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Production of a Fibrinolytic Enzyme in Bioreactor Culture by *Bacillus subtilis* BK-17

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Abstract *Bacillus subtilis* BK-17 which produces a novel protease with fibrinolytic activity was isolated from soybean paste. Bioreactor production of the enzyme was studied in order to optimize fermentation conditions such as medium concentration, pH, agitation speed, and temperature. Under most cultural conditions, enzyme production initially began when the cell growth stopped. The onset of the enzyme production was indicated by rapid increase in both dissolved oxygen (DO) and pH. Two- to three-times more concentrated medium than the flask optimum medium yielded higher enzyme production in the bioreactor fermentation. When the medium pH was controlled constant, pH 6.5 exhibited the highest activity in the range of 6.0 to 7.5, but the activity was similar to the case when the pH was initially adjusted to 7.5 and subsequently maintained within a relatively wide range of 6.4 to 7.8. Agitation speed did not affect the enzyme production with an exception of DO reaching zero. Fermentation time was reduced when temperature increased within the range of 25°C to 37°C. However, the highest activity, along with the slow decrease of the enzymatic activity after reaching the maximum value, was observed at 25°C. By shifting the temperature from 37°C to 25°C immediately after DO reached the minimum level, the high enzyme production of 1,100 U/ml along with the short fermentation period of 13 h could be obtained.

Key words: *Bacillus subtilis*, fibrinolytic enzyme, bioreactor optimization

Fibrin is the primary protein component of blood clots, which is formed from fibrinogen by thrombin (EC. 3.4.21.5). Under the normal body conditions, the formation and degradation of fibrin are properly controlled by the help of thrombin and plasmin. However, once the balance fails, blood clots accumulate in the blood vessels, resulting in myocardial infarction and other cardiovascular diseases. According to the recent statistics, the death toll caused by the circulatory diseases in Korea during the year of 1996 was as high as 24.6% [3]. These diseases are more prevailing among elderly people above 50 and categorized as the infirmities of age. Considering the recent age distribution being rapidly shifted to the advanced years, the death toll from these circulatory diseases is consequently expected to increase.

The thrombolytic agents which are currently being used or under clinical investigation in patients with an acute myocardial infarction include streptokinase, two-chain urokinase (tcu-PA), anisoylated plasminogen streptokinase activator complex (APSAC), recombinant tissue-type plasminogen activator (rt-PA), and recombinant single-chain urokinase-type plasminogen activator (rscu-PA) [7, 28]. Among these, strepokinase, APSAC, and tcu-PA are known to cause extensive systematic activation of the fibrinolytic system, but they have a side effect to destroy such plasma proteins as fibrinogen, factor V, and factor VIII [7]. The rt-PA and rscu-PA activate plasminogen preferrentially at the fibrin surface and the fibrin-associated plasmin is protected from rapid inhibition by antiplasmin. This makes the physiological plasmin activators highly specific toward fibrin and thus being used widely. However, they have a relatively short plasma half-life and should be administered repetitively in large quantities making it inconvenient and costly [7].

Recently, Sumi *et al.* have reported that, when urokinase was orally administered, a dog or a human could retain some fibrinolytic activity in the blood for more than 6 h [1, 21, 24-27]. A similar result has been reported with another fibrinolytic enzyme lumbrokinase, which was isolated from the earthworm [16]. These results suggest a

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new way of treating the thrombosis. When the enzyme is produced by food-grade microorganisms in a fermented food supply, it can be used to prevent the thrombosis along with the related diseases. In this respect, the fibrinolytic enzymes isolated from several fermented foods such as Natto [23], Shiokara [22], Chung-Kook-Jang [6], and Jeot-Gal [13] gained a special attention. In addition, a novel *B. subtilis* BK-17 producing a fibrinolytic enzyme was isolated from the soybean paste, a popular Korean traditional food [5, 12]. The enzyme designated as BK-17 was highly specific towards fibrin clots and directly degraded them. The enzyme was identified as a serine protease with the molecular weight of 31 kDa and had N-terminal sequences similar to those of subtilisin [12].

There have been many previous studies on the production of microbial proteases due to their industrial importance [6, 10, 15, 20, 29]. Under most cultural conditions, high carbohydrate concentrations or presence of readily usable nitrogen sources repressed the enzyme production [29]. In many *Bacillus* spp., the synthesis of the proteases occurred during the post-exponential phase and required triggering mechanism similar to those for the initiation of sporulation [6, 29]. In addition, enzyme activity decreased very rapidly at the late exponential phase of a batch culture and this phenomenon was a distinctive characteristic of protease fermentation. These results indicate that the protease fermentations share many common features. However, it is generally accepted that the optimal conditions established for one specific enzyme cannot be applied to another, since factors affecting protease production vary depending on the strains, the kind of proteases to be synthesized, and the medium compositions employed [29].

