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EFFECTS AND OPTIMIZATION OF SELECTION PRESSURE IN ENZYME PRODUCTION USING RECOMBINANT *E. coli*

Jung Heon LEE, Young Je YOO* and Kwan Hwa PARK**

Department of Chemical Engineering (**Department of Food Science and Technology), Seoul National University, Seoul 151-742, Korea

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Abstract – Selection pressure is widely used in stabilizing the plasmid of the recombinant microorganism. The effects of selection pressure on enzyme production were investigated using recombinant *E. coli* harboring plasmid pTA322. Optimum ampicillin concentration which maximizes the enzyme production was 150 μg/ml in flask culture and 50 μg/ml in the culture using 2-1 fermentor. Optimum concentration of selection pressure in a stirred fermentor was different from that in a flask due to different agitation effects. It was found from mathematical modelling that as the concentration of ampicillin increased, the plasmid-harboring cells with low plasmid copy number were damaged severely, while the specific production rate increased due to the high plasmid copy number in the microorganism.

INTRODUCTION

With the development of biotechnology and molecular biology, enzymes are being widely used especially for pharmaceuticals and catalysts in food and chemical industries [1, 2]. The cost of enzymes is an important factor in commercial applications of the enzyme. Recently recombinant DNA technology has been introduced for overproduction of the enzyme [3, 4]. When employing a recombinant microorganism for commercial production, the genetic stability of recombinant DNA should be considered [5]. Segregational instability and structural instability are the main causes for the loss of desired gene function which occurs during fermentation. The factors which affect the plasmid stability in a recombinant microorganism are the characteristics of plasmid vector, genetic characteristics of host cell, degree of genetic expression, culture temperature, limiting nutrient, dilution rate, and the environmental factors of reactor operation. Many results have been published on the relation between plasmid stability and environmental factors [6-9].

Many strategies such as adding an inducer or shifting a fermentation temperature have been reported to stabilize the plasmid during fermentation. Selection pressure has been widely adopted to maintain the plasmid harboring microorganism [10, 11]. The degree of selection pressure determines to some extent the plasmid content of the microorganism and the productivity of the bioprocess, and the plasmid content of microorganism determines the capability of the product biosynthesis [7]. In the case of transient growth of plasmid-harboring and plasmid-free cells in partially selective medium, the degree of selection pressure required for stable maintenance of plasmid-harboring cells was determined [8].

In this communication, the effects of selection pressure and the agitation condition of the bioreactor on the plasmid stability in recombinant *E. coli* are reported.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

1. Microorganism

E. coli harboring recombinant plasmid pTA322 [12] was used as a model system in the present study. *E. coli* HB101 was used as a host strain and pTA322 plasmid was constructed by cloning a thermostable α-amylase gene from *B. licheniformis* to pBR322 plasmid. The α-amylase producing *E. coli* cells contained a 7.4 kb plasmid(pTA322) which was composed of the vector pBR322 and a 3.1 kb EcoRI fragment of *B. licheniformis* DNA. The plasmid pTA322 contained also genes for ampicillin resistance.

2. Culture conditions

Media compositions were tryptone(glucose, starch,

Table 1. Cell growth and enzyme production of the recombinant microorganism for various nutrient compositions

Media*	Maximum cell concentration (g/l)	Maximum enzyme activity (units/ml)
Trypton	1.04	19
Glucose	0.29	nil
Starch	0.60	nil
Maltose	0.52	nil

*yeast extract and NaCl were also included.

or maltose) 10g/*l*, yeast extract 5g/*l*, and NaCl 5g/*l*. Recombinant *E. coli* was inoculated from agar slant and precultured in a 250 m*l* Erlenmeyer flask containing 100 m*l* of LB broth for 12 hours at 37℃ and 150 rpm using 50 μg/m*l* of ampicillin. The precultured solution was transferred to 5-1 fermentor containing 2-1 of LB broth and maintained at pH 7.0 and 37℃. The air flow rate was adjusted to 1.0 vvm and ampicillin was added to the fermentor just before inoculation. Tryptone and yeast extract were from Difco, ampicillin was from Sigma, and other chemicals used were reagent grade.

3. Assays

Cell mass concentration was obtained by measuring the optical density at 660 nm using a spectrophotometer(Kontron, Uvikon 930) and by comparing with calibration curve. α-Amylase activity was measured using the modified DNS method by Fuwa [13]. 1 Unit of the enzyme is defined as the amount enzyme which liberates 50 μg of maltose during 10 min at 30℃. The stability of recombinant cells was determined by plating method. Colony counts of appropriate dilutions of culture samples after incubating and spraying iodine solution on starch agar plate provided information on the number of total plasmid-harboring cells (white region) and plasmid-free cells (black region). Antibiotic stability was measured by agar-plating method. *E. coli* HB101 was spread over agar plate and antibiotic-loaded paper (diameter 5 mm) was placed on agar plate. After overnight incubation at 37℃, the antibiotic activity was determined by the area of clear region.

