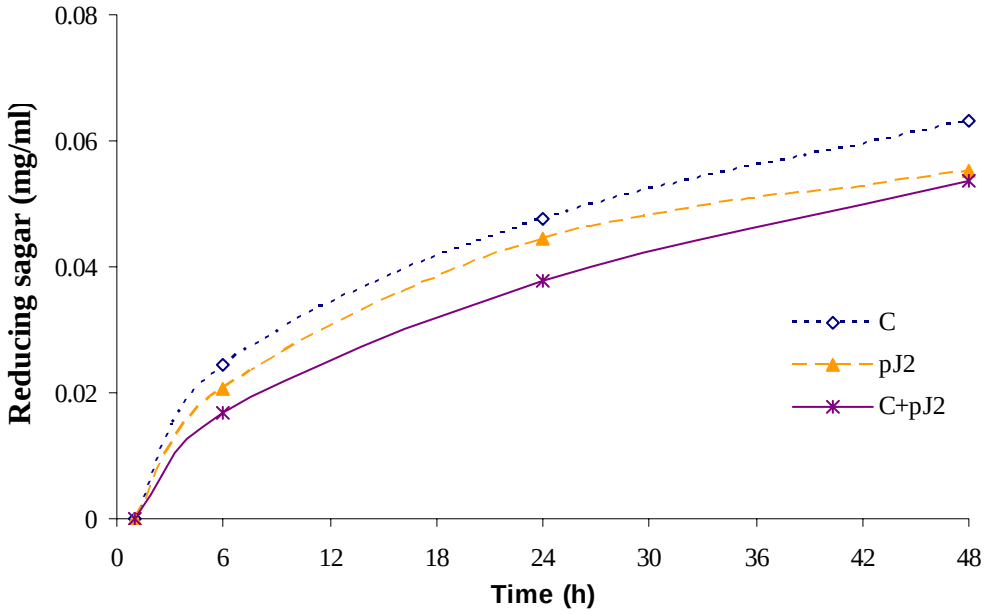

Figure 46 Grass powder degradation at various times by C: Crude enzyme, pJ2: the partial purified J2 and the combination of C + pJ2.

Considering the lower grass degradation by combination of C + J1 (Figure 45) or C + pJ2 (Figure 46), than separate C, J1 or pJ2. The crude enzyme was complex proteins it showed the highest 1,3-1,4-glucanase activity, other activities of xylanase, CMCase, dextinase, avicellulase and polygalacturonase could be detected as described in the previous study. Thus, reducing sugar released from grass degradation by the crude enzyme was from the concert action of these enzymes. Combination of 0.018 U of J1 or pJ2 to 0.018 U of crude enzyme could not enhance degradation activity of crude enzyme in C + J1 or C + pJ2 but the reduction of crude enzyme to a half portion caused lower reducing sugar released from grass degradation by both C + J1 and C + pJ2. Furthermore, grass degradation by C, C + J1 and C + pJ2 seemed to yield increased degradation with time. Unlike the action of individual J1 or pJ2, the degradation seemed to be constant after 24 h that would be from the limitation of β-glucan in grass suspension.