Static Characteristics of A Continuous Flow Bioreactor Containing Antibiotic-Resistant Recombinant Cells

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The steady-state behavior of a continuous bioreactor containing antibiotic-resistant recombinant cells has been investigated. Only the plasmid-free cell is susceptible to and killed by antibiotics. A Monod form of specific death rate was found to simulate quite well the experimental death rates of various cells due to antibiotics. The stability characteristics, including bifurcation of the possible steady states, are examined. Appropriate numerical illustrations for the steady-state characteristics have been provided. Theoretically, two coexistence steady states (CO), three partial washout steady states (PW), and one total washout steady state (TW) are feasible, but only one CO, one PW, and one TW were realized. When antibiotic consumption is not extremely significant the CO can exist over one or two ranges of dilution rates depending upon the antibiotic concentration in the feed. The CO is globally stable. Whenever the PW and/or the TW exist(s) together with the CO they are unstable. Sensitivity analyses for several key kinetic parameters have been made. The rate at which the plasmid-bearing cells revert to the plasmid-free cells has the most significant effect on the antibiotic susceptibility of the system. Some simplified optimization calculations for maximum profit have been carried out.

INTRODUCTION

Gene cloning is a powerful tool for enhancing production of metabolites. Plasmids are the most popular vectors for gene cloning in many types of microorganisms. Recombinant plasmids cloned with a specific gene and implanted in a bacterium cell enable the cell to produce a metabolite originating from the specific gene due to gene dosage effect. The plasmid-bearing cells may eventually be replaced by the plasmid-free cells which cannot produce the desired metabolite. There are two possible reasons for this; depression of the growth rate of the plasmid-bearing cells and plasmid instability due to segregation loss of plasmids during the cell divisions. Since extra energy is needed for the plasmid-bearing cells to produce the metabolite, its growth rate is usually lower than the plasmid-free cells. Segregation loss of the recombinant plasmids causes the reversion of the plasmid-bearing cells to the plasmid-free cells. The plasmid instability can also be caused by structural instability resulting from the loss of product gene from the plasmid. When the structural instability occurs, the existence of the plasmid in a cell as detected by the presence of a marker does not guarantee that the cell produces the desired product. Even a plasmid-bearing cell cannot produce the desired product, if it has no product gene in the plasmid. In the present study the structural instability of plasmid will not be considered.

The growth rate ratio of the plasmid-bearing to the plasmid-free cells can be controlled by the growth environments and the reversion rate of the plasmid-bearing cells is dictated by both the environments and the genetic functions of the recombinant plasmids. A theoretical study of the plasmid instability has been carried by Seo and Bailey for binary fission recombinant organisms. Some experimental studies on the effects of temperature and specific growth rate have been reported.

One of the promising methods for overcoming the plasmid instability and maintaining the plasmid-bearing cells is to use plasmids which remove substrate inhibition effect from the host, the plasmid-bearing cells. This method was suggested by Ryder and DiBiasio. Only the plasmid-free cells are susceptible to substrate inhibition and consequently, it acts as a selective pressure against the plasmid-free cells. One example is recombinant Methylophilus culture and a comprehensive analysis on this system has been reported. Another method is to use plasmids which give the host the ability to produce bacteriocins that are lethal to the plasmid-free cells. The plasmid-bearing cells are resistant to such bacteriocins. An engineering study on this method has been reported.

Utilization of plasmids which confer antibiotic resistance upon the host cells is another method. In this method, antibiotics play a strong selective pressure for
the plasmid-bearing cells by killing the plasmid-free cells. By selecting an effective plasmid which can give the host the ability to produce the desired metabolite as well as resistance to an antibiotic and operating a continuous bioreactor with a feed containing the antibiotic, the plasmid-bearing cells can be maintained in a high proportion. Some experimental study on the transfer of antibiotic resistance to bacterial cells have been reported. Use of an antibiotic is most effective method among the three mentioned so far for enhancing the plasmid stability. Antibiotic concentration can be adjusted readily by changing its concentration in the feed, which is not possible for a bacteriocin which is produced by the plasmid-bearing cells in the reactor. But, high cost of antibiotic is the major drawback of this method and an optimization for maximum profit is essential. There has not been any published report on the engineering study of this method.