In the present study, the production of the fibrinolytic enzyme BK-17 in bioreactor culture was studied. An attempt was made to optimize the nutritional and environmental conditions for the purpose of maximizing the culture broth enzymatic activity and minimizing the time of culture. Major parameters investigated include nutrient concentration, pH, agitation speed, and temperature. The deactivation of fibrinolytic activity during the cultivation was also investigated.

MATERIALS AND METHODS

Microorganism and Medium Composition

Bacillus subtilis BK-17 was isolated from the fermented soybean paste [12]. The medium for culture maintenance was LB agar which contains 10 g tryptone, 5 g yeast extract, 5 g NaCl, and 15 g agar in 1-liter distilled water. For the inoculum culture, the same LB-broth was used. A basal medium adopted for the enzyme production contained 15 g soybean flour, 5 g D-glucose, and 0.5 g Na_2HPO_4 in 1 l distilled water, which was developed from the flask experiments [5]. Soybean flour was kindly provided by CKD Pharmaceutical Co. (Seoul, Korea).

Fermentation Conditions

For the inoculum culture, the colonies maintained in the agar plate were taken into 10 ml L-broth in a 30 ml tube and cultivated in a shaking incubator (Model KMC-8450SF, Vision Scientific, Korea) at 37°C and 160 rpm for 5 h. The tube culture was then transferred into 100 mM L-broth in a 500 ml Erlenmyer flask and further incubated for about 7 h under the same condition. When the absorbance at 600 nm (A_{600}), determined by Spectronic 20+ (Milton Roy Co., U.S.A.), reached 1.8 at the end of exponential growth, the whole culture was transferred into a bioreactor.

A 5 l bioreactor (KF-500, Korea Fermentor, Korea) was equipped with a pH electrode (InPro 3000 pH combination electrodes, Mettler Toledo, U.S.A.) and DO electrode (Transmitter type 170, Mettler Toledo, U.S.A.). Fermentations were conducted under the conditions of temperature 37°C, air flow rate 1.3 vvm, agitation speed 800 rpm, and initial pH 7.5, unless specified otherwise. The liquid working volume was 3.0 l. When required, the culture pH was controlled by using 2.5 N HCl and 2.5 N NaOH.

Assay of Enzyme Activity

Quantitative analysis of fibrinolytic activity was carried out by the fibrin plate method of Astrup *et al.* [20] with some modifications [5], as follows: Ten ml of plasminogen-free fibrinogen solution containing 0.6% (w/v) bovine fibrinogen (Sigma) in 0.1 M borate buffer (pH 7.5) was mixed with 0.1 ml of thrombin solution (200 NIH U/ml; Sigma) in a petri dish (100×15 mm) and was solidified at room temperature. Then, three paper discs (Toyo Roshi Kaisha, Ltd.; dia. 8 mm) were placed on each plate, 100 µl of diluted sample solution was added onto each disc, and the plates were incubated at 37°C for 3 h. Clear zones were formed from the degradation of fibrin and the areas were converted into the unit of urokinase activity. One unit (U) was defined as the amount activating plasminogen to give 1.0 A_{275} when incubated for 1 min with α-casein as substrate.

Colony Forming Unit

Since the soy bean flour included in the production medium was not soluble, the cell concentration was determined by measuring the colony forming unit (CFU). After serial dilution of the culture broth, 100 µl was spread onto each of LB agar plates. Then, these plates were incubated at 37°C for 8-12 h, and the number of colonies in the plates with CFU of 30 to 200 were counted.

Glucose Concentration

Glucose concentration was analyzed by the DNS method [17]. Calibration curve was obtained in the range of 0.0 to

1.0 g/l by using a 1.0 g/l glucose standard solution (Sigma Chemical Co., U.S.A.). DNS reagent (10 g 2,3-dinitrosalicylic acid, 2 g phenol, and 10 g sodium sulfite in 1 l of 1% NaOH solution) was stored in an amber bottle at 4°C.

Tunneling Electron Microscope (TEM)

The culture was centrifuged, washed twice with distilled water, and fixed with 2.5% glutaraldehyde. The TEM photograph was taken at Baek Hospital, Pusan, Korea.

