Arch. Pharm. Res. 2(2), 79~83(1979)

Studies on the Cephalosporin C Biosynthesis by Fermentation

Kang Man Lee and Dewey Doo-Young Ryu

Korea Advanced Institute of Science, Seoul 131, Korea

(Received 12 May 1979)

Abstract□ Using *Cephalosporium acremonium* ATCC 14553, we studied on the important factors in fermentation. Those factors were: carbon and nitrogen sources, methionine effect, adsorbent effect, fermentation variables in a batch fermentation, effect of reuse of its cellular biomass as a reusable nutrient, and 2,4-dinitrophenol effect on cephalosporin C production.

Keywords□ Cephalosporin C—β-lactam antibiotic—factors on fermentation—*Cephalosporium acremonium* —effect of methionine—use of inert adsorbents.

Cephalosporin derivatives belong to β-lactam antibiotics and are produced by fermentation process and/or chemical modifications (e.g., ring expansion of β-lactam thiazolidine ring to β-lactam dihydro-thiazine ring). Cephalosporin C has become the most important raw material for 3-acetoxy cepham derivatives.

Cephalosporin C is the major product of *Cephalosporium acremonium* fermentation. Brotzu found the so-called Brotzu strain in 1948. The strains related to industrial production of cephalosporin C belong to the decendents of Brotzu strain. There are papers concerning strain improvements, its media composition, and fermentation variables[1~3]. There are, however, few practical data required for cephalosporin C fermentation, and we have undertaken this study with the ultimate purpose of developing a process technology for production of cephalosporin antibiotics.

EXPERIMENTAL

Microorganism

Cephalosporium acremonium ATCC 14553 was used. This strain was preserved in lyophilized vials and its stock culture was grown on Czaped-Dox agar at 25°C for two weeks with 5% lactose. The stock culture slant was stored at 0°–4°C.

For preparation of inoculum, one ml of the suspension of the washed and scraped stock culture was transferred into 30 ml of seed medium and was incubated for 72 hours at 27°±1°C and 300rpm. The seed medium concontained corn steep liquor 2.7 %, ammonium acetate 0.44 %, and sucrose 2.0 %.

Fermentation

The fermentation in shake-flask started with transferring one ml of the seed inoculum culture into 50 ml-fermentation medium and incubated at 27°C, 300 rpm for 5 days. The fermentation medium contained sucrose 4.0 %, soytone 1.0 %, C.S.L. 2.5 %, NH_4OAc 0.1%, DL-methionine 0.5%. The medium was

adjusted to pH 7.3. To determine a suitable carbon source, sugars such as glucose, sucrose, lactose, and starch were evaluated. Suitable nitrogen sources were selected to test for possible improvement of cell growth and antibiotic productivity. The nitrogen sources tested were corn steep liquor, soytone, beef extract, NH_4OAc, casitone, peptone, casein, trytone, yeast extract, and urea. DL-Methionine effect on cephalosporin C production was also tested. DL-Methionine concentration evaluated was in the range of 0.1 %–0.7 %.

Satisfactory fermentation conditions for 5 liter jar fermentor were specified as follows: inoculum size 5 %, working volume 3 liter, aeration 3–5liter/min, agitation 350 rpm, and temperature 26°±1°C.

Adsorbent Effect on Cephalosporin production

Various adsorbents (such as celite, Wakogel, cellulose, CMC, bentonite, methylcellulose, charcoal, and Hyflo-super cel) were tested by adding 0.1 % adsorbent to the fermentation medium. Also, the effects of charcoal and bentonite concentration in the range of 0.1–0.5 % were evaluated.

Reuse of Its Cellular Biomass

The filter-cake obtained from *Cephalosporium acremonium* fermented broth was washed twice with distilled water and dried in an oven (temp. 80–90°C). Pulverizing the dried cellular biomass in mortar the biomass (4g) was added to 20 ml of 2N H_2SO_4 and hydrolyzed at 120°C for 60 min. The hydrolyzed cellular biomass(HCB) was neutralized with 2N NaOH. With the hydrolyzed cellular biomass(HCB), comparative experiments were carried out. The control was the normal fermentation medium. The two kinds of HCB media used contained: i) HCB 15ml, sucrose 4%, and DL-methionine 0.5%; and ii) HCB 30ml, sucrose 4 %, DL-methionine 0.5 %. The media i) and ii) were added to distilled water to make up 50ml medium each and adjusted to pH 7.2 before autoclaving.

2,4-Dinitrophenol Effect on Cephalosporin C Production

2,4-Dinitrophenol (DNP) was added to fermentation medium in the concentration range (0.0015g–0.0045g/50ml) and incubated under the fermentation condition.

