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Effects of Carbon Sources and Other Process Variables in Fed-Batch Fermentation of Penicillin

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페니실린 발효 공정에 있어서 탄소원 및 다른 공정변수가 미치는 영향

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ABSTRACT

In the fed-batch fermentation of penicillin specific uptake rates of carbon source and ammonia nitrogen, and specific production rate of penicillin as the most important process variables were evaluated over the fermentation course and their effects on the productivity studied. As the results, glucose and lactose each as a major carbon source showed the following values, respectively; the specific uptake rates of 47~93mg hexose per gm-DCW per hr and 37~44mg hexose per gm-DCW per hr, the specific uptake rates of 4.6~6.8mg NH_3—N per gm-DCW per hr and 1.2mg NH_3—N per gm-DCW per hr and the specific production rates of 32~42 arbitrary unit per gm-DCW per hr and 46~50 arbitrary unit per gm-DCW per hr.

The productivity of penicillin could be improved by controlling the feed rates of glucose and ammonia nitrogen to meet the uptake rates.

INTRODUCTION

It was found during the early days of penicillin development in 1940's that glucose as a sole source of carbon was a poor substrate for penicillin production and lactose, on the other hand, supported excellent penicillin biosynthesis. The concept that the slow utilization of sugar is the key to penicillin formation has been substantiated by the subsequent experimental results that the continuous and slow feed of glucose can be a good replacement for the lactose (Hosler and Johnson, 1953; Fishman and Biryukov, 1974)

Thus, the penicillin fermentation which has been converted from the batch system to a fed-batch, in which the limiting carbon source is continuously fed to the batch culture (Pirt, 1974; Dunn and Mor, 1975) to increase the productivity, poses some fundamental problems in the optimization of fermentation process. The batch fermentation can be viewed as a dynamic process in which the environment is continuously

changing and the micorbial cells are continuously responding to these changes. These changes in cellular environment result in the significant changes in physiological state, and each point in time represents an instantaneous and transient relationship between the given environmental conditions and physiological state of cells. Consequently, for the design of an optimally controlled fed-batch fermentation process, it is desired to keep the environmental conditions constant in such a way that the various specific metabolic rates that support the maximum specific production rate of penicillin can be maintained (Young and Koplove, 1972). Hence, the control system must continuously monitor the change in cell biomass and adjust the supply of nutrients to meet the metabolic demand. However, the complexity of the fermentation system is enormous, and it is likely to take a long time before complete understanding of the physical, chemical, and biological properties of the fermentation system is achieved. Thus, in this study, we selected the following key parameters that were considered most important to the penicillin productivity (Ryu, 1977) and attempted to optimize the process with respect to those variables. These are specific production rate of penicilin, specific uptake rates of sugar, ammonia nitrogen, and phenylacetic acid and dissolved oxygen concentration.

MATERIALS AND METHODS

1. Microorganisms.

A mutant of production strain of *Penicillium chrysogenum* which gives 25,000 units/m*l* under normal fermentation conditions was used in these studies.

2. Fermentation and Media.

The abundant spores from the stock culture on an agar slant were suspended in 5m*l* sterile distilled water, and used to inoculate 100m*l* germination medium in a Sakaguchi flask. It was cultivated at 25°C for 2 days on areciprocating shaker (130 reciprocations per minute and 10 cm amplitude). The inoculum from the first germination flask was transferred to another 500m*l* germination medium and made a vegetative inoculum by cultivating 36 hours under the same conditions.

Start-up of the main fermentation began by inoculating the 10 liter fermentation medium in 20 liter Marubishi jar fermentor. Fermentation conditions were as follows unless otherwise stated: temperature was controlled at 25±1.0°C over the batch cycle, and *p*H, at 7.0±0.1 with 10% sulfuric acid and 10% ammonia water. Air was supplied through a ring sparger at a rate of one volume of air per volume of culture liquid per minute, and the agitation speed was stepped up by 100 rpm. increment from 500 rpm to 800 rpm as the apparent viscosity of the broth increases with the fermentation age. Rice bran oil was used as an antifoam agent when necessary. Potassium phenyl-acetate as a precursor was fed every 12 hours to give 0.025% w/v. As an additional nitrogen source, ammonium sulfate was supplied every 12 hours from 84 hours of age to give 0.15% w/v, and 150m*l* of the culture broth was sampled every 12 hours for analyses.

