Korean J. of Chem. Eng., 13(3), 266-274 (1996)

KINETIC MODEL FOR EFFECTS OF ETHANOL AND PHOSPHATE ON CELL GROWTH AND EMULSAN PRODUCTION IN *ACINETOBACTER CALCOACETICUS* RAG-1

Jeong-Woo Choi†, Hyun-Goo Choi, Sang-Baek Lee* and Won-Hong Lee

Department of Chemical Engineering, Sogang University, C.P.O. Box 1142, Seoul 121-742, Korea
*Department of Chemical Engineering, Cheju National University, Cheju, Korea
(Received 5 October 1995 • accepted 28 February 1996)

Abstract – A mathematical kinetic model was proposed to describe the cell growth and the emulsan production in batch cultivations of *Acinetobacter calcoaceticus* RAG-1. Ethanol and phosphate concentrations were chosen as the key variables, which affected the cell growth and emulsan production in the batch cultivations. The cell growth was inhibited by high concentrations of ethanol and was slightly affected by intracellular phosphate level. And the emulsan production was related to the intracellular phosphate level dependent upon the extracellular phosphate concentration. Kinetic model for the cell growth was formulated using the ethanol inhibition term and the intracellular phosphate level. The relationship between extracellular and intracellular phosphate level was expressed by the concept of active transport. Kinetic model for the emulsan production was represented using growth-associated term and intracellular phosphate inhibition term. Release of emulsan was presumed as the primary release from the surface of viable cell and the secondary release by cell lysis. The model predicted the experimental results with good agreement.

Key words: Emulsan, Acinetobacter calcoaceticus RAG-1, Kinetic Model, Ethanol Inhibition, Phosphate Effect

INTRODUCTION

Emulsan, a polyanionic heteropolysaccharide produced by *Acinetobacter calcoaceticus* RAG-1, is an excellent bioemulsifier which can be widely applied in many fields of oil-associated industries and consumer product industries [Reisfeld et al., 1972; Rosenberg et al., 1979a, b; Rosenberg et al., 1980; Kosaric et al., 1987]. The possible structure and properties of emulsan were investigated and described [Zuckerberg et al., 1979; Gutnick and Shabtai, 1987]. Emulsan is synthesized at bacterial cell wall and released subsequently from cell surface, but the mechanism and the factors which affect its release have not been clearly understood [Gutnick and Shabtai, 1987].

Nutrient composition frequently affects the cell growth and exopolysaccharide synthesis. In *A. calcoaceticus* RAG-1, ethanol is frequently used as the carbon source, which participates in cell growth, maintenance, and emulsan production [Schaefer, 1985; Wang and Wang, 1989]. It was reported that the high concentration of ethanol (above 10-16 g l^{-1}) had an inhibitory effect on the cell growth of *A. calcoaceticus* [Abbott, 1973; Abbott et al., 1973; Schaefer, 1985; Choi et al., 1996a]. Since emulsan can be produced in a mixed growth-associated pattern, cell growth can affect emulsan synthesis [Gutnick et al., 1980; Schaefer, 1985; Choi et al., 1996a].

Phosphate, one of the essential nutrients for microorganisms, has a profound effect on expression of genes and enzymes concerned with primary and secondary metabolisms [Toda and Yabe, 1979; Pazoutova et al., 1981; Bramble et al., 1991]. It is generally known that phosphate is transported into intracellular pool by active transport through bacterial cell membrane and then is utilized through the metabolism. Intracellular phosphate is closely related to the formation of high energy phosphate compounds, such as ATP (adenosine 5'-triphosphate) and UTP (uridine 5'-triphosphate), and the ratio of ATP to ADP (adenosine 5'-diphosphate) dominate whether the metabolism is activated or deactivated. Since the initial concentration of extracellular phosphate decides the intracellular phosphate level, it can affect the metabolism concerned with the cell growth and emulsan synthesis. The effect of phosphate for *A. calcoaceticus* RAG-1 is not well reported in literature. Thus, the major role of phosphate on cell growth and emulsan synthesis in *A. calcoaceticus* RAG-1 should be elucidated.

In this study, a mathematical kinetic model which can provide the information needed in establishing optimum operating strategy for emulsan production and composing the objective process for the control of the cultivation was developed by considering the interactive effects of ethanol and phosphate on cell growth and emulsan synthesis based upon the experimental results of the batch cultivations.

MATERIALS AND METHODS

1. Microorganism

The microorganism used in this study was a gram negative bacterium, *Acinetobacter calcoaceticus* RAG-1 (ATCC 31012).

2. Medium and Culture Conditions

The organism was cultured in a medium containing 7.3 g l^{-1} of KH_2PO_4, 16.9 g l^{-1} of K_2HPO_4, 0.5 g l^{-1} of $MgSO_4$, 4.0 g l^{-1} of $(NH_4)_2SO_4$ and trace-salts solution. Ethanol (16.0 g l^{-1}) was used as the carbon source. For stoke culture, 18.0 g l^{-1} of agar was used with the above medium, and for each agar plate a filter paper wetted with 200 μl of ethanol was placed on the back

†To whom all correspondences should be addressed.

cover, where the vapor of ethanol was used as the carbon source. The agar plates were incubated upside-down at 30°C for 2 d, and then the filter paper was removed. The strain was kept on agar plates at 4°C and transferred monthly. For shake flask culture, the liquid medium was sterilized in an autoclave at 121°C for 15 min and pH was adjusted to 7.0 before autoclaving. The magnesium and trace-salts solution were sterilized separately and then added to the shake flask after cooling along with the addition of ethanol.

From a petri dish, three loops of cells were transferred into 100 ml maintenance medium for 250 ml shake flask and cultured at 30°C and 180 rpm for 20 h. Three times of subculture were performed to stabilize cells before used as a seed culture for the experiments. Batch cultivations were performed in a 5 l jar reactor (B.E. Marubishi Ltd., MD-300) with an initial volume of 2 *l*. Before sterilize medium, pH was also adjusted 7.0. The temperature was 30°C, the agitation speed was 500 rpm with 0.5 vvm of air flow rate. To investigate the effects of ethanol and phosphate on cell growth and emulsan synthesis, the initial concentrations of ethanol and phosphate in the medium were varied in different experiments [Choi et al., 1996a].

