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# Production of Gentamicin by *Micromonospora purpurea* —Studies on Fermentation Variables—

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# *Micromonospora purpurea*에 의한 gentamicin 생성 —발효공정 변수에 대한 연구—

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## ABSTRACT

Using *Micromonospora* strain, gentamicin was produced by fermentation. The increase in gentamicin productivity was studied by strain improvement and systematic optimizaion of fermentation process variables. The productiviity of parent strain of *M. purpurea* (ATCC15835) was improved by selection of superior mutant after U. V. irradiation and induction of gnetamicin rseistance. Potato starch and soy bean meal were the best carbon and nitrogen sources for gentamicin fermentation, respectively. The optimum stimulating concentration of Co ion for gentamicin production was 0.006g $CoCl_2$ per liter of broth. Oxygen was found to be an important factor for gentamicin yield. The optimum *p*H for the cell growth and gentamicin production were 7.2 and 6.8 respectively. By controlling the *p*H, oxygen, and other conditions found in this study at the optimal conditions for cell growth and gentamicin production, the total productivity of gentamicin was increased significantly.

## INTRODUCTION

In 1963, it was discovered that *Micromonospora purpurea* and *Micromonospora echinospora* isolated from soil produced the gentamicin (1). Since the discovery of gentamicin, it has become one of the most important antibiotics and has been widely studied because of their broad spectrum activity against gram-positive and gram-negative bacteria. Gentamin is a basic, water soluble aminoglycoside antibiotic (2). It is the first antibiotic produced by genus *Micromonospora* for practical use. Besides the gentamicin, other antibiotics produced by *Micromonospora* have been discovered (3). Gentamicin derivatives and their combined dosage applicaiion have also studied(4, 5).

For gentamicin fermentation studies, only a few papers and patents have been reported(9~12, 18). In this paper, the results of our studies related to the pro-

duction of gentamicin are presented.

## MATRIALS AND METHODS

### 1. Microorganism

*Micromonospora purpurea* strain (ATCC 15835) was improved by mutation. Cultures were maintained by lyophilization or as frozen cells in skim milk(in equal part of mycelium broth and 11% skim milk by volume). These cultures were used in all experiments.

### 2. Media

The germination medium of the first and second stages and the fermentation media used were the same as those used by Lee *et al.* (7). Soy bean oil was used as antifoam. 500-ml flasks containing 80-100 ml medium were sterilized at 121°C for 20 minutes.

### 3. Fermentation variables

The 500-ml flask containing 70 ml of the germination medium was inoculaetd with a vial of lyophilized cells or a slant of a frozen cell. The flask was incubated for four days at 35°C on a rotary shaker at 280 rpm. Five percent of the germinated culture broth was transferred to the flask containing the same germination medium, and the flask was incubated for 48-72 hours at 28°C. In case of shake-flask fermentation, 5% of the second-stage germination broth was transferred to the 500-ml flask containing the fermentation medium. Fermentation lasted for six days at 28°C. Fermentation was also performed in a 5—1 jar fermentor (New Brunswick Scientific Co.). One hundred fifity ml of the second-stage inoculum was used to inoculate 3—1 working volume of the fermentation medium. Air flow rate was adjusted to 1 liter air /liter-broth/minute, and agitation was 400 rpm. *p*H was controlled by two point automatic *p*H controller(New Brunswick Scientific Co.).

### 4. Strain improvement

a. U. V. treatment

Homogeneous suspension of mycelium culture was prepared for U. V. irradiation. 0.1ml of of the appropriately dilutured culture broth was spread on an enriched agar plate(same as germaintion medium plus 1.5% agar). The 10 watt straight lamp of 275 nm U. V. source. The colonies that appeared after irradiation were counted, isolated, and germinaed by shaked culture for 3~4 days at 35°C Germinated mycelium was separated by filtration through sterilized glass wool. The filtrate was plated on enriched agar and incubated at 35°C. The single colony appeared was reisolated.

b. Induction of gentamicin-resistant mutants

The mycelium culture from a single colony was transferred to the germination broth containing 200~400 units gentamicin/ml broth. Transfer was repeated 3~4 times. This broth was spread on agar plate covered with 1ml of the solution containing 1,000 units gentamicin/ml and incubated at 35°C. The single colony appeared was isolated as the gentamicin resistant strain.