Media compositions were as follows: for sporulation medium, glycerol 1.5%, molasses 1.5%, peptone 1.0%, potassium dihydrogenphosphate 0.012%, magnesium sulfate 0.01%, and sodium chloride 1.0%, and 3% agar. Germination medium consisted of lactose 2.0%, glucose 2.0%,

corn steep liquor 6.0%, potassium dihydrogenphosphate 0.06%, magnesium sulfate 0.03%, and calcium carbonate 0.6%. The fermentation medium consisted of lactose 10%, corn steep liquor 6%, ammonium sulfate 0.6%, potassium dihydrogen phosphate 0.4%, sodium sulfate 0.3%, and calcium carbonate 1.2%. 1% rice bran oil was added to the fermentation medium, and *p*H was adjusted to 6.5 before sterilization. Medium was steam-sterilized at 120°C for 30 min.

3. Analytical methods

1) Benzylpenicillin: Benzylpenicillin in the filtrate was acidified with glycine buffer and extracted with butylacetate. The penicillin in the solvent layer was re-extracted with sodium bicarbonate solution and then the potency in the broth determined by the hydroxylamine method with this aqueous layer (Boxer and Everett, 1949). Blank value was obtained by a method of reagent inversion (Avanzini *et al.*, 1968).

2) Total reducing sugar: Broth filtrate containing lactose or sucrose was acid-hydrolyzed with sulfuric acid at 120°C for 30 minutes, and the hydrolyzate was neutralized with sodium hydroxide and then diluted with distilled water to give a solution of 0.5~2.0mg hexose equivalent per m*l*. Hexose in this solution was determined by the Shaffer-somogyi micro-method (Horwitz, 1975).

3) Ammonia Nitrogen: Broth filtrate was mixed with a saturated solution of potassium carbonate to evolve the gaseous ammonia, while the air through the concentrated sulfuric acid was being introduced to the mixture. Ammonia was collected and dissolved in boric acid solution with the mixed indicator of methyl red and brom-cresol green. Ammonia nitrogen in this solution was titrated with 0.01 *N* potassium biiodate solution.

4) Phenylacetic acid: Phenylacetic acid in the broth filtrate treated with potassium ferricyanide was acidified and extracted with toluene. The extracted phenylacetic acid was then determined by the modified Kapeller-Adler method (Pan and Perlman, 1954).

5) Cell concentration: A predetermined amount of culture broth was withdrawn just before any feeding and/or the changes were made. The sample was filtered and washed sufficiently with 0.1 *N* HCl and again with distilled water. The mycelial filter cake was dried at 100~105°C to a constant weight, and the dry cell weight determined.

6) Dissolved oxygen: Dissolved oxygen profile during the fermentation cycle was followed with a dissolved oxygen electrode (Marubishi), and 100% full scale reading was calibrated by immersing the electrode alternately in air-saturated water and sodium sulfite solution containing copper sulfate as a catalyst (Cooper *et al.*, 1944).

RESULTS AND DISCUSSION

1. Effect of carbon sources on *p*H and penicillin potency

The changes in uncont rolled *p*H and penicillin potency of the culture broth were shown in Table 1 when each of lactose, sucrose, glucose and soluble starch was used as a carbon source. Lactose medium showed relatively stable *p*H at about 7.2 over the fermentation period and its penicillin potency was increased steadily until 140 hours of age. The other sugars induced drastic changes in *p*H and gave the penicillin potencies less than 50% of those in lactose medium.

When the hydrolysis of lactose was metabolic rate-limiting step, the assimilation rate of the carbon source would be dependent on the rate of lactose hydrolysis. Consequently, this results in less accumulation of organic acid and carbon dioxide in the culture broth than those culture medium with the other carbon sources. This, then, could give the stable *p*H profile of the broth(Pan *et al.*, 1972). Since, the *p*H that is favorable to penicillin biosynthesis was reported to be in the range of 6.5 to 7.5 (Hosler and Johnson, 1953; Lurie and Levitov, 1967) and furthermore, excess amount of glucose induces the catabolite repression which inhibits the penicillin biosynthesis (Demain, 1968). Lactose is a preferable carbon source for the penicillin formation in batch fermentation in batch fermentation without accurate *p*H control as compared with the other carbon sources.

