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Optimal pH Profile in Rifamycin B Fermentation

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리파마이신B 발효생산의 최대화를 위한 pH변화의 최적화

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Abstract

The kinetic study of rifamycin B production in batch culture of *Nocardia mediterranei* was undertaken in part of our endeavor to optimize the fermentation condition.

The growth parameters such as μ_m and Ks values for nitrogen source were evaluated by employing Monod equation. From the experiments, μ_m and Ks were 0.15hr^{-1} and 8.35 g/l, respectively. The growth kinetics in batch culture was found successfully interpreted by logistic law, i.e., the initial specific growth rate and the maximum cell mass concentration were determined as function of pH and both found to have maxima. For the production of rifamycin B, a non-growth associated production kinetics was employed and the specific productivity as a function of pH was found to have two maximum points. The yield coefficient and the specific productivity were calculated as mean values in production phase. Utilizing these experimental data as a function of pH, the optimal condition for the rifamycin B production was discussed with regard to the pH effect on the cell growth and production of the antibiotic. As a result, growth phase at pH 6.5 and production phase at pH 7.0 were found to be recommended.

Introduction

Rifamycin B is an antibiotic produced by *Nocardia mediterranei* formerly assigned to *Streptomyces* species.

Since the discovery of rifamycin as announced by Sensi *et al.*,[1] detailed studies on the production of rifamycins have been done with regard to the optimal fermentation condition for the production of rifamycin B,[2] biogenesis,[3-4] chemical modification,[5-7] and antibiotic effect on the genetic basis.[8]

Rifamycin occupies a well defined place among the therapeutically useful antibiotics for its particular biological properties. Among these, the high activity against mycobacteria had led to intensive experimentation in the field of tuberculosis and leprosy. The most sophisticated and effective drug found till now is rifampicin, a semisynthetic drug made by the chemical modification of the starting material, rifamycin B. The effects on the production of rifamycin B in fermentation had been considered in various aspects. These include

the effect of aeration and agitation,[2] the effect of barbitals,[9-10] and the effect of various nutrients with the statistical approach to enhance the rifamycin productivity.[11] Although synthetic media had been used extensively in the study of biosynthetic pathways, it is not thought suitable for the wide application in industrial fermentation on account of the low yields obtained with them.

In view of the importance of rifamycins for the therapeutic purpose, it is somewhat surprising to note the scarcity of the informations concerning the fermentation conditions for the maximization of productivity. The rifamycin fermentation shows an interesting pH pattern. The initial neutral pH increases continuously to alkaline region in the initial growth phase, followed by the decrease to acidic pH at the beginning of the production phase.

The rise in pH in the initial growth phase is probably due to the disappearance of the carbohydrate and the metabolism of the intermediate organic acids accumulated, as well as the appearance of nitrogeneous materials metabolized from proteins. The characteristic pattern of pH changing behavior shows a way to improve the rifamycin B productivity by controlling or changing the pH during the fermentation.

In the present study, the effect of pH on microbial growth and the production of rifamycin B was carefully analyzed, and the optimal strategy of pH variation during fermentation was discussed with a view to maximize the rifamycin B productivity.

Materials and Methods

Materials and Equipments

Bacto-peptone, Bacto-agar, malt extract, and yeast extract were purchased from Difco Lab. (Detroit, Michigan, U.S.A.); glucose from Wako Chem. Co., Japan; Sodium barbiturate from Merck Chem. Co., Germany. Other chemicals were of the grade of Extra Pure from Kanto Chem. Ind. (Osaka, Japan).

750 ml baffled jar fermentor was used (N.B.S. Model C30, U.S.A.). Optical density was determined using Spectronic 20 (Baush & Lomb). The centrifuge used for harvesting the cell mass was from Clay-Adams, Inc.

Methods

From the parent strain of *Nocardia mediterranei*, a mutant strain of high rifamycin B production was isolated. This mutant strain was used throughout the experiment.

Seed culture medium was composed of Bacto-peptone, yeast extract, malt extract, glucose, and barbitrate. Culture was done in shaking incubator at 25-28°C for three days (Kook Je Sci. Co., Korea). The seed culture volume used for the inoculation into the main fermentation comprised 10% of the total liquid volume in the jar fermentor.

