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Production of α-Amylase using Aqueous Two-Phase System

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수성 2상계를 이용한 알파-아밀라제의 생산

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Aqueous two-phase fermentation system was tested for the overproduction of extracellular enzyme through α-amylase fermentation by *Bacillus amyloliquefaciens*. By employing aqueous two-phase system α-amylase activity showed 25% increase compared to the result using regular medium and no deactivation of the enzyme was observed. The presence of polyethylene glycol was observed to promote the enzyme production, while to inhibit the growth of the microorganism. It is recommended that polyethylene glycol be added during the log-growth phase and dextran be added after the enzyme activity reaches its maximum for efficient α-amylase fermentation and *in situ* recovery of the enzyme.

In recent years, many researches have been performed on the biosynthesis and application of enzymes of microbial origins. For overproduction of enzymes many different approaches, such as strain development by mutation or genetic engineering, optimization of bioreactor system, and development of efficient recovery and purification processes, have been studied.

Aqueous two-phase system have been recently developed for the separation of intracellular proteins from cell debris mixtures(1-5), enzymatic bioconversion(6), and acetone-butanol fermentation (7). The basis of separation by aqueous two-phase system is the selective distribution of substances between the phases formed by the incompatibility between polymers (e.g. polyethylene glycol) and polymer or salt(e.g. dextran or potassium phosphate). In this paper, feasibility of novel fermentation process employing aqueous two-phase system is reported for overproduction of the extracellular enzymes.

Materials and Methods

Strains and culture conditions

Bacillus amyloliquefaciens strain F (ATCC 23350) was used for α-amylase production. All the fermentations were performed in 250 m*l* flasks. 100 m*l* of medium was used and the microorganism was grown in incubating shaker with agitation speed of 150 RPM and at 37 °C. The composition of the nutrients used in the regular medium is as follows (in g/*l*): maltose, 10.0; ammonium phosphate, 5.0; yeast extract, 1.0; K_2HPO_4, 1.0; $MgSO_4 \cdot 7H_2O$ 0.5; sodium citrate, 0.5; $CaCl_2$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.1; $MnSO_4 \cdot H_2O$, 0.1.

Aqueous two-phase system

Polyethyleneglycol (PEG 6000, M.Wt. 6000, Sigma Chemical Co.) and Dextran T37(M. Wt. 15300, Sigma Chemical Co.) were used as components in aqueous two-phase system for the technical feasibility study of aqueous two-phase system for

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extracellular enzyme production. Two-phase system was constructed with 5%(w/w) of PEG 6000, 7% (w/w) of Dextran T37, and 88% (w/w) of fermentation broth.

Analytical methods

The optical density of cell mass was measured using a spectrophotometer at 650 nm. The dry cell mass was obtained from fermentation broth after centrifugation, washing, and drying overnight at 90 °C. The maltose concentration was measured by Nelson-copper method(8). α-Amylase activity was measured using a starch-iodine method(9). One α-amylase unit was defined as the amount of enzyme such that 1 mg of starch is hydrolyzed by 1 m*l* of α-amylase solution in 10 minutes at 25 °C and pH 5.9. Protease activity was measured using casein solution according to the method by Aiyappa *et al.* (10). One unit of protease was defined as the amount of enzyme which 1 μmole of tyrosine can be produced for 10 minutes at 37 °C and pH 7.5.

Results and Discussion

Aqueous two-phase system

Partition coefficient of α-amylase in the model system (5% PEG and 7% Dextran) was found to be 0.45 and the volume ratio (volue of top phase/volume of bottom phase) was 1.5. Therefore resulting yield of α-amylase in the top phase and in the bottom phase was 40.3% and 59.7% respectively. Most of PEG was distributed in the top phase and most of dextran was located in the bottom phase.

α-Amylase production using aqueous two-phase system

First experiment was performed using the regular medium without aqueous two-phase system. α-Amylase was produced during growth phase and early stationary phase. Maximum enzyme activity obtained was 820 unit/m*l* at 50 hours of incubation. The enzyme activity began to decrease after the enzyme activity reached a maximum point. Maximum cell density was 2.32 g/*l* and specific enzyme activity was 373 unit of enzyme/mg of dry cell mass.

