Chapter 3

Results and Discussions

1. Effect of cultural medium and environmental conditions on growth and fibrinolytic enzyme production

1.1 Effect of cultural medium

S. commune BL 23 was cultured in each medium (PDB, SDB, MY, MM, PYGM which initial pH of 6.0) after incubation at 30°C, 150 rpm for 7 days, the supernatant was used for determining fibrinolytic activity on fibrin plate and the result was shown in Figure 5. The production of fibrinolytic enzyme was affected by medium used and PYGM was the most supportive medium compared to MY, SDB, PDB and MM ($P<0.05$). The fibrinolytic activities were 486.27, 245.38, 226.23, 119.99 and 108.17 U, respectively. This is probably due to high nitrogen source (0.5% peptone and 2% yeast extract) in PYGM. The results correlated with previous work of El-Aassar *et al.* (1990) who demonstrated that peptone and yeast extract were the best nitrogen source for fibrinolytic enzyme from *P. chrysogenum* H9 and the enzyme activity was 219.6 μg tyrosine/mg protein. However, Hirasawa *et al.* (1997a) reported that corn steep liquor was a better nitrogen source than yeast extract for the fibrinolytic enzyme production from *S. commune* which yielded the enzyme activity of 342 mm^2/30 μl. Yeast extract is an excellent substrate for many microorganisms. It contains amino acid and peptides, water soluble vitamin and carbohydrate. Corn steep liquor is also a superb organic nitrogen source for microorganisms (Crueger and Crueger, 1990).

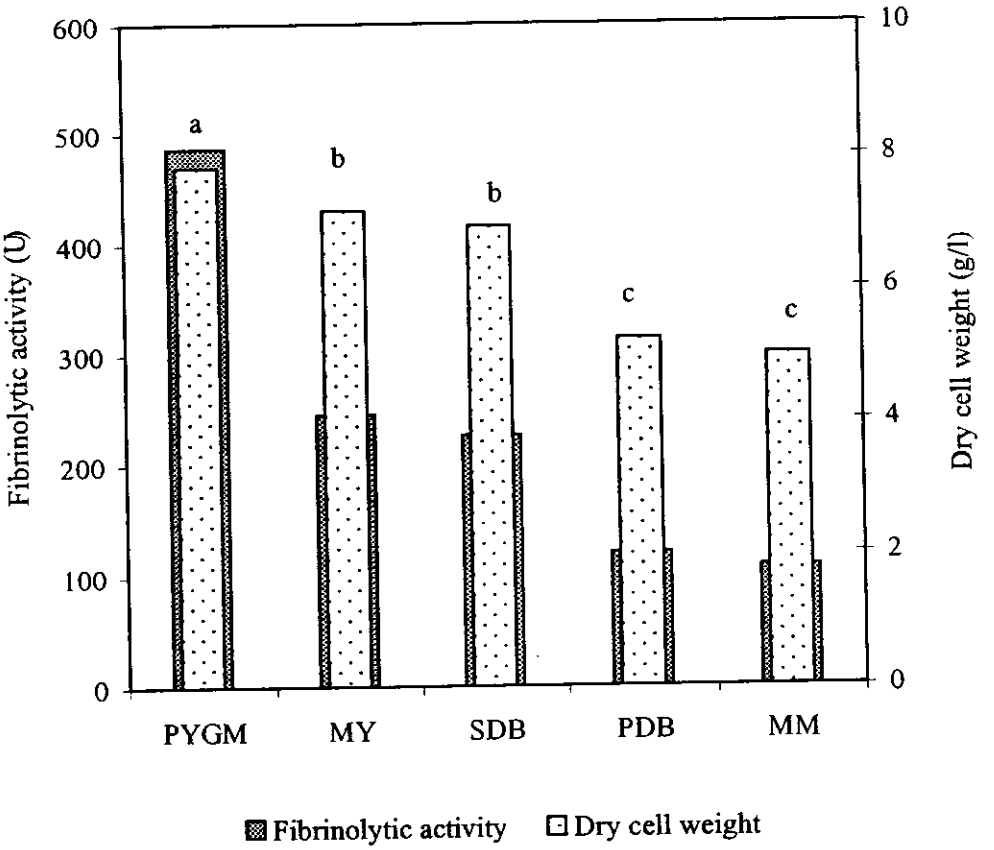

Figure 5 Effect of cultural medium (initial pH = 6) on fibrinolytic enzyme production of *S. commune* BL 23 cultivated at 30°C, 150 rpm for 7 days. Only fibrinolytic activity in different culture media were determined for significant comparison. The same alphabet meaned not significant different between groups ($p<0.05$).

1.2 Effect of incubation periods

S. commune BL 23 was cultured in the selected medium (PYGM, initial pH = 6.0). The culture broth was drawn 3, 7, 10, 12 and 14 days of incubation for cell dry mass and enzyme activity and the result was shown in Figure 6. Dry cell weight and the enzyme activity were 3.94 g/l and 450.79 U, respectively after 3 days of cultivation. On day 7, the cell growth reached stationary phase with 7.14 g/l of dry cell weight and the maximum enzyme activity was achieved at this stage (506.91 U). After 10 days of cultivation, the growth was in stationary phase and the enzyme activity decreased significantly. After 14 days of cultivation, the enzyme activity and dry cell weight were 206.59 U and 7.49 g/l, respectively. Therefore, incubation time for further experiments was 7 days.

1.3 Effect of pHs

S. commune BL 23 was cultured in PYGM with varying initial pH of 5.0, 6.0 and 7.0. As shown in Figure 7, fibrinolytic enzyme production was influenced by the initial pH of culture medium. The maximum dry biomass of 9.0 g/l was achieved at the initial pH of 7.0 while the maximum activity (489.46 U) was obtained from the medium with initial pH of 6.0 ($p<0.05$). At optimum pH for fibrinolytic enzyme production, dry cell weight of 7.51 g/l was achieved. The enzyme activity of 434.13 and 441.95 U were obtained at the initial pH of 5.0 and 7.0, respectively. The influence of pH on fungal growth and metabolism is complex. Fungi are characteristically tolerant of low pH and most have an optimum pH between 5.0 and 7.0 for growth (Berry, 1975). From the results reported by Hirasawa (1997a), *S. commune* was cultured in the basal medium containing 2% corn steep liquor with the initial pH of 5.5. Fibrinolytic enzyme activity of 342 mm^2/30 µl was obtained. El-Aasssar (1995) also reported the different initial pH of the medium for fibrinolytic enzyme production from *F. pallidoroseum* under submerged cultivation. The medium containing wheat bran or soya bean with the initial