3. Analytical Methods

Cell concentration was determined by a UV spectrophotometer (Shimadzu, UV-240) at 660 nm. Ethanol was measured by gas chromatography (GOW-MAC, GC model 69-550) equipped with a thermal conductivity detector using n-propanol as an internal standard. The GC column used was packed with porapak type Q (Waters, mesh 80-100) and was operated at 135°C with helium as the carrier gas flowing at a rate of 40 ml min^{-1}. Extracellular phosphate concentration was measured by the vanadomolybdo-phosphoric acid method with detection of optical density at 400 nm. For the determination of intracellular phosphate concentration, the same method was used after sonic disruption of cells and centrifugation. Extracellular emulsan concentration was determined by the phenol-sulfuric acid method with detection of optical density at 480 nm [Dubois et al., 1959; Wang and Wang, 1989]. The concentration of cell-bound emulsan was determined by the same method after EDTA treatment to release the cell-bound emulsan.

MODEL

In this study, ethanol and phosphate were chosen as the key variables affecting the cell growth and the emulsan production. Based on the experimental results of batch cultivation [Choi et al., 1996a], the kinetic model was developed as follows.

1. Ethanol and Phosphate Effects on Cell Growth

It was observed that ethanol had an inhibitory effect on cell growth of *A. calcoaceticus* RAG-1 [Schaefer, 1985; Choi et al., 1996a]. The cell growth was expressed by considering the ethanol inhibition and the effect of intracellular phosphate as follows.

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

where μ is the specific growth rate (h^{-1}).

Since the cell growth was inhibited by high concentration of ethanol and was slightly affected by intracellular phosphate level [Choi et al., 1996a], the rate expression of cell growth was

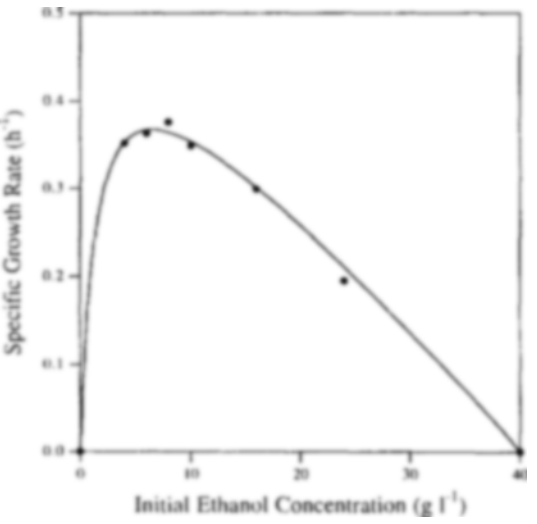

Fig. 1. Inhibitory effect of ethanol on specific growth rate of *A. calcoaceticus* RAG-1.

formulated based on the combination of a limiting substrate utilization kinetics for the ethanol and effect of intracellular phosphate on cell growth rate as

$$\mu = \mu_s f(P_i) \tag{2}$$

where μ_s is the specific growth rate related to ethanol (h^{-1}); $f(P_i)$ is the function of growth activation by intracellular phosphate; and P_i is the intracellular phosphate level ($g\ g^{-1}$).

The specific growth rate, μ_s, was determined from the experimental data at various initial ethanol concentrations with a fixed initial extracellular phosphate concentration, 12.1 gl^{-1}. As shown in Fig. 1, the specific growth rate data was well represented by the inhibition kinetics, Eq. (3), which was proposed by Luong [1987]

$$\mu_s = \frac{\mu_{S,max} S}{K_s + S}\left(1 - \frac{S}{S_{max}}\right)^{n_s} \tag{3}$$

where $\mu_{S,max}$ is the maximum growth rate in ethanol utilization (h^{-1}); S is the ethanol concentration ($g\ l^{-1}$); K_s is the saturation constant ($g\ l^{-1}$); S_{max} is the maximum substrate concentration above which the cell growth is completely inhibitied ($g\ l^{-1}$); and n_s is the constant.

The effect of intracellular phosphate on the specific growth rate, $f(P_i)$, was determined from the experimental data of batch cultivations at different initial concentration of extracellular phosphate with a fixed initial ethanol concentration, 8.0 $g\ l^{-1}$. The correlation between the μ/μ_s and intracellular phosphate level at each 3 h interval in the exponential growth phase was evaluated as shown in Fig. 2. The mathematical expression of the function, $f(P_i)$, was proposed to represent the experimental results by the authors as

$$f(P_i) = \mu_{Pi,max}\left[1 - \exp\left\{-K_{Pi}\left(\frac{P_i}{P_{i0}}\right)^2\right\}\right] \tag{4}$$

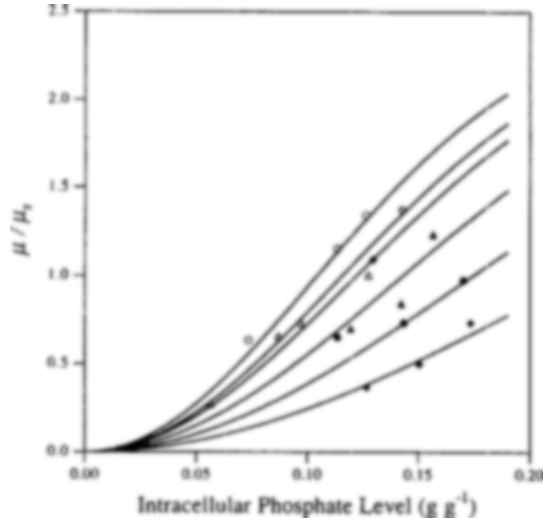

Fig. 2. The plot of μ/μ_s vs. P_i for various initial extracellular phosphate concentration at 8.0 gl^{-1} of initial ethanol concentration.
Symbols: Initial extracellular phosphate 6.05 gl^{-1} (△); 12.10 gl^{-1} (○); 18.15 gl^{-1} (◇); 24.20 gl^{-1} (▲); 30.25 gl^{-1} (●); 36.30 gl^{-1} (◆)

