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MODELING OF CELL GROWTH AND *phoA*-DIRECTED EXPRESSION OF CLONED GENES IN RECOMBINANT *Escherichia coli*

Pyong Kyun Shin*, Ja Hyup Koo, Woo Jong Lee and Jin Ho Seo†

Department of Food Science & Technology, Research Center for New Biomaterials in Agriculture.
Seoul National University, Suwon 441-744
*Korea Institute of Science and Technology, Seoul 136-650, Korea
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Abstract—A mathematical model was formulated to describe growth and cloned protein production in the recombinant *Escherichia coli* cells containing *phoA*-directed expression systems. Kinetic parameters for the strains with two fusion genes (*phoA-lacZ* either on the chromosome or on a multicopy plasmid and *phoA-amyE* on a multicopy plasmid) were estimated and compared to analyze the effects of cloning site (chromosome and plasmid), product type (*E. coli* β-galactosidase and *Bacillus subtilis* α-amylase), and culture temperature on the cell's behavior. The presence of a multicopy plasmid reduced the specific growth rate and the phosphate uptake rate of the cell, both by 10%, compared with those of the chromosome-integrated strain. The overexpression of *B. subtilis* α-amylase decreased the specific growth rate and the glucose consumption rate more than the β-galactosidase overproduction system. The presence of multiple copies of the *phoA* promoter on either an intact *phoA* gene or the fusion gene reduced both the repression and derepression efficiencies. Culture temperatures showed a significant effect on α-amylase production. A temperature of 30℃ is more desirable than 37℃ for α-amylase production in the recombinant *E. coli* containing the *phoA* promoter.

Key words: *Modeling, phoA, α-Amylase, Recombinant E. coli*

INTRODUCTION

The use of a regulated promoter in an expression system allows tight control of cloned protein levels through manipulation of growth conditions so that competitive interactions between the cloned DNA and host cell's chromosome can be avoided. Secretion of a product protein across the cytoplasmic membrane can reduce the degradation of the protein by cellular proteases present in the cytoplasmic space of the cell. Therefore, these two aspects are of central importance in maximizing the productivity of a recombinant DNA system.

The *Escherichia coli phoA* gene can be a very useful system for the production of a desired protein as it provides both a regulated promoter and a signal sequence. The *phoA* promoter is repressed in the presence of inorganic phosphate in the medium while derepressed when inorganic phosphate is exhausted. In addition, the *phoA* signal sequence can direct the cloned protein across the cytoplasmic membrane to the periplasm of *E. coli*. Because of these characteristics many studies have used the *E. coli phoA* expression system for production of valuable proteins such as human epidermal growth factor, human growth hormone, human interferon-α , and α-neo-endorphine [Oka et al., 1985; Yoda et al., 1987].

The expression of *Bacillus subtilis* α-amylase from the *phoA-amyE* fusion gene in a recombinant *E. coli* system was investigated under various environmental conditions [Shin et al., 1992]. Environmental parameters studied were growth temperature and pH. The effect of chloramphenicol on protein stability was also examined.

†Corresponding author.

This study is concerned with formulation of a mathematical model to describe the *E. coli phoA*-directed expression systems. Kinetic parameters involved in the model were estimated and compared to elucidate the effects of cloning sites (chromosome-integration and multicopy plasmid), product types (a native cytoplasmic protein (β-galactosidase) and a foreign secreted protein (α-amylase)), and culture temperatures on growth and product formation of the recombinant *E. coli* cells.

MATERIALS AND METHODS

1. Bacterial Strains and Plasmids

E. coli K-12 strain BW3414 [Δ(*argF-lac*)205 (U169) *pho*-499] was used as a host. The *phoA-lacZ* fusion gene was transformed into the host strain (BW13704) by using the plasmid pDK110 and integrated into the chromosome (BW13706) by using a phage λ. The plasmid pDK110 and the strain BW13706 was described elsewhere [Shin and Seo, 1990]. Plasmid pAMYA which contains the *phoA-amyE* fusion gene was introduced into the host strain (BW 3414/pAMYA) by transformation [Shin et al., 1992].