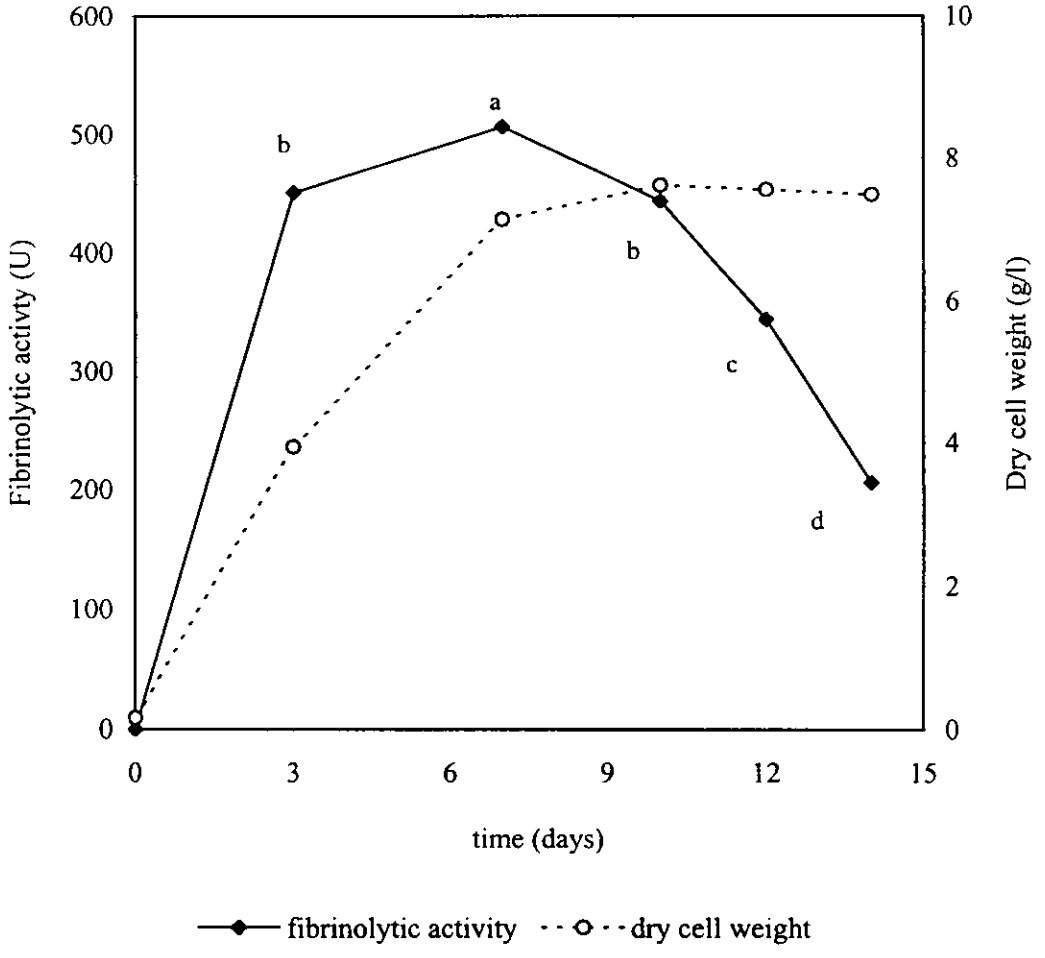

Figure 6 Effect of incubation period on growth and fibrinolytic enzyme production of *S. commune* BL 23 cultivated in PYGM medium (initial pH = 6) at 30°C and 150 rpm. Only fibrinolytic activity in different incubation period were determined for significant comparison. The same alphabet meaned not significant different between groups ($p<0.05$).

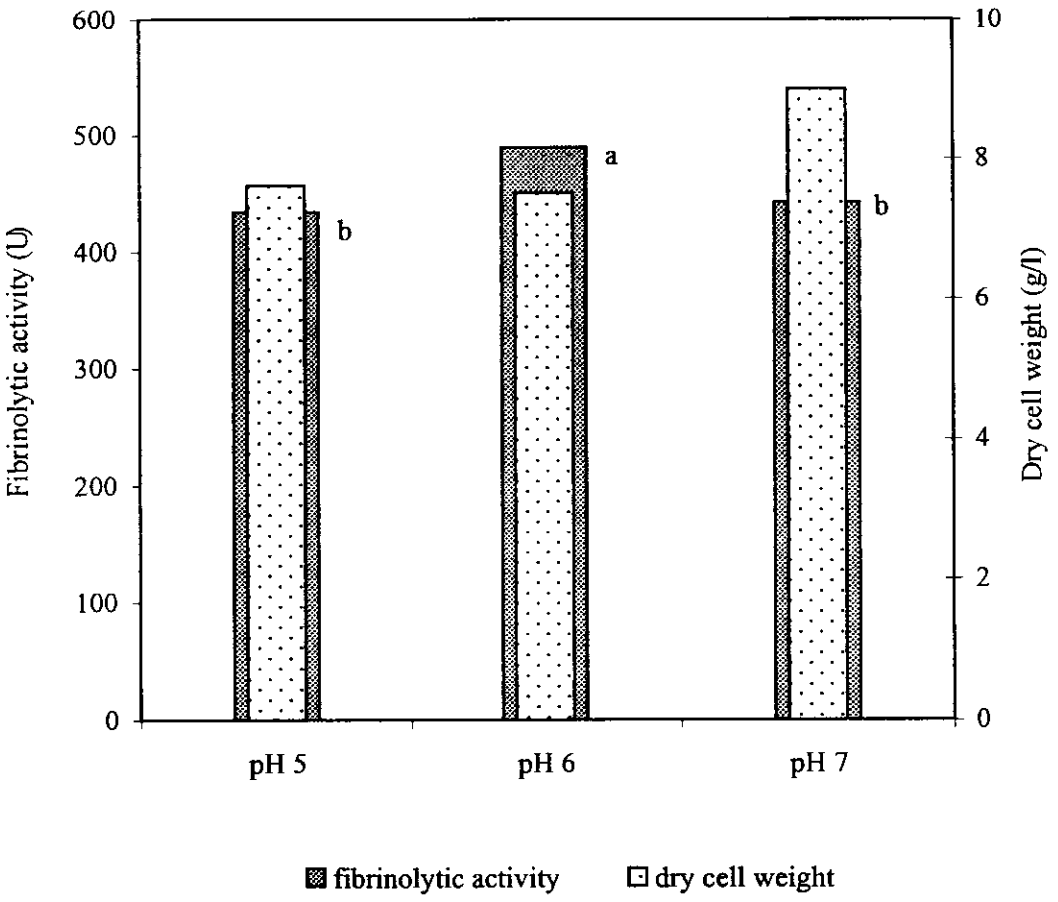

Figure 7 Effect of initial pH of PYGM medium on growth and fibrinolytic enzyme production of *S. commune* BL 23 cultivated at 30°C, 150 rpm for 7 days. Only fibrinolytic activity in different initial pH of medium were determined for significant comparison. The same alphabet meaned not significant different between groups ($p<0.05$).

pH of 6.5 supported the maximal growth and the enzyme activity of 3.67 U/ml (culture broth) was achieved. *P. chrysogenum* H9 was also cultured in the medium with the initial pH of 6.5 for fibrinolytic enzyme production (El-Aasssar *et al.*, 1990). The initial pH of cultivated medium affected other enzyme production of *S. commune*, for example the production of cellobiose dehydrogenase (CDH) by *S. commune* AS 5.391 depended on initial pH of the medium. CDH activity was observed in the pH range of 4.0-6.5. The maximum enzyme production was occurred at pH 4.5 (Fang *et al.*, 1999).

1.4 Effect of temperatures

S. commune BL 23 was cultured in PYGM (initial pH = 6.0) and was incubated at the temperatures of 25, 30 and 35°C. The result in Figure 8 showed that the maximum dry cell weight (9.74 g/l) and the highest enzyme activity (575.22 U) were obtained at 35°C ($p<0.05$). Dry cell weight of 8.79 g/l and the enzyme activity of 441.75 U were obtained at 30°C. Dry cell weight obtained at 25°C and 35°C was nearly the same but the enzyme activity gained from 25°C was much low than that from 35°C. Hirasawa *et al.* (1997a) investigated fibrinolytic enzyme production from *S. commune* which was incubated at 20-30°C for two weeks and the enzyme activity of 342 mm^2/30 µl was obtained. However, *P. chrysogenum* H9 was cultured at 28°C for fibrinolytic enzyme production (El-Aasssar *et al.*, 1990). The production of fibrinolytic enzyme from *F. pallidoroseum* was affected by incubation temperature. At 25 and 30°C, the maximum enzyme activity was obtained after 4 days, while 10 days were needed at 20°C. The temperature of 55°C was suitable for the enzyme production from thermophilic *Streptomyces megasporus* SD5 (Chitte and Dey, 2000). From the results, the incubation temperature for further study was 35°C.