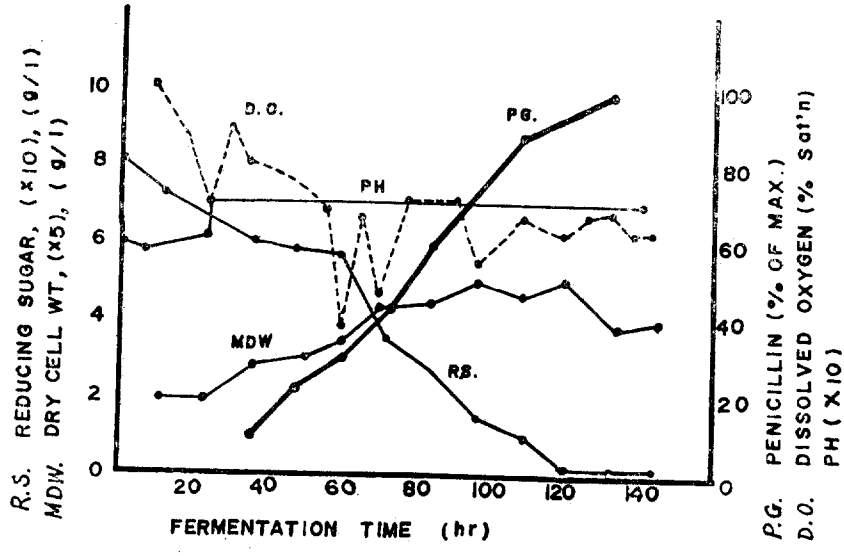

Fig. 1. Concentration profile in penicillin fermentation with lactose

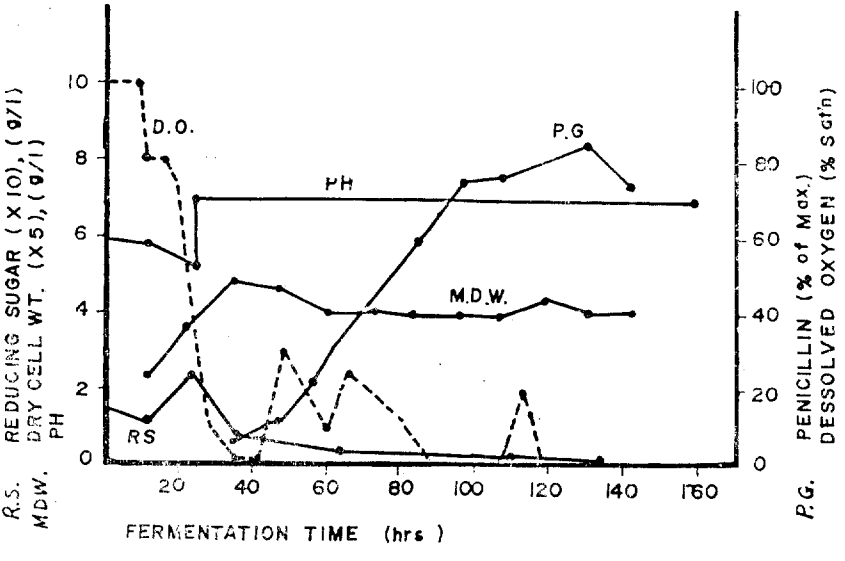

Fig. 2. Concentration profile in penicillin fermentation with sucrose feeding

2. Comparison of the process variables in lactose and sucrose-fed medium

Concentration profiles during fermentation in 20 liter fermentor were shown in Fig. 1 and Fig. 2, where lactose and sucrose were used as a source of carbon respectively. In the latter case, a given amount of sucrose was fed every 12 hours so that the total amount of sugar supplied was the same as that in lactose medium. Comparison of the process variables in these two systems showed that the maximum potency of penicillin in sucrose-fed medium reached 86% of that in lactose medium. This productivity was 2.4 times as large as that in shake-flasks.

Dissolved oxygen concentration in the broth of lactose medium decreased gradually with the increase in cell concentration and was maintained at the level of 65% saturation during the idiophase or penicillin production phase. Sucrose-fed medium, however, showed an abrupt fall in dissolved oxygen in the initial phase of batch cycle and thereafter the condition of insufficient oxygen supply persisted. Frequent peaks of dissolved oxygen was due to the increase in agitation speed.

When the total amount of sugar consumed by microorganism is plotted against the fermentation age, a slope of the curve shows a uptake rate of sugar on the volumetric basis. The uptake rate of sugar in lactose medium was estimated as 0.6~0.8g hexose per liter per hour and that of sucrosefed medium as 0.4~0.5g hexose per liter per hour. Dividing the uptake rate of sugar by the cell concentration at the time gives a specific uptake rate of sugar which is of physiological importance and represents the amount of sugar consumed by unit amount of microorganism in unit time. It was determined as 38 to 75 mg hexose per gm-DCW per hour in lactose medium, and 25 to 33.4mg hexose per gm-DCW per hour in sucrose-fed medium. This smaller value in the latter case might be due to the limited supply of sugar and, in part due to insufficiency of dissolved oxygen.