2. Medium and Cultivation

An MOPS (3-[N-Morpholino] propanesulfonic acid) minimal medium [Neidhardt et al., 1974] containing 2 g/L of glucose was employed for this study. Cells were grown at 37℃ and pH 7.4 under an aerobic condition in a fermentor (New Brunswick Scientific, Model F-1000). Initial concentrations of inorganic phosphate in the growth medium were changed to induce expression from the cloned *phoA* gene (1.0 mM K_2HPO_4 for repression and 0.1 mM K_2HPO_4 for derepression). β-Glycerophosphate (Sigma) was used as an organic phosphate source. For a selective culture, ampicillin (Sigma) was added at 100 mg/L. Detailed experimental

procedures were already described elsewhere [Shin et al., 1992].

MATHEMATICAL MODELING

1. Cell Growth

Monod growth kinetics relates cell growth to a limiting nutrient concentration in the medium. When the concentration of the limiting nutrient falls to zero in the medium, cells can no longer grow. Glucose and other carbon sources belong to this type of nutrients. But cells require another type of nutrients for their growth. Nyholm [1976] called these 'conservative substrates' since these nutrients are stored within the cell rather than consumed irreversibly. Vitamins and many inorganic ions such as inorganic phosphate belong to the conservative substrates. When a conservative substrate becomes a limiting nutrient, cells still can grow without it in the medium but at a slow growth rate. In this case, Monod growth kinetics cannot describe cell growth on the conservative substrate.

Nyholm [1976] suggested a mathematical model for the growth of microorganisms under limitation by conservative substrates. According to his model, the intracellular concentration of the limiting substrate is the key element for cell growth and can be divided into two parts: the absolute (structural) and excess (functional) substrates. The former corresponds to the cell's minimum substrate requirement while the latter can be used for growth. He expressed the specific growth rate as a function of the excess part of the limiting substrate. Based upon the conservative substrate concept, Toda and Yabe [1979] developed a model equation similar to Nyholm's for the phosphate-limited growth of *Saccharomyces carlsbergensis*. They used a different functional form from Nyholm's to account for the dependency of the specific growth rate upon the excess of the limiting substate.

In this work, the model equation developed by Nyholm was modified for the growth of the recombinant *E. coli* cells under phosphate limitation. Simple mass balance equations were formulated for the change of the limiting substrate concentration in the medium and inside the cell. Mass balance equations for cell mass (X), glucose (S), extracellular inorganic phosphate (P) and intracellular inorganic phosphate (P_i) were given,

$$\frac{dX}{dt} = \mu X \tag{1}$$

$$\frac{dS}{dt} = -\frac{\mu X}{Y} - mX = -\frac{1}{Y^{app}}\frac{dX}{dt} \tag{2}$$

$$\frac{dP}{dt} = -vX = v_m \frac{P}{K_p + P} X \tag{3}$$

$$\frac{dP_i}{dt} = v - \mu P_i \tag{4}$$

where

$$\mu = \mu_m \left(\frac{S}{K_s + S}\right) f_s(P_i) \tag{5}$$

It is assumed that an intracellular inorganic phosphate concentration remains at its maximum value (P_i^m) as long as inorganic phosphate is present in the medium. It is also assumed that there exists a certain value of intracellular phosphate concentration (P_i^t) at which the transition of both cell growth and product formation occurs. In the cell mass balance equation, two different specific growth rates μ_{m1} and μ_{m2} are used to account for the transition between nonlimiting and limiting conditions as follows:

$$\mu_m = \mu_{m1} \text{ at } P_i > P_i^t$$
$$\mu_m = \mu_{m2} \text{ at } P_i \le P_i^t$$

$f_s(P_i)$ in Eq. (5) represents the dependence of inorganic phosphate concentration on cell growth, which is expressed by Eq. (6):

$$f_s(P_i) = \left(\frac{P_i - P_i^a}{P_i^m - P_i^a}\right) \tag{6}$$

where P_i^a in the absolute concentration of inorganic phosphate required for cell's viability.