4. Shear rate calculation

The Reynolds number of the fermentor was calculated from the following equation;

$$N_{Re} = \frac{D_a^2 n \rho}{\eta} \quad (1)$$

where, D_a, n, ρ and η are impeller diameter, rotational speed, liquid density, and viscosity, respectively. The viscosity and the density of liquid were obtained from Perry's handbook [14]. The average shear rate of liquid was

$$\left(\frac{\partial u}{\partial y}\right)_{ave} = 11n \quad (2)$$

and the maximum shear rate of liquid was

$$\left(\frac{\partial u}{\partial y}\right)_{max} = 19n. \quad (3)$$

RESULTS AND DISCUSSIONS

1. Nutrient effects

To determine the effects of media, flask cultures were performed using different media. Table 1 shows the effects of medium. Cell growth was the highest in the medium containing tryptone and the enzyme activity was only observed in the medium containing tryptone (LB media). In the presence of glucose, maltose or starch, the enzyme activity was not observed. Results indicate that the enzyme biosynthesis was repressed very strongly by catabolite [15]. Since tryptone and yeast extract were major components in LB media, the effects of nutrient compositions were investigated. Recombinant *E. coli* harboring plasmid pTA 322 was cultivated using the medium containing yeast extract. Without yeast extract, no growth was observed. As the concentration of yeast extract increased, more growth of the microorganism was observed. However no remarkable effects were observed by changing the concentration of tryptone. Therefore yeast extract was considered to contain an essential nutrient for the growth of the recombinant *E. coli* and can be regarded as a limiting nutrient, even though it was not clear what component in yeast extract was limiting.

2. Ampicillin effects

The recombinant microorganism was cultivated at various ampicillin concentrations. As shown in Table 2, the highest cell mass was obtained without selection pressure and the highest enzyme activity was obtained when initial ampicillin concentration was 150 μg/m*l* in flask culture. In the experiments using 2-1 stirred fermentor, maximum enzyme activity was obtained when initial ampicillin concentration was 50 μg/m*l* as shown in Table 3. As the ampicillin concentration increased, the specific enzyme activity increased while the growth of the recombinant microorganism decreased. Since the differences in cell mass and the enzyme activity were minor above 50 μg/m*l* of ampicillin concentration in the fermentations using stirred fermentor, experiments were repeated and almost the same results were obtained. However, when ampicillin con-

Table 2. Effects of ampicillin in flask culture

	Ampicillin concentration (μg/ml)				
	0	50	100	150	200
Cell mass (g/l)	0.94	0.95	0.89	0.87	0.79
Enzyme activity (unit/ml)	11.0	18.0	19.1	20.1	18.1
Specific enzyme activity (unit/mg of cell)	11.7	18.9	21.5	23.3	22.9

Table 3. Effects of ampicillin in 2-1 stirred fermentor

	Ampicillin concentration (μg/ml)				
	0	50	100	150	200
Cell mass (g/l)	1.56	1.52	1.27	1.16	no growth
Enzyme activity (unit/ml)	23.2	49.3	48.9	47.3	–
Specific enzyme activity (unit/mg of cell)	14.9	32.4	38.5	40.8	–

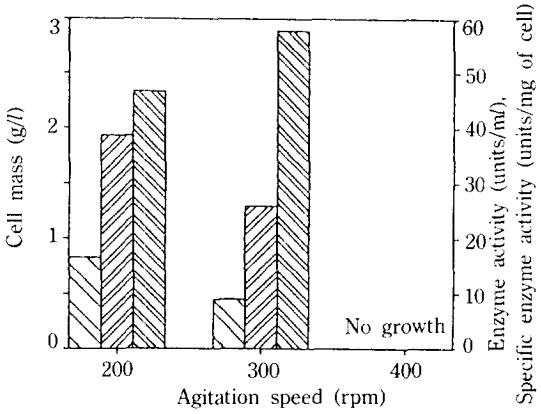

Fig. 1. Effects of agitation speeds on the cell growth and enzyme biosynthesis in 2-1 fermentor culture(ampicillin conc. = 200 μg/ml).
▩: cell mass (g/l), ▨: enzyme activity (units/ml), ▧: specfic enzyme activity (units/mg of cell)

centration was 200 μg/ml, no growth of the recombinant *E. coli* was detected. Since the environmental conditions, especially agitation effect, between flask culture and the culture using a stirred fermentor were different, agitation effect can be postulated to cause the microorganism's resistance to antibiotic differently for different agitation conditions at the same antibiotic concentrations.