Results and Discussion

Bioreactor Fermentation Under Flask Optimal Conditions

Figure 1 shows the time course profiles of bioreactor fermentation under the flask optimal conditions [5]. The exponential cell growth was observed right from the start without any lag time and continued for 6 h. The maximum growth rate was about 1.10 h^{-1}. The initial enzyme activity appeared at approximately 5 h when the cell growth almost ceased, reached a maximum (470 U/ml) at 9 h, and decreased rapidly thereafter. Glucose consumption was very rapid during the exponential growth period, but slowed down when the growth stopped. Change in the cell morphology during the fermentation was shown in Fig. 2 *B. subtilis* BK-17 had endospores during the active production period of the fibrinoloytic enzyme. This result, along with the contents of Fig. 1, indicates that the enzyme can be classified as a secondary metabolite, as in the case of many other proteases [15, 20, 22].

Figure 1 also shows the change in DO and pH during the bioreactor culture. The DO level decreased exponentially during the growth period but showed a sudden increase upon the cessation of growth. This suggests that oxygen requirement of BK-17 varies depending on the growth

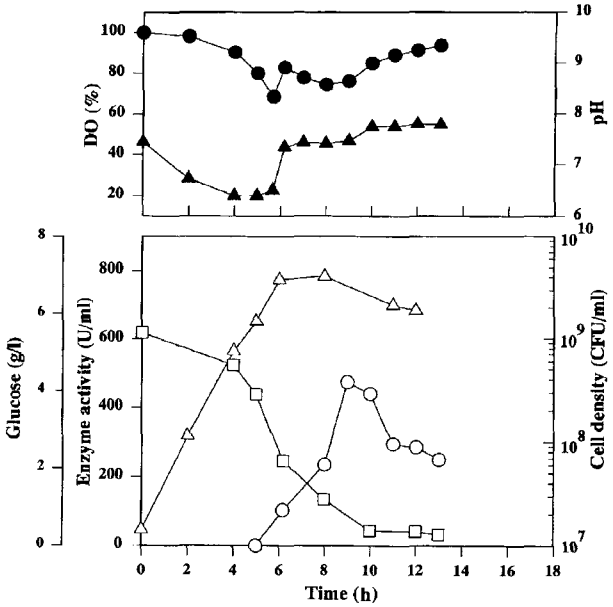

Fig. 1. Time course profiles of cell growth (△), enzyme activity (○), glucose concentration (□), pH (▲), and DO (●) in bioreactor culture under flask-optimal conditions.

stage and DO is an useful parameter for monitoring the fermentation process. The pH of the medium was initially adjusted to 7.5 and maintained in the range of 6.4 to 7.8. The decrease in pH during the initial growth stage is caused by the production of organic acids from glycolysis, as observed previously in the flask culture [5]. Since unusually low pH inhibits the enzyme production [8, 9], pH has to be maintained above 6.4.

Compared to the flask experiments, the fermentation time of bioreactor with maximum enzymatic activity could be shortened by more than 6 h. However, the maximum activity (470 U/ml) was considerably lower than that of the

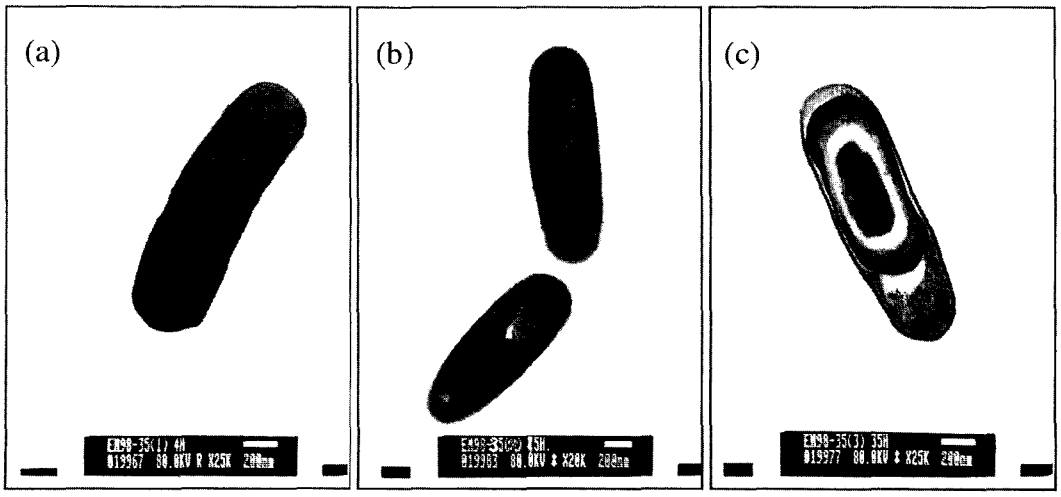

Fig. 2. Transmission electron micrographs of *Bacillus subtilis* BK-17 cells at (a) 4 h (mid-exponential phase), (b) 10 h (stationary phase), and (c) 35 h (death phase).