### 5. Analytical Method

Determination of gentamicin in the fermentation broth was carriedout according to the methods of Grove and Randall (1955). An aliquot of the whole broth was acidified to *p*H 2.7~2.5 with 6N $H_2SO_4$ and shaken for 30 minutes and centrifuged about ten minutes at 10,000 rpm. Collected supernatant was neutralized to

*p*H 7. 8—8.0 with $NaHCO_3$ solution. Neutralized broth was tested for gentamicin activity by agar diffusion method using the *Staphylococcus aureus*(ATCC 65389p) as the test organism

Cell growth was measured by packed cell volume and dry cell weight. The residual calcium carbonte in the fermentation was solubilized with 1N HCl to prevent an error caused by insolubility of such salts. Packed cell volume was determined by centrifuging the pretreated broth in a graduated centrifuge tube at 5,000 rpm for twenty minutes, and reading the volume of packed cells. Dry cell weight was determined by immediately filtering at 105°C until constant weight was obtained. Carbohydrate in the fermentation broth was determined by anthrone methone method as the total carbohydrate (13).

**6. Recovery and identification**

From ion exchange chromatography of the fermentation broth, partially purified gentamicin eluate was prepared by the method of Lee, *et al.* (7). Based on the relative mobility of the sample and that of the gentamicin standard, the biologically active eluate was identified as gentamicin.

## RESULTS AND DISCUSSION

**1. Result of strain improvement**

The productivity of gentamicin was improved by single colony isolation. This method improved the productivity by as much as 45 percent (Table 1). The efficacy

Table 1. Strain Improvement by UV I rradiation

| | Final *p*H | D.C.W. | Method of Treatment | Relative Activity |
|---|---|---|---|---|
| Wild strain | 7.11 | 20.26 | Natural Selection | 1.0 |
| Mutant G-1* | | | U.V. | 1.2 |
| Mutant G-2 | | | U.V. | 1.27 |
| Mutant G-3 | | | Induction of the resistance | 1.42 |
| Mutant G-4 | 6.81 | 26.0 | U.V. | 1.6 |

*G: Generation

of U.V. irradion depended on experimental conditions of cells at the time of mutagenic treatment and the culture conditions prior to the mutagenic treatment. The cells during the logarithmic growth phase are more sensitive to the mutagenic treatment than that of the stationary phase and *M. purpurea* was irrradiated during the logarithmic growth. The sur vival rate was about 1~2%.

The resistance inducing method resulted in highly homogeneous culture, and gentamicin productivity increased by 15 percent. We assumed that gentamicin had a selective lethal effect on the natural population of the low activity. The mechanism involved in induction of resistance against its own metabolite is not known. However, the results obtained with some other antibiotic producers indicated that the final concentration of metabolite might be regulated by the metabolism resembling feed back control (14). The differences in the biosynthetic activities

of various strains may depend on the degree of their resistances to their own metabolites.

These high yielding mutants showed characteristics which were different from those of wild strain (Table 2). Colors of colony and culture broth was intense and these criteria were employed in selecting the high-yielding mutants. Also, the high yielding mutants showed better growth than the wild strain.

