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Effect of Methionine on Cephalosporin C Production in a Fluidized-bed Bioreactor

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유동층 생물반응기에서 세파로스포린 C 생산에 대한 메치오닌의 영향

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Effects of methionine on cephalosporin C(CPC) production in a fluidized-bed bioreactor were investigated using bioparticles of *Cephalosporium acremonium*. Since methionine was found to be an important metabolic regulator on the synthesis of cephalosporin C, the effects of its concentration in the cuture broth and feeding mode to the bioreactor were studied. It was observed that the presence of initial methionine was essential for higher cephalosporin C production and there existed an optimal content of methionine. Carbon consumption rate also increased significantly under the presence of methionine. Production of cephalosporin C was most active when methionine was exhausted in the broth; however its additional feeding did not enhance the antibiotic production in the fluidized-bed bioreactor as much as expected. It was therfore considered important to feed an optimal content of methionine at the early operating stage for a higher cephalosporin C production in a fluidized-bed bioreactor. An interesting thing to note was that titre of the antibiotic with reused bioparticles was about 2 times higher in the methionine containing medium than that without methionine. Therefore repeated use of bioparticles, with an optimal content of methionine, was believed to be very useful to enhance to process productivity.

An interesting feature in cephalosporin C fermentation is that methionine has a stimulatory effect on the biosynthesis of cephalosporin C (1-4). Ott *et al.* (2) confirmed that the yield of cephalosporin C as well as penicillin N were enhanced by the addition of methionine. The essential role of methionine was considered as a sulfur donor for the β-lactam antibiotic (5).

Although it is clear that methionine sulfur is a good source of the sulfur atom of cephalosporin C, there still exist arguments to support the idea that methionine is a metabolic regulator: Norleucine, a nonsulfur analogue of methionine, was reported to be able to replace methionine for the stimulation of cephalosporin C production (6) whereas the metabolic intermediates closer to cehalosporin C such as homocysteine, cystathionine, and cysteine were reported to be less effective for the antibiotic production (7). Furthermore, it was shown in a resting cell system that methionine had a greater stimulatory effect on cephalosporin C production when it presented in the growth phase rather than the production phase (8). These observations suggest that methionine may affect, directly or indirectly, the intrinsic cephalosporin C synthetase system of the microorganism.

A fluidized-bed bioreactor system has been developed for antibiotic production (9, 10), in which

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stable development and maintenance of bioparticle activity were the most important operational factors for the bioreactor system. Bioparticles of *C. acremonium* developed by using celite as a support material showed relatively poor stability (10) compared to those of *P. crysogenum* (9).

We have investigated the effect of methionine on cephalosporin C production in a fluidized-bed bioreactor using the bioparticles of *C. acremonium* and the results are reported in this article.

Materials and Methods

Microorganism

A mutant strain of *Cephalosporium acremonium* ATCC 20339, N-10, was used throughout the investigation. It was kindly supplied by Dr. T.I. Mheen of Microbial Technology Laboratory of Genetic Engineering Center, KIST.

Media used and preparation of bioparticles

The strain was maintained on an agar slant containing 1.0% (w/v) peptone, 0.5% beef extract and 0.25% NaCl. The medium for seed flask culture consisted of 2.0% dextrose, 1.0% yeast extract and 1.0% peptone. The chemically defined medium used for the main fermentation was prepared by mixing the following constituents in one liter of distilled water; sucrose, 36g; dextrose, 27g; ammonium sulfate, 77.5g; DL-methionine, 5g; oleic acid, 1.5g; Fe $(NH_4)_2SO_4 \cdot 6H_2O$, 0.15g, and 135 m*l* of a salt mixture which consisted of KH_2PO_4, 204g; K_2HPO_4, 208g; Na_2SO_4, 22.7g; $MgSO_4$, 4.9g; $CaCl_2$, 0.4g; $ZnSO_4 \cdot 7H_2O$, 0.4g; $MnSO_4 \cdot 4H_2O$, 0.4 and $CuSO_4 \cdot 5H_2O$, 0.1g in 1.8 liter of distilled water. Sucrose, dextrose, and ammonium sulfate were autoclaved separately. Spores of *C. acremonium* were formed by incubating the microorganism at 28°C for 7-8 days on an agar plate containing 0.5% dextorse, 0.5% yeast extract, 0.005% peptone, 0.005% $MgSO_4 \cdot 7H_2O$, 0.0046% KH_2PO_4, and 0.01% K_2HPO_4.

