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Penicillin Fermentation using a Carrier-supported Mycelial Growth

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담체에 고정화된 균사체 증식을 이용한 페니실린의 발효

박상경 · 김정회 · 박영훈

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A carrier-supported mycelial growth of *Penicillium chrysogenum* was applied to penicillin fermentation system. Among various materials tested, celite was found to be most effective for both spore adsorption and bioparticle development. Hyphal growth through pore matrices of the material showed strong anchorages and provided highly stable biofilm growths. When 5-10% celite was employed, both cell growth and penicillin production were observed to increase significantly comparing to the dispersed filamentous growth. Specific productivity of penicillin, however, was found to be kept almost constant at a value of 1,900 unit/g cell/hr. A semicontinuous fermentation in a fluidized-bed reactor, using the carrier-supported biofilm growth, was conducted successfully although free mycelia appeared in the late phase of the fermentation made the reactor operation difficult. Control of the size of bioparticles was considered as a major operating factor to maintain the reactor productivity at a desired level.

Applications of immobilized living microorganisms to the production of various microbial metabolites are of increasing interest and have been reported for several cases.[1-5] In most cases, however, productivity of the reactor system was far below the conventional operation employing dispersed phase growth. Lower productivities were mainly due to the immobilization techniques and reactor operating patterns. Cell entrapment[1-3] technique has caused severe mass transfer limitations for both substrate and product(s). Fixed-bed operation of biofilm particles, applied for citric acid production, also suffered from severe oxygen transfer limitation to yield lower productivity.[5] These situations could have been corrected by using a fluidized bed type biofilm fermentor in which bioparticles are fluidized by both liquid growth medium and air.

Authors[6-8] have analyzed a continuous, aerobic, fixed-film bioreactor by simulating the behavior of penicillin production in a three-phase fluidized-bed. Rigorous mathematical models were developed for a fluidized-bed fermentor. Steady-state[6,8] and dynamic behavior[7] of the fermentor system were appraised in terms of penicillin productivity and outlet concentration. The results showed that penicillin production reached a maximum with processing time, but subsquently decreased as cell mass accumulated and substrate deficiencies occurred. It was also predicted that the maximum level could be maintained for increased operating times if the substrate supply was continuously increased. The duration of the prolonged production was a direct function of the rate of increase and the operating time at which the increase was initiated. Oxygen transfer capacity of the reactor

was also of crucial importance to the effectiveness of a feeding strategy. The simulation study not only provided valuable information for design and operation of the fermentor system, but also elucidated that it was one of the most promising bioreactor system for production of various microbial secondary metabolites.

Experimental verification of the model simulated behavior has been tried. As the first effort, a development of stable bioparticles using celite as an inert carrier was studied in our laboratory. In this technique, spores of *Penicillium chrysogenum* were first entrapped into celite particles and therefrom stable bioparticles with active biofilm growth were developed. Penicillin production using the bioparticles in a fluidized-bed fermentor was investigated and the results are reported in this article.

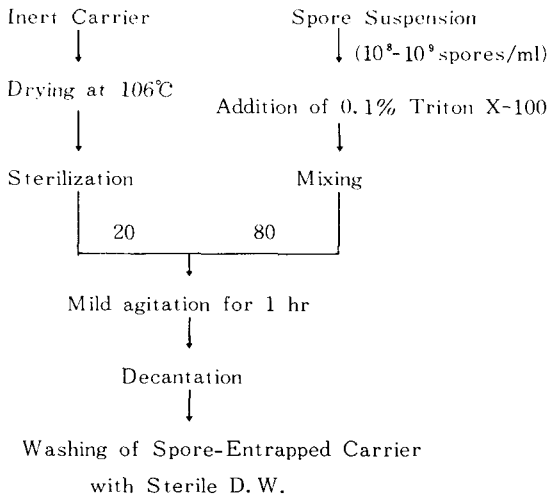

Fig. 1. Procedure of spore entrapment.

MATERIALS AND METHODS

Microorganism and media

P. chrysogenum ATCC 26818 was used in this study. The strain was maintained on an agar slant containing 1.0% (w/v) glycerol, 1.5% sucrose, 0.75% corn steep liquor, 0.75% bacto peptone, 0.009% KH_2PO_4, 0.0075% $MgSO_4 \cdot 7H_2O$, 0.00075% $CuSO_4 \cdot 5H_2O$, 0.6% NaCl and 0.0003% $FeCl_3 \cdot 6H_2O$.