Glucose is consumed for cell growth (Y) and maintenance (m). Glucose consumption was lumped by using the apparent yield coefficient, Y_{app}.

2. Cloned-gene Expression

Yagil and Yagil [1971] formulated a model equation for the repression of alkaline phosphatase in *E. coli* based upon the operon model which relates the synthesis of regulatable enzymes to cellular effector concentrations. The cellular effector can be either an inducer or a corepressor depending upon the regulation mode. They considered inorganic phosphate in the medium as an effector and correlated the rate of alkaline phosphatase synthesis with an inorganic phosphate level in the medium. This model successfully described the repression of alkaline phosphatase in *E. coli* but cannot explain the derepression of the enzyme in the absence of inorganic phosphate in the medium. Toda and Yabe [1979] also applied the operon model for the production of acid phosphatase in yeast which is also under the control of inorganic phosphate in the medium. They used the excess intracellular phosphate as an effector. Although this model equation succeeded in describing the expression of acid phosphatase in yeast, the parameter values in the model equation were found to be very unstable during parameter estimation from our experimental results. In this study, we used a simple growth-associated product formation kinetics. The model equation for product formation (E) is

$$\frac{dE}{dt} = K_E \frac{dX}{dt} \tag{7}$$

where

$$K_E = K_{E2} + (K_{E1} - K_{E2}) f_E(P_i)$$

It is assumed that an intracellular phosphate concentration affects the product formation only through cell growth when its level falls below P_i^t. A critical intracellular phosphate level (P_i^t) was used for product formation instead of using P_i^a for cell growth. $f_E(P_i)$ represents effects of inorganic phosphate concentration on product formation and was expressed as follows:

$$f_E(P_i) = \left(\frac{P_i - P_i^t}{P_i^m - P_i^a}\right)^n \quad \text{for } P_i > P_i^t$$
$$f_E(P_i) = 0 \quad \text{for } P_i \le P_i^t \tag{8}$$

RESULTS

1. Cloning Site Effect

Kinetic parameters were estimated with the SimuSolv software (Modeling and simulation software, The Dow Chemical Co., Midland, Michigan) and summarized in Table 1. The accuracy of the parameter values and the validity were confirmed with different initial inorganic phosphate concentration (data not shown).

Table 1. Estimated parameter values for the recombinant *E. coli* strains producing either β-galactosidase (BW13704) or α-amylase (BW3414/pAMYA)

Parameter		BW13706[1] (37°C)	BW13704[2] (37°C)	BW3414/pAMYA[3] (37°C)	BW3414/pAMYA (30°C)
Cell growth	μ_{m1}	0.68	0.63	0.63	0.36
	μ_{m2}	0.30	0.28	0.075	0.05
Glucose	Y	1.30	1.11	1.07	1.05
	m	0.095	0.034	0.099	0.077
	Y^{app}	0.62	0.75	0.44	0.35
Extracellular phosphate	V_m	0.27	0.24	0.244	0.15
	K_p	0.001	0.001	0.001	0.001
Intracellular phosphate	P_i^a	0.144	0.144	0.144	0.144
	P_i^m	0.38	0.38	0.37	0.37
	P_i^l	0.30	0.30	0.31	0.31
Product (alkaline phosphatase)	K_{E1}	10.5	6.6	6.35	6.5
	K_{E2}	4970	828	9860	9050
	n	0.36	0.71	0.378	0.69
Product (β-galactosidase)	K_{E1}	5.0	1294	-	-
	K_{E2}	8598	22300	-	-
	n	0.25	0.21	-	-
Product (α-amylase)	K_{E1}	-	-	1.87	0.0
	K_{E2}	-	-	34.3	162
	n	-	-	0.024	0.0
	k_d	-	-	0.018	0.0

[1]The strain contains the *phoA-lacZ* fusion gene on the chromosome.
[2]The strain contains the *phoA-lacZ* fusion gene on a multicopy plasmid.
[3]The plasmid pAMYA contains the *phoA-amyE* fusion gene.