flask experiments (1,400 U/ml). In addition, decrease in the enzymatic activity after reaching the maximum was remakable in the bioreactor culture. Therefore, it is noticed that the bioreactor optimization should focus not only on increasing the maximum enzymatic activity but also reducing the enzyme degradation. The latter is especially important from the practical standpoint, since the off-line analysis for enzymatic activity takes a relatively long time (refer to Materials and Methods) and cannot be used to determine the most productive harvest time.

Effect of Medium Concentration

Figure 3 shows the effect of medium concentration. The maximum enzyme activity was higher with two- (2×) to three-fold (3×) concentrated media (800 U/ml). However, the rapid deactivation after the activity reached its peak was about the same in all three cases. The time when the enzyme production started was also the same at approximately 5 h, regardless of the medium concentration. Considering the previous reports [15, 20, 22] that the production of most proteases began after some nutrients were depleted, the present result was somewhat unexpected. Glucose concentration did not appear to be related to the initiation of the enzyme production in BK-17. However, the depletion of glucose coincided with the time for the maximum activity and might have affected the cessation of enzyme production and/or the subsequent degradation of the activity. Although not shown in Fig. 3, final cell density rose with the increasing medium concentration but the cell growth rate was about the same in the range of 1.04 to 1.14 h^{-1}. With a concentrated medium, the step increase in DO was also discovered when the cell growth stopped. The minimal DO levels were 69%, 41% and 27% for 1×, 2×, and 3× medium, respectively.

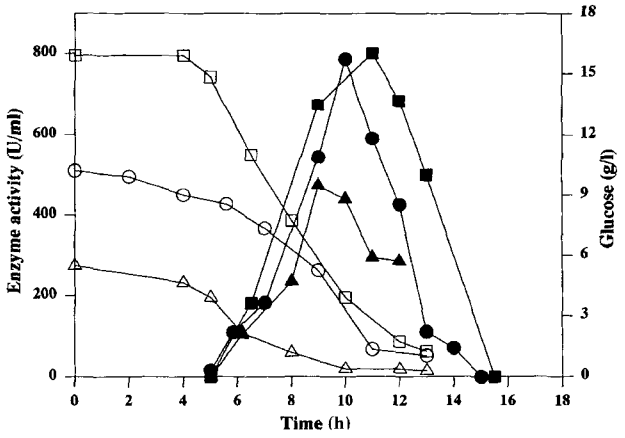

Fig. 3. Effect of medium concentration on enzyme activity (closed symbols) and glucose concentration (open symbols).
Basal medium (▲), two-fold (●), and three-fold (■) concentrated media were tested.

Effect of medium concentration was further studied by conducting fed-batch cultures (data not shown). A bioreactor culture was started with 1× medium and either whole medium or glucose alone was fed intermittantly from 5 h to 12 h. The total amount of medium components added was adjusted to be the same as that of 3× medium or 1× medium plus 3× glucose. For both cases, neither a higher activity nor lower deactivation rate compared to the 3× medium was obtained. These results indicate that the depletion of medium component(s) might not be the major reason for the cessation of the fibrinolytic enzyme or the rapid deactivation of the enzyme, at least under the given conditions. The following experiments were performed with 2× medium.

Effect of Culture Medium pH

Effect of pH was studied in the range of 6.0 to 7.5. When maintained at a constant level, pH 6.5 exhibited the maximum enzyme production of 790 U/ml. At the beginning, cell growth was very similar, but became slower in the late growth period as the medium pH shifted further away from 6.5. At pH 7.5, cell growth was normal but enzyme production was extremely low. This indicates that the production of the fibrinolytic enzyme with BK-17 might not be closely correlated with the cell growth. Although