Ampicillin deactivations during 24 hours of fermentation were observed to be 5-8% of the original activities. To clarify the agitation effect in the stirred fermentor, cultures were performed for different agitation speeds with 200 μg/ml of ampicillin. Fig. 1 shows the effects of agitation speed on cell growth and enzyme production. When agitation speed was 200 and 300 rpm, respectively, the cell growth and the enzyme biosynthesis were observed even at 200 μg/ml ampicillin. Cell growth and enzyme biosynthesis were higher at 200 rpm compared to the results at 300 rpm. When agitation speed was 200 rpm, the mode of antibiotic action was appeared to be milder than that of 300 rpm.

3. Mathematical analysis

To characterize quantitatively the effects of ampicillin on the fermentation of recombinant microorganism, a mathematical model was used. On the growth of the recombinant microorganism, a reported model [16, 17] as shown in equations (4) to (7) was employed. The model assumes that the recombinant cells fail to grow with a probability β(apparent segregation coefficient) at division due to low plasmid copy number. The enzyme biosynthesis rate as in equation (8) was assumed to follow the Luedeking-Piret model and to be also inhibited by the enzyme activity in the cell. This assumption was made since the enzyme was intracellular and the specific production rate of the enzyme showed a decreasing tendency at the end of the fermentation.

The growth rates of plasmid-harboring cells(X^+) and plasmid-free cell (X^-) were expressed as follows:

$$\frac{dX^+}{dt} = (1-\beta)\mu^+ X^+ \tag{4}$$

$$\frac{dX^-}{dt} = \mu^- X^- + \beta\mu^+ X^+ \tag{5}$$

$$\mu^+ = \frac{\mu_m^+ S}{K_s^+ + S}, \text{ and } \mu^- = \frac{\mu_m^- S}{K_s^- + S} \tag{6}$$

$$S = S_0 - \frac{1}{Y_{x/s}^+}(X^+ - X_0^+) - \frac{1}{Y_{x/s}^-}(X^- - X_0^-) \tag{7}$$

, where S_0, $Y_{x/s}$ and X_0 denote the initial limiting substrate concentration, cell yield coefficient and initial cell mass, respectively. Superscript + and − denote plasmid harboring cells and plasmid-free cells, respectively.

The rate of product formation can be therefore described as follow:

$$\frac{dE}{dt} = \alpha X^- + \gamma\frac{dX^+}{dt} \cdot \frac{1}{K_i + E} \tag{8}$$

, where α, γ and K_i denote the non-growth associated production constant, growth associated constant, and product repression constant, respectively.

Table 4. Estimated parameter values in the model for various ampicillin concentrations (agitation speed = 400 rpm)

Amp. conc.	0 μg/ml		50 μg/ml	100 μg/ml	150 μg/ml	200 μg/ml
	X^-	X^+	X^-	X^+	X^+	X^+
μ_m	1.05	1.0	0.99	0.98	0.97	no growth
K_s	1.6	1.5	1.4	1.0	0.8	–
$Y_{x/s}$	0.303	0.294	0.294	0.294	0.294	–
α	0.0	4	6	7	8	–
γ	0.0	500	950	1150	1200	–
K_r	0.0	5.5	5.5	5.5	5.5	–
β	0.16		0.015	0.18	0.27	

Parameter values for various ampicillin concentrations in the model equations were obtained from experimental results and also by regression analysis as shown in Table 4. Fig. 2 showed the time courses of the fermentation when no selection pressure was used. Simulated results using estimated model parameter values fitted the experimental results well. Fig. 2 showed also a fraction of plasmid-harboring cells during fermentation. Model prediction showed the same trend as the experimental results obtained using plating method. When selection pressure was 50 μg/ml or more, plasmid free cells were not detected by agar plating method. When ampicillin concentration was 50, 100, and 150 μg/ml, respectively, the simulated results using estimated model parameter values fitted the experimental results well, too (figures not shown).