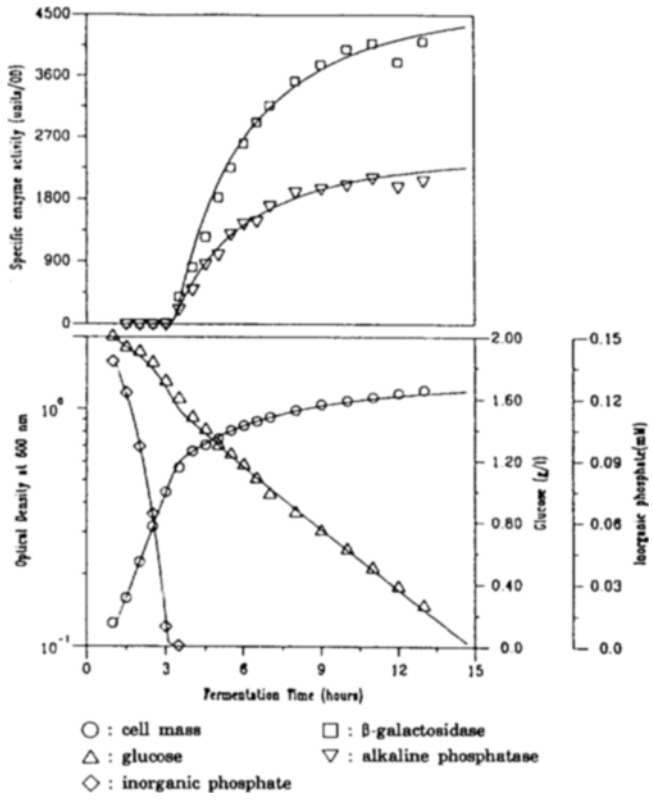

Fig. 1. Comparison of the simulation results with experimental data for the chromosome-integration strain (BW13706) grown at 37°C in the MOPS medium with a low inorganic phosphate (0.1 mM). Lines represent simulation results.

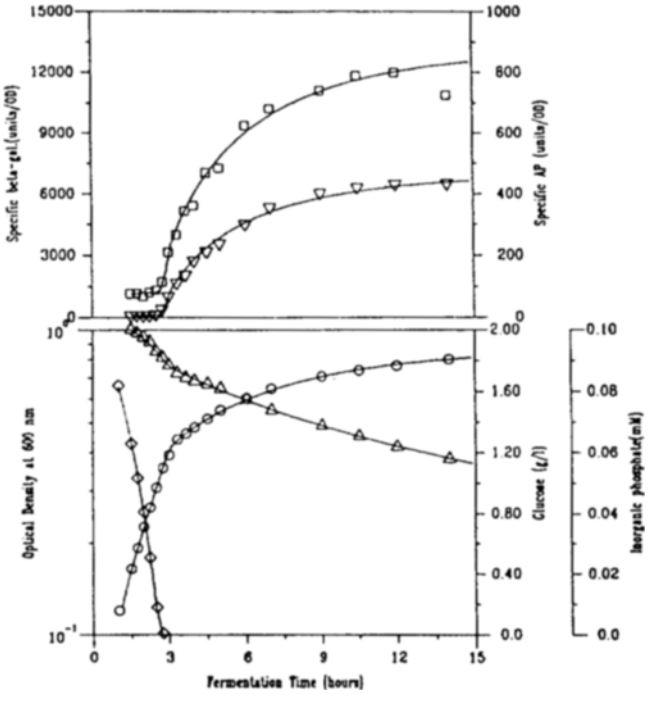

Fig. 2. Comparison of the simulation results with experimental data for the plasmid-bearing strain (BW13704). Grown conditions and symbols are the same as Fig. 1.