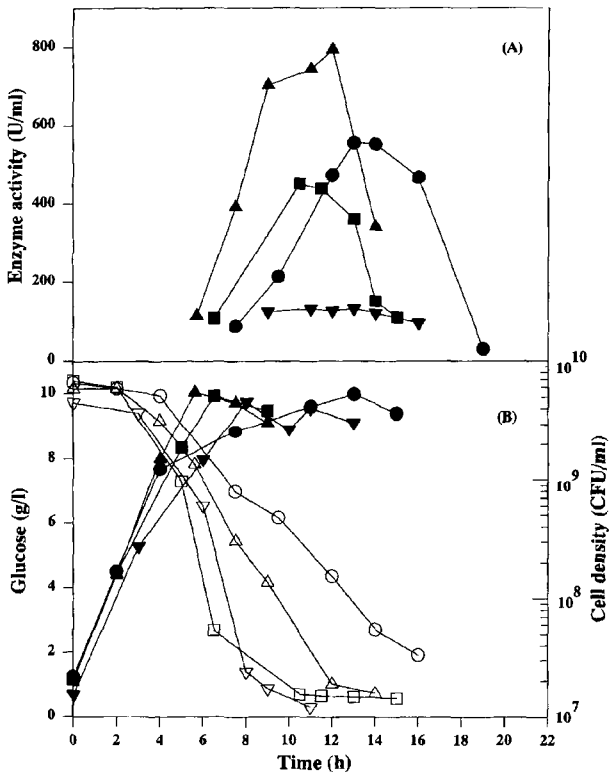

Fig. 4. Effect of pH on enzyme production (A), cell growth (B, closed symbols), and glucose concetration (B, open symbols).
pH 6.0 (●), pH 6.5 (▲), pH 7.0 (■), and pH 7.5 (▼).

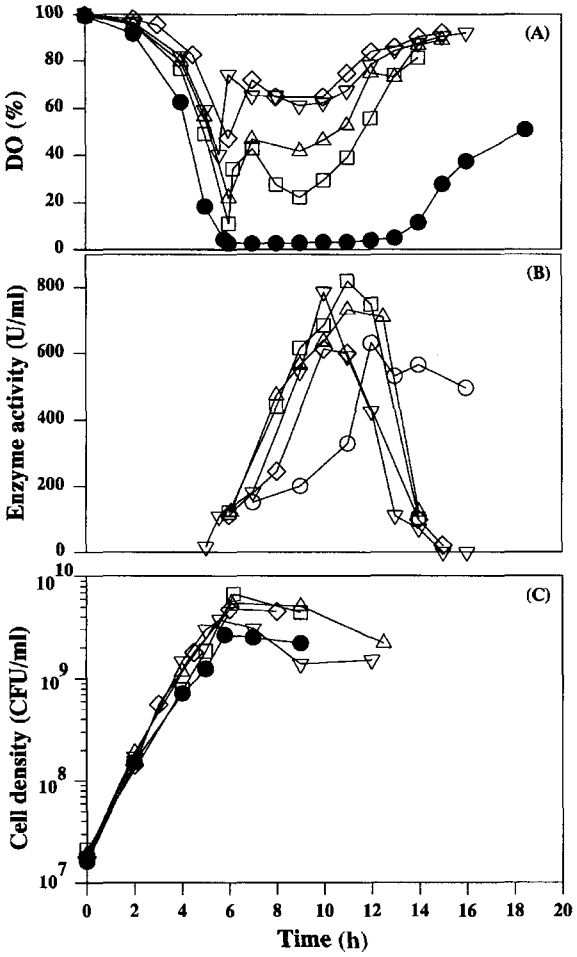

Fig. 5. Effect of agitation speed on DO pattern (A), enzyme production (B), and cell growth (C).
500 rpm (○), 600 rpm (□), 700 rpm (△), 800 rpm (▽), and 900 rpm (◇).

pH 6.5 gave the best result tested, the production level was not higher than the case in whose pH was controlled in a relatively wide range of 6.4 and 7.8 with an initial pH 7.5 (Fig. 3). Therefore, subsequent experiments were carried out with an initial pH of 7.5 and subsequently controlled in the wide range of 6.4 to 7.8.

Effect of DO Concentration

Effect of DO concentration was studied by varying the agitation speed of impeller in the range of 500-900 rpm.