From Table 4, the growth rate of the plasmid-free cell obtained from the host strain was higher than that of plasmid-harboring cell. When there was no selection pressure, approximately 16% of the plasmid-harboring cells lost their plasmid and became plasmid-free cell. When ampicillin concentration was 150 μg /ml, apparent segregation coefficient, β, was 0.27. This shows that 27% of the cells are plasmid free cells and low plasmid copy numberd cells, which could not survive under the given selection pressure. Plasmid copy number has been known to vary as the cells differentiate depending on environmental conditions [18]. As the concentration of the selection pressure increased, the apparent segregation coefficient of the microorganism increased. This trend can be applied for the cases of ampicillin concentrations higher than 100 μg/ml. It is not clear why the apparent segregation coefficient has a low value when the ampicillin concentration was 50 μg/ml. However, the plasmid-harboring cells with low plasmid copy number seems to be damaged severely by the high concentration of the selection pressure. The growth rate of plasmid harboring cell decreased as the selection pressure increased. It was possibly due to the differences in plasmid

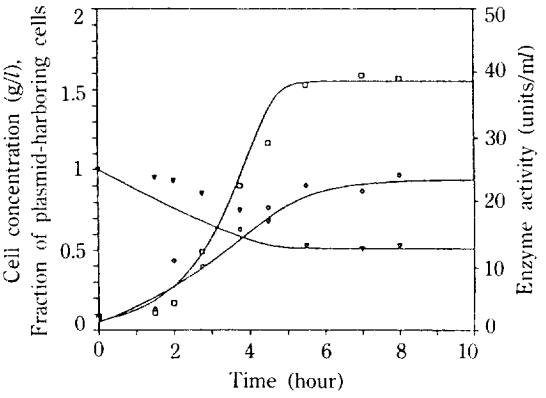

Fig. 2. Experimental data and simulation results when no selection pressure was applied (Dots are experimental data and lines are from simulation).
□: cell mass (g/l), ◇: enzyme activity (units/ml), ▽: fraction of plasmid harboring cells

copy number of the recombinant cell. Since high activity of β-lactamase can be expected with high content of plasmid, high copy numbered plasmid-harboring cells can survive under the high selection pressure. This explains that the specific production rate increased as the selection pressure increased because high plasmid copy numbered cell could only grow in the broth. Separate experimental verifications could support the results, which are left for further study.

Table 5 lists the estimated parameter values in the model equation for various agitation speeds and the ampicillin concentrations of 200 μg/ml. The agitation speed of the fermentor alters the effects of selection pressure. As the agitation speed increased, the plasmid-harboring cells with low plasmid copy number were damaged severely and the growth rate decreased as described above. And growth and non-growth associated constants were increased slightly. The average shear and maximum shear rates increases linearly with agitation speed according to eqs (2) and (3). The

Table 5. Estimated parameter values in the model for various agitation speeds (ampicillin concentration =200 μg/ml)

	Agitation speed		
	200 rpm	300 rpm	400 rpm
μ_m	0.96	0.95	no growth
K_s	0.7	0.65	–
$Y_{x/s}$	0.29	0.29	–
α	8.5	8.8	–
γ	1220	1230	–
K_i	5.5	5.5	–
β	0.48	0.75	1.0
Reynolds No.	28445	42667	56889
Average shear rate	36.7	55.0	73.3
Maximum shear rate	63.3	95.0	126.7

agitation effect coupled with ampicillin effect on the recombinant *E. coli* can be thus described to the shear effect coupled with ampicillin effect. Even though the detailed mechanisms of antibiotic effects coupled with agitation were not clarified enough, the agitation speed during fermentation is an important operating parameter when ampicillin concentration is high.

CONCLUSION

Optimum ampicillin concentration which maximizes enzyme production by recombinant *E. coli* was 150 μg/ml in flask culture and 50 μg/ml in the culture using 2-l stirred fermentor. Optimum concentration of selection pressure in a stirred fermentor was different from that in a flask due to agitation effects. As the concentration of ampicillin increased, the plasmid-harboring cells with low plasmid copy number seemed to be damaged severely, while the specific production rate increased due to the high plasmid copy number in the microorganism.

NOMENCLATURE

D_i : impeller diameter [m]
E : produced enzyme activity [units/ml]
K_i : product inhibition constant [units/ml]
K_s : limiting substrate inhibition constant [g/l]
n : agitation speed [RPS]
S : concentration of limiting substrate [g/l]
S_o : initial concentration of limiting substrate [g/l]
t : time [h]
X^+ : plasmid-harboring cell concentration [g/l]
X : plasmid-free cell concentration [g/l]
$Y_{x/s}$: yield of plasmid-harboring cells on limiting substrate
$Y_{x/s}$: yield of plasmid-free cells on limiting substrate

Greek Letters

α : non-growth associated constant [(units/ml)^2/(g cell/l)/h]
β : apparent segregation coefficient [dimensionless]
γ : growth associated constant [(units/ml)^2/(g cell /l)]
η : viscosity [Pa · S]
μ_m : maximum specific growth rate [h^{-1}]
μ : specific growth rate [h^{-1}]
ρ : density [kg/m^3]

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