Steady state behavior of continuous recombinant cultures fed with a medium containing an antibiotic will be examined in the present article. The stability characteristics, including possible occurrence of static bifurcation and Hopf bifurcation of the possible steady states are investigated. Sensitivity analyses for several kinetic parameters, such as susceptibility of the plasmid-free cells to antibiotic, are carried out. An optimization to find the optimal antibiotic feed concentration for maximum profit are described briefly for a specific set of system parameters.

MODELLING OF CELL DEATH OR DEPRESSED GROWTH BY ANTIBIOTICS

Antibiotics act on cells in many ways to kill them or depress their growth, and the modes of antibiotic actions may be classified as follows:\textsuperscript{29} (1) type I: inhibitors of bacterial and fungal cell wall synthesis; (2) type II: antibiotics affecting function of membrane; (3) type III: inhibitors of nucleic acid synthesis; and (4) type IV: inhibitors of ribosome function. Type I antibiotics cause a decrease in bacterial growth, a decrease in viability, and cell lysis by inhibiting enzymatic reaction steps for key cell wall components. It was assumed for many years that the cell wall growth involved a balance between the controlled activity of hydrolytic enzymes to create new growth points and the synthetic activity. Consequently, if synthesis was inhibited while the activity of the hydrolysis continued, a defective wall would soon result and the cell would ultimately disintegrate from osmotic pressure. Type II antibiotics include surfactants\textsuperscript{30} and some hydrolytic enzymes such as lysozyme. Cytolysis may occur even when the amount of a surfactant is insufficient to give a monomolecular layer over the whole cell. The surface energy of the cell membrane is greatly reduced by the presence of a surfactant and this increases cell permeability and the tendency of the membrane-forming molecules to disperse into the surrounding medium to the extent that the integrity of the membrane may be lost. If the layer external to the cytoplasmic membrane is removed, as for example by lysozyme, the membrane is exposed and ruptures from osmotic pressure and the cells undergo lysis. Type III antibiotics prevent cells from growing by interfering with the synthesis of nucleic acids in several different ways. Type IV antibiotics interfere with protein synthesis.\textsuperscript{31} Thus, faulty proteins are synthesized and these proteins are incapable of sustaining vital cell functions. In a short time the cells are killed. Modes of actions for a wide variety of antibiotics in practical use including penicillins are described in details in the references cited earlier.\textsuperscript{29-31}

Adsorption of antibiotic molecules to the surfaces of cells or to some enzymes in cells can be considered to be the governing mechanisms for the actions of the antibiotics, types I-IV. If this were true, then there would exist a saturation effect of antibiotics and a Monod form in terms of antibiotic concentration would be a good choice for the specific death rate expression for the cells killed by an antibiotic. For a continuous culture in which cells exposed to an antibiotic are growing on a limiting substrate, the following mass balance equation for the cells can be used to describe the effect of the antibiotic on their death:

\[
\frac{dX}{dt} = -DX + \mu(S)X - \phi(B)X
\] (1)

Type I antibiotics affect both cell growth and death (\(\mu\) and \(\phi\)), types II and IV affect cell death (\(\phi\)), and type III affects cell growth (\(\mu\)). In this model, however, antibiotics were assumed to affect only cell death on the basis that when only viable cells are of interest, decrease in growth rate due to antibiotics can be qualitatively replaced by increase in death rate.

Some batch experimental results on the effect of sub-MIC (Minimum Inhibitory Concentration) of antibiotics on bacteria have been reported.\textsuperscript{32} Using the data from those experiments, the kinetic parameters of cell death by antibiotics were estimated. In these experiments, the inoculum sizes were ca. \(1 \times 10^6\) CFU (Colony Forming Units)/mL and the substrate concentrations were so high that the cells grew exponentially during the 5-h runs when no antibiotic was added. In this case, the specific growth rate remains constant as in eq. (2). A Monod form of specific death rate expression, eq. (3), was used.

\[
\mu(S) = c_1
\] (2)

\[
\phi(B) = \frac{c_2B}{C_2 + B}
\] (3)

Antibiotic consumption by the cells killed was assumed negligible. Since the media used in those experiments were well buffered, antibiotic decomposition was neglected. In this case, antibiotic concentration remains
constant and no material balance equation for the antibiotic needs to be solved. An inoculum size of \(1 \times 10^6\) CFU/mL is roughly equivalent to \(2 \times 10^{-4}\) g/L biomass concentration with an assumption that an individual cell weighs ca. \(1 \times 10^{-12}\) g and its water content is ca. 80%. Using the data from the runs without antibiotics, \(c_1\) was estimated first, then \(c_2\) and \(C_2\) were determined simply by goodness of fit. Figure 1 shows that a Monod form of specific death rate can simulate the experimental data reasonably well.