Fermentations in shake flasks were carried out at 26°C on a reciprocal shaking incubator (100 strokes/min). For the operation of fluidized-bed bioreactor, bioparticles were developed according to the method described in the previous works (9, 10).

Celite (560, Johns-Manville Corp., Denver, Co, USA) was used as a support material for the spore entrapment. Celite particles in a diameter range of 150-300 μm were collected by using an appropriated sieve. After sterilized by autoclaving at 121°C for 15 min, the celite particles were dried overnight at 90°C. Spore suspension with a concentration of approximately 10^7 spores/m*l* was added to the pretreated celite particles such that celite constituted 20% (w/v) of the suspension. Spore cocncentration was determined by using a haemacytometer. Spore carrying celite was then aseptically transferred to a flask containing the seed culture medium and incubated at 26°C on a reciprocal shaker. Bioparticles harvested after 48 hr cultivation in shake flask were washed with sterile distilled water and then aseptically transferred to a fluidized-bed bioreactor such that the original amount of celite for bioparticles constituted 10% (w/v) of the culture broth. The structure and arrangement of the reactor system was the same as the one used in the previous work (9).

The volume of culture broth was kept 350 m*l* with an aeration rate in a range of 10-15 vvm: dissolved oxygen could be maintained above 40% of the saturation level with the air supplying rate.

Cell mass

To determine the dry weight of free cells, a 5 m*l* portion of culture broth was washed twice with saline and once with distilled water on a filter paper, and dried in an oven (90°C) to a constant weight. To determine the dry weight of immobilized cell, bioparticles were taken from the bioreactor, and free cells were carefully removed by washing. The biopparticles were then dried to a constant weight. After burnt in a furnace (500°C) for the three hours, the residual was reweighed. The difference of these weights was counted as the dry weight of the cell mass attached to the bioparticles.

Determination of glucose and sucrose

Glucose was measured by a DNS method (11) after appropriate dilutions were made with distilled water. Sucrose was measured as following: 1 m*l* of sample containing appropriate range of sucrose was mixed with 20 μl of 2 N HCl solution and boiled for 30 min. Glucose thus liberated was then assayed as the total sugar by DNS method after neutralizing the solution to pH 7.0 with NaOH solution. Sucrose

Table 1. Effect of initial methionine concentration on the productivity of cephalosporin C.

Initial methionine conc. (%)	Methionine depletion time (hr)	Glucose consumption rate (g/hr)	CPC appearance time (hr)	Max. CPC productivity (10^{-4} g/lhr)
0	0	0.118	229	3.58
0.1	120	0.281	108	5.59
0.3	120	0.281	120	9.21
0.5	150	0.250	132	6.25
0.7	+[a]	0.250	157	4.35
0.9	+[a]	0.250	157	3.81

a: + represents that methionine was not completely exhausted until the end of fermentation.

concentrations were obtained by subtracting the portion of glucose from the total sugar.

Determination of methionine

Methionine concentration was measured by a modification of the method of Matsmura *et al.*(4) in which a thin layer chromatography (TLC) technique was used. Proteineous substances were removed by precipitation with trichloroacetic acid before methionine was separated on a TLC plate. 5 μl sample was dropped on a TLC plate using a hamilton syringe and dried. It was then developed in a solvent system of n-butanol-acetate-water (4:1:2). Methionine on the chromatogram was detected by spraying with ninhydrin, and eluted with 75% ethanol. Methionine levels of the eluted solutions were then estimated from the optical density measurements at 540 nm. Control sample of methionine used for this experiment was DL-methionine purchased from Sigma Chem. Co.