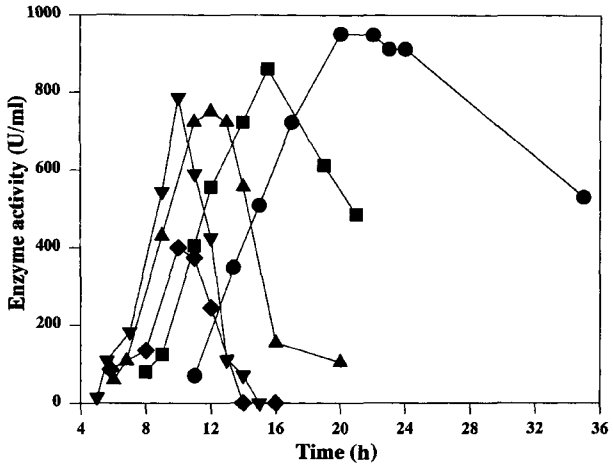

Fig. 6. Effect of temperature on enzyme production.
25°C (●), 30°C (■), 34°C (▲), 37°C (▼), and 40°C (◆).

Cell growth rate was initially the same regardless of the agitation speed but reduced with the decreasing speed because of lowered DO as the fermentation proceeded. Except for 500 rpm, the markedly rapid increase in DO was observed when the growth stopped. The trend of enzyme production was almost the same for 600-900 rpm, although the highest level was somewhat lower at 900 rpm. In the case of 500 rpm, the activity increased slowly and the maximum level was lower than those of 600-800 rpm. However, the deactivation rate of the enzyme after reaching the maximum was much slower, indicating that the low DO alleviates the serious enzyme deactivation.

Effect of Fermentation Temperature

Effect of the fermentation temperature was summarized in Fig. 6 and Table 1. When the temperature was reduced in the range of 25°C to 40°C, cell growth rate decreased from 1.18 h^{-1} to 0.44 h^{-1} and the exponential growth period indicated by the minimum DO increased from 5.8 h to 13.5 h. Activation energy of the cell growth was estimated to be 15.2 kcal/mol. The highest enzymatic activity of 950 U/ml was obtained at 25°C, which is approximately 20% higher than at 37°C. The minimal DO level also increased with the decreasing temperature. This is attributed to the reduction of oxygen consumption rate of the cells as well as the enhanced oxygen solubility at low temperature.

Table 1. Effect of culture temperature on cell growth and enzyme deactivation rate.

Culture temperature	Maximum specific growth rate μ_{max} (h^{-1})	Minimum DO (%)	Time at minimum DO (h)	Enzyme deactivation rate (U/ml·h)
25°C	0.44	72	13.5	28
30°C	0.66	60	8.8	71
34°C	0.97	50	6.7	97
37°C (control)	1.14	41	5.8	180
40°C	1.18	38	5.83	100

The different rate of enzyme deactivation after its peak draws a special attention. It was high at 37°C but rapidly dropped with the decreasing temperature. As a result, it took only 4 h to lose the activity completely at 37°C but at 25°C, more than 50% of the maximum activity remained even after 15 h. Activation energy for the denaturation (E_a') of the enzyme was estimated to be 25.8 kcal/mol, which is somewhat lower than the typical value of 40-70 kcal/mol [4].

Two-Step Cultivation with Temperature Shift

In Fig. 7, the experiment with temperature shift was conducted. During the growth period, the temperature was maintained at 37°C to optimize the cell growth, while during the production period, it was reduced to 25°C to minimize the deactivation of the enzyme produced. The time point for the temperature change was determined based on DO variation. A high activity of 1,100 U/ml along with slow deactivation rate are quite noticeable, although the time for the maximum activity was delayed for a shorter period than the case of 37°C.

Rapid deactivation of extracellular proteases during the late period of *Bacillus* fermentation is a well-known problem [6, 10, 11, 29]. The deactivation of the present fibrinolytic enzyme might be due to three mechanisms such as denaturation, degradation by other proteases, and autolysis by other proteases which have been suggested [6]. However, since the activation energy of the thermal deactivation (E_a') of the present enzyme is relatively low and purified enzyme is highly stable up to 50°C [12], denaturation and autolysis seem to be less attributable than the degradation by other proteolytic enzymes. Among the degrading enzymes, there can be two kinds, i.e., the intracellular enzymes released from cell lysis during sporulation and the extracellular alkaline proteases which are produced during the stationary period. Under most culture conditions in the present study, the considerable cell lysis did not occur during the early deactivation period when enzyme deactivation was most significant. This indicates that the rapid degradation of the fibrinolytic enzyme is most likely associated with alkaline proteases. Further research should be carried out to elucidate the exact mechanism.