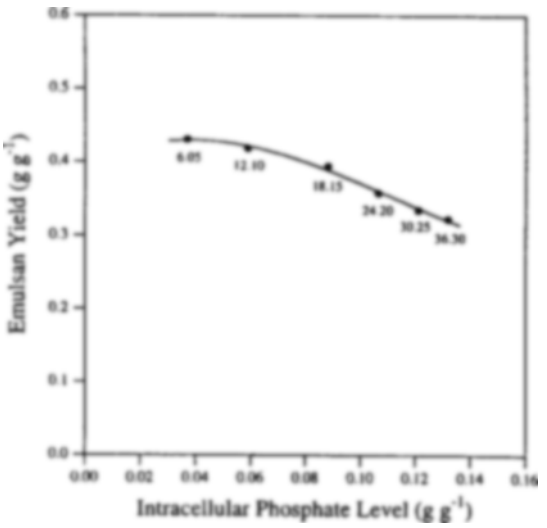

Fig. 3. The emulsan yield as a function of intracellular phosphate level in stationary phase (at 33 hr). The numbers below the data points represent the initial extracellular phosphate concentrations in the medium (unit: gl^{-1}).

where $\mu_{Pi,max}$ is the constant; K_{Pi} is the growth index which represents the degree of growth activation by intracellular phosphate; and P_{i0} is the limit value of intracellular phosphate level which does not contribute to the specific growth rate (g g^{-1}).

By substituting Eqs. (3) and (4) into Eq. (2), the overall specific growth rate is then formulated as

$$\mu=\frac{\mu_{max}S}{K_s+S}\left(1-\frac{S}{S_{max}}\right)^{n_s}\left[1-\exp\left\{-K_{Pi}\left(\frac{P_i}{P_{i0}}\right)^2\right\}\right] \tag{5}$$

where μ_{max} represents the combination of $\mu_{S,max}$ and $\mu_{Pi,max}$ (h^{-1}). μ_{max}, S_{max}, K_S, n_S, and P_{i0} were determined as 0.7820, 40.0, 1.5571, 0.3059, and 0.06, respectively, in Fig. 1 and Fig. 2.

2. Phosphate Effect on Emulsan Production

The specific production rate of emulsan can be expressed as a combination of a growth-associated term and a non growth-associated term.

$$v=v_{GA}+v_{NGA} \tag{6}$$

During the exponential growth phase, the emulsan production is proportional to the cell growth. Thus, growth-associated production term was represented by

$$v_{GA}=k_{e1}\,\mu \tag{7}$$

where k_{e1} is the factor related growth-associated production (g g^{-1}).

After the growth was ceased, during the stationary phase, the emulsan production was primarily affected by the intracellular phosphate level dependent upon extracellular phosphate concentration in the cultivation medium. In the cultivations with various initial phosphate concentration, the emulsan yield was represented as a function of intracellular phosphate level in the stationary phase (at the 33 h) as shown in Fig. 3. To represent this experimental results, non growth-associated production term can be expressed as

$$v_{NGA}=\frac{k_{e2}P_i}{K_{Pi1}+P_i}\exp\left(-\frac{P_i}{K_{Pi2}}\right) \tag{8}$$

where k_{e2} is the rate constant of non growth-associated production (g g^{-1} h^{-1}); K_{Pi1} is the saturation constant of intracellular phosphate (g g^{-1}); and K_{Pi2} is the inhibition constant of intracellular phosphate on emulsan production (g g^{-1}). The formulation of Eq. (8) was originally proposed to correlate product inhibition and thereafter suggested to describe substrate inhibition [Luong, 1987; Mulchandani and Luong, 1989]. In this study, this kinetic equation was used to express the inhibition effect of intracellular phosphate on polysaccharide synthesis. And overall specific production rate is then expressed by substituting Eqs. (7) and (8) into Eq. (6).

$$v=\left[k_{e1}\mu+\frac{k_{e2}P_i}{K_{Pi1}+P_i}\exp\left(-\frac{P_i}{K_{Pi2}}\right)\right] \tag{9}$$

3. Dry Cell Weight

The dry cell weight could be represented in terms of the cell growth and the cell degradation. Substituting Eq. (5) into Eq. (1) and introducing the cell degradation and lag time, t_{lag},

$$\frac{dX}{dt}=\left[\left\{1-\exp\left(-\frac{t}{t_{lag}}\right)\right\}\frac{\mu_{max}S}{K_s+S}\left(1-\frac{S}{S_{max}}\right)^{n_s}\left[1-\exp\left\{-K_{Pi}\left(\frac{P_i}{P_{i0}}\right)^2\right\}\right]-k_d\right]X \tag{10}$$

where X is the dry cell weight (g l^{-1}); k_d is the specific death

rate constant (h^{-1}); and t_{lag} is the lag time (h), which was determined as 6 h in our experiments.

4. Ethanol Consumption

Ethanol, as carbon and energy source, is utilized in the metabolism concerned with cell growth, maintenance, and product formation. Therefore, the rate equation of ethanol consumption can be expressed in terms of the consumption for cell growth, cellular maintenance, and product formation as

$$\frac{dS}{dt} = -\left[1-\exp\left(-\frac{t}{t_{lag}}\right)\right]\frac{\mu}{Y_{X/S}}X - m_s X - \left[k_{e1}\left\{1-\exp\left(-\frac{t}{t_{lag}}\right)\right\}\mu + \frac{k_{e2}P_i}{K_{Pi1}+P_i}\exp\left(-\frac{P_i}{K_{Pi2}}\right)\right]\frac{1}{Y_{E/S}}X \quad (11)$$

where $Y_{X/S}$ is the yield coefficient (g g $^{-1}$); m_s is the maintenance coefficient (g g $^{-1}$ h $^{-1}$); and $Y_{E/S}$ is the yield coefficient for emulsan (g g $^{-1}$). $Y_{X/S}$, m_S, and $Y_{E/S}$ were determined from the experimental results and stoichiomatric evaluation as 0.9242, 0.2116E-2, and 0.9428, respectively [Schaefer, 1985; Choi et al., 1996a].

5. Active Transport of Phosphate

It has been known that the active transport of extracellular phosphate is performed by carrier molecules in the cellular membrane, and the carrier molecules might be considered as transport proteins. A transport protein forms, specifically and reversibly, a complex with the target molecules and the undergoes conformational changes, which result in the release of the molecule on the intracellular side of the membrane. The rate expression of active transport can be formulated as followings

$$C + P_e \xrightarrow{K_{Pe}} CP_e \xrightarrow{k_{Pe,max}} C + P_i \quad (12)$$

where C is the free carrier molecule (g g $^{-1}$); CP_e is the extracellular phosphate-carrier molecule complex (g g $^{-1}$); P_e is the extracellular phosphate concentration (g l $^{-1}$); $k_{Pe,max}$ is the maximum rate constant of active transport for phosphate (h^{-1}); and K_{Pe} is the saturation constant of extracellular phosphate (g l^{-1}).