Assay of cephalosporin C

Cephalosporin C was assayed by a bioassay technique using *Alcaligenes faecalis* ATCC 8750 as a test organism (12). The standard sample of cephalosporin C used for the assay was potassium salt purchased from Sigma Chem. Co.

Results and Discussion

Shake-flask culture using free cell

As a preliminary experiments, the effect of initial methionine content in the culture meidum on cephalosporin C production was investigated in shake-flasks, and the result was shown in Fig. 1 and summarized in Table 1.

It is first noticed that glucose consumption rate is higher when methionine is present in the broth. It is also intersting that the productivity of cephalosporin C is rather sensitive to the amount of methionine present than the glucose consumption rate. In methionine-containing medium, it is apparent that glucose is assimilated at a faster rate; while in a medium without methionine, the triggering of the antibiotic synthesis seems to be delayed to some extent. This observation agrees well with the previous report by Matsumura *et al.* (4) that the level of intracellular methionine affects directly the antibiotic synthesis; it should take some time to reach a certain level of intracellular methionine. Matsumura *et al.* showed in their study that intracellular level of methionine was the highest when the microorganism started to synthesized the antibiotic.

Increase of methionine concentrations up to 0.3% resulted in higher productivity of cephalosporin C but its concentrations higher than 0.7% rather seemed to inhibit the syenthesis of the antibiotic.

On the other hand, methionine was added during the fermentation as in Fig. 1(A) (dashed-line) to determine whether its presence was important to consumption of glucose and subsequent synthesis of the antibiotic. It was noted that the carbon consumtion was accelerated and the synthesis of cephalosporin C commenced earlier by the addition of methionine.

Thus there existed an optimal content of methionine and its presence in the early phase of the fermentation was considered important to achieve higher productivity.

Shake-flask culture using bioparticles

Cephalosporin C production with bioparticles was investigated in a series of shake-flask experiments, and the result was summarized in Table 2. The bioparticles developed for 2 days in a shake-flask culture were washed with sterile water and transferred to the production medium in such a way that celite weight of the bioparticles constituted 10% (w/v) of the broth volume. Growth of immobilized cell reached its maximum after 24 hr, and then the cell concentration decreased gradually as the ratio of free cell to immobilized cell increased. This was due