**SYSTEM MODEL**

Only one limiting substrate was assumed. The model for the bacterium-plasmid system of interest must include the competition between the plasmid-bearing and plasmid-free cells for the limiting substrate. The rate of plasmid loss (or reversion of the plasmid-bearing cells to their unaltered phenotype) was considered to be proportional to the growth rate of the plasmid-bearing cells. In other words, the fraction of the plasmid-bearing cells at the stage of fission that revert can be considered to be constant without respect to their growth rate. Antibiotics were assumed to affect only cell death rate, not cell growth rate. Schematically, this system can be represented by

\[
S \underset{X_+}{\xrightarrow{\mu(S)}} X_+, S \xrightarrow{X_-} M, X_- \xrightarrow{B} X_{-\text{dead}}
\]

The unsteady state mass balance equations for the two cell types, the limiting substrate, the antibiotics, and the metabolite product for a continuous stirred tank bioreactor fed with a sterile feed may be expressed as

\[
\dot{X}_- = -DX_+ + \mu_-(S)X_- + \alpha \mu_+(S)X_+ - \phi(B)X_-
\]

\[
\dot{X}_+ = -DX_+ + (1 - \alpha) \mu_+(S)X_+ - \sigma_-(S)X_- - \sigma_+(S)X_+
\]

\[
\dot{S} = D(S_F - S) - \sigma_-(S)X_- - \sigma_+(S)X_+
\]

\[
\dot{B} = D(B_F - B) - \psi(B)X_- - \eta(B)
\]

\[
\dot{M} = -DM + \pi(S)X_+
\]

The specific growth rates and the specific substrate consumption rates for the plasmid-free and the plasmid-bearing cells were assumed to be of Monod form:

\[
\mu_-(S) = \frac{k_1S}{K_1 + S}
\]

\[
\mu_+(S) = \frac{k_2S}{K_2 + S}
\]

\[
\sigma_-(S) = \frac{k_3S}{K_3 + S}
\]

and

\[
\sigma_+(S) = \frac{k_4S}{K_4 + S}
\]

The decrease in the growth rate of the plasmid-bearing cells due to incorporation of recombinant plasmids can be realized by using a larger value of saturation constant in the specific growth rate expression for the plasmid-bearing cells than that of the plasmid-free cells.

As shown earlier a Monod form of death rate expression was found to be a good choice for the death by antibiotics of a relatively wide concentration range. In actual situation, the practical antibiotics concentration

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**Figure 1.** Effect of sub-MIC (minimum inhibitory concentration) of antibiotics. (Experimental data are taken from ref. 32. The numbers stand for the ratios of antibiotic concentrations to MIC): (a) *Proteus mirabilis* exposed to amoxicillin (MIC = \(1.0 \times 10^{-3}\) g/L, \(c_1 = 0.95\) h\(^{-1}\), \(c_2 = 440\) h\(^{-1}\), \(C_2 = 0.22\) g/L); (b) *Proteus mirabilis* exposed to ampicillin (MIC = \(2.0 \times 10^{-3}\) g/L, \(c_1 = 0.95\) h\(^{-1}\), \(c_2 = 2.4\) h\(^{-1}\), \(C_2 = 1.7 \times 10^{-4}\) g/L); (c) *E. coli* exposed to ampicillin (MIC = \(6.0 \times 10^{-3}\) g/L, \(c_1 = 1.15\) h\(^{-1}\), \(c_2 = 2.83\) h\(^{-1}\), \(C_2 = 1.2 \times 10^{-4}\) g/L).
range will be very low compared with the MIC values used earlier. In this limiting range, the specific death rate will be linear to antibiotics concentration, making further analysis much simpler. A linear specific death rate and a linear specific antibiotics consumption rate were used for the plasmid-free cells:

$$\phi(B) = k_5B$$  (13)

and

$$\psi(B) = k_6B$$  (14)

A first-order decomposition rate expression was used for the antibiotics:

$$\eta(B) = k_7B$$  (15)

Since $M$ does not appear in eqs. (4)–(7), only the first four equations need to be considered for multiplicity and stability analysis.