Samples of 10 ml broth were centrifuged at 3000 rev/min for 15 mins and the dry cell weight of mycelium was determined. Growth is expressed on the basis of dry cell weight (D.C.W. g/l).

Since rifamycin B has a yellow color, spectrophotometric assay such as glucostat method and Somogi-Nelson method could not be used. Supernatant obtained by centrifugation was analyzed by modified Somogi-Nelson method. This method gave practically accurate results, the error being within ±1%.

Supernatant was analyzed by Folin-Lowry method for protein determination. Rifamycin B interfered with this protein assay rendering protein assay during production phase immaterial. If not stated otherwise, rifamycin B assays were carried out spectrophotometrically since the presence of small amounts of other components with different antibiotic activities made the microbiological assay difficult in interpretation.[12] Supernatant was diluted and stabilized by acetate buffer, and was made colorless by reducing the rifamycin B to rifamycin 0. This solution was used as blank. Optical density was measured at 425 nm.

Results

Determination of μ_m and K_s for Protein

Protein as nitrogen source was used at a limiting level for cell growth, while maintaining glucose, the carbon source, at the saturation level so that the glucose was not the limiting factor for growth. One molecule of nitrogen is inserted into a rifamycin B molecule as revealed in molecular formula. In trophophase the cell growth is the main objective, and is controlled by nitrogen source at the limiting level. In order to investigate the effect of the

nitrogen source at the limiting level, chemostat culture was adopted and operated at various dilution rates. Three levels of protein concentration around the K_s value were chosen as the one in the feed to the chemostat.

As usual with most antibiotic fermentations a low specific growth rate was necessary for the optimal rifamycin B production during the production phase.[16] As mentioned in the previous section the interference of rifamycin B in protein assay resulted in the difficulties in the control on the protein concentration in the fermentor. If the fermentation volume had been large enough to sample without affecting the fermentor working volume to any significant degree, another assay system would have been introduced and the optimal control for the protein concentration could have been made.

The maximum specific growth rate μ_m and the half saturation constant K_S with respect to the concentration S of the limiting nutrient, protein, in the Monod equation (1)

$$\mu = \frac{\mu_m S}{K_S + S} \quad (1)$$

were obtained from the double reciprocal plot in Fig. 1. The values were 0.15hr^{-1} and 8.35 g/l, respectively.

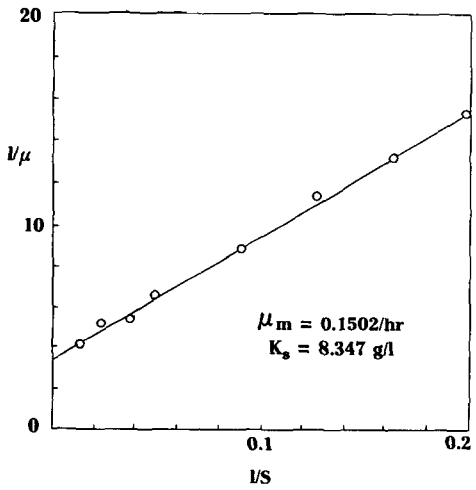

Fig. 1. Determination of K_s and μ_m for protein by double reciprocal plot.

Cell Growth

Although the vegetative form of the strain *Nocardia mediterranei* is mycelial type, pellets were not formed during the fermentation because of high aeration rate and vigorous agitation. The Monod equation regarding the mycelium as isolated single cell was successfully applied for the interpretation of experimental data.

The protein was expected to be hydrolyzed by the action of protease at the initial stage of fermentation, and the ammonium ion, the metabolic product of protein, is thought responsible to the pH rise in culture. As shown in Fig. 2, the cell growth is dependent on the pH value. A slight acidic pH (pH 6.5) was found favorable for the growth rate, while the decreasing cell growth was observed at high pH. The initial optimal condition may be weakly acidic, but the optimal pH condition is nearly neutral at idiophase. The cell growth did not coincide with the rifamycin B production. In many cases of antibiotic production, rate is weakly dependent on cell growth rate.