Analytical Methods

Dry cell weight was measured by drying the cell mass at 100°–105°C for 24 hours. Specific oxygen uptake rate was measured by using Gilson respirometer under the fermentation temperature. In addition to cephalosporin C, some penicillin N and trace of cephalosporin P were produced in the fermentation broth. Cephalosporin P was not active against the test organism used for assy.[4] To inactivate penicillin N(largely by its conversion to penicillic acid), a sample of the culture fluid was brought to pH 2.5–3.0 with 1M H_3PO_4 and kept at 37°C for 3hr. The pH of the stirred solution was then adjusted carefully to 6.5–7.0 with 1N NaOH. The cephaloporin C in the sample was virtually unaffected by this procedure and was subsequently assayed by the disc diffusion method. In this experiment, test organism was *Salmonella typhimurium.*

RESULTS AND DISCUSSION

The ability of the microbial strain for

Table I: Identification of cephalosporin fermentation products with Kieselgel GF 254 thin layer chromatography

Sample	Rf value*	Compound
Standard	0.30	Cephalosporin C
1 spot	0.30	Cephalosporin C
2 spot	0.48	Penicillin N
3 spot	0.83	Cephalosporin P

*Solvent system, BuOH:CH_3COOH:H_2O=4:1:4(V/V)

Table II: Selection of carbon source

Carbon source (4 %)	Cell growth*	Broth relative activity
Glucose	++	100.0
Sucrose	+++	163.1
Lactose	++	135.7
Starch	+	116.7

* +++ represents very good growth.
* + represents poor growth.

cephalosporin C production was positively identified by thin layer chromatography of the fermented broth. The products identified were cephalosporin C, penicillin N, and cephalosporin P. Table I shows the results of TLC detected by UV and iodometric method.[5] Among the carbon sources evaluated, sucrose was found to be the best.

Table III: Selection of nitrogen sources

Nitrogen source (3 %)	Cell growth*	Broth relative activity
C. S. L.	+++	130.7
Soytone	+++	102.6
Yeast. ext.	+++	100
Beef ext	++	.
casitone	++	.
Peptone	+	.
Tryptone	++	.
Caseine	−	.
Urea	−	.
NH OAC	(+)	.

* (−) represents no growth.

The nitrogen sources have been recognized as important sources of precursors and more readily assimilable nutrients. Corn steep liquor and soytone were found to be better than the others as shown in Table III.

It was reported that methionine played a significant role in cephalosporin synthesis, as lysine did for penicillin synthesis[6]. Optimum concentration of DL-methionine was determined for the maximum production of cephalosporin C. As the concentration of DL-methionine was increased, the production of cephalosporin C was enhanced (Table IV).

Figure 1 shows the profile of fermentation variables obtained from 5-liter scale fermentation. The pH varied from the initial pH 6.8~6.9 to the final pH 7.9~8.0. The maximum cell concentration was about 8 g/liter. The specific oxygen uptake rate reached the maximum value(1.4 m mole O_2/g-cell/hr) at 60hr. During the period of cephalosporin C production, the values of specific oxygen uptake rate varied in the range of 0.9~1.2m mole O_2/g-cell/hr. The production of cephalosporin C started at about 72 hr culture time and reached maximum at 120 hr. These facts confirmed that the idiophase was the production phase of cephalosporin C.

Various adsorbents were tested whether they had the positive effect or not on the fermentation[7]. Among the adsorbents, charcoal and bentonite showed more positive effects on cephalosporin C production as shown in Table V. It was postulated that adsorbent would be porous and could provide a very large surface area for the cell growth and that