Sucrose, one of the carbon sources disadvantageous to penicillin formation in batch sytem, was recognized as a promising sugar for the replacement of lactose in the penicillin production medium when a well-controlled feeding technique is used. It has been reported that the penicillin biosynthetic mechanism of cells was not damaged when, at least 15 to 30% saturation level of dissolved oxygen was maintained during the production phase (Shu, 1972). The not so well-controlled feeding of sucrose irrespective of cell concentration caused uncontrolled growth resulting in the excessive increase in cell concentration (Fig.2), and the rapid utilization of sucrose required much more oxygen (Johnson, 1946). This, consequently, caused the dissolved oxygen concentration to become a limiting factor in the fed-batch fermentation (Shu, 1972).

Therefore, when readily assimilable sugar is fed it is recommended that a continuous feeding or frequent feeding shouldbe used so that the undesirable effect due to the excessive amount of sugar can be avoided.

3. A relationship between glucose feeding and *p*H control

As shown in Table 1, glucose lowered *p*H of the broth and later raised it. With this property of glucose, attempts were made to control *p*H of the cultute broth only with glucose feeding. Fig. 3 shows the profiles of *p*H and penicillin potency during the fermentation with the feeding of glucose as a sole source of carbon in 5 liter fermentor. The *p*H of the culture

Tabel 1. Chanes in pH and the penicillin potency when 4 kinds of sugar were used as a sole source of carbon.

Sugar		Age(hrs)						
		0	36	62	86	112	140	160
lactose	*p*H	6.5	7.2	7.5	7.3	7.6	7.1	7.2
	penicillin* potency	—	—	19	43	75	100	92
glucose	*p*H	6.5	6.0	5.6	6.4	7.8	8.5	8.6
	penicillin* potency	—	—	14	37	51	32	33
sucrose	*p*H	6.5	6.0	5.6	6.0	6.3	8.4	8.5
	penicillin* potency	—	—	14	37	33	21	40
soluble starch	*p*H	6.5	6.1	5.7	5.8	7.5	8.5	8.6
	penicillin* potency	—	—	10	23	23	23	14

* % value with reference to the maximum potency of penicillin in lactose medium.

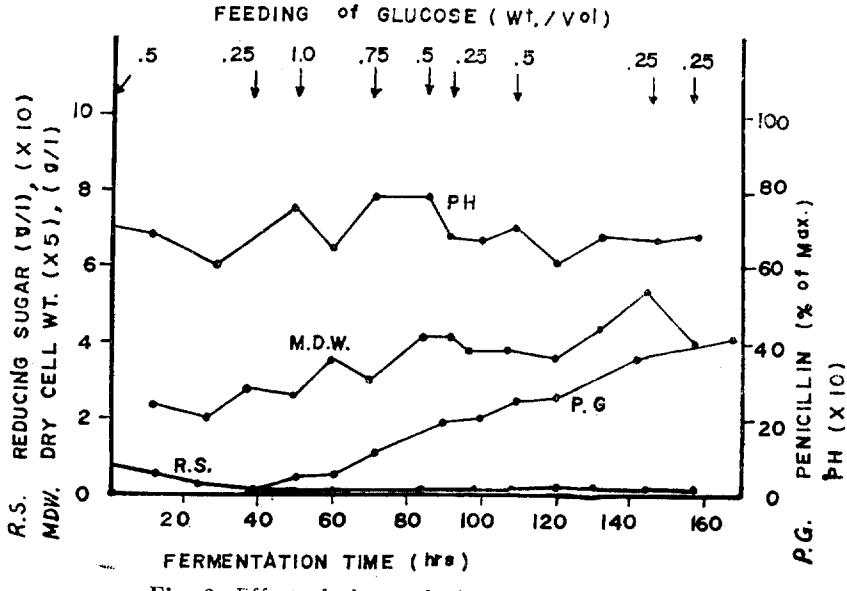

Fig. 3. Effect of glucose feeding on the *p*H profile

broth was measured and glucose feeding was executed when *p*H began to rise beyond the set point, *p*H 7.0. Although considerable fluctuations of *p*H were observed duringthe first 90-hour period, comparatively stable *p*H was observed in the range of 6.5 to 7.0. Residual reducing sugar was scarcely detected after 40 hours of age and cell concentration increased only slightly beyond 40 hours, although there was a minor fluctuation on account of sugar-limiting condition.