The effect of cloning sites on cell growth and cloned-gene expression was compared for the chromosome-integrated (BW13706) and plasmid-bearing (BW13704) strains. Kinetic parameters estimated from the experimental results were summarized in Table 1 (column 1 and 2). As shown in Figs. 1 and 2, the model successfully described the behavior of cell growth and product formation for the two strains.

The presence of a multicopy plasmid is deleterious to cell growth regardless of the inorganic phosphate levels in the medium. Furthermore, it also appears to cause a decrease in the phosphate uptake into the cells. The multicopy plasmid-containing strain utilizes a carbon source (glucose) more efficiently than the chromosome-integrated strain as indicated by an increase in the apparent glucose yield (Y^{app}). The differences in glucose consumption rates between the two strains are even more evident after phosphate depletion (Figs. 1 and 2). As Y and m were observed to be very unstable during parameter estimation, we simply compared the apparent yield Y^{app}, which is the ratio of cell mass produced to glucose consumed. Y^{app} of the BW13704 strain is higher than that of the BW13706 strain, suggesting that the former uses glucose for cell growth more efficiently than the latter. The reason for this observation is not clear, though we assume that the presence of a multicopy plasmid somehow disturbs the glucose utilization machinery.

The enzyme alkaline phosphatase (AP) was expressed from a single copy of the *phoA* gene in the chromosome in both strains while β-galactosidase was expressed from the *phoA-lacZ* fusion gene either in the chromosome or the plasmid. When inorganic phosphate was present in the medium, the expression of both

phoA and *phoA-lacZ* genes was repressed and hence the K_{E1} value represents the minimum expression rate constant under repression. When inorganic phosphate is exhausted in the medium, both genes are initiated to express at a maximum rate and so K_{E2} indicates the maximum expression rate constant under derepressed conditions. As the *phoA-lacZ* fusion gene is present one or two copies in the chromosome-integrated strain along with a single copy of the *phoA* gene, we may consider the K_{E1} and K_{E2} values corresponding AP and β-galactosidase expression in this strain as the repression and derepression efficiencies, respectively, because AP was expressed from a single copy of the *phoA* gene.

The K_{E1} value of β-galactosidase in the plasmid-bearing strain is more than 200 times greater than that of the chromosome-integrated strain. This implies that the repression efficiency decreased by this ratio in the plasmid-bearing strain compared with that in the chromosome-integrated strain. The presence of multiple copies of the fusion gene disturbs significantly the regulation of *phoA* gene expression in *E. coli*. The K_{E1} value of AP in the plasmid-bearing strain decresed only by 40% compared with that in the chromosome-integrated strain. The K_{E2} value of AP in the plasmid-bearing strain is only one-fourth that of the chromosome-integrated strain. The K_{E2} value of β-galactosidase in the plasmid-bearing strain is three times larger than that of the chromosome-integrated strain. It is estimated that the plasmid-bearing strain has about a 40 times higher number of the fusion gene than the chromosome-integrated strain. Therefore, this analysis reveals that the enhancement of K_{E2} value is not proportional to the increase of the copy number of the fusion gene. Rather the derepression efficiency of the fusion gene decreased greatly due to the presence of multiple copies of the fusion gene. The decrease in the derepression efficiencies of both AP and β-galactosidase for the plasmid-bearing strain compared with those for the chromosome-integrated strain suggests the limitation of the *phoA* gene expression machinery in this strain since many gene products are involved in the expression of the *phoA* gene in *E. coli* [Toda and Yabe, 1979].

