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HIGH CELL DENSITY CONTINUOUS CULTURE OF *Escherichia coli* PRODUCING PENICILLIN ACYLASE

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SUMMARY

We studied high cell density continuous culture (HDCC) of a recombinant(*E. coli*HB101(pPAKS2)) and a mutant(*E. coli* ATCC 11105) strains of *E. coli* producing penicillin acylase(PA). Using pure oxygen, high cell density up to 95 g/l was obtained without significant inhibition by a main byproduct, acetic acid. The operation was simple and productivity was several times higher than those of conventional batch and continuous culture. Dissolved oxygen level and CO_2 concentration were important variables, and glucose concentration was naturally regulated in HDCC.

INTRODUCTION

Continuous operation and high cell density are desirable for the high productivity of fermentation. However, continuous operation has been practised mainly for physiological studies rather than for industrial purposes because of the possibilities of strain degeneration and contamination It has been usually operated at a low cell concentration (Melling, 1977). A high cell density culture requires sufficient oxygen supply, continual feeding of substrates without inhibition, and minimal formation of inhibitory byproducts (Landwall and Holme, 1977). As means of high cell density, culture dialysis (Landwall and Holme, 1977), fed-batch (Mori et al., 1979) , and recycle culture (Rogers et al., 1980) were tried. These systems achieved high cell density that could not be obtained with a conventional batch method. But fed-batch is the only method currently under commercial practice. The other two methods present some operational difficulties or require some subsidiary equipment that

are not economical. If continuous culture can be operated at high cell density, it will have a great advantage in productivity without any additional apparatus. So far HDCC has been studied only for producing single cell protein (Hofmann and Heisel, 1984). Although *E. coli* has been widely used as a host of gene cloning, high density cultivation of this microbe was difficult due to the inhibitory byproduct formation such as acetic acid (Landwall and Holme, 1977). From our experiences of membrane recycle culture (Lee and Chang, 1988), we discovered that the most important factors for high density culture were the minimization of an inhibitory byproduct (acetic acid), glucose limitation, and adequate D.O. control. High cell density or high medium concentration did not present any problems. So we practised HDCC where glucose could be naturally limited.

In the present study, we report on HDCC of a recombinant and a mutant strain of *E. coli* producing PA. PA is an industrially important enzyme that hydrolyses penicillins into 6-aminopenicillanic acid (6-APA) used as a precursor of semisynthetic antibiotics(Vandamme and Voets, 1974).

MATERIALS AND METHODS

Bacterial strains: *E. coli*HB101(pPAKS2) and *E. coli* (ATCC 11105) were used. The former is a genetically engineered strain for producing penicillin acylase. In this strain, PA production is not repressed by glucose and need not be induced with phenylacetic acid (Kim et al., 1983). But the reverse is true for the latter.
Media: The medium YL1 consisted of (g/l): yeast extract 5, casamino acid 5, phenylacetic acid 1, K_2HPO_4 0.5, $MgSO_4.7H_2O$ 0.1, $CaCl_2$ 0.01, trace metal 0.1 ml. And YL2 (g/l): glucose 10, yeast extract 5, casamino acid 5, K_2HPO_4 0.5, $MgSO_4.7H_2O$ 0.2, $CaCl_2$ 0.02, Vit.B1 0.001, trace metal 0.1 ml. Trace metal solution contained the following minerals in g/l 5N-HCl: $FeSO_4.7H_2O$ 10, $MnSO_4.nH_2O$ 10, $AlCl_3.6H_2O$ 10, $CoCl_2$ 4, $ZnSO_4.7H_2O$ 2, $Na_2MoO_4.2H_2O$ 2, $CuCl_2.2H_2O$ 1, H_3BO_4 0.5.
Experimental setup: Jar fermenter(500 ml) of New Brunswick Scientific Co. was used. Hollow fiber(I.D.=1.5mm, O.D.=2.5mm, molecular weight cut-off=50,000) was purchased from Berghof Co.(Germany) and the cartridge was made in our laboratory and its total surface area was 600 cm^2. Schematic diagram of the whole setup was shown in Figure 1.
Assay: Cell concentration was determined by measuring optical density at 600 nm(Beckman Spectrophotometer model 35). Glucose was assayed enzymatically with glucose analyzer kit(Yeongdong Pharm. Co.). Organic acids were analyzed with FID gas chromatography(Gow Mac 750, column; 10% Carbowax 20M on Chromosorb WAX 80/100 mesh). We measured CO_2 concentration with Orsat gas ana-

lyzer(Hays Republic Co.) and D.O. with a galvanic type D.O. probe(Tokyo Rikakikai Co.). Viscosity was measured with Ostwald viscometer. PA activity was determined by measuring 6-APA spectrophotometrically that was produced from the reaction in a 25 mg/ml penicillin-G solution in a 50 mM phosphate buffer(pH=7.5) at 37°C. One unit was defined as activity producing 1 μ-mole 6-APA/hr.