Basic fermentation medium consisted of 3.0% corn steep liquor, 0.5% ammonium sulfate, 0.3% KH_2PO_4, 0.025% $MgSO_4 \cdot 7H_2O$, 0.5% $CaCO_3$, 1.0% soybean oil, 0.025% polyethylene glycol, 0.98% phenylacetic acid and 2% (v/v) of a trace metal mixture (which was prepared by mixing 0.3% $FeSO_4 \cdot 7H_2O$, 0.25% $CuSO_4 \cdot 5H_2O$ and 1.0% $ZnSO_4 \cdot 7H_2O$). Lactose and glucose were autoclaved separately and added before inoculation to the concentrations indicated in the text. 0.5 ml of potassium phenylacetic acid solution (concentration 3.91%) was added to the culture at every 24 hours upto 5 times during the fermentation period.

Spore Entrapment

Various porous support materials were tested for spore adsorption: celite (Johns-Manville Corp., San mateo, CA, USA), Dowex macroporous glass resin (Sigma Chem. Co., St. Louis, MO, USA), ceramic particles and glass beads.

Spore suspension was prepared by adding sterile distillied water to spores developed on an agar slant medium in a 1.0 liter tissue culture bottle. Spore concentration of the suspension adjusted to $10^8 - 10^9$ spores/ml. 0.1% Triton X-100 was added to the suspension to prevent aggregation of the spores. The procedure for spore entrapment was summarized in Fig. 1. The amount of spores adsorbed on the carrier was determined by the difference between the spore concentration in the inoculum suspension and that remained in the supernatant. Spore concentration was determined by using a hemacytometer (AO Brightline, USA).

Shake Flask Cultivation

The spore-adsorbed carrier material was washed thoroughly with sterile distilled water to remove free spores. Forty milliliters of fermentation medium was added to 250 ml Erlenmeyer flasks containing the washed spore-carrying materials and incubated at 25°C on a rotary shaker (New Brunswick Scientific, New Brunswick, NJ, USA). The shaking speed was fixed at 250 rpm throughout this study.

Fluidized-bed Fermentor Operation

Bioparticles with attached biofilm growth, harvested after 72hr growth in a shake flask (bioparticle size was ca. 500μm), were transferred to a fluidized-bed fermentor schematically described in Fig. 2. Internal diameter and height of the reactor were 75 mm and 200 mm, respectively. The column was made of Pyrex glass tube and supported by a 1.5 mm thick sintered stainless steel plate filter with an average pore size of 20μm. The working volume of the fermentor was kept at 300 ml. Sterile air was continuously supplied from the bottom of the reactor. Temperature was maintained at 25°C by controlling the water temperature of a bath in which the reactor was immersed. During the operation, dissolved oxygen concentration was monitored by an oxygen probe (New Brunswick Scientific) and was controlled, if necessary, by adjusting the air flow rate.

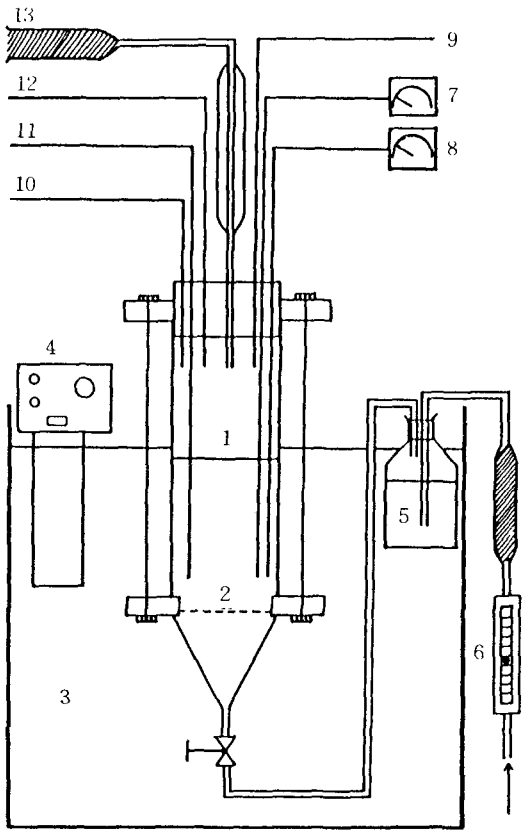

Fig. 2. Diagram of a fluidized bed reactor.