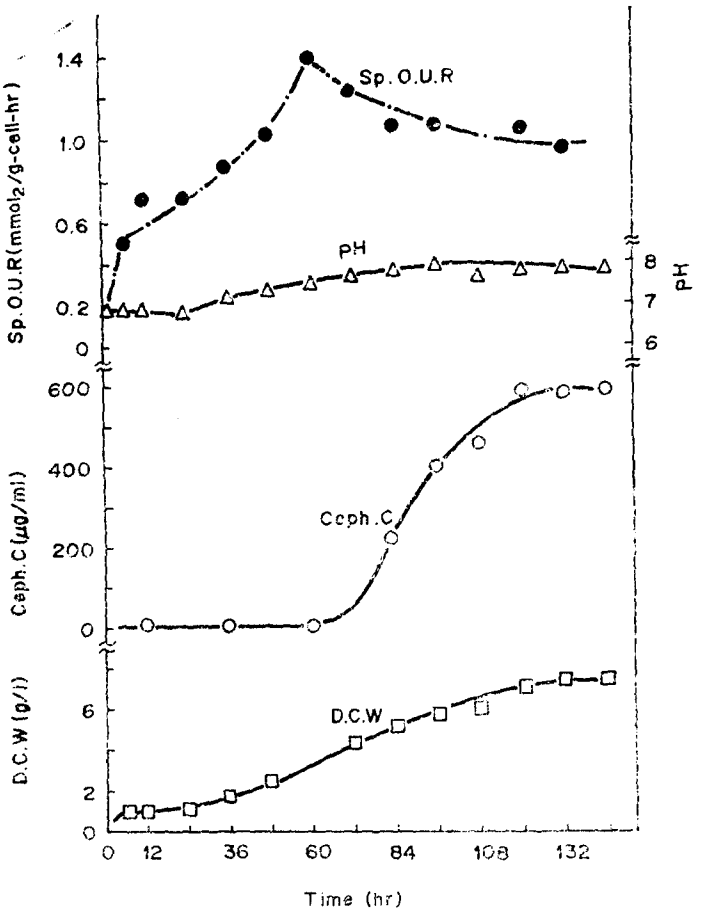

Fig. 1: The profiles of fermentation variables in cephalosporin C fermentation

the homogeneous growth in the fermentation broth could result. This would result in better cell growth without any mass transfer limitation for nutrients and oxygen.

The test results of the effects of charcoal and bentonite concentrations showed that 0.1 % charcoal was more effective and 0.5 % in the case of bentonite(Table VI). *Cephalosporium acremonium*, in particular, grows slowly. It needs novel approaches to better medium and fermentation design for cell growth and antibiotic production.

Cellular biomass of *Cephalosporium acremonium* was treated and reused as a nutrient source. Table VII showed the effect of reused mycelial nutrient on the cephalosporin C production. The treated cellular biomass would

Table IV: Effect of DL-methionine concentration.

DL-Methionine(%)	Broth relative activity
0.1	100
0.3	129
0.5	153
0.7	160

Table V: Effects of adsorbents on cephalosporin C production

Adsorbent	Broth relative activity
*Control	100
Celite	88
Wako-gel	97
Cellulose	94
Bentonite	117
Methyl Cellulose	105
Charcoal	120
Hyflo-super Cel	100

* Control without an addition of any adsorbent.

Table VI: Effect of adsorbent concentration

Adsorbent	Broth relative activity		
	0.1%	0.3%	0.5
Charcoal	100	91	89
Bentonite	100	102	116

Table VII: Effect of reuse of cellular biomass

Madia	Broth relative activity
*Control	100
HCB 15	125
HCB 30	106

*Control used the fermentation medium

not be enough for a good cell growth as a complete nutrient. But supplementation of other nitrogen sources gave more cell growth and higher antibiotic productivity.

2,4-Dinitrophenol affects the electron transport system in the eucaryotic energy metabolism. This compound was expected to give some effects on cephalosporin C production since it is related to the biosynthetic energy metabolism and carbohydrate metabolism. But its effect was not always positive as compared to the control(without addition of DNP), and the results of 2,4-dinitrophenol on cephalosporin C productivity was inconclusive.

CONCLUSION

Cephalosporin C was prepared by improving fermentation conditions. The yield obtained was about 1.5~2.8 g/liter. Positive effect of methionine on cephalosporin C biosynthesis was confirmed. A new method of increasing surface area for submerged culture by the use of inert adsorbent particles was successfully tested and an increased productivity of cephalosporin C was observed.

LITERATURE CITED

1) Demain, A. L. and Newkirk, J. F., Biosynthesis of cephalosporin C. *Appl. Microbiol.* **10,** 321(1962).
2) Caltrider, P. G. and Niss, H. F., Role of methionine in cephalosporin synthesis, *Appl. Microbiol.* **14,** 746(1966).
3) Drew, S. W. and Demain, A. L., The obligatory role of methionine in the conversion of sulfate to cephalosporin C. *Eur. J. Appl. Microbiol.* **1,** 121 (1975).
4) Kavanagh, F., Cephalosporin C in *Analytical Microbiology,* Academic Press, New York, p. 165, (1963).
5) Nara, T., Misawa, M., Okachi, R., and Yamamoto, M., Enzymatic synthesis of D(-)-α-aminobenzylpenicillin (I). Selection of penicillin acylase producing bacteria, *Agr. Biol. Chem.* 35, 1676(1971).
6) Demain, A. L., Newkirt, J. F., and Hendlin, D., Effect of methionine, norleucine, and lysine derivatives on cephalosporin C formation in chemically defined media, *J. Bacteriol.* **85,** 339(1962).
7) Metz, B. and Kessen, N. W. F., The growth of molds in the form of pellets. *Biotech. Bioeng.* **19,** 781(1977).