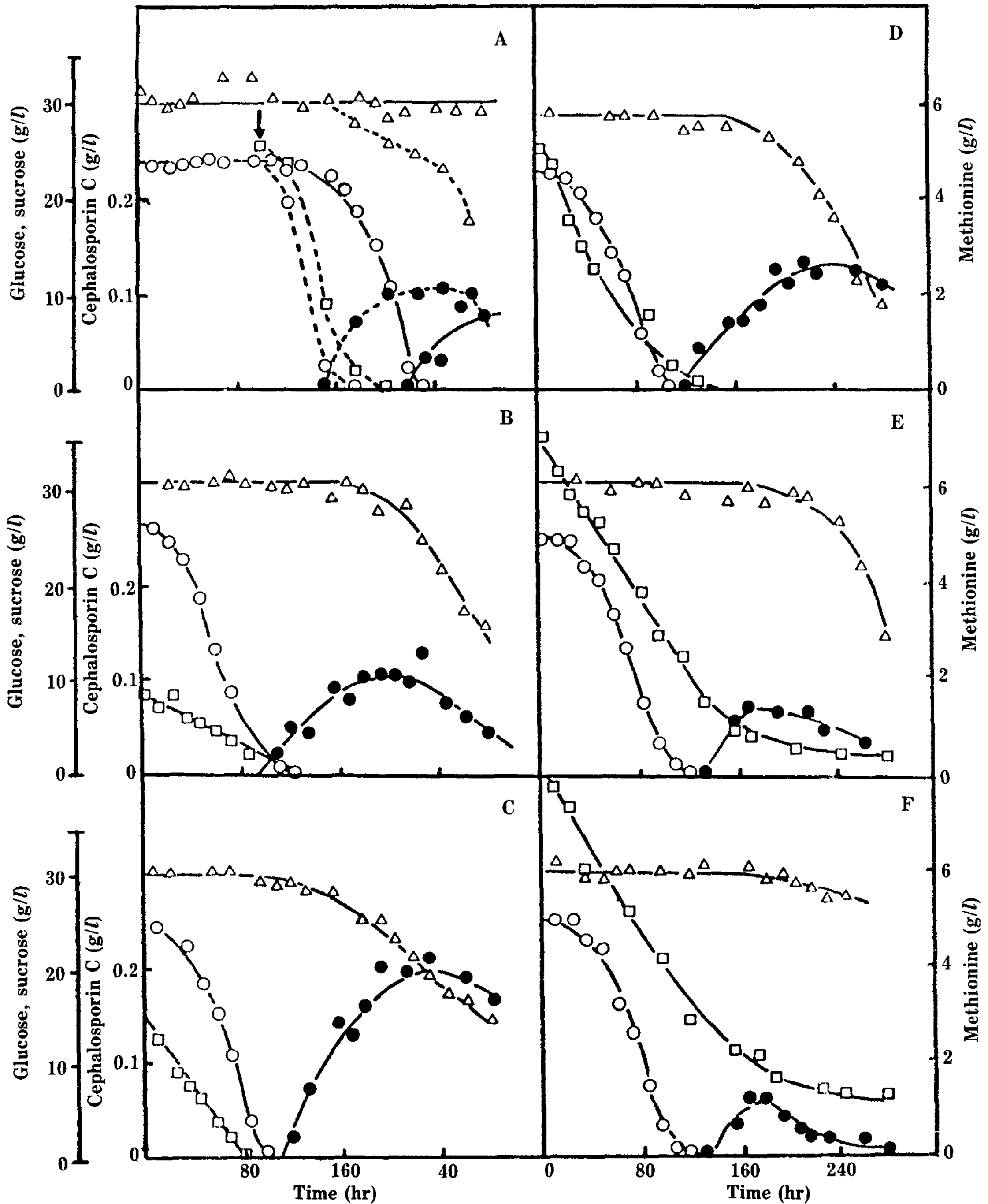

Fig. 1. Effect of initial methionine concentration on CPC production in shake-flask culture. (A: 0%, B: 0.1%, C: 0.3%, D: 0.5%, E: 0.7%, F: 0.9. In A, arrow indicates the addition time of methionine (5g/*l*). In this case, each profile is shown by the dashed line).
(-○-: glucose, -△-: sucrose, -□-: methionine, -●-: CPC)

to the morpholgical differentiation of the microorganism. From the microscopic examination, there existed four types of the morphology in *C. acremonium*: filamentous hyphae, swollen hyphae,

Table 2. Shake-flask culture using bioparticles.

Fermentation time (hr)	Immo. cell (g/l)	Free cell to immo. cell	Residual methionine	CPC titre (g/l)	Predominant morphology of immo. cell
0	6.1	0	5.0	0	long filamentous hyphae
24	13.6	0.04	3.1	0	long filamentous or swollen hyphae
48	11.4	0.14	0.6	trace	long swollen hyphae
72	6.2	0.98	0	0.111	short swollen hyphae
95	5.3	1.50	0	0.131	short swollen hyphae
119	4.8	1.76	0	0.117	arthrospore
144	4.4	.01	0	0.142	arthrospore
177	4.2	2.18	0	0.090	arthrospore