2. Product Effect

To compare the influence of cloned proteins on cell growth and product formation, two different proteins: a native and cytoplasimic protein (β-galactosidase, BW13704) and a foreign and secreted protein (*B. subtilis* α-amylase, BW3414/pAMYA) were used as model proteins. The structural genes of both proteins were fused with a gene fragment containing the promoter and signal sequence of the *E. coli phoA* gene. Consequently, the expression of both fusion genes is under the control of inorganic phosphate concentration in the medium. Furthermore, α-amylase is secreted into the periplasmic space by the direction of the *phoA* signal sequence, while β-galactosidase can not be secreted out of the cytoplasm. Two fusion genes (*phoA-lacZ* and *phoA-amyE*) were cloned on multicopy plasmids and introduced into the same host strain, BW3414. Kinetic parameters for cell growth and cloned-gene expression of both BW13704 and BW3414/pAMYA strains are summarized in Table 1 (columns 2 and 3).

In the presence of inorganic phosphate in the medium, the specific growth rates μ_{m1} for the BW3414/pAMYA strain and the BW 13704 strain containing the *phoA-lacZ* fusion gene on the plasmid were the same, while μ_{m2} the specific growth rate after phosphate depletion, of the former strain was only one-fourth that of the latter strain. Since the expression of both fusion genes is repressed in the presence of inorganic phosphate in the medium and derepressed without phosphate, the difference in specific growth rate in the derepressed condition suggests that the overproduction of α-amylase is more deleterious to the cell growth than that of β-galactosidase. The maximum inorganic phosphate uptake rates V_m for both strains were almost the same, which supports our previous observation that the presence of multicopy plasmids did not affect the phosphate uptake by the cell. Examination of glucose utilization showed a big difference in the apparent yield for both strains. The apparent yield Y^{app} for the α-amylase producing strain (BW3414/pAMYA) is only 60% of that for the β-galactosidase producing strain (BW13704), indicating the overproduction of α-amylase affects the cell growth more severely than the glucose uptake.

The mode of AP expression in the BW3414/pAMYA strain is very different from the trend observed in the β-galactosidase producing strains (BW13704 and BW13706). The previous studies have shown that as the number of the *phoA* promoters inside a cell from both the intact *phoA* gene and fusion gene (*phoA-lacZ*) increases, the maximum expression rate of alkaline phosphatase (AP) from a single copy of the *phoA* gene decreases because of the limitation of the *phoA* gene expression machinery. In other words, the derepression efficiency for a *phoA* gene decreases as the number of the *phoA* promoters inside a cell increases. Since the copy number of the pAMYA plasmid was estimated as three to five copies, the K_{E2} value of AP for the α-amylase producing strain is expected to be between those of the BW13706 and BW 13704 strains based upon the above trend. But it was found to be two times a larger value of K_{E2} than that of the BW13706 strain. Even though the overexpression of a secreted protein α-amylase prevents cell growth it has little effect on the gene expression machinery. As a consequence, the K_{E2} value of AP increases significantly for the α-amylase producing strain. Another difference in the AP expression behavior of the α-amylase producing strain compared with that of the β-galactosidase producing strain is the decrease in the AP expression rate about three hours after phosphate depletion. The AP expression rate remained constant more than 9 hours after phosphate depletion in the BW13704 strain.

The first order decay term was inserted to accout for the instability of α-amylase, whereas such term was not considered for both AP and β-galactosidase as these proteins are natural to *E. coli*.

3. Temperature Effect

To see the effects of culture temperature on the cell growth and a foreign protein expression in *E. coli*, we compare the kinetic parameters for a strain containing the *phoA-amyE* fusion gene on a multicopy plasmid (BW3414/pAMYA) grown at two different temperatures: 30℃ and 37℃. This strain produces *B. subtilis* α-amylase under the control of inorganic phosphate in the medium. The estimated kinetic parameters at two different temperatures are summarized in Table 1 (columns 3 and 4). Figs. 3 and 4 illustrate the experimental data and the simulation results. As discussed in the previous section, the model equations described the experimental results very well except for AP expression behavior.