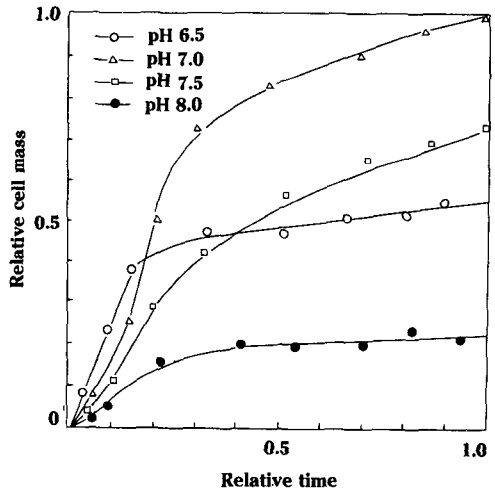

Fig. 2. Batch Progress Curves for Cell Mass.

For the expression of the cell growth, a logistic law was introduced as Equation (2) as discussed in the later section of determination of optimal pH profile. The values of initial specific growth rate and final cell mass are shown in Fig. 6.

Glucose Utilization

For rifamycin B fermentation the glucose consumption rate was constant at a given pH condition. Probably, the consumption rate at trophophase might be equal to that of idiophase.

At pH 6.5 the glucose consumption rate was much

faster than the other cases, but rifamycin production rate was very poor. From this point of view, pH 8.0 was thought to be very good condition on the sole basis of rifamycin B productivity on the glucose consumed. But pH 8.0 cannot be recommended for the maximization of overall rifamycin B productivity considering the very low production rate at this pH.

Most of the glucose was consumed in the idiophase, and the consumption amounted to 90% of the initial glucose. Therefore, utilization of glucose at production phase was an influencing factor for the rifamycin B production. At pH 7.0 and pH 7.5, the rates of glucose consumption were nearly identical as shown in Fig. 3.

From this point of view, the optimal pH condition in production phase was found to be in the range of pH 7.0-7.5.

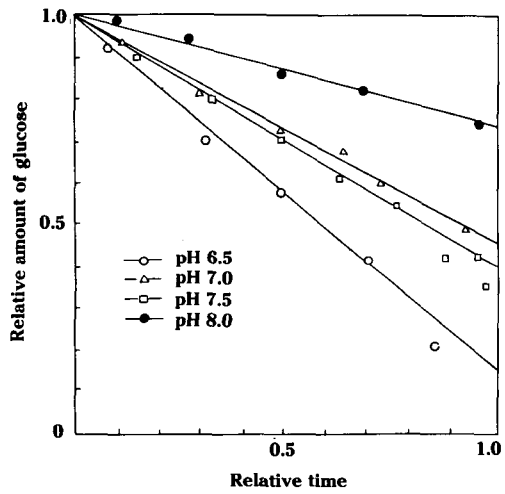

Fig. 3. Batch Progress Curves for Glucose Consumption.

Rifamycin B Production

In anbitiotic production by *Streptomyces* species, the progress is divided into two phases, trophophase and idiophase. A trophophase is a growth phase, and a idiophase is a production phase. Between these two phases, there exists the transition state. Such shape also appeared in batch progress curve of rifamycin B production (Fig. 4).

In rifamycin B fermentation, there was a time lag between the beginning of trophophase and the beginning of idiophase. This time lag is a transition state, when the primary metabolites stimulate the enzyme system or

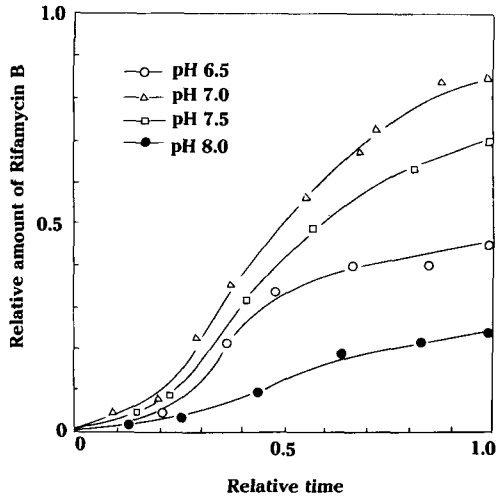

Fig. 4. Batch Progress Curves for Rifamycin B Production.

genetic assembly for the production of rifamycin B. At transition state, antibiotic may be accumulated inside the cell. In *N. mediterranei*, cells at transition state included a small amount of rifamycin B, which was identified by sonification of the cells of transition state. But antibiotic activity scarcely existed in fermentation broth. From this, in transition state, optimal control of environmental condition is thought to be very important for the enhancement of the antibiotic productivity.