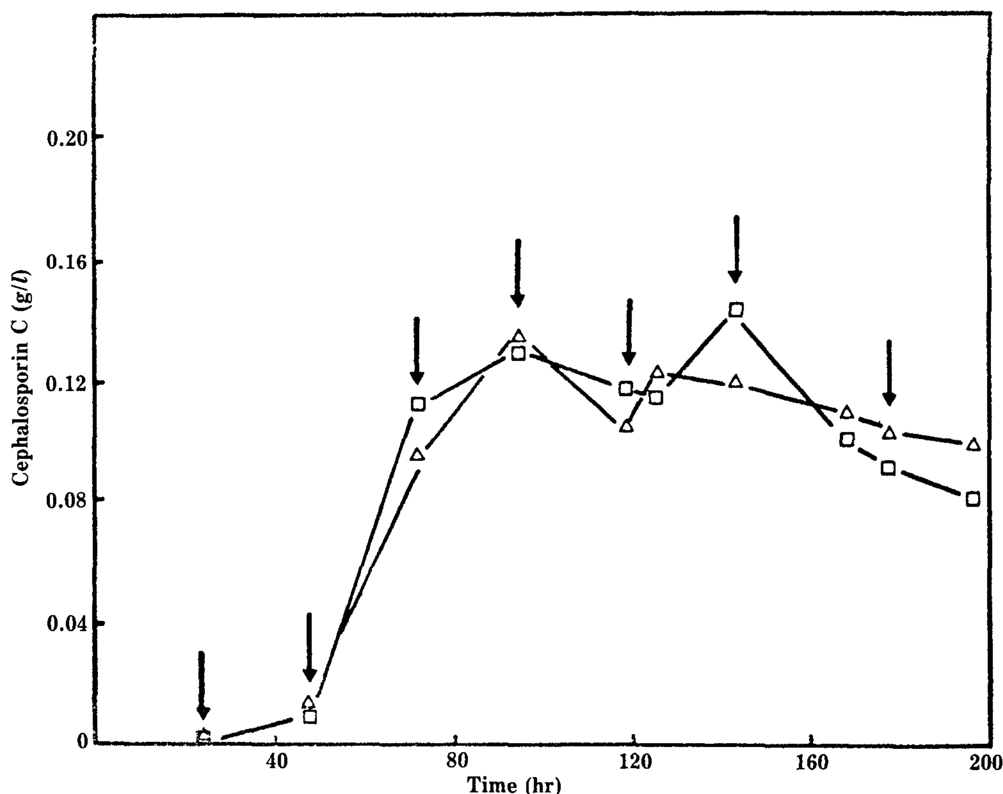

Fig. 2. Time profiles of CPC production using bioparticles in shake-flask culture.
Arrows indicate the removal of free cell.
(-□-: removal of free cell, -△-: no removal of free cell)

arthrospore and conidia. The differentitation of filamentous hyphae into the swollen hyphae and arthrospore occurred on the bioparticles with time (10). This morphological differentation of the microorganism was therefore considered most responsible for the loss of biofilm and for the increase of free cells in the culture broth. On the other hand, production of cephalosporin C increased steeply when methionine in the broth was exhausted. It was also apparent that the increase coincided with the morphological trasition of the cells from long swollen hyphal forms to short swollen ones.

In order to elucidate the degree of contribution by the free cell to the antibiotic production, fermen-