When a culture temperature lowered from 37℃ to 30℃, the specific growth rate in the presence of phosphate decreased 43% while the specific growth rate without phosphate did 33%. Since cells grow very slowly without phosphate in the medium, the effect of temperature on cell growth is more profound when inorganic phosphate is present in the medium. The phosphate uptake rate also decreased about 40% with decreasing culture tempera-

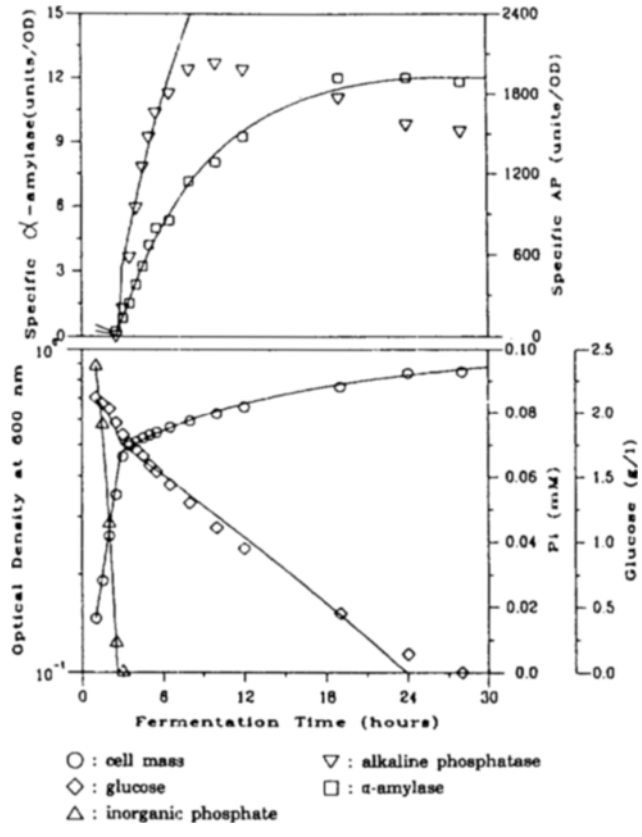

Fig. 3. Comparison of the simulation results with experimental data for the α-amylase producing strain (BW3414/pAMYA) grown at 37°C. Other growth conditions are the same as Fig. 1.

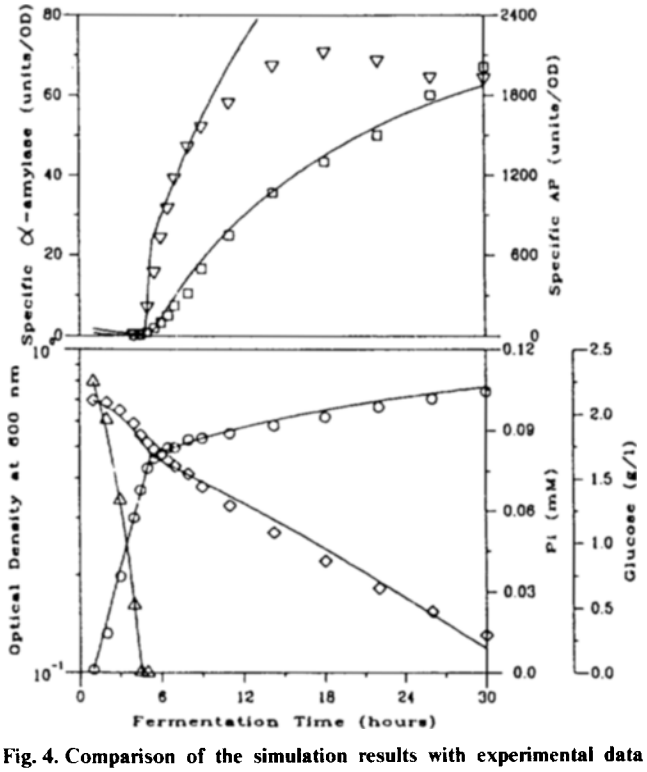

Fig. 4. Comparison of the simulation results with experimental data for the recombinant strain BW3414/pAMYA grown at 30°C. Other growth conditions and symbols are the same as Fig. 3.

tures. So did the apparent yield Y^{app} by 20%.