Culture condition: Culture temperature was 28°C and pH was maintained at 7.0 with 4N-NH_4OH solution. D.O. level was manually controlled at 50-60% of air saturation with air, oxygen enriched air or pure oxygen according to the oxygen demand and gas flow rate was regulated at 1 vvm.

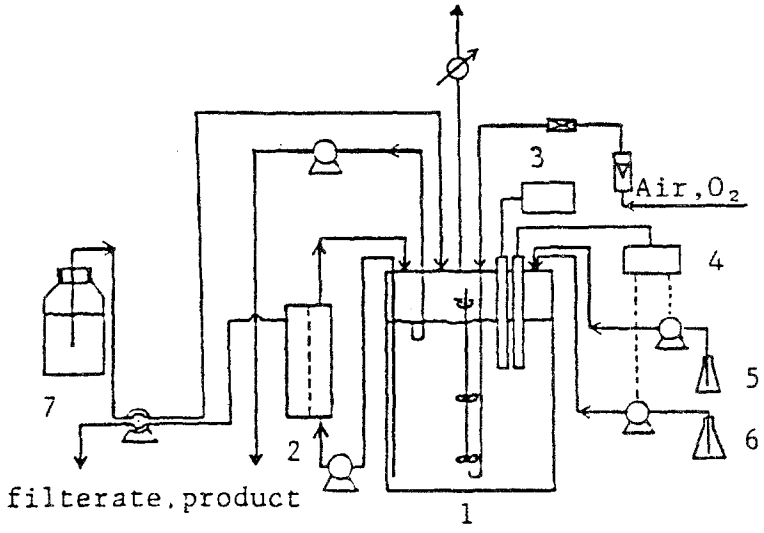

Figure 1. Schematic diagram of the experimental setup: 1.fermenter, 2.filter, 3.D.O. monitor, 4.pH controller, 5. acid solution, 6.alkali solution, 7.medium bottle.

RESULTS AND DISCUSSION

Figure 2 shows a typical experimental run of high cell density continuous culture with *E. coli*HB101(pPAKS2). Batch culture was performed for the first 16.5 hours and cell concentration reached 5.4 g/l. Glucose, acetic acid and propionic acid concentrations were 0.5, 0.9, and 0.5 g/l, respectively. Then we started membrane recycle culture to increase cell mass. During the recycle culture acetic acid and propionic acid concentrations increased to 3 and 1 g/l, respectively. Glucose concentration was 0.5-0.1 g/l at the early operation and was limited after 23 hours. When the cell concentration reached about 83 g/l, we started continuous run at a dilution rate of 0.06 hr^{-1}. The cell concentration decreased to 64

g/l. The acetic acid and propionic acid concentrations were maintained at around 0.5 and 1 g/l, respectively. During the continuous culture glucose concentration remained at nearly zero. It is thought that the reduced acetic acid formation resulted from the glucose limitation which could be naturally obtained in continuous culture.

We emphasize that membrane recycle culture was used to speed up the cell mass build-up in the fermenter. Fed-batch culture could also be used as well for this purpose (data not shown here). Cell concentrations in HDCC are shown in Figure 3 as a function of dilution rate. Cell concentration with 15xYL2 was about 95 g/l while that with 10xYL2 was 64 g/l, which indicates that the supplied glucose was converted to cell mass stoichiometrically. The run with 20xYL2 produced so high a solid content up to 120 g/l that we could not continue the run because of high viscosity, poor mixing, and insufficient oxygen supply. The cells were washed out at a dilution rate of 0.18 hr^{-1} which was less than the maximum specific growth rate (0.28 hr^{-1}) at the batch culture. The early washout may be due to the combined influences of growth inhibitory factors such as high CO_2 concentration, O_2 toxicity, and high osmotic pressure of cell broth. Gas phase CO_2 concentration increased up to 28 % in this particular experiment, which was found to be a function of gas flow rate, medium concentration, and dilution rate. Even though D.O. was maintained at about 50 % air saturation, use of pure oxygen may give an inhibitory effect on cell growth. While O_2 and CO_2 had influences on specific growth rate and yield, that of CO_2 was more significant (Fig.4). As D.O. is not maintained at high level even with pure oxygen, CO_2 would be a main operational variable to be controlled in high density culture. Cell broth may exert high osmotic pressure which gives a negative effect on cell growth because of high medium concentration used. In fact, cell growth was retarded at flask culture with the centrifuge supernatant of broth with nutrients supplemented. Another possibility was that foam might be one of the causes of cell washout (Thomas and Winkler, 1977).