Table 2. Cultural and Morphological Characteristics of the Mutants

| | Mutants (G-3 and G-4) | Wild strain |
|---|---|---|
| Colony size | 2—5mm | 1—3mm |
| Colony morphology | irregular umbilicate raised undulate-labate | round~irregular umbilicate, flat entire~undulate |
| Color of colony | brown~orange | purple |
| Germination rate | rapid | slow |
| Pigment of broth culture | light orange~orange | purple |

**2. Optimization of fermentation medium**

a. Selection of the medium

Among the fermentation media tested the medium reported by Lee, *et al.*, gave the best cell growth and gentamicin productivity. The synthetic medium used by Wagman, *et al.* (11) was not so good for cell growth.

b. Carbon source

Table 3. Gentamicin Productivity on Different Medium

| Media | D.C.W. (mg/ml) | Final *p*H(1) | Relative(2) Activity | Relative(3) Spec. Activity |
|---|---|---|---|---|
| French Patent(18) | 8.89 | 6.34 | 0.22 | 0.5 |
| Wagman, *et al.* (11) | 3.45 | 6.27 | 0.06 | 0.36 |
| Lee, *et al.* (7) | 20.5 | 7.09 | 1.00 | 1.00 |

(1) Initial *p*H was adjusted to 7.2 before sterilization.
(2) Activity based on unit volume of broth.
(3) Activity basedon unit amount of cells.

Table 4. Effect of Different Carbon Source on Gentamicin Production

| Carbon source* | Final *p*H | D.C.W. (mg/ml) | Relative Activity | Relative Spec. Activity |
|---|---|---|---|---|
| Potato starch | 7.23 | 17 | 1.00 | 1.00 |
| Glucose | 7.17 | 13.2 | 1.03 | 1.38 |
| Sucrose | 7.07 | 14.8 | 0.77 | 0.88 |
| Corn starch | 7.13 | 15.2 | 0.93 | 0.86 |
| Lactose | 8.01 | 4.0 | 0.13 | 0.56 |

* Carbohydrate was substituted on a weight basis in place of potato starch in medium(7).

As shown in Table 4, potato starch was the best carbon source for the cell growth, and glucose for the gentamicin production. Among the carbon sources tested, lactose was the worst for both the cell growth and gentamicin production. From these results, it was assumed that *M. purpurea* could easily and rap idly hydrolyze the starch to glucose and the glucose is used as the carbon source for gentamicin. Although potato starch gave slightly lower productivity than glucose, it was selected as the best carbon source for industrial use because of their relatively low cost and supporting good growth.

c. Nitrogen source

Among the nitrogen source evaluated, soy bean meal and soy bean flour were better than corn steep liquor (Table 5).

d. Effects of metal ions

Addition of $Mg^{+2}$ did not improve the growth and productivity. An addition of more than 20 mM of $Mg^{+2}$ ion decreased the productivity as shown in Table 6-Ⅰ. Addition of about 5 mM-20 mM of $Mg^{+2}$ to the fermentation medium was reported increase the aminoglycoside antibiotics productivity(16). Our medium was made of soy bean meal and soy bean flour which contains about 0.55% of $Mg^{+2}$ of whole meal. This amount of $Mg^{+2}$ was enough for the cell growth and metabolism. Additional amount of $Mg^{+2}$ (as $MgSO_4$) in the medium might slightly inhibit the productivity by lowering the *p*H of the medium and causing other complex reactons.

Table 5. Effect of Different Nitrogen Source on Gentamicin Prodction

| Nitrogen source | Final *p*H | P.C.V. (ml/10ml broth) | Relative Activity | Relative Spec. Activity |
|---|---|---|---|---|
| Soybean meal | 7.04 | 3.1 | 1.00 | 1.00 |
| Soybean flour | 6.98 | 3.0 | 0.87 | 0.90 |
| Corn steep liquor | 6.34 | 3.0 | 0.5 | 0.26 |