The total quantity of carrier molecules, C_0, is given by

$$[C_0] = [C] + [CP_e] \quad (13)$$

and the dissociation or saturation constant is expressed as following.

$$K_{Pe} = \frac{[C][P_e]}{[CP_e]} \quad (14)$$

Then Eq. (13) is rearranged by modifying Eq. (14).

$$[C] = \frac{[C_0]}{(1+[P_e]/K_{Pe})} \quad (15)$$

The latter step in Eq. (12) is an irreversible process and the rate controlling, therefore, the overall rate is represented as

$$k_{Pi} = k_{Pe,max}[CP_e] \quad (16)$$

and the final formulation is then expressed by substituting Eqs. (14) and (15) into Eq. (16).

$$k_{Pi} = \frac{k_{Pe}P_e}{K_{Pe}+P_e} \quad (17)$$

where

$$k_{Pe} = k_{Pe,max}[C_0]$$

In the above equaton, k_{Pe} is the rate constant of active transport (g g $^{-1}$ h $^{-1}$).

By plotting K_{Pi} vs. initial extracellular phosphate concentration as shown in Fig. 4, the relationship between the cell growth activation and the initial extracellular phosphate concentration can be established. The empirical correlation formulated as a ratio of the active transport, k_{Pi}/k_{Pi0}, was proposed to describe the relationship between K_{Pi} and the initial extracellular phosphate concentration because the intracellular phosphate level to determine the degree of cell growth activation depended upon the active transport of extracellular phosphate. Based upon the experimental results, the mathematical expression of K_{Pi} can be proposed by the authors as

$$K_{Pi} = \frac{k_{Pi}}{k_{Pi0}}\left(1-\frac{P_e}{P_{e,max}}\right)^{n_{Pe}} = \frac{k_{Pe}P_e}{K_{Pe}+P_e}\left(1-\frac{P_e}{P_{e,max}}\right)^{n_{Pe}} \quad (18)$$

where k_{Pi0} is the reference value of active transport rate to make the K_{Pi} value as a dimensionless form, which was assumed to be 1.0 (g g $^{-1}$ h $^{-1}$); $P_{e,max}$ is the maximum extracellular phosphate concentration above which no growth activation exists, which was determined from the earlier experiments as 55.0 (g l $^{-1}$) [Choi et al., 1996a]; and n_{Pe} is the constant. K_{Pe}, k_{Pe}, and n_{Pe}, were estimated as 41.1458, 0.1281, and 2.2969, respectively, in Fig. 4.

6. Distribution of Phosphate

It was observed that the cell growth and the emulsan production were affected by intracellular phosphate level related with extracellular phosphate concentration. The extracellular

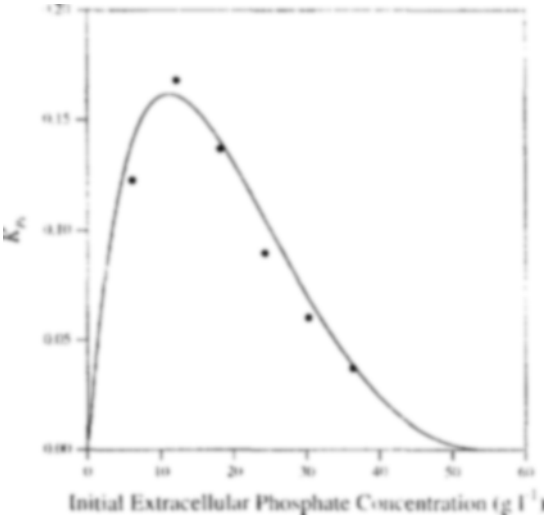

Fig. 4. The plot of K_{Pi} value vs. initial extracellular phosphate concentration.

phosphate concentration can be expressed as

$$\frac{dP_e}{dt} = -\frac{k_{Pe} P_e}{K_{Pe} + P_e} X + (Y_{P/X} + P_i)\, k_d X \tag{19}$$

where $Y_{P/X}$ is the yield coefficient (g g^{-1}). The first and the second term on the right hand side of Eq. (19) represent the uptake of extracellular phosphate by active transport and the return of phosphate to the medium by cell degradation, respectively.

The dynamic changes of the intracellular phosphate level should be balanced as the following equation.

$$\frac{d(XP_i)}{dt} = -\frac{dP_e}{dt} - Y_{P/X}\frac{dX}{dt} - m_{Pi} X - Y_{P/Et}\, \nu X \tag{20}$$

The first two terms of the above equation were previously described by Pazoutova et al. [1981]. In this study, two terms, the utilization concerned with the metabolisms of maintenance and product formation, were added as represented in Eq. (20). Differentiating the left hand side of Eq.(20) and dividing the both sides by X, the differential balance on intracellular phosphate level is expressed as Eq. (21)

$$\frac{dP_i}{dt} = -\frac{1}{X}\frac{dP_e}{dt} - (Y_{P/X} + P_i)\frac{1}{X}\frac{dX}{dt} - m_{Pi} - Y_{P/Et}\, \nu \tag{21}$$

where $Y_{P/X}$ is the yield coefficient (g g^{-1}); m_{Pi} is the maintenance coefficient (g g^{-1} h^{-1}); and $Y_{P/Et}$ is the yield coefficient for emulsan (g g^{-1}). $Y_{P/X}$, m_{Pi}, and $Y_{P/Et}$ were determined from the experimental results as 0.2573E-2, 0.2001E-1, and 0.3002, respectively [Choi et al., 1996a].

Consumption rate of intracellular phosphate could be expressed as two formulations that depend on whether extracellular phosphate is limited or not limited. When extracellular phosphate in limited, most of phosphate in medium is transported into intracellular side of the cell and then is utilized. Therefore, the consumption rate of intracellular phosphate could be expressed as Eq. (21).