**IDENTIFICATION OF STEADY STATES**

The steady states are identified from the solution of eqs. (4)–(7) with the left sides of those equations set to zero. The steady-state relations among various concentrations are

$$[D - \mu_{-}(S) + \phi(B)]X_{-} = \alpha \mu_{+}(S)X_{+}$$  (16)

$$[(1 - \alpha) \mu_{+}(S) - D]X_{+} = 0$$  (17)

$$D(S_F - S) = \sigma_{-}(S)X_{-} + \sigma_{+}(S)X_{+}$$  (18)

$$D(B_F - B) = \eta(B) + \psi(B)X_{-}$$  (19)

Three types of steady states are possible for this system. The steady-state types are

(CO) coexistence: $X_- > 0; X_+ > 0; B > 0$; and $(1 - \alpha) \mu_{+}(S) - D > \mu_{-}(S) - \phi(B)$

(PW) partial washout: $X_- > 0; X_+ = 0; B > 0$; and $D = \mu_{+}(S) - \phi(B)$  (20)

(TW) total washout: $X_- = X_+ = 0; S = S_F$; and $D(B_F - B) = \eta(B)$

For the coexistence steady state, the steady-state value of $S$ is determined from eq. (17), which is used to obtain the steady-state values of $X_-; X_+,$ and $B$ by solving simultaneously eqs. (16), (18), and (19). After some manipulation a quadratic equation in terms of $X_-$ can be obtained and therefore, two coexistence steady states are feasible. Each coexistence steady state is identified as

(CO1): $X_- = X_{-1}(S); X_+ = X_+(X_-, S);$ and $B = B(X_-, S)$  (21)

(CO2): $X_- = X_{-2}(S); X_+ = X_+(X_-, S);$ and $B = B(X_-, S)$

where, $X_{-1}$ and $X_{-2}$ are the roots of the quadratic equation and

$$X_{-1} > X_{-2}$$  (22)

For the partial washout steady state, only eqs. (16), (18), and (19) need to be solved. These three equations are coupled and must be solved simultaneously. A cubic equation in terms of $X_-$ can be obtained and thus three partial washout steady states are possible. Each state is identified as

(PW1): $X_- = X_{-1}; S = S(X_-);$ and $B = B(X_-)$  (23)

(PW2): $X_- = X_{-2}; S = S(X_-);$ and $B = B(X_-)$

(PW3): $X_- = X_{-3}; S = S(X_-);$ and $B = B(X_-)$

where $X_{-1}, X_{-2},$ and $X_{-3}$ are the roots of the cubic equation in $X_-$, which results from eqs. (16), (18), and (19):

$$X_{-1} > X_{-2} > X_{-3}$$  (24)

For the total washout steady state

(TW): $X_- = X_+ = 0; S = S_F$; and $B = \frac{DB_F}{D + k_7}$  (25)

and only one steady state is possible.

Equations (4)–(7) can be rewritten into dimensionless form as

$$\frac{dx_1}{d\tau} = -x_1 + DaR_1(x_3)x_1 + \alpha DaR_2(x_3)x_2$$  (26)

$$\frac{dx_2}{d\tau} = -x_2 + (1 - \alpha)DaR_2(x_3)x_2$$  (27)

$$\frac{dx_3}{d\tau} = -x_3 + DaR_3(x_3)x_1 + DaR_4(x_3)x_2$$  (28)

$$\frac{dx_4}{d\tau} = \beta - x_4 - DaR_4(x_3)x_1 - DaR_5(x_3)x_4$$  (29)

with the help of the dimensionless variables

$$x_1 \equiv X_-/S_F; x_2 \equiv X_+/S_F; x_3 \equiv (S_F - S)/S_F; x_4 \equiv B/S_F; \beta \equiv B_0/S_F; \tau \equiv Dt;$$

$$Da \equiv \sigma_{-}(S_F)/D; R_1(x_3) \equiv \mu_{-}(S)/\sigma_{-}(S_F); R_2(x_3) \equiv \mu_{+}(S)/\sigma_{+}(S_F);$$

$$R_3(x_3) \equiv \sigma_{-}(S)/\sigma_{+}(S_F); R_4(x_3) \equiv \sigma_{+}(S)/\sigma_{+}(S_F);$$

$$R_5(x_3) \equiv \eta(B)/[\sigma_{+}(S_F)]$$

**STABILITY AND BIFURCATION ANALYSIS**

The local stability characteristics of each steady state are examined by linearizing eqs. (26)–(29) around a
steady state. The linearized equations take the form