For comparison *E. coli*(ATCC 11105) producing PA from chromosomal DNA was also cultured (Fig.5). Since YL1 medium did not contain glucose, acetic acid formation was minimal (<0.2 g/l) compared to the case of the recombinant strain. At the specific growth rate of 0.3 hr^{-1}, dissolved oxygen level dropped to zero even with pure oxygen and vigorous agitation. In that case maximum productivity was limited by oxygen transfer rate. As heat removal was a troublesome problem even in this small fermenter, it would be an important design parameter in a large scale fermenter for high density culture. Unlike other recombinant cells, *E. coli*HB101(pPAKS2) was very stable and its specific activity was

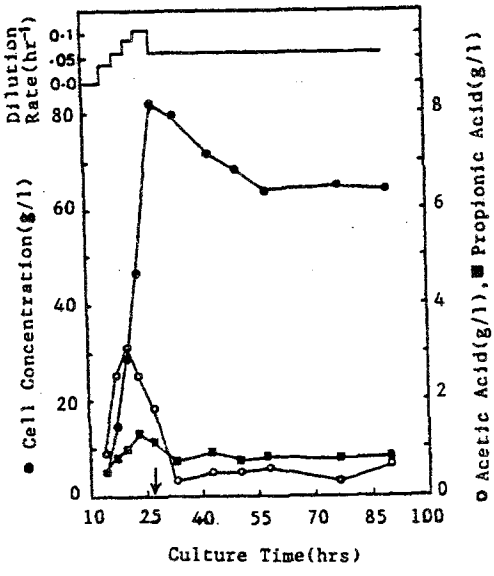

Figure 2. Cell and organic acid concentration changes when culture mode was changed from recycle to continuous mode(↓) in culture of E. coliHB101(pPAKS2). Medium:10xYL2.

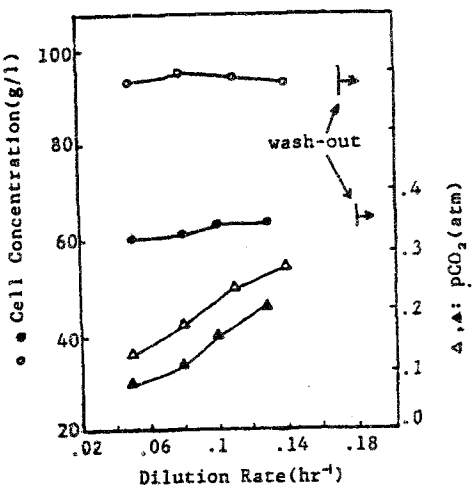

Figure 3. Cell and CO_2 concentration changes with dilution rate in continuous culture of E. coliHB101(pPAKS2). Medium:10xYL2(●,▲),15xYL2(o,Δ).

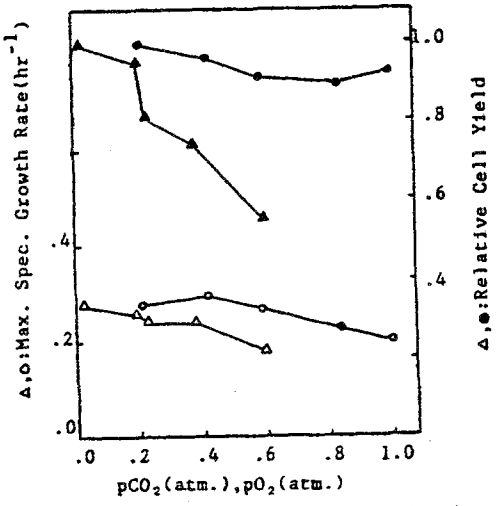

Figure 4. Effects of pCO_2(▲,Δ) and pO_2(●,o) on the specific growth rate and cell yield in batch culture of E. coliHB101(pPAKS2) with YL2 mudium.

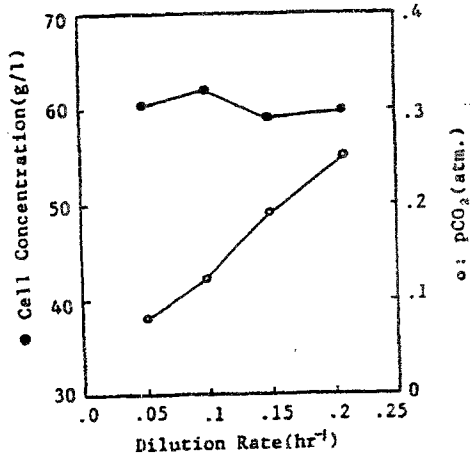

Figure 5. Cell and CO_2 concentration cnanges with dilution rate in continuous culture of E. coli ATCC11105. Medium:25xYL1.

about 6 units/mg throughout the experiment. In the present experiment, we obtained the maximum productivity of 13 g/l.hr, which could have been increased further by optimization. It is more than ten times higher than that of simple batch culture. HDCC need not be necessarily operated for a long time at the risk of degeneration and contamination. Several batches or equivalent generations will guarantee higher productivity than any other culture methods. Moreover, HDCC is advantageous because it does not require any auxiliary apparatus such as a membrane filter.

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