1. Fluidized bed reactor 2. Stainless filter
3. Water bath (500×250×400nm)
4. Temperature controller
5. Humidifier 6. Air flowmeter
7. DO controller 8. pH controller
9. Medium feeding line 10. Antifoam feeding line
11. Sampling line 12. Spore port
13. Exhaust air line with air filter and condenser

Analytical Methods

Concentration of penicillin was determined by both biological and chemical assay methods. *Staphylococcus aureus* ATCC 6538P was used as a test organism for a biological assay.[9] A chemical assay technique, hydroxylamine-ferric ammonium sulfate method, was conducted according to the procedure described elsewhere.[10,11]

Sugar concentration was determined by dinitrosalicylic acid (DNS) method.[12]

Dry cell weight was determined after the filtered sample was dried at 105°C to a constant weight.

Penicillin G standard (sodium salt) was purchased from Sigma Chem. Co. and phenylacetic acid from Aldrich Chem. Co., Milwaukee, WI, USA. Corn steep liquor was obtained from Miwon Co., Seoul, Korea. Other chemicals were of Extra Pure grade and purchased from Wako Pure Chemical Co., Osaka, Japan.

RESULTS AND DISCUSSION

Selection of Proper Carrier Materials

Adsorption of spores of *P. chrysogenum* on support carriers was tested at a spore concentration of 5.5×10^6/ml for various inert materials listed in MATERIALS AND METHODS section. As shown in Fig. 3, celite 560 was superior to the others; 80% of the spores were adsorbed within 3 hours of contact. The rapid adsorption kinetics of celite could be explained by an active mechanistic capillary suction of macro- and micropores of the material. Physical and structural properties of celite 560 reported were as following[13]: specific pore volume, 2.22cm^3/g; specific surface area, 1.26m^2/g; bulk density, 0.32g/cm^3; apparent density, 1.16g/cm^3; porosity, 0.72; average particle size, 400-500μm.

High porosity and the pore diameter (1-15μm) of the celite particle were considered to be important factors contributing to effective entrapping (or adsorption) of the spores. A bioparticle with attached mycelial biofilm growth was demonstrated in Fig. 4: it was envisaged that the surface mycelia shown in Fig. 4 (C-D) were propagated outwards

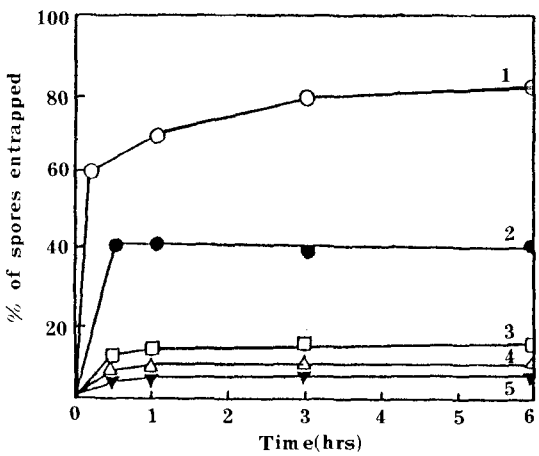

Fig. 3. Spore adsorption kinetics of carrier materials tested.

1. Celite 560
2. Dowex macroporous glass resins
3. Ceramic particles
4. Glass beads (40/80mesh)
5. Glass beads (20/90 mesh).

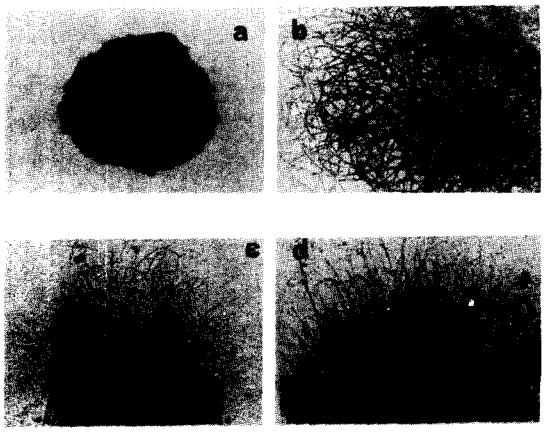

Fig. 4. Microphotographs of growth forms of *P. chrysogenum*.

a) Inert celite 560 (×50)
b) Dispersed filamentous growth (×70)
c) Biofilm growth on celite (×50)
d) Biofilm growth on celite (×70)

from the germinated spores. It was also observed that the bioparticles developed in such a manner rendered very stable biofilm growth during the fermentation period.