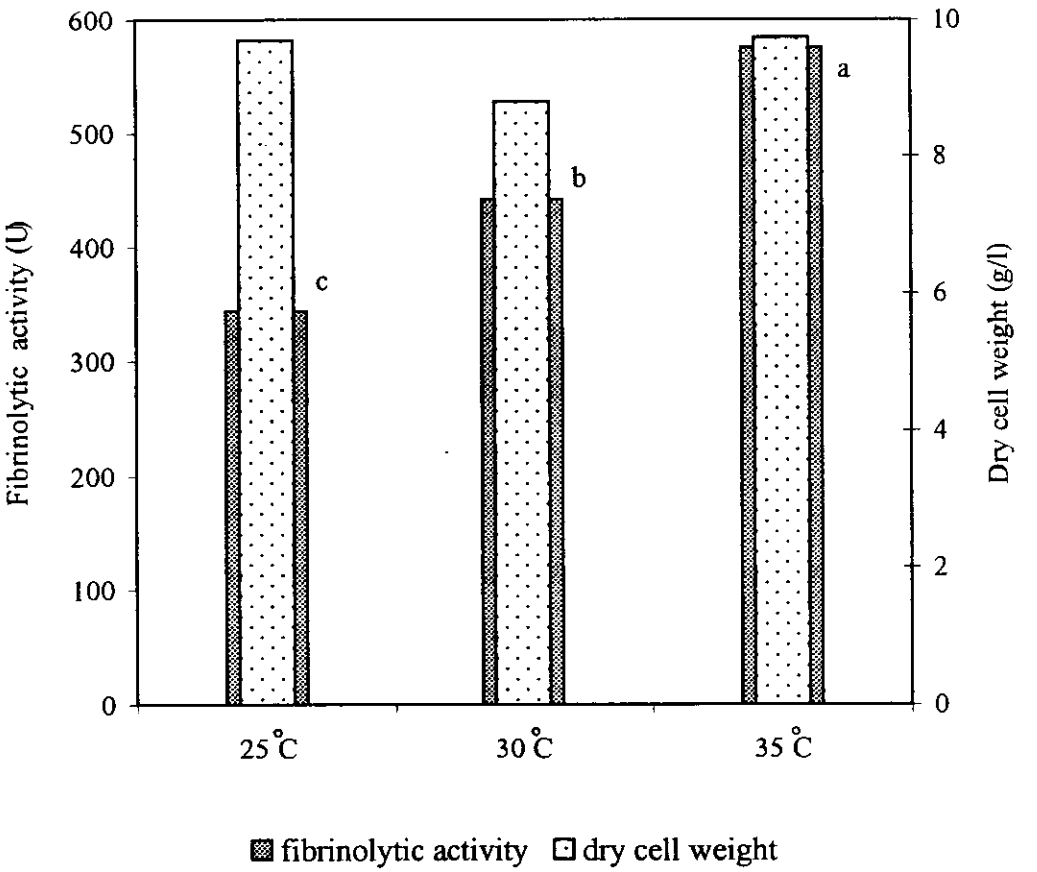

Figure8 Effect of temperature on growth and fibrinolytic enzyme production of *S. commune* BL 23 cultivated in PYGM with initial pH of 6.0 at 150 rpm for 7 days. Only fibrinolytic activity in different temperature were determined for significant comparison. The same alphabet meaned not significant different between groups ($p<0.05$).

1.5 Effect of shaking speed

S. commune BL 23 was cultured in PYGM (initial pH = 6.0) at 35°C with varying shaking speed of 150, 200 and 250 rpm. The maximum dry cell weight of 9.79 g/l was achieved at 200 rpm, followed by 8.62 and 7.65 g/l at shaking speed of 150 and 250 rpm, respectively. While the maximum enzyme activity of 492.06 U was achieved at 150 rpm, followed by 290.03 and 259.04 U ($P<0.05$) at shaking speed of 250 and 200 rpm, respectively as shown in Figure 9. It is possible that the higher of the shaking speed may fragment fungal mycelium. Similar to the study of El-Aassar, *et al.* (1990), fibrinolytic enzyme production of *P. chrysogenum* H9 in the static culture was higher than the free shaken culture.

1.6 Time course of growth and fibrinolytic enzyme production under optimal conditions

S. commune BL 23 was cultured in the optimal medium (PYGM, initial pH = 6.0) at 35°C and 150 rpm. The culture broth was drawn after 3, 5, 7, 10, 12 and 14 days of incubation to determine dry cell weight and enzyme activity. The result was shown in Figure 10. The dry cell weight of 5.50 g/l and the enzyme activity of 492.64 U were obtained after 3 days cultivation. The maximum dry cell weight of 8.93 g/l and the enzyme activity of 576.73 U were achieved on day 7 when the fungus was almost stationary phase of growth. The enzyme activity and cell growth rapidly declined and reached 91.63 U and 5.94 g/l, respectively after 14 days incubation. These results might be an affect of autolysis. Autolysis is considered as the last stage of culture development even when it is observed in parts of hyphae during the stationary phase. The main cause of lysis may be occurred from disturbance of the organells, an accumulation of toxic metabolites, physical, chemical and enzyme influences which disturb the intracellular structure of the cell wall, as well as a lack of nutrients, especially of sources of energy (Fencyl, 1978). In addition, pH of the culture broth was measured in this study. The pH value