When extracellular phosphate is not limited, the level of intracellular phosphate becomes higher and all phosphate transported is not utilized. Thus, fraction related to the growth in intracellular phosphate consumption, α, should be multiplied to the second term on the right hand side of Eq. (21).

$$\frac{dP_i}{dt} = -\frac{1}{X}\frac{dP_e}{dt} - \alpha(Y_{P/X} + P_i)\frac{1}{X}\frac{dX}{dt} - m_{Pi} - Y_{P/Et}\, \nu \tag{22}$$

Substituting the expression for the emulsan production, Eq. (9), and the active transport of extracellular phosphate, Eq. (17), into Eq. (22), the utilization rate of the intracellular phosphate for non-limiting case is given by

$$\frac{dP_i}{dt} = -\frac{k_{Pe} P_e}{K_{Pe} + P_e} - \alpha(Y_{P/X} + P_i)\mu - m_{Pi} - Y_{P/Et}\left[k_{e1}\left\{1 - \exp\left(-\frac{t}{t_{lag}}\right)\right\}\mu + \frac{k_{e2} P_i}{K_{Pi1} + P_i}\exp\left(-\frac{P_i}{K_{Pi2}}\right)\right] \tag{23}$$

7. Distribution of Emulsan

Emulsan is subsequently produced in two steps, synthesis at cell layer and release from cell surface. It should be considered that emulsan is released by two mechanisms, the primary release from surface of viable cell and the secondary release by cell lysis. Thus, the rate expression of cell-bound emulsan is expressed with the synthesis at the bacterial cell wall and the release from the cell surface of viable cells and by cell lysis as following equation

$$\frac{dE_b}{dt} = \left[k_{e1}\left\{1 - \exp\left(-\frac{t}{t_{lag}}\right)\right\}\mu + \frac{k_{e2} P_i}{K_{Pi1} + P_i}\exp\left(-\frac{P_i}{K_{Pi2}}\right) - k_r\right] X - \xi k_d X \tag{24}$$

and

$$\xi = \frac{E_b}{X}$$

where k_r is the rate constant of emulsan released from the cell surface of viable cell (g g^{-1} h^{-1}); and ξ represents the cell-bound emulsan per dry well weight (g g^{-1}).

Rate of free emulsan release in medium is expressed in terms of the primary and the secondary release.

$$\frac{dE_f}{dt} = k_r X + \xi k_d X \tag{25}$$

The total production rate is obtained by combining Eqs. (24) and (25).

$$\frac{dE_t}{dt} = \frac{dE_b}{dt} + \frac{dE_f}{dt} = \left[k_{e1}\left\{1 - \exp\left(-\frac{t}{t_{lag}}\right)\right\}\mu + \frac{k_{e2} P_i}{K_{Pi1} + P_i}\exp\left(-\frac{P_i}{K_{Pi2}}\right)\right] X \tag{26}$$

8. Parameter Estimation

All parameters were known from the earlier experiments [Choi et al., 1996a] except k_d, k_{e1}, k_{e2}, K_{Pi1}, K_{Pi2}, k_r, and α. The unknown parameters in proposed model were estimated with Nonlinear Parameter Estimation Package (NONLIN) [Metzler et al., 1974] while Eqs. (10), (11), (19), (23), (24), (25), and (26) were solved simultaneously with Hamming's modified predictor-corrector method [IBM Corporaion, 1970; Maron, 1982]. The estimated values of model parameters are shown in Table 1.

Table 1. The estimated values of the kinetic model parameters

Parameter	Dimension	Value
k_d	h^{-1}	0.1425 E-2
k_{e1}	g g^{-1}	0.1088
k_{e2}	g g^{-1} h^{-1}	0.7212 E-1
K_{Pi1}	g g^{-1}	0.7829 E-1
K_{Pi2}	g g^{-1}	0.1308
k_r	g g^{-1} h^{-1}	0.1872 E-1
α		0.2060 E-1

RESULTS AND DISCUSSION

Fig. 5(a) and (b) show the experimental and model predicted cell growth and ethanol uptake behavior, respectively. It can be concluded that the proposed model accurately represents the experimental data of cell growth and ethanol consumption for given three cases of initial conditions. The cell growth, after about 6 h lag time, increased exponentially and then ceased as ethanol was completely depleted. The ethanol was consumed rapidly and most of it was converted to cell mass and emulsan.

Fig. 6(a) and (b) represent the experimental data and model prediction of extracellular and intracellular phosphate level, respectively. It was observed that the proposed model accurately represents the experimental data. Extracellular phosphate was decreased slowly durig the exponential growth phase but decreased more rapidly thereafter. Intracellular phosphate level decreased more rapidly during the exponential growth phase compared with during the stationary phase. The rapid decrease of intracellular phosphate during the exponential growth phase could be explained as follows. It has been known that the large amount of ATP is required for the biosynthesis of sugar compounds and their subsequent incorporation into polysaccharide. When acetyl-CoA is used as the starting material instead of glucose, 3 to 4-fold amount of ATP is required in polysaccharide synthesis [Sutherland and Norval, 1970; Troy et al., 1971; Jarman and Pace, 1984]. In *A. calcoaceticus* RAG-1, it can be considered that ethanol as carbon source is oxidized to acetate and subsequently converted to acetyl-CoA, and then acetyl-CoA is used as a precursor for biosynthesis of cellular compound and polysaccharide [Schaefer, 1985]. Additionally, UTP is also essentially required in the synthetic pathway of polysaccharide [Sutherland and Norval, 1970]. Therefore, large amount of phosphate was essentially required for cell growth and emulsan production in *A. calcoaceticus* RAG-1 culture.