As shown in Fig. 4, pH 7.0 is the best condition for the production of rifamycin B. At pH 6.5, the production rate of antibiotic did not correspond with the cell growth rate.

Determination of Optimal pH Profile

To determine the time course of optimal pH profile, following parameters were taken as criteria.

(A) *cell growth.*

$$\frac{dX}{dt} = kX\left(1 - \frac{X}{B}\right) \quad (2)$$

$$\frac{dX}{dt} = a \quad (3)$$

X : cell mass (g/l)
t : fermentation time (hr)
k : initial specific grow rate (hr^{-1})
B : final cell mass (g/l)
a : *growth constant* (g/l-hr)

Equation (3) was adopted to production phase.

(b) rifamycin B production.

$$\frac{dR_B}{dt} = K'X + R_B U \qquad (4)^{(13)}$$

$$\frac{dR_B}{dt} \cong b \cong K' \qquad (5)$$

R_B : rifamycin B concentration (g/l)

K' : specific productivity in idiophase (R_B/g D.C.W.-hr)

b : production constant (g/l-hr)

Equation (5) was deduced from (4) by assuming that specific growth rate was very small in the production phase.

(C) miscellaneous properties.

$$\bar{Y}_{R_{B/S}} = \frac{B_{B.f} - R_{B.pi}}{S_i - S_{pi}} \qquad (6)$$

$\bar{Y}_{R_{B/S}}$: mean yield coefficient

$R_{B.f}$: final concentration of rifamycin B

$R_{B.pi}$: initial concentration of rifamycin B in the production phase

S_i : initial concentration of glucose

S_{pi} : initial concentration of glucose in production phase

$$\bar{V} = b/\bar{X} \qquad (7)$$

$\bar{V}$: mean specific productivity

$\bar{X}$: mean value of cell mass in production pahse

From the above consideration, following results are deduced. In Fig. 5, the effect of pH on mean yield coefficient, $Y_{R_{B/S}}$ and mean specific productivity, $\bar{V}$ is appeared. The curve of mean yield coefficient has maximum at pH 7.0 and minimum at pH 6.5. Among the parameters involved in rifamycin B fermentation, mean yield coefficient is thought most important on the economic aspect. Therefore pH 7.0 is the most economic point for production of rifamycin B. Although $\bar{V}$ was maximum at pH 6.5, the condition is poor since mean yield coefficient was very low. Other pH condition were not better. Since $\bar{V}$ is based on unit cell mass, cell mass is an important parameter.

Considering the cell mass, parameters such as k,B, and a were introduced. The initial specific growth rate (k) at the trophophase is an important criterion for the assessment of fermentation process since the rifamycin B

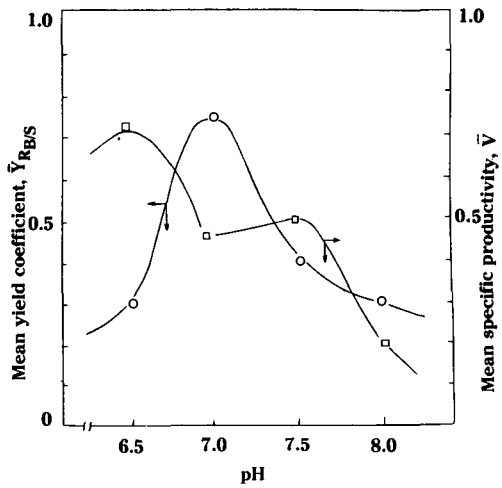

Fig. 5. Effect of pH on the Mean Yield Coefficient and the Specific Productivity.