The kinetic parameters for both AP and α-amylase expression showed totally different dependencies upon temperatures. The K_{E2} value of AP was kept almost constant regardless of temperature. However, the K_{E2} of α-amylase increased five times as temperature decreases. Furthermore, the first order decay constant k_d of α-amylase was found to be 0.018 at 37℃, while it was almost equal to zero at 30℃.

DISCUSSION

Model equations were formulated to describe the behavior of the *E. coli phoA*-based expression systems. The kinetic parameters were estimated for the strains with two fusion genes (*phoA-lacZ* and *phoA-amyE*) to compare (1) cloning site effects (chromosome and multicopy plasmid), (2) product effects (*E. coli* β-galactosidase and *B. subtilis* α-amylase), and (3) culture temperature effects on the cell growth and product formation in *E. coli*.

The presence of a multicopy plasmid reduced the specific growth rate by 10% and this effect was more severe when the cell expressed a secreted foreign protein α-amylase. In the latter case, the specific growth rate decreased by more than 70% compared with that of the strain (BW13706) which expressed a native β-galactosidase from the *phoA-lacZ* fusion gene on the chromosome. A multicopy plasmid also caused the decrease in the phosphate uptake rate by 10%. However, the plasmid-bearing strain (BW13704), which produces β-galactosidase utilized glucose more efficiently than the chromosome-integrated strain (BW13706), whereas the α-amylase producing strain (BW3414/pAMYA) showed a 30% decreased glucose consumption yield compared with the chromosome-integrated strain (BW13706).

In the case of product formation, the presence of the multiple copies of the *phoA-lacZ* fusion gene reduced the derepression efficiency of AP (represented by K_{E2}) by 5 times and the regulation efficiency of β-galactosidase (represented by K_{E1}) by 200 times compared with those of the chromosome-integrated strain. The K_{E2} value of AP for the BW3414/pAMYA strain increased more than two times compared with that of the chromosome-integrated strain (BW13706), suggesting that the expression and secretion of α-amylase retarded cell growth significantly, while the gene expression machinery seemed to be unaffected.

When a culture temperature changed from 37℃ to 30℃ for the α-amylase producing strain (BW3414/pAMYA), all the cell's activities decreased significantly except for the K_{E2} value of α-amylase. A decrease in temperature enhanced the K_{E2} value for α-amylase by 5 times. Furthermore, the enzyme α-amylase seems to be unstable at 37℃, as indicated the increased value of k_d. This observation is very interesting since α-amylase is known to be thermal stable.

A multicopy plasmid is usually favorable for overexpression of a target protein and the chromosome-integrated seems to be more efficient for the regulation of gene expression. When a foreign secreted protein is produced, its effects on the cell growth are more severe than that of a native protein and as a result, the cell producing a foreign secreted protein shows totally different behavior in cell growth and product formation from the recombinant cell overproducing a native protein. These comparisons along with the effects of growth conditions on product formation may provide some useful information on optimal production of a foreign protein using a *phoA*-directed expression system in *E. coli*.

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NOMENCLATURE

K : saturation constant [mol/*l* or g/*l*]
K_E : product formation constant [units/ml/O.D.]
m : specific maintenance rate for carbon source [g/l/hr/O.D.]
n : experimental constant in Eq. (9)
P : inorganic phosphate concentration [mol/*l*]
S : carbon source (glucose) concentration [g/*l*]
V : specific phosphate uptake rate [mol/*l*/hr/O.D.]
X : cell mass [O.D.]
Y : cell yield factor based on carbon source [O.D./g/*l*]

Greek Letter

μ : specific growth rate [hr^{-1}]

Superscripts

a : absolute requirement
app : apparent

Subscripts

i : intracellular
m : maximum
p : phosphate
s : carbon source
1 : under repression
2 : under derepression

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