Batch cultivations with various initial ethanol concentration were done at a fixed initial concentration of extracellular phosphate as shown in Fig. 6(a) and (b). But the intracellular phosphate level was different though the initial extracellular phos-

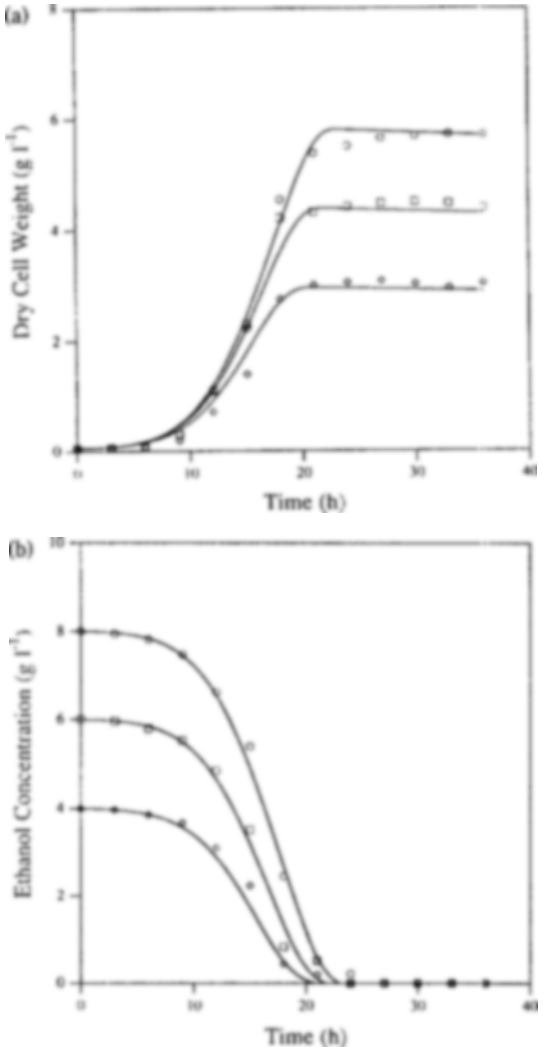

Fig. 5. The experimental data and the model prediction of (a) dry cell weight (b) ethanol consumption.
Symbols: Initial ethanol 4.0 g l^{-1} (◇); 6.0 g l (□); 8.0 g l^{-1} (○); Model Prediction (—).

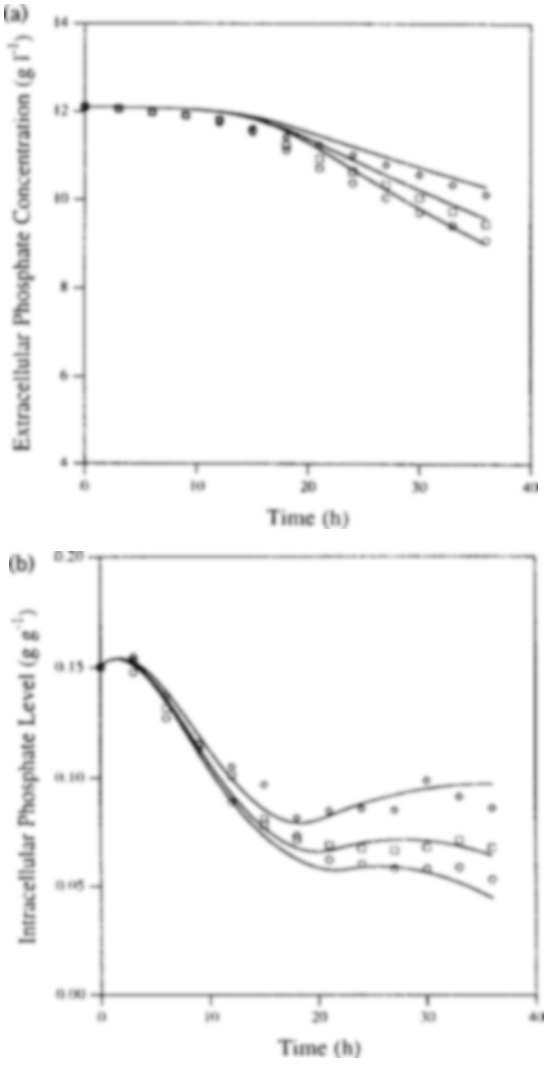

Fig. 6. The experimental data and the model prediction of (a) extracellular phosphate (b) intracellular phosphate.
Symbols: Initial ethanol 4.0 g l^{-1} (◇); 6.0 g l^{-1} (□); 8.0 g l^{-1} (○); Model Prediction (—).

phate concentration the same. Since a large amount of intracellular phosphate are required for biosynthesis of precursors essential for subcellular components and emulsan during the exponential growth phase, intracellular phosphate was rapidly decreased even though the active transport rate of extracellular phosphate was high. During the early exponential growth phase, the intracellular phosphate level was similar to one another, but during the late exponential growth and stationary phase, the intracellular phosphate level of the cultivation with low initial ethanol concentration was higher than that of the cultivation with high initial ethanol concentration. The reason for this phenomena might be the earlier ceasing of the cell growth at the lower initial ethanol concentration compared to the cultivation with the higher initial ethanol concentration.