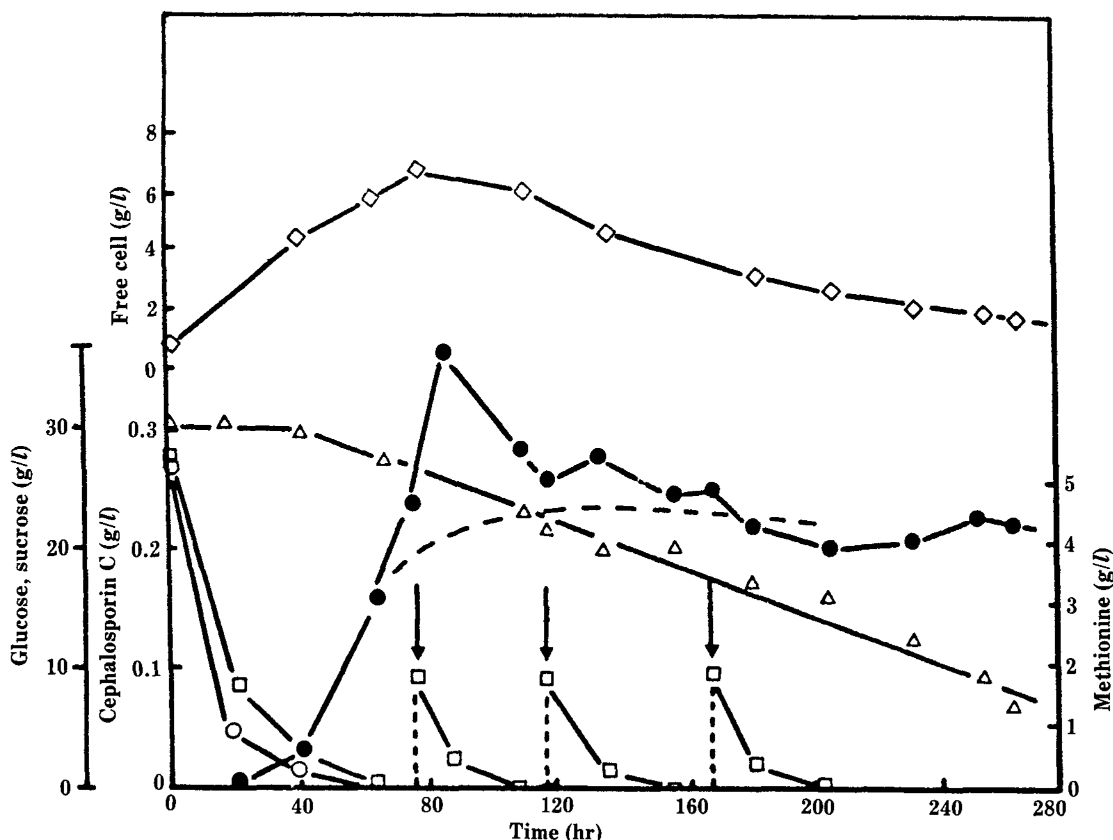

Fig. 3. Intermittent addition of methionine to fluidized-bed bioreactor with fresh bioparticles. Arrows indicate the time of methionine addition. A control experiment was shown by the dashed line. (-○-: glucose, -△-: sucrose, -□-: methionine, -●-: CPC, -◇-: free cell)

tation was carried out only by bioparticles after free cells were carefully removed from the broth. From Fig. 2, it can be noted that free cells do not contribute to cephalosporin C production in this bioreactor system.

Intermittent additions of methionine in fluidized-bed bioreactor using bioparticles

It was noted in batch cultures that the maximum production rate of cephalosporin C occurred when methionine become limiting in the broth. Thus it was considered quite possible to extend the production phase if the bioreactor was operated at such methionine-limiting condition.

Fig. 3 shows the effect of intermittent additions of methionine on cephalosporin C Production. Because Production of Cephalosporin C was negatively affected when excess methionine existed in medium (Table 1), 0.2% methionine was fed in to the medium upon its exhaustion. When the first addition was made right after the initial methionine was exhausted, production rate of the antibiotic was observed to increase to some extent. However, second and third additions practically had no further effect on the antibiotic production. The reason was probably because that methionine stimulated the synthesis of the antibiotic only when added at the early stage of the fermentation. A similar result was reported by Matsumura *et al.* (3); they found that extra addition of DL-methionine in the production phase did not increase the accumulation of cephalosporin C. Decrease in cephalosporin C concentration at the prolonged operation is probably due to CAH (cephalosporin C acetylhydrolase) (13) activity which is able to hydrolase cephalosporin C to deacetoxycephalosporin C.