Table 6. Effects of Different Metal Ions on Gentamicin Production

6-Ⅰ. $Mg^{+2}$ ion

| Concentration of added Mg ion(mM) | Final *p*H | D.C.W. (mg/ml) | Relative Activity | Relative Spec. Activity |
|---|---|---|---|---|
| 0 | 7.04 | 22.0 | 1.00 | 1.00 |
| 5 | 6.89 | 19.0 | 0.87 | 0.98 |
| 10 | 6.99 | 19.8 | 0.95 | 1.05 |
| 20 | 6.88 | 22.0 | 0.6 | 0.6 |

6-Ⅱ. $Co^{++}$ ion

| Concentration of added Co ion(g/l) | Final *p*H | D.C.W. (mg/ml) | Relative Activity | Relative Spec Activity |
|---|---|---|---|---|
| 0 | 6.98 | 22.2 | 0.16 | 0.14 |
| 0.0013 | 7.09 | 20.21 | 1.00 | 1.00 |
| 0.006 | 7.11 | 20.26 | 1.40 | 1.40 |
| 0.01 | 7.07 | 20.0 | 1.31 | 1.33 |

The stimulating effect of $Co^{+2}$ ion was already reported and patented(17). Optimum stimulating concentration of $Co^{+2}$ was 0.006g $CoCl_2$/liter broth(Table 6-Ⅱ).

### 3. Oxygen effect

Oxygen uptake rate by M. purpurea was determined, to supply the adequate oxygen during gentamicin fermentation. Maximum oxygen uptake rate was 6.25 mM $O_2$/liter-broth/hr at 48 hours after inoculation. Thereafter, oxygen uptake rate decreased and levelled off, which coincided with cessation of cell growth as shown in Figure 1.

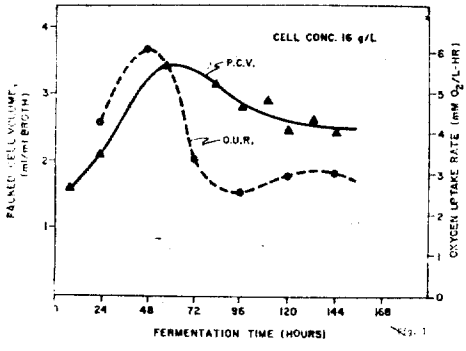


Figure 1. Packed cell volume and oxygen uptake rate of *M. purpurea* during gentamicin fermentation

The effect of oxygen supply was studied by fermenting in the 500ml flasks containing different volumes of the fermentation medium. Freedman determined the oxygen absorption rates under the similar operating conditions by using the sulfite oxidation method(15). As shown in Figure 2, the amount of oxygen supplied was an important factor gentamicin fermentation. Gentamicin productivity was more sensitive to the oxygen than to growth. The optimum broth volume for the gentamicin production was 70ml in 500 ml flask. The oxygen absorption rate was 40mM $O_2$/liter-broth/hr under the conditions of 70ml culture volume in 500 ml flask and 280 rpm shaking.

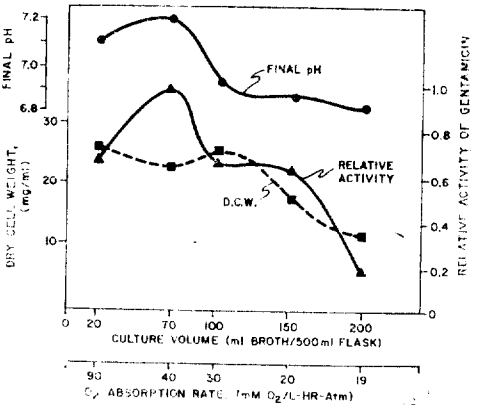


Figure 2. Effect of oxygen supply on cell growth and gentamicin fermention

### 4. Effect of initial *p*H of the medium

Table 7 shows the effect of various initial *p*H of the fermeentation broth. Optimum initial *p*H for the cell growth and gentamicin production were 7.3 and 6.8—6.9, respectively. Our result was slig-