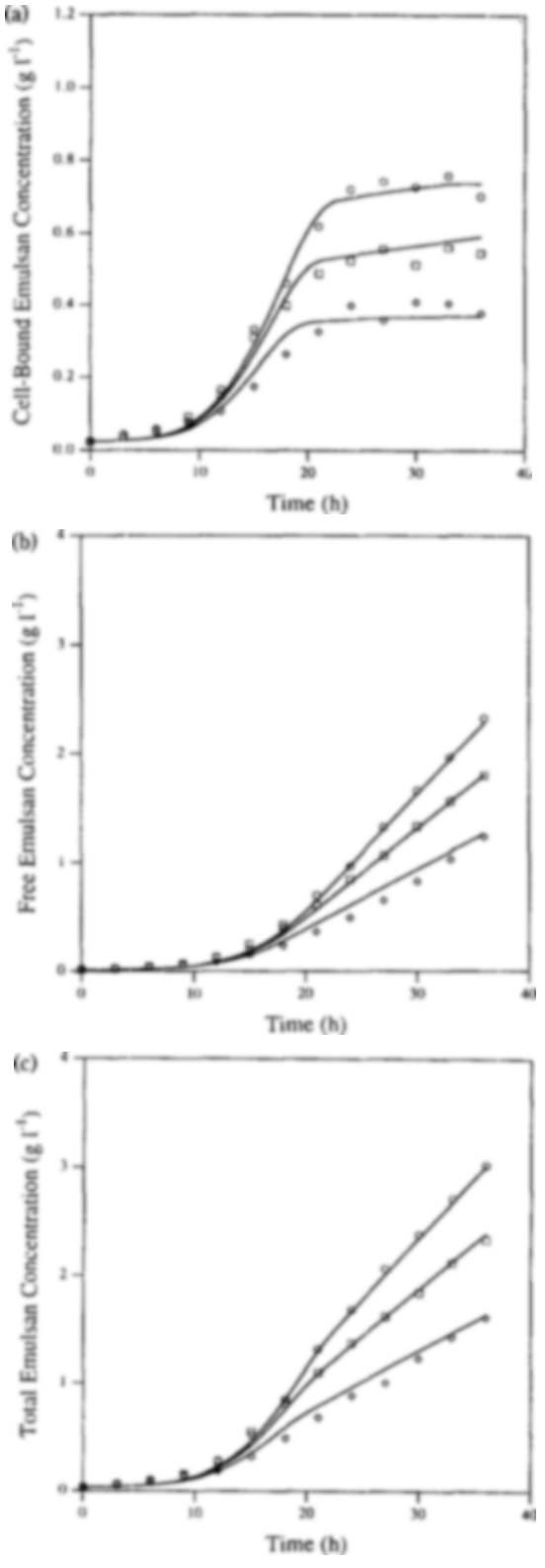

Fig. 7. The experimental data and the model prediction of (a) cell-bound emulsan production (b) free emulsan production (c) total emulsan production.
Symbols: Initial ethanol 4.0 g l^{-1} (◇); 6.0 g l^{-1} (□); 8.0 g l^{-1} (○); Model Prediction (—).

The experimental and model prediction cell-bound emulsan, free emulsan and total emulsan synthesis over the time course of batch cultivations are shown in Fig. 7(a), (b), and (c), respectively. Cell-bound emulsan synthesis was proportional to cell growth during the exponential growth phase and then continued during the stationary phase, however, it was appeared as if the synthesis nearly ceased. It might be due to the continuous release of cell-bound emulsan to cultivation medium during the stationary phase. The free emulsan was rarely detected during the early exponential growth phase, but it was increased drastically after late-exponential growth phase, and at the final state, above 75% of total emulsan synthesized existed as the extracellular form. Total emulsan synthesis was maximized during the mid- and late-exponential growth phase, and then the synthesis rate was slightly decreased and became nearly constant.

The proposed kinetic model was used to establish the operating strategy for the enhancement of emulsan production and to compose the objective process for the control of the fed-batch cultivation. Based on the model derivation using experimental data as shown in Figs. 1 and 4, enhancement of emulsan production can be achieved by maintaining of ethanol and phosphate at optimal range. Thus a fed-batch cultivation with continuous feeding of ethanol and phosphate to keep the optimal levels might be considered as the most feasible operating mode for emulsan production. By composing the objective process based on the process model, control of the ethanol concentration in a fed-batch cultivation of *A. calcoaceticus* RAG-1 using feedback-assisted iterative learning control algorithm was perfórmed to enhance emulsan production by the authors [Choi et al., 1996b].

CONCLUSIONS

The proposed mathematical kinetic model expressed as a function of ethanol and phosphate, which included the interactions between the effects of ethanol and phosphate on cell growth and emulsan synthesis, represented successfully the experimental results of dry cell weight, ethanol uptake, extracellular and intracellular phosphate level, and emulsan synthesis and release. It can be found that the roles of ethanol and phosphate on the cell growth and emulsan synthesis are complicate and interactive and the appropriate intracellular phosphate level existed for each growth phase. It can be also found that the emulsan synthesis is mainly dependent upon the cell growth rate during the exponential growth phase and the intracellular phosphate level during the stationary phase. The

results suggest that the enhancement of emulsan production can be achieved by maintaining of ethanol and phosphate at optimal range, and that a fed-batch cultivation with continuous feeding of ethanol and phosphate might be considered as the most feasible operating mode for emulsan production.

NOMENCLATURE

C : free carrier molecule [g g^{-1}]
C_0 : total carrier molecule [g g^{-1}]
CP_e : extracellular phosphate-carrier molecule complex [g g^{-1}]
E_b : cell-bound emulsan [g l^{-1}]
E_f : free emulsan [g l^{-1}]
E_t : total emulsan [g l^{-1}]
$f(P_i)$: function of growth activation by intracellular phosphate
k_d : specific death rate [h^{-1}]
k_{e1} : factor related to the growth-associated production [g g^{-1}]
k_{e2} : rate constant of non growth-associated production [g g^{-1} h^{-1}]
k_{Pe} : rate constant of active transport [g g^{-1} h^{-1}]
$k_{Pe,max}$: maximum rate constant of active transport [h^{-1}]
K_{Pe} : saturation constant of extracellular phosphate [g l^{-1}]
k_{Pi} : active transport rate [g g^{-1} h^{-1}]
k_{Pi0} : reference value for active transport rate [g g^{-1} h^{-1}]
K_{Pi} : growth index [k_{Pi}/k_{Pi0}]
K_{Pi1} : saturation constant of intracellular phosphate [g g^{-1}]
K_{Pi2} : production inhibition constant [g g^{-1}]
K_S : saturation constant of ethanol [g l^{-1}]
m_{Pi} : maintenance coefficient for intracellular phosphate [g g^{-1} h^{-1}]
m_S : maintenance coefficient for ethanol [g l^{-1} g^{-1} h^{-1}]
n_{Pe} : constant in Eq. (18)
n_S : inhibitory index of ethanol
P_e : extracellular phosphate [g l^{-1}]
P_i : intracellular phosphate [g g^{-1}]
S : ethanol [g l^{-1}]
t : time [h^{-1}]
t_{lag} : lag time [h]
X : dry cell weight [g l^{-1}]
Y_{EtS} : yield coefficient of total emulsan to ethanol [g g^{-1}]
Y_{XS} : yield coefficient of cell to ethanol [g g^{-1}]
Y_{PX} : yield coefficient of phosphate to cell [g g^{-1}]
Y_{PEt} : yield coefficient of phosphate to emulsan [g g^{-1}]

Greek Letters

α : factor related to the growth in intracellular phosphate consumption
μ : specific growth rate [h^{-1}]
μ_{max} : maximum growth rate [h^{-1}]
$\mu_{s,max}$: maximum growth rate by ethanol contribution [h^{-1}]
$\mu_{Pi,max}$: constant in growth rate Eq.
ν : specific production rate [g g^{-1} h^{-1}]
ν_{GA} : growth-associated production rate [g g^{-1} h^{-1}]
ν_{NGA} : non growth-associated production rate [g g^{-1} h^{-1}]
ξ : cell-bound emulsan per dry cell weight [g g^{-1}]

REFERENCES

Abbott, B. J., "Ethanol Inhibition of a Bacterium (*Acinetobacter calcoaceticus*) in Chemostat Culture", *J. Gen. Microbiol.*, **75**, 383 (1973).