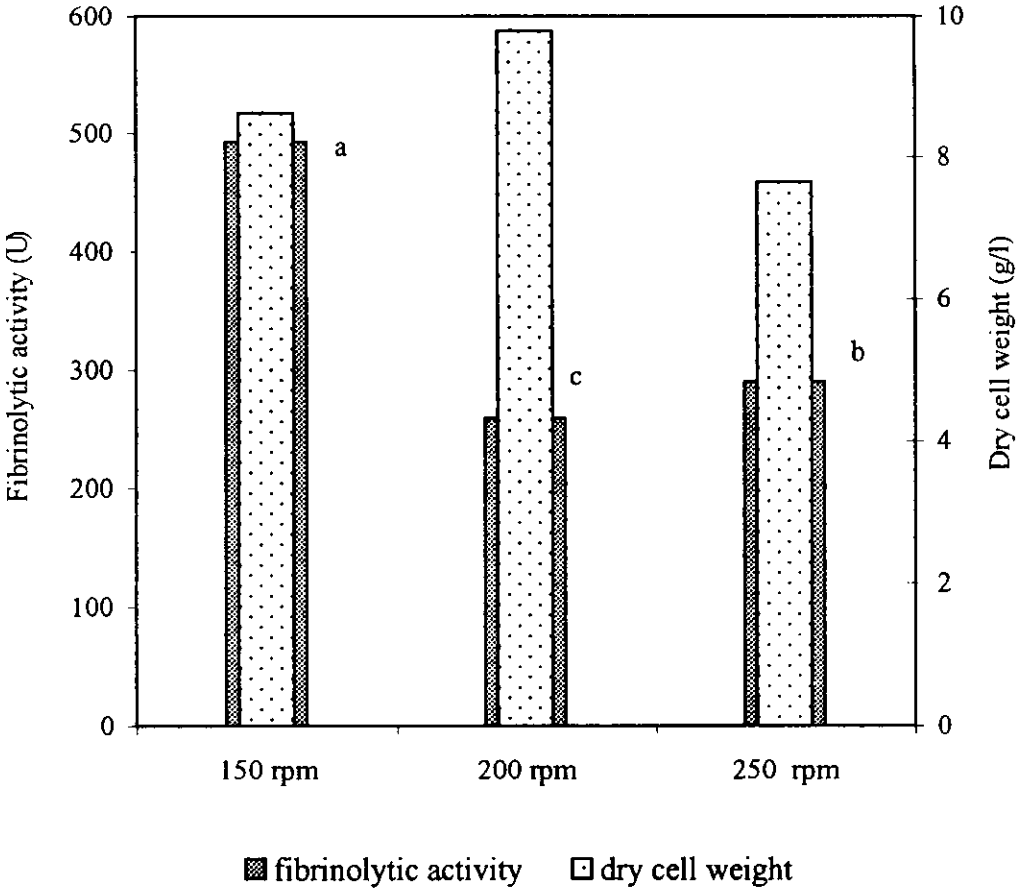

Figure 9 Effect of shaking speed on growth and fibrinolytic enzyme production of *S. commune* BL 23 cultivated in PYGM with initial pH of 6.0 at 35°C for 7 days. Only fibrinolytic activity in different shaking speed were determined for significant comparison. The same alphabet meaned not significant different between groups ($p<0.05$).

was maintained for 5 days and then was gradually increased to pH 8.0 after 12 days of cultivation. It was similar to the previous report, El-Aassar *et al.* (1990) showed that the maximum fibrinolytic activity was obtained when the cell growth was in stationary phase. The pH of the culture broth of *P. chrysogenum* H9 was also shifted to alkaline (pH 8.0) after 2-3 days of incubation. It was confirmed that the maximum fibrinolytic enzyme activity and dry cell weight were obtained in the stationary phase of the fungal growth. Addition to the study of El-Aassar (1995) and Tao *et al.* (1997), the enzyme activity of *F. pallidoroseum* and *F. oxysporum* were raised with an increasing of culture time and the optimal fermentation time for the enzyme production was 4 days in solid state fermentation. Then, prolonged incubation periods decreased the enzyme activity in both of the fungi.

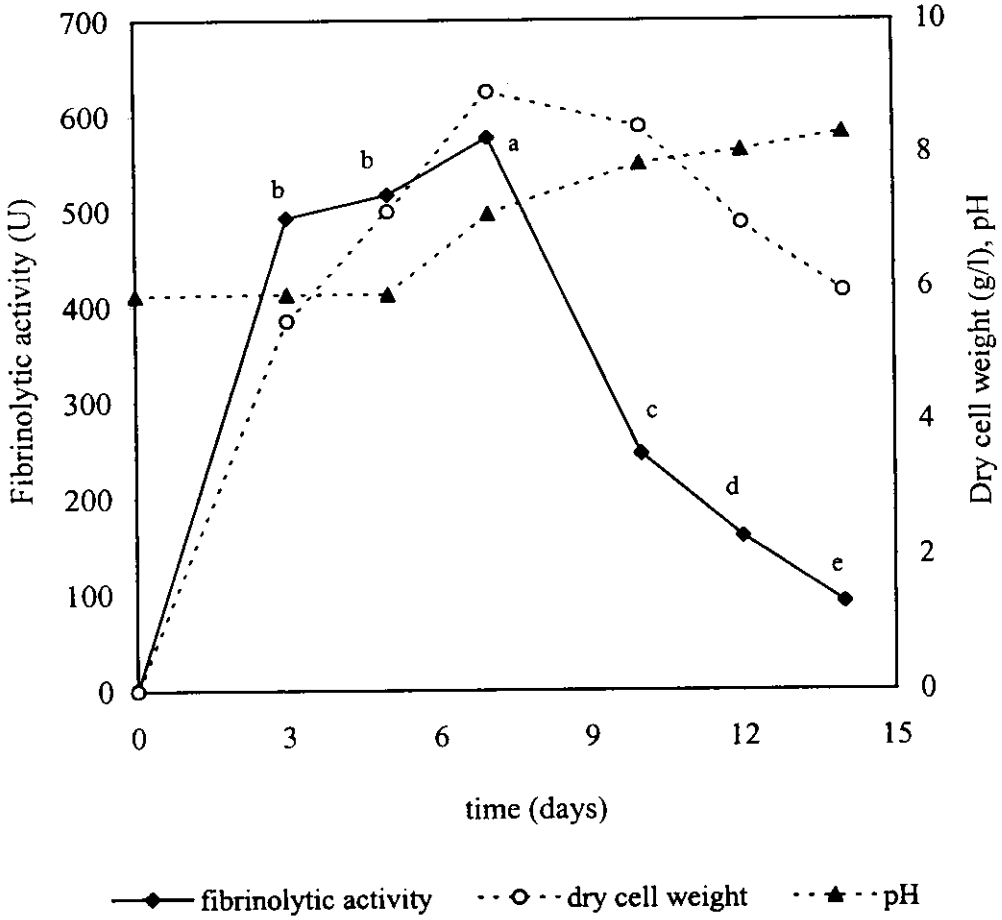

Figure 10 Time course of growth and fibrinolytic enzyme production of *S. commune* BL 23 cultivated in PYGM medium with initial pH of 6.0 at 35°C and 150 rpm. Only fibrinolytic activity in different incubation period were determined for significant comparison. The same alphabet meaned not significant different between groups ($p<0.05$).

2. Purification of fibrinolytic enzyme

2.1 Ammonium sulfate precipitation

Crude enzyme (culture broth) had the fibrinolytic activity of 19,889 units/ml and specific activity of 4,887 units/mg protein (with the protein concentration of 4.07 mg/ml). The culture broth was precipitated by various percentage of saturated $(NH_4)_2SO_4$. Enzyme activity and specific activity were determined after each step, and the results were shown in Table 3. At 40-60% salt saturation, the polymer was appeared in the mixture. After centrifugation of the polymer, the mixture was further salt precipitation. The appropriate salt saturation was 60-80% which showed the highest fibrinolytic activity of 150,720 units/ml and maximum specific activity of 141,256 units/mg protein and protein concentration was 10.67 mg/ml. At 60-80% saturation of $(NH_4)_2SO_4$, most of fibrinolytic enzyme was salting out. However, small amount of the fibrinolytic enzyme activity of 3,063 units/ml and specific activity of 1,721 units/mg protein were detected from the supernatant. El-Aassar (1995) purified fibrinolytic enzyme from *F. pallidoroseum* by fractional precipitation of the crude enzyme with ammonium sulfate (25% to 90%). The fraction precipitated from 50-60% saturation showed the highest specific fibrinolytic activity yielding 37.6% of the original activity. Choi and Shin (1998) purified fibrinolytic enzyme from the crude extract of the *P. ostreatus* fruiting body with 80% $(NH_4)_2SO_4$ and the specific activity of 10 units/mg protein was obtained.

2.2 Dialysis

After salt removal by dialysis, enzyme solution of 14.5 ml was obtained. It was found that the enzyme activity and specific activity after dialysis were decreased. The activity and specific activity of fibrinolytic enzyme were 323,847 units/ml and 12.85×10^4 units/mg protein, respectively. Ammonium sulfate might affected the enzyme activity. Then, three ammonium salts ($(NH_4)_2SO_4$, $NH_4H_2PO_4$ and NH_4Cl) and two sulfate salts

($MgSO_4.7H_2O$ and Na_2SO_4) at the concentration of 20% (w/v) were added into the culture broth and investigated the influence of ammonium ion and sulfate ion on the enzyme activity. The activity of fibrinolytic enzyme was determined after incubation at 4°C for 4 h and the results were shown in Table 4. It was found that all ammonium salts enhanced enzyme activity compared to the crude enzyme. But in case of sulfate salts, the fibrinolytic activity was could be detected only from $(NH_4)_2SO_4$ and $MgSO_4$ whereas the activity could not be done in Na_2SO_4 since it caused polymer formation in the culture broth. Ammonium sulfate was generally used for protein precipitation. From these results it can be clearly stated that $(NH_4)_2SO_4$ showed highly influence on the fibrinolytic activity. This might be the reason that the enzyme activity determined after dialysis was lower than those presented during salt precipitation.