Penicillin potency of the harvested broth was 40% of that obtained from lactose medium in 20 liter jar fermentor.

Therefore it was confirmed that glucose

feeding could control *p*H and im prove penicillin yield somewhat although the relationship between the glucose feeding and the productivity need to be further studied.

4. Comparison of the process variables in lactose and glucose-fed medium

Fig. 4 and Fig. 5 presents the concentration profiles during the fermentation in 20 liter fermentor, where lactose and glucose were used as a source of carbon respectively. In batch fermentation with lactose medium, the *p*H was controlled at 7.0 ± 0.1 with $5N$ sulfuric acid and $5N$ potassium hydroxide solution from 24 hours of age, and the precursor was fed every 6 hours give 0.018% w/v at the instant of feeding. Additional ammonium sulfate was not supplied. On the other hand, in glucose-fed batch fermentation, the control of *p*H

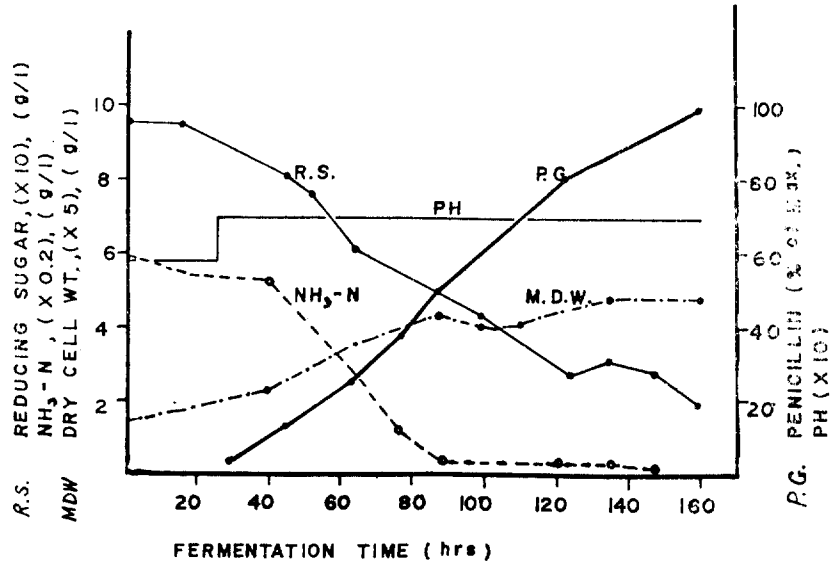

Fig. 4. Concentration profile in penicill in fermentation with an excess amount of lactose

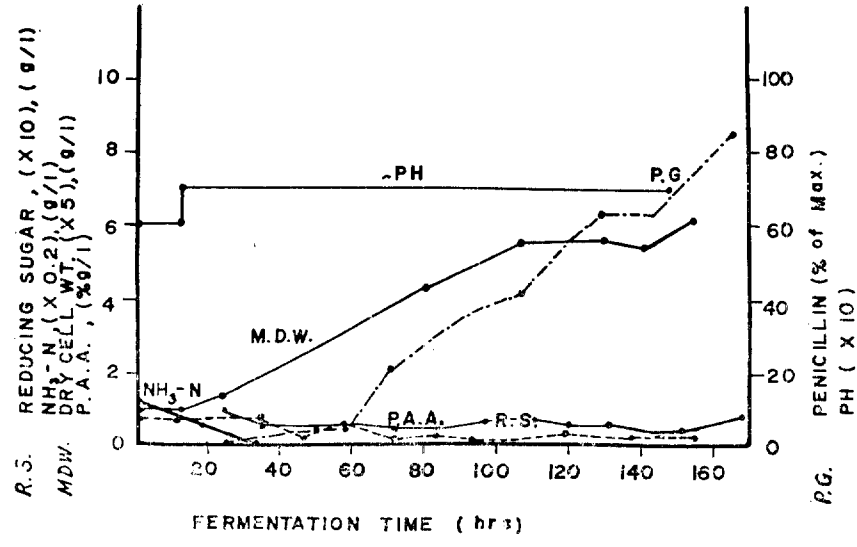

Fig. 5. Concentration profile in penicillin fermentation with glucose feeding

Table 2. Rates and specific rates in penicillin fermentation with lactose and glucose-fed medium.