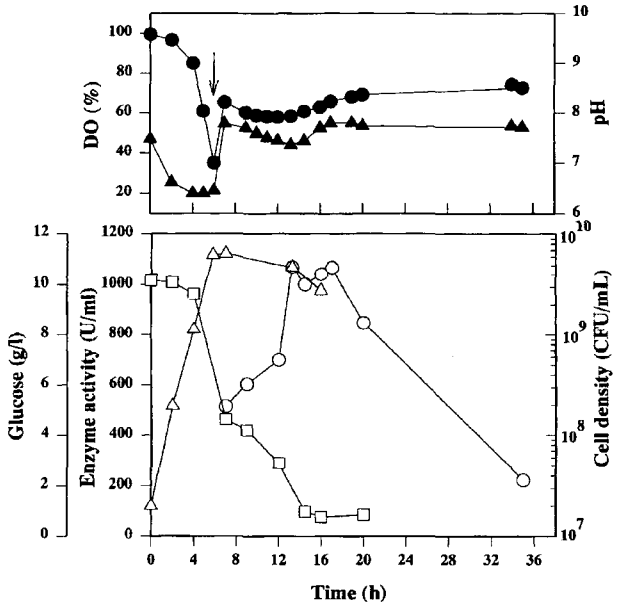

Fig. 7. Time course profiles of cell growth (△), enzyme activity (○), glucose concentration (□), pH (▲), and DO (●) in temperature-shift experiment.
Arrow indicates the time for temperature shift from 37°C to 25°C.

Various methods to stabilize the proteases were also suggested in the literature. Use of different carbon and/or nitrogen sources, different C/N ratio, or addition of stabilizing agents such as metal ions and polymeric materials are good examples [6, 29]. In the present study, the effect of cultivation temperature was only investigated in detail. By simply reducing the temperature in the stationary phase, the degradation rate greately decreased to less than one-sixth. This also seemed to contribute to enhancing the maximum activity to 1,100 U/ml. The technique of changing culture temperatures between growth phase and production phase has already been well established for the secondary metabolites in many fermentations and was clearly effective for the production of fibrinolytic enzyme BK-17 in the present study. For the further improvement, more extensive works in combination with changing other factors such as medium components and environmental conditions should be carried out.

In summary, bioreactor optimization was carried out in the present study with an emphasis on the culture temperature. The maximum activity of the fibrinolytic enzyme could be increased by more than three times. However, it should be pointed out that the maximum activity obtained from the bioreactor culture was still considerably lower than that from the flask culture. This indicates need of much research to be conducted for the further optimization of the bioreactor production of BK-17.

Acknowledgments

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References

1. Abe, T., M. Kazama, T. Kinoshita, I. Naito, T. Ogushi, Y. Yoshimura, J. Teruya, and N. Shimizu. 1982. Shift of fibrinolysis system at oral administration of urokinase in human subjects: Double blind cross over study. *Blood and Vessels (in Japanese)* **13:** 472-479.