2.3 Anion-exchange column chromatography

The dialyzed enzyme solution was applied into DEAE-Sephacel column. The other proteins were washed out with 20 mM Tris-HCl, pH 7.0 then eluting the required fibrinolytic enzyme with a linear gradient of 0-0.5 M NaCl in the same buffer. The result showed that fibrinolyic enzyme was eluted in a broad range which covered protein from the fraction number 87-158 (Figure 11). The high enzyme activity fractions (number 90-145) were pooled giving the total volumn of 597 ml. The unabsorbed enzymes first emerged from the column and the protein from the fixed charges was displaced by the ability of counterions (salts) (Deutscher, 1990). The specific activity and the purity of the enzyme were summarized in Table 5. The total enzyme activity and percentage yield of fibrinolytic enzyme were $7.04x10^6$ units and 36.47%, respectively. The purification factors of the fibrinolytic enzyme was increased 86 fold.

Table 3 Ammonium sulfate precipitation of fibrinolytic activity at various salt saturation

Fraction	Volumn (ml)	Protein (mg/ml)	Activity (units/ml)	Specific activity (units/mg)
Crude enzyme	200	4.07	19,889	4,887
0-20% sat. $(NH_4)_2SO_4$ (supernatant)	211	3.80	27,367	7,202
20-40% sat. $(NH_4)_2SO_4$ (supernatant)	210	3.13	28,134	8,988
40-60% sat. $(NH_4)_2SO_4$ (supernatant)	186	2.85	19,846	6,964
60-80% sat. $(NH_4)_2SO_4$ (supernatant)	184	1.78	3,063	1,721
60-80% sat. $(NH_4)_2SO_4$ (precipitate)	1.5	10.67	150,720	141,256

Table 4 Effect of ammonium salts and sulfate salts on fibrinolytic activity

Salts	Activity (U)	Percentage
Crude enzyme	612.98	100
$(NH_4)_2SO_4$	976.19	159
$NH_4H_2PO_4$	861.34	141
$MgSO_4.7H_2O$	821.46	134
NH_4Cl	809.86	132
Na_2SO_4	nd*	nd*

nd* = not detected

Table 5 Summary of fibrinolytic enzyme purification steps

Purification Steps	Volumn (ml)	Protein conc. (mg/ml)	Activity (units/ml)	Specific activity (units/mg)	Total activity (units)	Yield (%)	Purity (fold)
Crude extract	1,000	4.21	19,302	4.59×10^3	19.30×10^6	100	1
80% sat. $(NH_4)_2SO_4$	11.0	4.73	1,660,934	35.12×10^4	18.27×10^6	94.65	77
Dialysis	14.5	2.52	323,847	12.85×10^4	4.69×10^6	24.33	28
DEAE-Sephacel	597	0.03	11,794	39.31×10^4	7.04×10^6	36.47	86

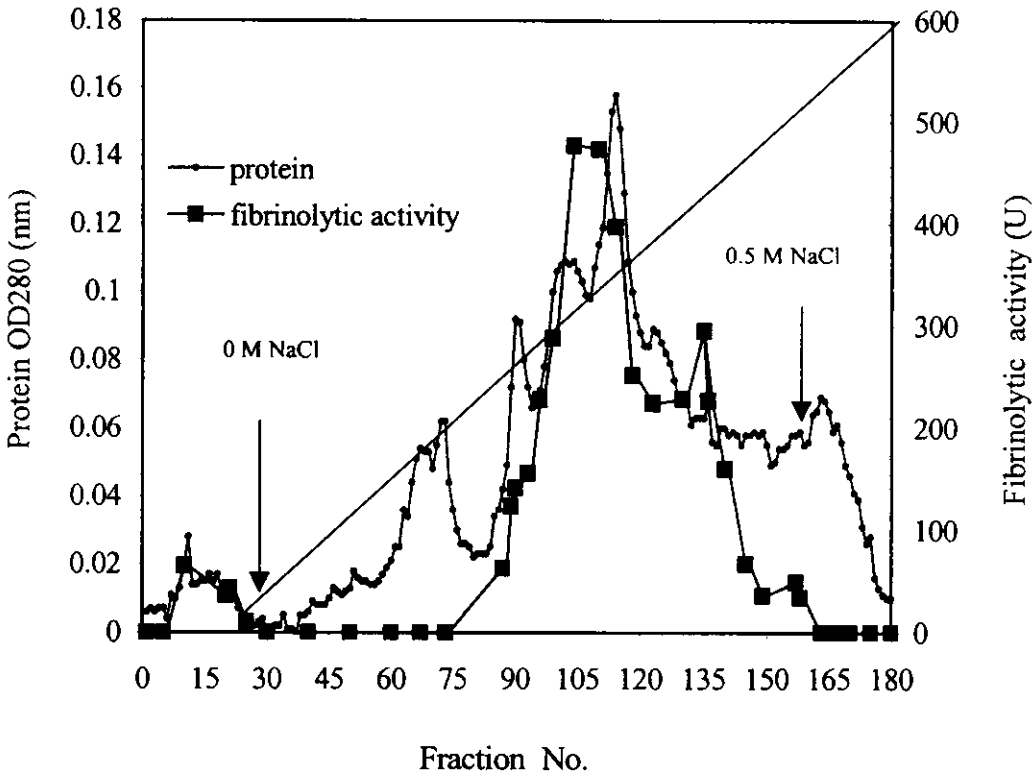

Figure 11 Anion exchange column chromatography on DEAE-Sephacel of fibrinolytic enzyme of *S. commune* BL 23 using linear gradient 0-0.5 M NaCl. The sample applied on the column (2.5x15 cm) was washed with a linear gradient of 0-0.5 M NaCl in 20 mM Tris-HCl, pH 7.0 in 4.0 ml fractions at the flow rate of 0.4 ml/min.

2.4 Gel electrophoresis

The enzyme solutions from each step of purification including ammonium sulfate precipitation, dialysis and DEAE-Sephacel column were detected for the purity using Native PAGE method which presented the characteristics of the native proteins. Although, the crude enzyme (culture broth) contained the various proteins but the protein band (Lane1) could not clearly observed, which may resulted from a lower protein content (about 2 μg/15 μl/well). During ammonium sulfate precipitation (at 40-60% salt saturation), the polymer was appeared in the mixture and then, it was separated by centrifugation. This was possible that the many proteins may be entrapped together in the gel. Then, the supernatant was further salt precipitation (at 60-80% salt saturation), most of fibrinolytic enzyme was salting out. The enzyme still showed many protein bands after dialysis. After DEAE-Sephacel column chromatography, the enzyme appeared as a broad band of protein (Figure 12). This might be resulted from the collection of the many enzyme fractions (fraction No. 90-145). The broad band of protein on Native PAGE was detected for the fibrinolytic enzyme activity on the fibrin plate. Gel was cut into 27 pieces, each piece was 2 mm width and was put onto the fibrin plate. After incubation for 18 h, it was found that the gel pieces number 16 to 21 corresponded to the band which showed fibrin hydrolysis (Figure 13). This partially purified enzyme fraction was used for further characterization.