Biomass Growth and Penicillin Production by Bioparticles

To compare the biomass growth and penicillin production by using bioparticles to the conventional dispersed filamentous growth, a series of shake flask culture experiments was designed.

First, since the number of bioparticles in the fermentation broth would be proportional to that of celite particles added, the effects of celite concentration on the biomass growth and penicillin production were investigated. As shown in Fig. 5(A), a higher biomass growth was observed for a higher celite concentration. It was also observed that free mycelia in the culture broth were reduced when celite concentration increased. Gbewonyo and Wang [13] had reported a similar observation that increasing the concentration of carrier material enabled the microbial cultures to maintain a higher proportion of cells on the carrier, reducing free cell concentration in the broth. Thus upon increasing the carrier concentration, substrate and oxygen uptake by bioparticles were anticipated to be more facilitated, resulting in higher cell growth. Actually the final cell mass and the maximum specific growth rate for the case of 15% celite were increased 1.4 and 1.6 times, respectively, comparing to those without carrier material.

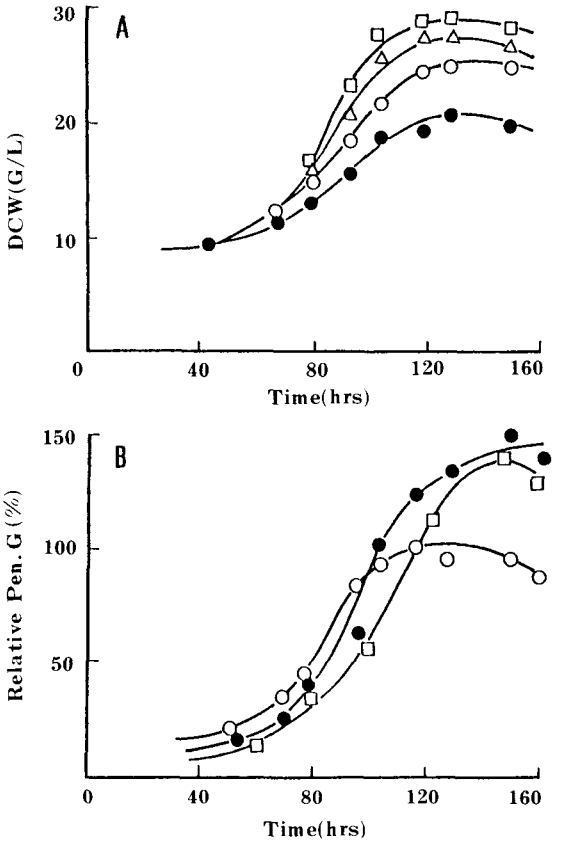

Fig. 5. Time-profiles of biomass growth(A) and penicillin production(B) for various celite concentrations.

(○) Control; dispersed filamentous growth
(●) 5% (□) 10% (△) 15% celite

Penicillin production was also increased by employing bioparticles (see Fig. 5(B)). But it should be noted that the maximum specific penicillin production rate (that is, the amount of penicillin produced by 1 g dry cell weight per hour), Q_p^{max}, was kept almost constant. These observations were summarized in Table 1.

The increased biomass growth and penicillin production with bioparticles could be explained by the reduced viscosity of culture broth and consequent improvement in the nutrient (including oxygen) mass transfer capacity of the fermentor.

Little changes in the value of Q_p^{max} in Table 1. might also indicate that the biofilm thickness at the corresponding fermentation times were thin enough not to cause any substrate or oxygen limitation within the biofilm. Subsequent increase in the biofilm thickness would cause such limitations and it would be followed by a reduction in the penicillin production rate. It should be noted that the

Table 1. Comparison of parameters related to biomass growth and penicillin production.

Parameters \ Fermentation method	Control	5% celite	10% celite
Final cell mass (g/l)	18.05	24.72	27.53
Final Pen. G production (IU/ml)	2,100	3,150	2,900
μ_{max} (hr^{-1})	0.020	0.025	0.031
Q_p^{max} (IU/g-cell-hr)	1,880	1,970	1,856

decrease in penicillin production rate after Q_p^{max} was reached, were expected in a mathematical simulation study,[6,7,8] that is, the portion of biofilm starved by the substrate and/or oxygen was increased with increasing biofilm thickness.