Table 7. Effect of Initial of Initial *p*H of the Fermentation Medium on Gentamicin Production

| Initial *p*H* | Final *p*H | D.C.W. (mg/ml) | Relative Activty | Relative Spec. Activity |
|---|---|---|---|---|
| 6.51 | 6.91 | 22.4 | 0.94 | 0.92 |
| 6.80 | 7.26 | 21.9 | 1.00 | 1.00 |
| 6.88 | 7.18 | 21.4 | 0.90 | 0.92 |
| 7.02 | 7.05 | 24.8 | 0.76 | 0.68 |
| 7.15 | 7.12 | 24.5 | 0.56 | 0.52 |
| 7.3 | 7.05 | 26.1 | 0.54 | 0.45 |

* Initial *p*H was measured after inoculation.

htly different from that of Abou-Zeid, *et al.* (9). The optimum *p*H that they found was 7.0—7.5 for both the growth and gentamicin production. This difference is attributed to the differ ent medium used.

**5. Experiments with controlled *p*H**

The production of gentamicin started at end of the growth phase and continued idiophase shown in Figure 3. Optimum *p*H for the cell growth differed from that of the production phase of gentamicin as

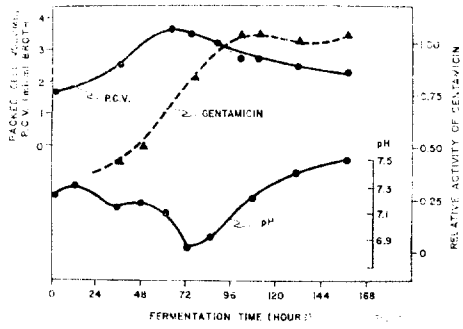


Figure 3. Concentration profile of batch gentamicin fermentation

shown in Table 6. Based on these results, two different optimum *p*H for cell growth and gentamicin production were used during fermentation The *p*H was controlled with 15% (v/v) $NH_4OH$, 50% glucose solution and 1N $H_2SO_4$ as *p*H controlling agent. The *p*H was maintained at 7.2 during the growth phase until 48~55 hours after inoculation. After this period, *p*H was adjusted to 6.9(Figure 4). By employing the *p*H control, total productivity of gentamicin was increased by 20% as compared to that of the uncontrolled batch operation.

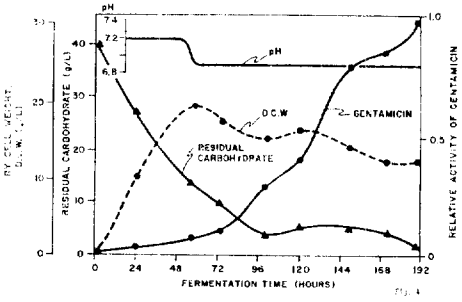


Figure 4. Gentamicin fermentation kinetics with pH control

## 적 요

*Micromonospora purpurea* 균주를 사용한 발효에 의해 gentamicin이 생산되었다. Gentamicin의 생산역가를 높이기 위해 균주의 개량과 여러가지 중요한 발효공정 변수의 최적화가 시도되었다. ATCC에서 들어온 야생균주(ATCC 15835)는 계속적인 자외선 처리와 고농도 gentamicin에 대한 저항성을 유도함으로써 개량되었다. 발효배지 중 가장 좋은 탄소원과 질소원으로는 potato starch와 soy bean meal이 각각 선택되었고, gentamicin의 생산역가에 큰 자극효과가 있는 $Co^{+2}$ ion의 최적농도는 0.006 g/*l* broth였다. 또한 gentamicin 생산역가는 산소공급량에 크게 영향을 받았고, 균주생장과 gentamicin 생성시기의 최적 *p*H는 다르게 나타났으며, 각각 7.2와 6.8—6.9였다. 발효과정중 *p*H를 균주생장과 gentamicin 생성 모두에 최적인 조건으로 조절함으로써 총 gentamicin 생산량은 *p*H를 조절하지 않은 회분발효보다 약 20%만큼 증가되었다.

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