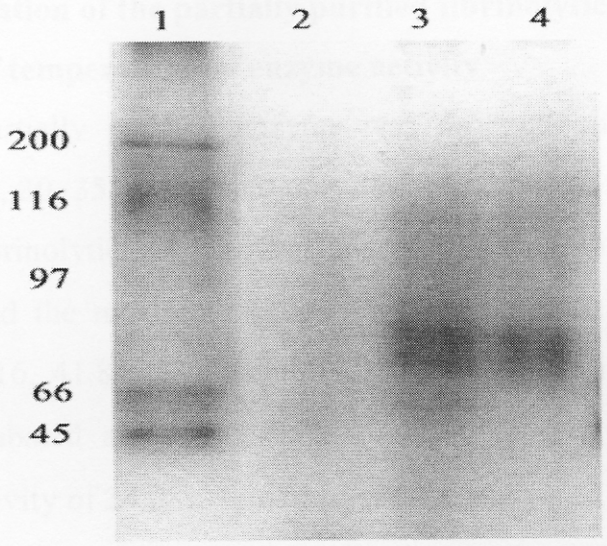

Figure 12 Native-polyacrylamide gel electrophoresis of protein fractions obtained during purification of fibrinolytic enzyme of *S. commune* BL 23. Lane 1, protein standard; 2, crude enzyme; 3, dialysis; 4, DEAE-Sephacel.

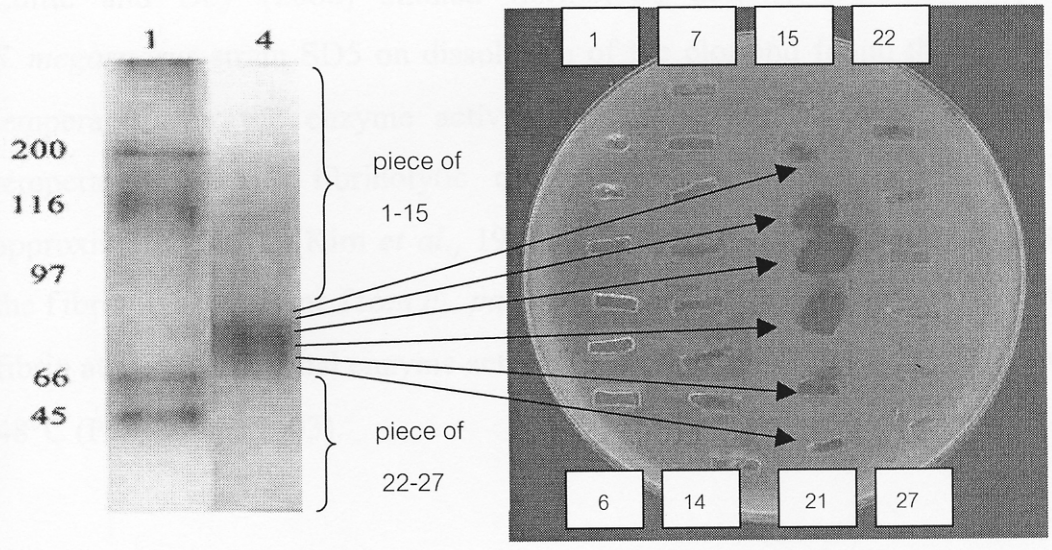

Figure 13 Partially purified fibrinolytic enzyme activity on fibrin plate after running native polyacrylamide gel electrophoresis.

3. Characterization of the partially purified fibrinolytic enzyme

3.1 Effect of temperature on enzyme activity

The partially purified enzyme was dropped onto fibrin plate and incubated at 25, 30, 35, 40, 50 and 60°C for 18 h and the result was shown in Figure 14. Fibrinolytic activity was increased when increasing incubation temperature and the maximum activity was obtained at 50°C. The relative activity of 38.16, 41.84, 58.18 and 76.08% were obtained, when the fibrin plate was incubated at 25, 30, 35 and 40°C, respectively. However, the fibrinolytic activity of 24.08% remained at 60°C.

The optimal temperature (50°C) for the hydrolysis of fibrin from *S. commune* BL 23 was lower than those of fibrinolytic enzyme from *B. subtilis* IMR-NR1 (55°C) (Chang *et al.*, 2000) and the enzyme from *Bacillus* sp. strain CK 11-4 (70°C) (Kim *et al.*, 1996). However, the optimum temperature for fibrinolytic activity in this strain was higher than other results. Chitte and Dey (2000) studied fibrinolytic activity from thermophilic *S. megasporus* strain SD5 on dissolution of the clot and found that optimum temperature for the enzyme activity was 37°C for 2 min. The optimal temperature of the fibrinolytic enzyme from *Bacillus* sp. KA38 was approximately 40°C (Kim *et al.*, 1997). El-Aassar (1995) also reported that the fibrinolytic enzyme from *F. pallidoroseum* was highly active on human fibrin at 40°C. Maximal enzyme activity from *B. amyloliquefaciens* DC-4 was 48°C (Peng *et al.*, 2003).

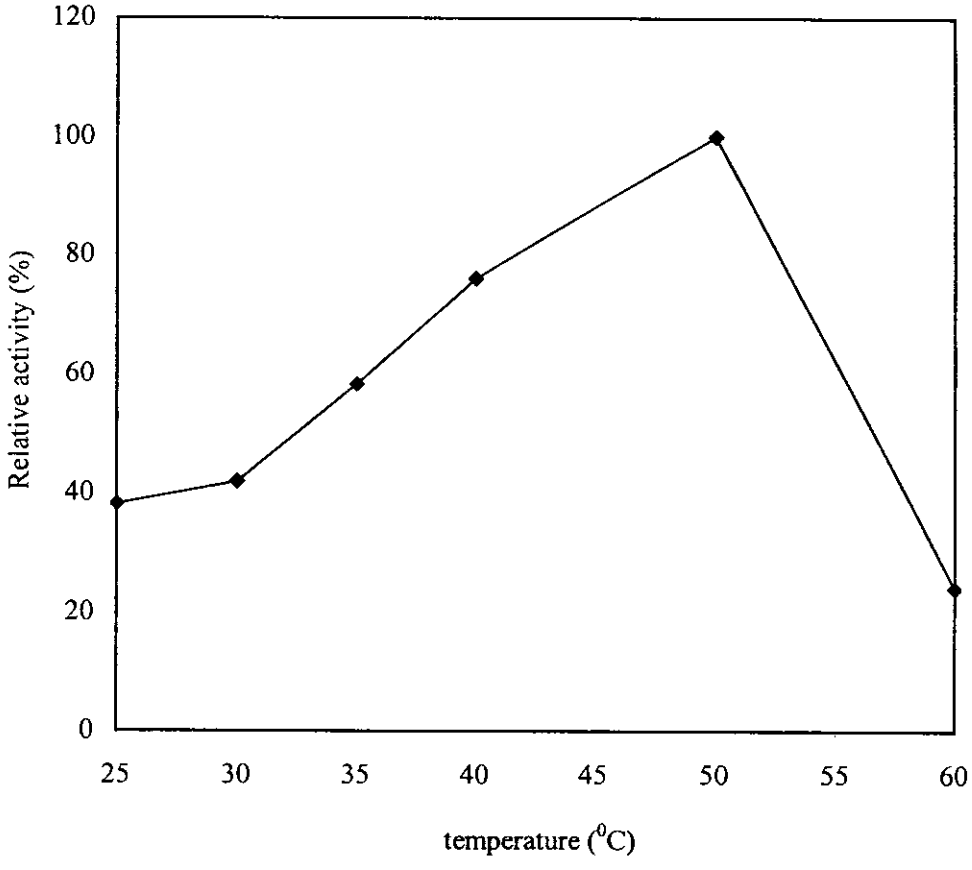

Figure 14 Effect of temperature on enzyme activity, the partially purified enzyme of *S. commune* BL 23 was incubated at various temperature for 18 h.