Operation of A Fluidized-bed Fermentor

To improve the penicillin production with bioparticles, a fluidized-bed type fermentor shown in Fig. 2 was operated in a semicontinuous mode; 75ml of the culture broth was taken for analyses and the same amount of fresh medium was added at every 6 hours to keep a constant working volume. A satisfactory mixing of bioparticles and culture broth in the reactor was achieved by feeding sterile air through the sintered stainless steel filter plate at a rate of 10-15 vvm.

A typical result of the operation was shown in Fig. 6. It was noted that both biomass growth and penicillin production were accompanied with the carbon source consumption in an early phase of the fermentor operation. However, after about 45 hours, penicillin production became to decrease rapidly while the consumption of lactose was still significant. The size of bioparticles at this period reached upto a diameter of 1000μm and seldom increased further. Free filamentous mycelia also appeared in the broth at this stage. From these observations, it was anticipated that the substrate was being used for biomass build-up and cell maintenance rather than product biosynthesis during this period. In a model simulation study,[7] however, a similar trend of penicillin production and biofilm growth was predicted, although it was conducted only for a continuous operation of such a fermentor. Major reasons for such a drastic fall of product formation were suggested as the substrate and/or oxygen deficiencies inside the biofilm. Since it is desirable to maintain a product formation rate at a high level as long as possible, a careful and effective measure should be taken to correct this situation. In this regard, control of the size of bioparticles (that is, the thickness of biofilm) was considered to crucial importance, as discussed in the previous papers,[6,7,8] for a successful operation of the reactor. Control of the size of bioparticles by nutrient limitation was proposed as one of the most promising techniques for such a purpose.[6-8,14,15] This could involve not only carbon source, but nitrogen and phosphate limitations. Development of suitable feeding strategies was thus considered important as suggested by Park *et. al.*[7]

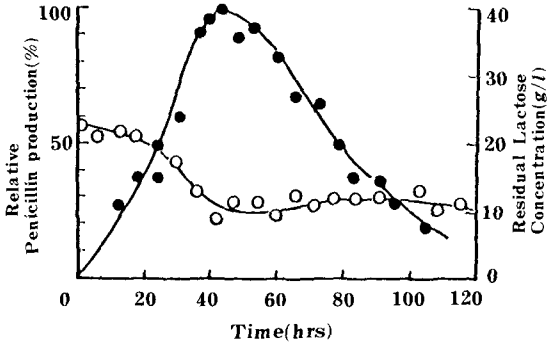

Fig. 6. Time-profiles of penicillin production and residual lactose concentration during a semicontinuous operation of the fluidized bed fermentor.
2%(w/v) lactose containing medium was used. (●) penicillin: (○) residual lactose concentration

A typical difficulty in the operation of a fluidized-bed fermentor employing bioparticles was the development of free cells (filamentous mycelia) in the late phase of fermentation; clogging of the mycelia onto the filter plate made aeration and mixing in the reactor more difficult. The studies on a fluidized-bed formentor with bioparticles should therefore be directed to the minimization of free cell development and more precise size control of the bioparticles. However, the physiological state of bioparticles should also be taken into consideration since the production of microbial secondary metabolities like penicillin was to be affected by the specific growth rate of the microorganism as well as its environmental conditions.[16]

ACKNOWLEDGEMENT

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요 약

새로운 페니실린 발효방법으로서, *Penicillin chrusogenum*의 포자를 담체의 미세구멍 조직에 흡

착시킨후 배양하여 얻은 균사증식담체를 이용하여 페니실린 발효를 시도하였다. 고정화 담체로써는 Celite가 가장 효율적이었으며, 이때 균사는 담체의 내부에서 부터 포자가 발아하여 담체의 표면에서 증식하기 때문에 매우 안정된 biofilm growth 를 얻을 수 있었다. 프라스크 배양에서 담체의 양을 5～10% 첨가하였을 때 세포의 증식과 페니실린의 생산이 전통적인 dispersed filamentous growth 에 의한 발효방법보다 현저히 증가되었다. 그러나 두 경우 모두 세포의 활성은 1900 unit/mg-cell·hr로 일정하였다. 균사증식 담체를 이용하여 유동층 발효조에서 반연속식으로 발효를 진행시킨 결과 페니실린의 연속생산이 가능함을 발견하였다. 그러나 발효조내에서 biofilm growth시 film두께의 조절과 페니실린 생산성을 일정수준으로 오랫동안 유지시킬 수 있는 방법이 앞으로 연구되어야 할 것으로 판단되었다.

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