C sourse variables		Volumetric rate*		Specific rate**	
		lactose	glucose	lactose	glucose
Consumption	Hexose	550—1000	1310—1360	37—44	47—93
	NH_3-N	20	125	1.2	4.6—6.8
	P.A.A	—	29.5—31.1	—	1.05—1.76
Production	Penicillin	630—1060	730—960	46—50	32—42

*The unit is mg (or arbitrary unit) per liter per hour.

**The unit is mg (or arbitrary unit) per gm-DCW per hour.

was started from 12 hours of age and the precursor was fed in the same manner as in the lactose medium. Additional ammonium sulfate was fed to give 0.075% w/v at the instant of feeding every 6 hours from 60 hours of age. Glucose feed rate was controlled at about 1.5 gm hexose per liter per hour. This feed rate was greater than the mean consumption rate of sugar in batch fermentation performed previously with lactose medium (Fig. 1).

The results of analyses of Fig.4 and Fig. 5 are summarized in Table 2. Batch fermentation with lactose sho wed that the sugar uptake rate was 0.55 to 1.00 gm hexose per liter per hour and the specific uptake rate of sugar was 37 to 44 mg hexose per gm-DCW per hour. However, glucose-fed batch fermentation showed 1.31 to 1.36 gm hexose per liter per hour and 47 to 93 mg hexose per gm-DCW per hour respectively. Thus, the ratio of specific consumption rate of glucose to lactose was about 1.28 to 2.12.

Consumption rate of ammonia nitrogen found was about 0.02 gm NH_3-N per liter per hour and the specific uptake rate of the nitrogen was about 1.2 mg NH_3-N per gm-DCW per hour during 40 to 88 hours of age in batch fermentation with lactose medium, and thereafter the residual ammonia nitrogen fell nearly to zero. On the other hand, 0.125 gm NH_3-N per liter per hour and 4.6—6.8mg NH_3-N per gm-DCW per hour in glucose fed-batch fermentation. Thus, the amount of ammonia nitrogen required for the utilization of unit amount of glucose was 2.65 to 3 times greater than that for lactose.

Penicillin production rate estimated was 0.63 to 1.06 arbitrary unit per m*l* per hour and the specific production rate of penicillin found was 0.046 to 0.050 arbitrary unit per mg-DCW per hour during 51 hours of age in batch fermentation with lactose. The corresponding values found with glucose fed batches were 0.73 to 0.96 arbitrary unit per m*l* per hour and 0.032~0.042 arbitrary unit per gm-DCW per hour respectively during 48 hours to 168 hours of age. Consequently, penicillin productivity of the glucose-fed batch fermentation was only about 80% of that with lactose.

Based on the relative productivity of penicillin and relative requirements for carbon and nitrogen sources, an important conclusion one may draw is that the use of lactose is far more economical than the use of glucose.

Mycelial dry weight in both fermentations reached the maximum after 100 hours of age, about 24 gm-DCW per liter in

lactose medium and 28 gm-DCW per liter in glucose medium.

From Fig. 5 the precursor uptake rate was estimated as 29.5~31.1 mg phenylacetic acid per liter per hour and the specific uptake rate of precursor found was 1.05 to 1.76 mg P.A.A. per gm-DCW per hour during the penicillin production phase.

The above results obtained so far suggests that if well-controlled supply of substrates such as glucose, sucrose ammonia or precursor was made just to meet the demand, the penicillin productivity could be increased by prolonging the optimal fermentation condition. For the purpose of further improvement of penicillin productivity, more detailed study on the quantitative physiology should be conducted.

摘 要

페니실린의 fed-batch 발효 공정에서 가장 중요한 공정변수인 주탄소원과 암모니아성 질소의 비소비율과 페니실린의 비생산율을 공정 전반에 걸쳐 조사하여 생산성에 미치는 영향을 연구하였다.

그 결과 포도당, 유당을 각각 주탄소원으로 사용했을 경우 당의 비소비율은 47~93mg hexose/gm-DCW/hr 및 37~44mg hexose/gm-DCW/hr, 암모니아성 질소의 비소비율은 4.6~6.8mg NH_3-N/gm-DCW/hr 및 1.2mg NH_3-N/gm-DCW/hr, 페니실린의 비생산율은 32~42 arbitrary unit/gm-DCW/hr 및 46~50 arbitrary unit/gm-DCW/hr였다.

이와 같이 산출된 비소비율에 맞추어 포도당과 암모니아의 공급속도를 조절하여 줌으로써 페니실린의 생산성을 향상시킬 수 있었다.

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