3.2 Effect of pH on enzyme stability

The partially purified fibrinolytic enzyme was kept in the various buffer solutions at room temperature for 20 min and 48 h. Results on the pH stability of the enzyme were shown in Table 6. Fibrinolytic enzyme from *S. commune* BL 23 was stable in the broad pH range of 5.0-11.0 at room temperature (28°C) for 20 min while during the same period of time, fibrinolytic enzyme of *S. commune*, reported by Hirasawa *et al.* (1997b), was stable in the narrow pH range of 6.5-8.0 compared to these results. After 48 h of incubation, the enzyme was stable in the narrow pH range of 6.0-9.0. It was observed that over 70% of the enzyme activity remained. However, Its activity was lost when it kept at the pH value of 3.0 for 48 h. These results were similar to fibrinolytic enzyme from *F. pallidoroseum* which was fairly stable in a pH range of 6.5-9.0 (40°C, 15 min) with 80% activity remained. While it showed less stability in acidic pH values (El-Aassar, 1995). Moreover, fibrinolytic enzyme from thermophilic *S. megasporus* strain SD5 was also stable in a pH 6.0-9.0 (55°C, 30 min) (Chitte and Dey, 2000). Fibrinolytic enzyme from *Bacillus* sp. strain CK 11-4 was very stable in the pH range of 7.0-10.5 at 30°C for 20 h with 80% residual activity. Above pH 11.0, the enzyme stability abruptly decreased (Kim *et al.*, 1996). The fibrinolytic enzyme from *Bacillus* sp. KA38 was stable at pH range of 7.0-9.0 for 12 h (at 40°C) with 70% residual activity but it became unstable out of this range (Kim *et al.*, 1997).

Table 6 Effect of pH on enzyme stability, the partially purified enzyme of *S. commune* BL 23 was incubated at room temperature (28°C) for 20 min and 48 h.

	Relative activity (%)	
pH	20 min	48 h
3.0	31	0
4.0	62	43
5.0	71	55
6.0	88	70
7.0	92	93
8.0	86	89
9.0	92	77
10.0	95	49
11.0	72	6
control*	100	100

*The partially purified enzyme solution in the buffer solution (pH 7.0) in the ratio of 1:1 was used as the control.

Buffers used were 0.2 M citrate buffer (pH 3.0-6.0), 0.2 M Tris-HCl buffer (pH 7.0-8.0) and 0.2 M glycine-NaOH buffer (pH 9.0-11.0).

3.3 Effect of temperature on enzyme stability

The partially purified enzyme was incubated for 20 min at 30, 37, 40, 50 and 60°C and determined for the retained activity. Fibrinolytic enzyme activity was stable in a broad range of temperature from 30-60°C (relative activity > 70%), but its activity was decreased rapidly from 92.13% to 65.55% at 70°C (data not showed). This results indicated that enzyme tended to denature at 70°C which differred from the results reported by Choi and Kim (2001) who studied the fibrinolytic protease from *B. amyloliquefaciens* DJ-4 and found that the enzyme was stable between 65-70°C for 30 min as 70% of its activity still remained. The stability of fibrinolytic enzyme obtained from this study showed in the same temperature range (60°C, 20 min) of *L. sulphureus* and *Coliorus* sp. reported by Hirasawa (1997b). However, in these results, *S. commune* showed slightly lower temperature (50°C) in retaining its activity comparing to that reported 60°C by the same author. Then, the enzyme solution was incubated at 40, 50 and 60°C for 48 h and the result was shown in Figure 15. Fibrinolytic enzyme was stable with 85.31% residual activity after incubation for 3 h at 60°C. The activity of the enzyme significantly declined and its activity was lost at 60°C within 48 h. This results showed higher temperature comparing with fibrinolytic enzyme from *Bacillus* sp. KA38 which was stable up to 40°C (> 80% remained activity) (Kim *et al.*, 1997) and the enzyme from *B. subtilis* IMR-NK1 which was stable at the same temperature for 40 min (Chang *et al.*, 2000). The fibrinolytic enzyme from thermophilic *S. megasporus* strain SD5was resistant to broad range of temperature ranging from 37-60°C for 30 min but it denatured at 70°C (Chitte and Dey, 2000).

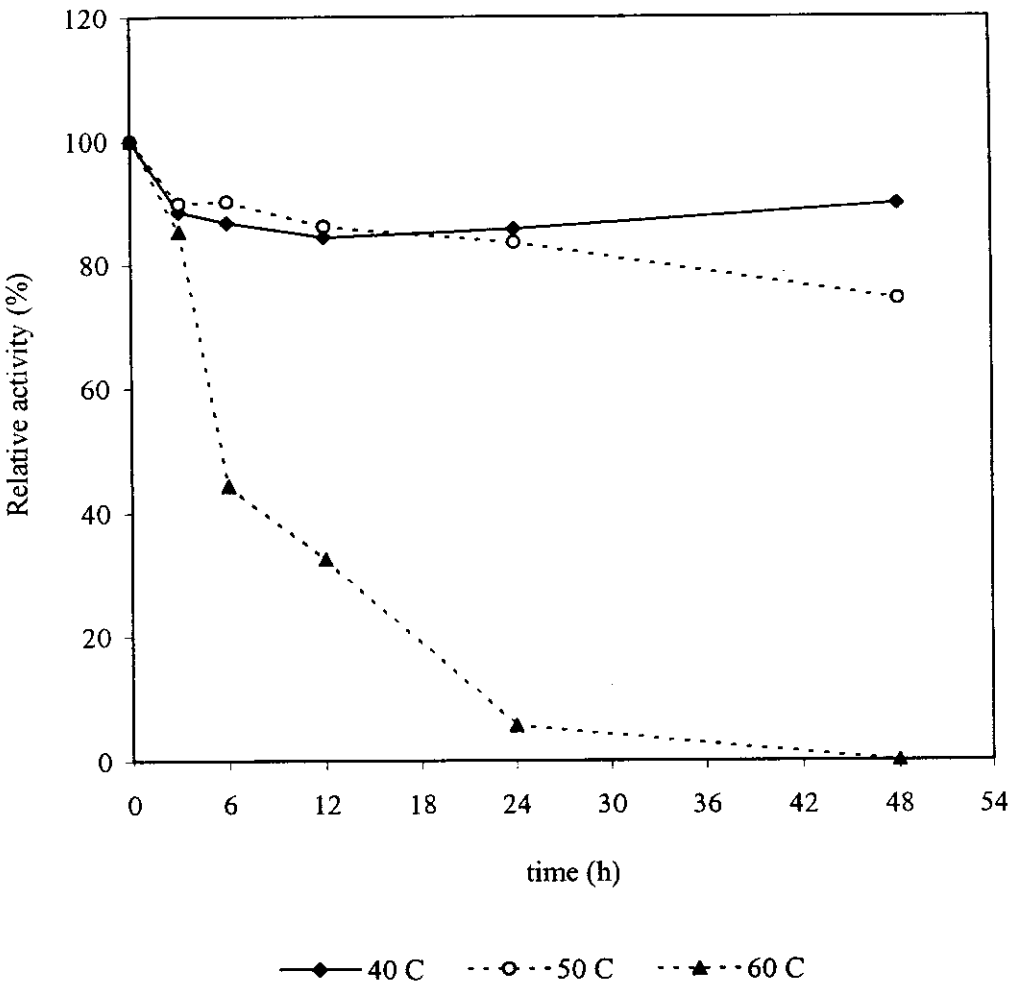

Figure 15 Effect of temperature on enzyme stability, the partially purified enzyme of *S. commune* BL 23 in the buffer solution (pH 7.0) was incubated at 40, 50 and 60°C for 48 h.