Reuse of bioparticles in fluidized-bed bioreactor

Table 3. Batch operation of fluidized-bed bioreactor with reused bioparticles

	W/ methionine	W/O methionine
Max. CPC titre (g/l)	0.294	0.145
Max. free cell conc. (g/l)	4.00	3.95
Sucrose consumption rate (g/l.hr)	0.055	0.025

One of the greatest merits to use immobilized cells is the repeated and prolonged use of biomass. Reuse of bioparticles was thus examined. After the bioparticles were used once, they are washed three times and transferred back to the fludized-bed bioreactor. And then the experiment was progressed in fresh medium with and without methionine at the same condition as described previously. The result was summarized in Table 3. It was noted that when reused bioarticles were employed, the concentration of free cells increased at a much slower rate than the fresh bioparticles. Microscopic observation of the culture broth revealed that the predominent morphology of bioparticle and free cells was short swollen hyphae and their fragments, respectively. An interesting thing to note was that the antibiotic titre in the methionine containing medium was about 2 times higher than that without methionine. When methionine was excluded from the culture medium, production of the antibiotic by the reused bioparticles significantly decreased with a reduced sucrose consumption rate. From this, methionine is still believed to play an important role as an effector in cepphalosporin C fermentation. The results suggests us that the process productivity in a fluidized-bed bioreactor system can significantly be improved by repeated use of bioparticles. Thus operation of the bioreactor in such a manner as fill-and-draw technique shall succesfully be employed for such purposes. A large scale operation of the bioreactor system upto 20 liter capacity is currently being planned and the result shall be reported shortly.

Conclusion

It the shake-flask culture with free cells it was observed that the presence of initial methionine was essential for higher cephalosporin C. It was also noted that there existed an optimal content of methionine since inhibitory effect on the cephalosporin C production by excess methionine was evident. In the shake-flask culture with bioparticles, production of cephalosporin C was most active when methionine in the broth was exhausted. It was also apparant that high production rate of the antibiotic coincided with the morphological transition of the cells from long swollen hyphal forms to short swollen ones. To test a possibility to extend the production phase, additional feeding of methionine to a limiting concentration was made, but it did not enhance the antibiotic production as much as expected. This may support the previous observations that intracellular pool of methionine exerts the major effect on the induction of cephalosporin C synthetase system of the microorganism. Therefore, it was considered important to feed an optimal content of methionine at the early operating stage for higher cephalosporin C production in a fluidized-bed bioreactor. Furthermove, reuse of bioparticles is believed to be a useful tool to enhance the process productivity in the bioreactor operation. With reused bioparticles, it was also considered essential to feed methionine at an optimized content to improve the antibiotic production.

요 약

Cephalosporium acremonium 균주로 미생물 고정화 증식입자를 제조하여 유동층 생물반응기에서 세파로스포린 C 생산에 대한 메치오닌의 영향에 대하여 연구하였다. 메치오닌은 세파로스포린 C 생합성에 매우 중요한 대사조절인자로 알려져 있는데, 본고에서는 초기 발효배지 내 메치오닌 농도의 영향과 이를 토대로 생물반응기에 메치오닌을 주입하였을 때 세파로스포린 C 생산에 미치는 메치오닌의 영향을 관찰하였다. 초기 배지 내에 존재하는 메치오닌에 의해 세파로스포린 C 생산성이 증가하였으며, 메치오닌의 최적양(0.3-0.5 w/v%)이 존재하였다. 또한 메치오닌에 의해 탄소원의 소모속도가 증가됨이 관찰되었다. 배지내 메치오닌이 고갈될 때 세파로스포린 C 생산이 극대화되었으나 유동층 생물반응기에서 메치오닌을 부가적으로 첨가하였을 때 그 효과는 기대한 수준에 미치지 못했는데 이는 생체내 메치오닌의 축적과 관계가 있는 것으로 판단되었다. 또 고정화 증식입자를 사용하였을 때 세파로스포린 C 생산

에 양호한 결과를 얻었는데 이 경우에도 적정량의 메치오닌 투여가 생산성에 중요한 인자임을 발견하였다. 따라서 유동층 반응기의 운전에 의해 세포로스포린 C 생산성을 향상시키기 위해서는 초기 운전 단계에서 최적양의 메치오닌을 주입하는 것이 가장 중요하며 고정화 증식입자의 사용에 의해 공정생산성을 획기적으로 증대시킬 수 있다고 판단되었다.

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