A two-phase culture medium composed of 88% of regular medium, 5% of PEG, and 7% of Dextran was used for α-amylase production. Cell mass was located at the bottom phase and enzyme activity was observed both at the bottom phase and top phase. Maximum cell density was 1.9 g/*l* and maximum enzyme activity in the top phase and in the bottom phase was 629 unit/m*l* and 1360 unit/m*l* respectively. Considering the volume ratio of 1.5, average α-amylase activity in the whole culture broth was 921 unit/m*l* and specific enzyme activity was 460.7 unit/mg of dry cell mass. Cell density was 17% lower but total enzyme activity was 12.4% higher in the aqueous two-phase fermentation sys-

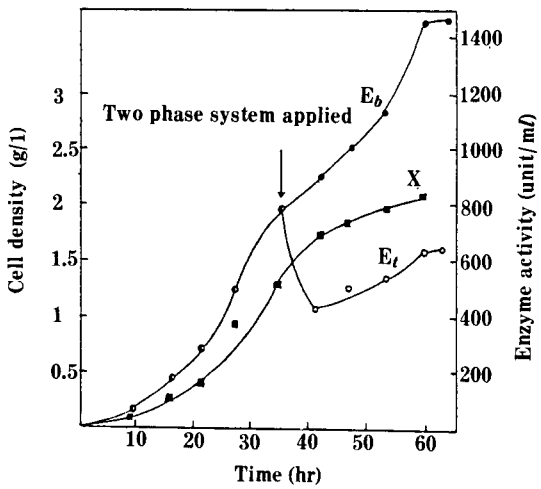

Fig. 1. Production of α-amylase with *Bacillus amyloliquefaciens* when aqueous two-phase system was applied at 35 hours of fermentation.

◑ : α-amylase activity before aqueous two-phase system was applied,
● : α-amylase activity in the bottom phase,
○ : α-amylase activity in the top phase,
■ : cell density.

Table 1. Comparison of maximum α-amylase activity from different fermentation systems. *1: Aqueous two-phase system was formed at the fermentation time t = 0. *2: Aqueous two-phase system was formed at the fermentation time t = 35 hours.

Fermentation system	X_{max} (g/*l*)	E_{max} (unit/m*l*)		Remarks
Regular medium	2.3	820		100 %
Aqueous		Top	629	
two-phase system	1.9	Bottom	1360	
(t = 0) *1		Average	921.4	112.4 %
Aqueous		Top	625	
two-phase system	2.0	Bottom	1425	
(t = 35) *2		Average	954	116.4 %

tem compared to the results in the ordinary fermentation system.

Since lower cell density in the two-phase system was due to the two-phase system, two-phase system was constructed at the middle of growth phase to minimize the two-phase effect on cell growth. As shown in Fig. 1, cell density reached 2.0 g/l and maximum enzyme activity in the top phase and in the bottom phase was 625 unit/ml and 1425 unit/ml respectively. Average enzyme activity obtained was 954 unit/ml, which was 16.4% higher compared to the result (820 unit/ml) obtained in the ordinary fermentation system. The above results are summarized in Table 1. Furthermore no deactivation of the enzyme was observed in the aqueous two-phase syste. Enzyme deactivation after cell growth was, in many cases, severe in ordinary fermentation system.

Effects of aqueous two-phase system

As a next step, experiments were performed to find out the causes of higher enzyme activity obtained in the two-phase system. First, various levels of PEG were added to the medium to find the effects of PEG on the fermentation. As shown in Fig. 2, maximum enzyme activity increased as the concentration of PEG increased, while maximum cell density showed a tendency of decline. With 5% of PEG in the medium, maximum enzyme activity increased by 14% compared to the result obtained from regular medium. PEG is generally known to promote the fluidization of protoplast and transportation of substances through cell wall and thus being used for protoplast fusion (11). The increase of α-amylase activity seems due to these effects of PEG, even though the detailed mechanisam of PEG on cell growth and α-amylase biosynthesis has not been clarified.

Since no deactivation of the enzyme was observed in the two-phase fermentation system, various effects on enzyme deactivation were considered. Many papers have been published on the deactiva-

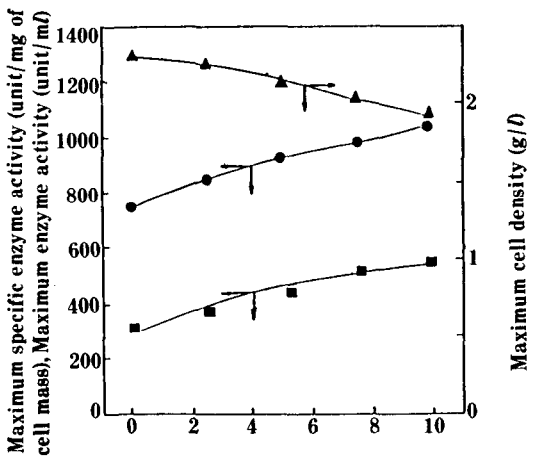

Fig. 2. Effects of PEG on α-amylase biosynthesis.
● : maximum enzyme activity (unit/ml),
▲ : cell density (g/l),
■ : maximum specific enzyme activity (unit/mg of cell mass).