3.4 Effect of metal ions and chemical reagents on enzyme activity

The effect of metal ions and chemical reagents on fibrinolytic activity from *S. commune* BL 23 were presented in Table 7. Fibrinolytic enzyme from *S. commune* BL 23 did not inhibit by 1 mM PMSF and 10 μg/ml SBTI. The residual activity were 121 and 127%, respectively. SBTI at 100 μg/ml was also not affected on the enzyme activity. These results indicated that fibrinolytic enzyme from this strain was not serine protease.

EDTA at 1 mM reduced fibrinolytic activity by 32% (68% remained activity) and its activity was decreased when EDTA concentration was increased (22% remained activity). The concentration of 1 mM EDTA was reported to inhibit 80% of fibrinolytic enzyme of *F. pallidoroseum* and its activity was lost when the enzyme was treated with 10 mM EDTA. This result indicated the possibility of the presence of reactive metal ion in the active site of the enzyme (El-Aassar, 1995). Two fibrinolytic enzymes from *F. oxysporum* N.R.C.1 were also inhibited with EDTA (Abdel-Fattah *et al.*, 1993). Similarly, the fibrinolytic enzyme from *Bacillius* sp. KA38 which characterized as metalloprotease was highly repressed by 1 mM EDTA with 14% remained activity (Kim *et al.*, 1997). Fibrinolytic enzyme activity from *Streptomyces* sp. Y405 was inhibited by 1 mM EDTA. In addition, its activity was also inhibited by 10 mM PMSF and 1 mM lysine. This report suggested that the enzyme is a metalloprotease and serine protease and the lysine binding site might play a role in the activity (Wang, 1999).

The enzyme activity was strongly inhibited with 1 mM of 1, 10-phenanthroline (a metalloprotease inhibitor). This is harmony with the results of the fibrinolytic activity from *P. ostreatus* (Choi and Shin, 1998) and *Bacillius* sp. KA38 (Kim *et al.*, 1997). It is suggested that the enzyme is a metalloprotease.

Table 7 Effect of metal ions and chemical reagents on fibrinolytic activity from *S. commune* BL23

Reagents	Final concentration (mM)	Relative activity (%)
$HgCl_2$	1	0
$ZnCl_2$	1	48
$CuSO_4$	1	71
$FeCl_3$	1	82
NaCl	1	85
KCl	1	86
$CaCl_2$	1	96
$CoCl_2$	1	110
EDTA	1	68
EDTA	10	22
1,10 Phenanthroline	1	23
PMSF	1	121
SBTI	10 μg/ml	127
SBTI	100 μg/ml	114
control	-	100

According to the above results, the effect of metal ions on fibrinolytic activity from *S. commune* BL 23 was also studied. Ca^{2+}, K^{+}, Na^{+}, Fe^{3+}, Cu^{2+} and Zn^{2+} at the concentration of 1 mM partially inhibited fibrinolytic activity and the residual activity were 96, 86, 85, 82, 71 and 48%, respectively. Its activity was totally inhibited by Hg^{2+}. However, fibrinolytic enzyme showed its stability when Co^{2+} was added. The similar results was also observed from *F. oxysporum* N.R.C.1 (Abdel-Fattah, 1993). Moreover, El-Aassar (1995) reported that the Co^{2+} at the concentration of 1 mM activating fibrinolytic

activity of *F. pallidoroseum*. In addition, fibrinolytic activity from *P. ostreatus* was recovered in the order of effectiveness by Zn^{2+}, Co^{2+} and Cu^{2+} (Choi and Shin, 1998).

3.5 Enzyme stability after prolonged incubation

The partially purified enzyme solution was incubated at 30°C for 5, 15, 25, 40 and 60 days. The result was shown in Figure 16. The enzyme was stable at pH 7.0, 30°C for 60 days with the remained activity over 90%. The fibrinolytic activity from *L. sulphureus* and *Coriolus* retained at 60°C for 20 min, however, the activity from *S. commune* retained at 50°C. The fibrinolytic enzymes from *S. commune* and *Coriolus* sp. were stable at -15°C for 12 months. On the contrary, when kept the enzyme at 37°C fibrinolytic activity from *S. commune* decreased within one day but it took 3 days for *Coriolus* sp. While the stability of the enzyme from *L. sulphureus* at -15°C and 37°C were 12 months and 6 month, respectively but the activity decreased at 0-10°C for 5 months (Hirasawa *et al.*, 1997b).

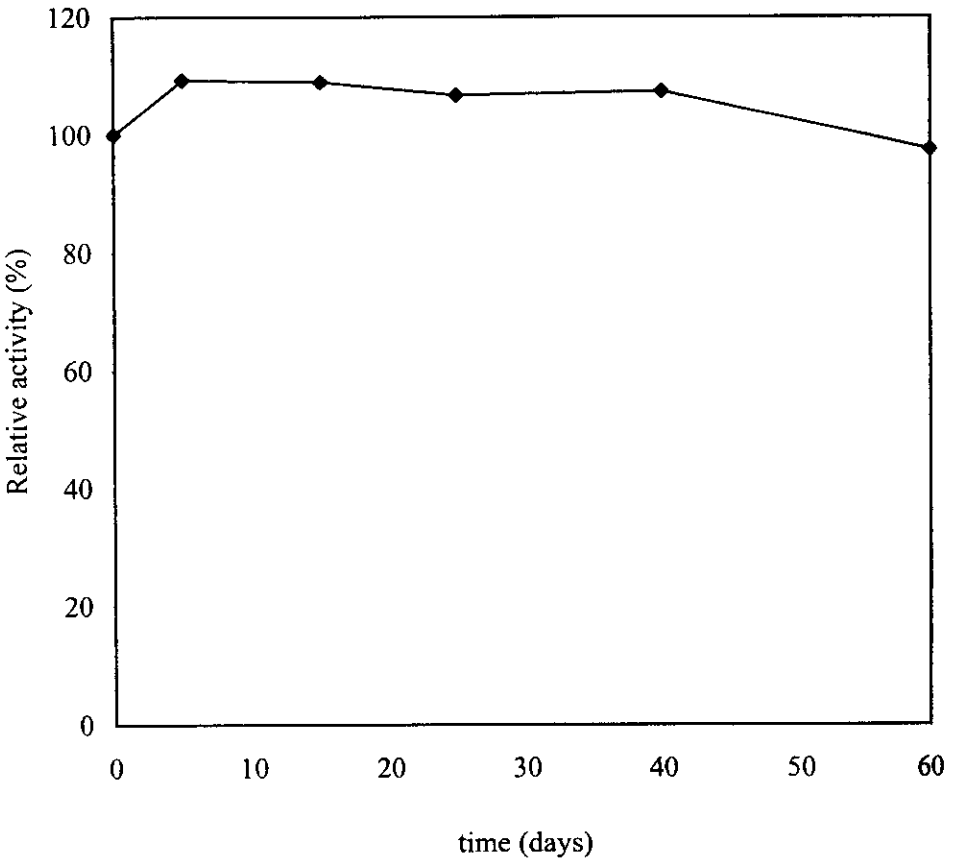

Figure 16 The enzyme stability from *S. commune* BL 23 after prolonged incubation time in the buffer solution (pH 7.0) at 30°C for 60 days.