2. Astrup, T. and S. Müllertz. 1952. The fibrin plate method for estimating fibrinolytic Activity. *Arch. Biochem. Biophys.* **40:** 346-351.
3. Anonymous. 1997. Statistics of '96 death toll in Korea, Department of Population Analysis, The Bureau of Statitics, Korea.
4. Bailey, J. E. and D. F. Ollis. 1986. *Biochemical Engineering Fundamentals*, p. 140. 2nd ed. McGraw-Hill, New York, U.S.A.
5. Choi, W.-A., J.-W. Lee, K.-H. Lee, and S.-H. Park. 1998. Effects of enviromental and nutritional conditions on fibrinolytic enzyme production from *Bacillus subtilis* BK-17 in flask culture. *Korean J. Biotechnol. Bioeng.* **13**: 491-496.
6. Chu, I.-M., C. Lee, and T.-S. Li. 1992. Production and degradation of alkaline protease in batch cultures of *Bacillus subtilis* ATCC 14416. *Enzyme Microb. Technol.* **14:** 755-761.
7. Collen, D., H. R. Lijnen, and H. K. Gold. 1991. Towards better thrombolytic therapy. *Progress in Cardiovascular Diseases* **34:** 101-112.
8. Haavik, H. I. 1974. Studies on the formation of bacitracin by *Bacillus licheniformis*: Effect of glucose. *J. Gen. Microbiol.* **81:** 383-390.
9. Haavik, H. I. 1974. Studies on the formation of bacitracin by *Bacillus licheniformis*: Role of catabolite repression and organic acids. *J. Gen. Microbiol.* **84:** 321-326.
10. Hanlon, G. W., N. A. Hodges, and A. D. Russell. 1982. The influence of glucose, ammonium and magnesium availability on the production of protease and bacitracin by *Bacillus licheniformis*. *J. Gen. Microbiol.* **128:** 845-851.
11. Hubner, U., U. Bock, and K. Schugerl. 1993. Production of alkaline serine protease subtlisin Carlsberg by *Bacillus licheniformis* on complex medium in a stirred tank reactor. *Appl. Microbiol. Biotechnol.* **40:** 182-188.
12. Jung, Y.-K., I.-S. Kong, and S.-H. Park. 1996. Fibrinolytic enzyme isolated from *Bacillus subtilis*. Korea Patent Number 96-8674.
13. Kim, H.-K., G.-T. Kim, D.-K. Kim, W.-A. Choi, S.-H. Park, Y.-K. Jeong, and I.-S. Kong. 1997. Purification and characterization of a novel fibrinolytic enzyme from *Bacillus* sp. KA38 originated from fermented fish. *J. Ferm. Bioeng.* **84:** 307-312.
14. Kim, W., K. Choi, Y. Kim, H. Park, J. Choi, Y. Lee, H. Oh, I. Kwon, and S. Lee. 1996. Purification and characterization of a fibrinolytic enzyme produced from *Bacillus* sp. strain CK-14 screened from Chungkook-Jang. *Appl. Environ. Microbiol.* **62:** 2482-2488.
15. Levisohn, S. and A. I. Aronson. 1967. Regulation of extracellular protease production in *Bacillus cereus*. *J. Bacteriol.* **93:** 1023-1030.
16. Mihara, H., H. Sumi, T. Yoneta, H. Mizumoto, R. Ikeda, M. Seiki, and M. Maruyama. 1991. A novel fibrinolytic enzyme extracted from the earthworm, *Lumbricus rubellus*. *Jpn. J. Physiol.* **41:** 461-472.
17. Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31:** 426-428.
18. Nakamura, T., Y. Yamagata, and E. Ichishima. 1992. Nucleotide sequence of the subtilisin NAT gene, *aprN*, of *Bacillus subtilis* (natto). *Biosci. Biotech. Biochem.* **56:** 1869-1871.
19. Priest, F. G. 1977. Extracellular enzyme synthesis in the genus *Bacillus*. *Bacteriol. Rev.* **41:** 711-753.
20. Riedel, K., A. Schroeter, P. Liebs, J.-P. Graba, M. Hecker, and D. Schrapel. 1987. Formation of extracellular neutral proteinase and the stringent response in *Bacillus subtilis*. *Folia Microbiol.* **32:** 96-100.
21. Sasaki, K., S. Moriyama, H. Sumi, N. Toki, and K. C. Robbins. 1985. The transport of ^{125}I-labeled human high molecular weight urokinase across the intestinal tract in a dog model with stimulation of synthesis and/or release of plasminogen activators. *Blood* **66:** 67-75.
22. Schaeffer, P. 1969. Sporulation and the production of antibiotics, exoenzymes, and exotoxins. *Bacteriol. Rev.* **33:** 48-71.
23. Sumi, H., H. Hamada, H. Tsushima, H. Mihara, and H. Muraki. 1987. A novel fibrinolytic enzyme (Nattokinase) in the vegetable cheese natto: A typical and popular soybean food in the Japanese diet. *Experientia* **43:** 1110-1111.
24. Sumi, H., M. Maruyama, T. Yoneta, and H. Mihara. 1983. Activation of plasma fibrinolysis after intrarectal administration of high molecular weight urokinase and its derivative. *Acta Haematol.* **70:** 289-295.
25. Sumi, H., M. Seiki, N. Morimoto, H. Tsushima, M. Maruyama, and H. Mihara. 1985. Plasma fibrinolysis after intraduodenal administration of urokinase in rats. *Enzyme* **33:** 121-127.
26. Sumi, H., N. Toki, K. Sasaki, and K. C. Robbins. 1980. Oral administration of urokinase. *Thromb. Res.* **20:** 711-714.
27. Toki, N., H. Sumi, K. Sasaki, I. Boreisha, and K. C. Robbins. 1985. Transport of urokinase across the intestinal tract of normal human subjects with stimulation of synthesis and/or release of urokinase-type proteins. *J. Clin. Invest.* **75:** 1212-1222.
28. Verstraete, M., H. R. Lijnen, and D. Collen. 1995. Thrombolytic agents in development. *Drugs* **50:** 29-42.
29. Ward, O. P. 1985. Proteolytic enzymes, pp. 789-815. *In* M. Moo-Young (ed.), *Comprehensive Biotechnology*, vol. **3**. Pergamon Press, Oxford, U.K.