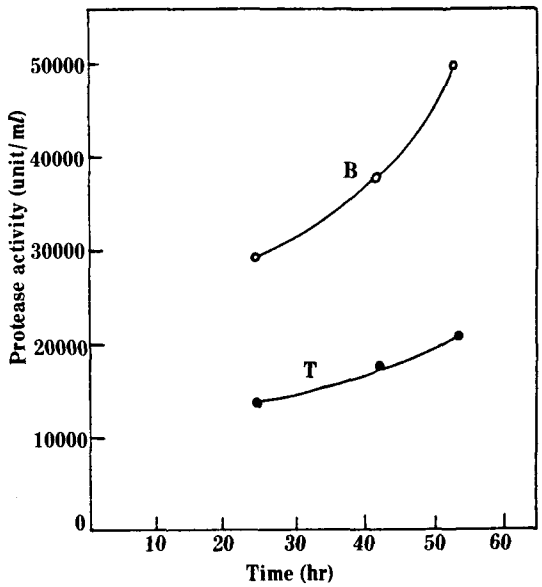

Fig. 3. Protease activities in a aqueous two-phase fermentation system.
○ : protease activity in the bottom phase,
● : protease activity in the top phase.

Table 2. Comparison of maximum α-amylase activity obtained from a suggested novel fermentation system. *: PEG was added at t=35 hours and dextran was added at t=60 hours of fermentation.

Fermetation system	X_{max} (g/l)	E_{max} (unit/ml)		Remarks
Ordinary fermentation system (no aqueous two-phase system)	2.30	800		100 %
Suggested aqueous two-phase fermentation system*	2.03	Top	653	
		Bottom	1510	
		Average	995.8	124.5 %

tion of enzymes. Temperature, extracellular protease, and autolysis during spore formation(12-14) were suggested as plausible causes of enzyme deactivation. Since temperature effect is not governing compared to the degree of deactivation in the fermentation broth(15), protease effect on α-amylase activity was investigated. As shwon in Fig. 3, protease activities were observed both in the top phase and in the bottom phase, and protease activities in both phases showed similar trends with α -amylase activities in both phases (Fig. 1). Therefore the presence of protease can not explain the stability of α-amylase in the two-phase fermentation system. Since cells also exist in the bottom phase where α-amylase located, the stability of α-amylase can not be explained. Even though no clear explantation has been made, it is certain that two-phase system contributes to the stability of α-amylase.

Development of a novel fermentation method

Since PEG promoted α-amylase biosynthesis, while inhibiting the growth of *Bacillus amyloliquefaciens,* a novel fermentation method was suggested to increase the fermentation efficiency; (i) grow the microorganism initially using regular medium, (ii) when cell density increases to a certain level, add PEG to the fermentation broth to increase α-amylase biosynthesis rate, and (iii) when enzyme activity reaches its maximum, add dextran and construct a aqueous two-phase system.

By applying this strategy for α-amylase production, α-amylase activity increased by 24.5% as shown in Table 2 and no deactivation was also observed. Even though the detailed physiological effects and mechanism of the aqueous two-phase fermentation system are not clarified enough, aqueous two-phase fermentation system developed in this research provides many desirable schemes, such as increased enzyme activity, prevention of enzyme deactivation, and a possible means of enzyme separation. The enzyme in the PEG-rich top phase can be recovered using ultrafiltration separation technique and PEG can be recycled for economic operation (16). By finding also optimum formula of two-phase system, it is possible to isolate most of the enzyme in the top phase, and by finding optimum times for the addition of PEG and dextran, α-amylase activity can be increased further, which are left for further research.

요 약

세포외 효소를 효율적으로 생산하기 위한 새로운 발효시스템으로서 수성 2상계를 시험하였다. 실험대상으로는 *Bacillus amyloliquefaciens* 균주에 의하여 α-amylase를 생산하는 시스템을 선정하였다.

Polyethylene glycol 5%, dextran 7%, 그리고 발효액이 88% 포함된 수성 2상계 발효시스템에 의하여 α-amylase의 생합성은 25% 정도 증가하였으며 효소의 비활성화는 관찰되지 않았다. 또한 Polyethylene glycol 이 효소의 생합성을 증진시키는 것으로 관찰되었다. 효소의 생합성을 증가시키며 동시에 효소를 발효액으로부터 분리하기 위하여는 polyethylene glycol을 성장도중에, dextran은 효소의 활성이 최고에 달한 후에 발효액에 가하는 발효방법이 바람직하다.

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