Abbott, B. J., Laskin, A. I. and McCoy, C. J., "Growth of *Acinetobacter calcoaceticus* on Ethanol", *Appl. Microbiol.*, **25**, 787 (1973).

Bramble, J. L., Graves, D. J. and Brodelius, P., "Calcium and Phosphate Effects on Growth and Alkaloid Production in *Coffea arabica*: Experimental Results and Mathematical Model", *Biotechnol. Bioeng.*, **37**, 859 (1991).

Choi, J. W., Choi, H. G. and Lee, W. H., "Effects of Ethanol and Phosphate on Emulsan Production by *Acinetobacter calcoaceticus* RAG-1", *J. Biotechnol.*, **45**, 217 (1996a).

Choi, J. W., Choi, H. G., Lee, K. S. and Lee, W. H., "Control of Substrate Feed Rate in a Fed-batch Cultivation of *Acinetobacter calcoaceticus* RAG-1 Using Feedback-assisted Iterative Learning Algorithm", *J. Biotechnol.*, in press (1996b).

Dubois, M., Gillies, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F., "Colorimetric Method for Determination of Sugars and Related Substances", *Anal. Chem.*, **28**, 350 (1959).

Gutnick, D. L., Bayer, E. A., Rubinowitz, C., Pines, O., Shabtai, Y., Goldman, S. and Rosenberg, E., "Emulsan Production in *Acinetobacter* RAG-1", Proceeding of the Sixth International Fermentation Symposium, Advances in Biotechnology, Vol. 3, Fermentation Product, Moo-Young, M., Robinson, C. W. and Vezina, C., eds., Pergamon Press, Toronto, (1980).

Gutnick, D. L. and Shabtai, Y., "Exopolysaccharide Bioemulsifier", Biosurfactants and Biotechnology, Kosaric, N., Cairns, W. L. and Gray, N. C. C., eds., Marcel Dekker, Inc., New York, (1987).

IBM Corporation, Technical Publications Department, "System/360 Scientific Subroutine Package Programmer's Manual", 5th ed., New York, (1970).

Jarman, T. R. and Pace, G. W., "Energy Requirement for Microbial Exopolysaccharide Synthesis", *Arch. Microbiol.*, **137**, 231 (1984).

Kosaric, N., Cairns, W. L. and Gray, N. C. C., "Biotechnology and Surfactant Industry", Biosurfactants and Biotechnology, Kosaric, N., Cairns, W. L. and Gray, N. C. C., eds., Marcel Dekker, Inc., New York, (1987).

Luong, J. H. T., "Generalization of Monod Kinetics for Analysis of Growth Data with Substrate Inhibition", *Biotechnol. Bioeng.*, **29**, 242 (1987).

Maron, M. J., "Numerical Analysis: A Practical Approach", Macmillan Publishing Co., New York, (1982).

Metzler, C. M., Elfring, G. L. and McEwen, A. J., "A Package of Computer Programs for Pharmacokinetic Modeling", *Biometric.*, **30**, 562 (1974).

Mulchandani, A. and Luong, J. H. T., "Microbial Kinetics Revisited", *Enzyme Microb. Technol.*, **11**, 66 (1989).

Pazoutova, S., Votruba, J. and Rehacek, Z., "A Mathematical Model of Growth and Alkaloid Production in the Submerged Culture of *Claviceps purpurea*", *Biotechnol. Bioeng.*, **23**, 2837 (1981).

Reisfeld, A., Rosenberg, E. and Gutnick, D. L., "Microbial Degradation of Crude Oil: Factors Affecting the Dispersion in Sea Water by Mixed and Pure Culture", *Appl. Microbiol.*, **24**, 363 (1972).

Rosenberg, E., Zuckerberg, A., Rubinowitz, C. and Gutnick, D. L., "Emulsifier of *Arthrobacter* RAG-1: Isolation and Emulsifying Properties", *Appl. Environ. Microbiol.*, **37**, 402 (1979a).

Rosenberg, E., Perry, A., Gibson, D. T. and Gutnick, D. L., "Emulsifier of *Arthrobacter* RAG-1: Specificity Hydrocarbon Substrate", *Appl. Environ. Microbiol.*, **37**, 409 (1979b).

Rosenberg, E., Zosim, Z., Belsky, I. and Gutnick, D. L., "Interaction of *Acinetobacter* RAG-1 Emulsan with Hydrocarbon", Proceeding of the Sixth International Fermentation Symposium, Advances in Biotechnology, Vol. 3, Fermentaton Product, Moo-Young, M., Robinson, C. W. and Vezina, C., eds., Pergamon Press, Toronto, (1980).

Schaefer, E., "Synthesis and Release of Emulsan by *Acinetobacter calcoaceticus*", Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, MA (1985).

Sutherland, I. W. and Norval, M., "The Synthesis of Exopolysaccharide by *Klebsiella aerogenes* Membrane Preparations and the Involvement of Lipid Intermediates", *Biochem. J.*, **120**, 567 (1970).

Toda, K. and Yabe, I., "Mathematical Model of Cell Growth and Phosphastase Biosynthesis in *Saccharomyces carlsbergensis* under Phosphate Limitation", *Biotechnol. Bioeng.*, **21**, 487 (1979).

Troy, F. A., Frerman, F. E. and Heath, E. C., "The Biosynthesis of Capsular Polysaccharide in *Aerobacter aerogenes*", *J. Biol. Chem.*, **246**, 118 (1971).

Wang, S. D. and Wang, D. I. C., "Cell Adsorption and Local Accumulation of Extracellular Polysaccharide in an Immobilized *Acinetobacter calcoaceticus* System", *Biotechnol. Bioeng.*, **34**, 1261 (1989).

Zuckerberg, A., Diver, A., Perry, Z., Gutnick, D. L. and Rosenberg, E., "Emulsifier of Arthrobacter RAG-1: Chemical and Physical Properties", *Appl. Environ. Microbiol.*, **37**, 414 (1979).