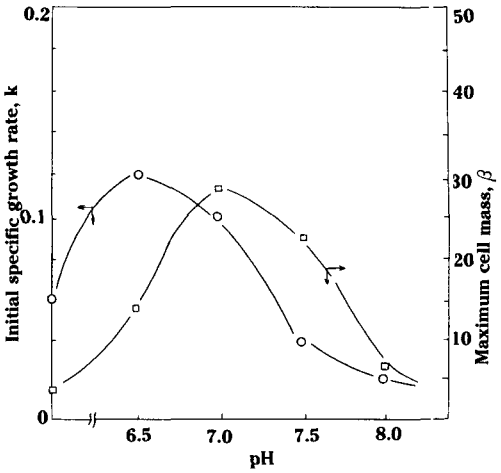

Fig. 6. Effect of pH on the Initial Specific Growth Rate and the Maximum Cell Mass.

productivity in idiophase mainly depended upon the cell mass. k has maximum value at pH 6.5. At pH 7.0, k is lower than the value at pH 6.5 by 13%. Considering the above experimental results initial pH of the fermentation should be pH 6.5 (Fig. 6). In linear growth phase where the antibiotic production is prominent, the cell mass increased about 20-30% of the final mass. In contrast to pH 6.5 where cell growth is negligible in linear growth phase, 29% of the final cell mass was produced in linear growth phase at pH 7.0. The value a has a maximum at pH 7.0 as appeared in Fig. 7. The effect of pH on the specific productivity of rifamycin B in idiophase is shown in Fig. 8.

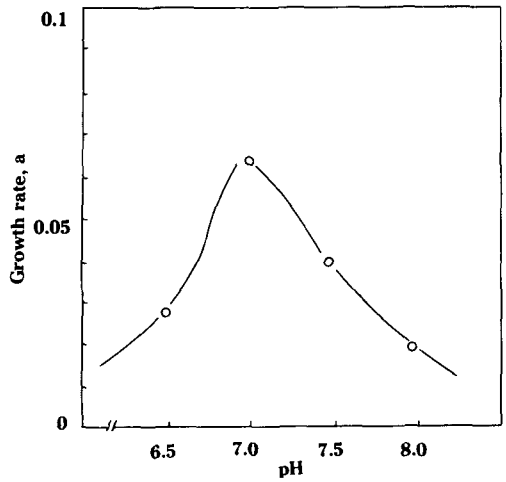

Fig. 7. Effect of pH on the Growth Rate in Production Phase.

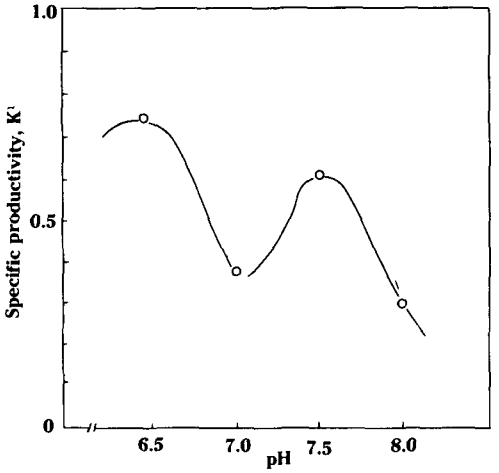

Fig. 8. Effect of pH on the Specific Productivity in Production Phase.

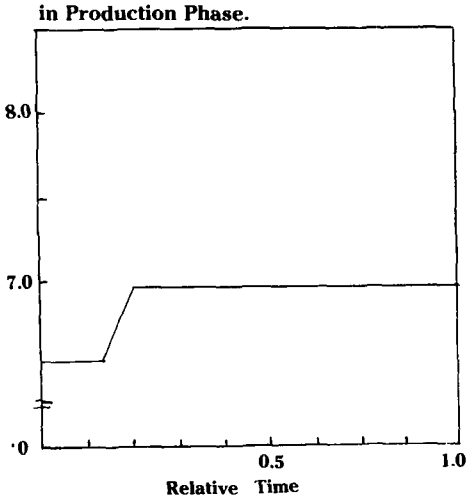

Fig. 9. Optimal pH Profile during the Batch Fermentation.

From the above experimental and theoretical results, the optimal strategy of pH during the rifamycin B fermentation is shown in Fig. 9. In a separate experiment we executed the fermentation following the above pH strategy. The result was very encouraging. The daily productivity of rifamycin B increase about 14% comparing with that of pH 7.0 throughout the fermentation.

The temperature dependency of rifamycin B production is well noted.[14,15] It ia also an important factor for the present purpose, and is under current investigation.

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