$$\frac{dX}{dt} = PX$$ (31)

where

$$X = [\{x_1 - x_{1i}\}, \{x_2 - x_{2i}\}, \{x_3 - x_{3i}\}, \{x_4 - x_{4i}\}]^T$$ (32)

and

$$P \equiv \{p_0\} = \begin{bmatrix}
\{DaR_1(x_{3i}) - DaR_5(x_{3i}) - 1\} & DaR_2(x_{3i}) & 0 \\
0 & \{(1 - \alpha)DaR_2(x_{3i}) - 1\} & DaR_4(x_{3i}) \\
-DaR_6(x_{4i}) & 0 & DaR_1(x_{3i}) + 1 \\
DaR_1(x_{3i})x_{1i} + DaR_2(x_{3i})x_{2i} & -DaR_5(x_{4i})x_{1i} & 0 \\
\{(1 - \alpha)DaR_2(x_{3i})x_{2i}\} & DaR_4(x_{3i})x_{2i} & 0 \\
0 & \{(DaR_6(x_{4i})x_{1i} + DaR_1(x_{3i})x_{1i}) - 1\} & 0 \\
\end{bmatrix}$$ (33)

The primes denote ordinary differentiation with respect to $x_3$ or $x_4$. A steady state is locally asymptotically stable if and only if the real parts of all eigenvalues of matrix $P$ are negative. The local stability characteristics of the steady states are discussed below.

**Coexistence Steady States (CO1, CO2)**

The steady-state substrate concentration satisfies the relation

$$(1 - \alpha)d_\mu(S) - D = 0$$ (34)

The characteristic equation is

$$\lambda^4 + a_1\lambda^3 + a_2\lambda^2 + a_3\lambda + a_4 = 0$$ (35)

where

$$a_1 = -(p_{11} + p_{33} + p_{44})$$

$$a_2 = (p_{11}p_{33} + p_{44}p_{11} + p_{33}p_{44} - p_{14}p_{41} - p_{31}p_{53} - p_{31}p_{32})$$ (36)

$$a_3 = (-p_{11}p_{33}p_{44} + p_{14}p_{33}p_{41} + p_{44}p_{33}p_{13} + p_{33}p_{12}p_{31})$$

and

$$a_4 = p_{23}(-p_{31}p_{14}p_{31} + p_{14}p_{31}p_{41} + p_{44}p_{12}p_{31})$$

The necessary and sufficient conditions for the local asymptotic stability of CO are obtained using the Routh–Hurwitz criteria. These conditions are

$$a_1 > 0; a_3 > 0; a_4 > 0; \text{and} \quad (a_1a_2 - a_3a_3 > a_1a_4)$$ (37)

Static bifurcation occurs for CO when at least one of the eigenvalues becomes zero while the other eigenvalues have negative real parts. There are three possibilities for this. One eigenvalue will become zero if

$$a_4 = 0; a_1 > 0; a_3 > 0; \text{and} \quad a_1a_2 - a_3a_3 > 0$$ (38)

Two eigenvalues will become zero at the same time if

$$a_3 = a_4 = 0; a_1 > 0; \text{and} \quad a_2 > 0$$ (39)

Three eigenvalues will become zero at the same time if

$$a_2 = a_3 = a_4 = 0 \text{ and } a_1 > 0$$ (40)

When more than one eigenvalues become zero, the derivative of each eigenvalue with respect to the bifurcation parameter (in this article, $Da$) has to have the same sign for static bifurcation to occur.

For periodic solution (Hopf bifurcation), one pair of eigenvalues has to cross the imaginary axis while the others have negative real parts. The necessary condition for one pair of eigenvalues to cross the imaginary axis is

$$a_1 > 0; a_4 > 0; a_2 \geq 2\sqrt{a_4}; \text{and} \quad (a_1d_2 - a_3a_3 = a_1d_4)$$ (41)

and the sufficient condition is

$$\left\{ \left[ \frac{a_4}{(a_1)^2} - 1 \right] + \frac{a_1}{a_1} \frac{da_5}{da_1} - \frac{a_3}{a_3} \frac{da_3}{da_1} \right\} \neq 0$$ (42)

where $Da^*$ denotes the value of $Da$ at which Hopf bifurcation occurs.

From eqs. (33) and (16), it can be seen that $p_{11}$ is always negative, while eqs. (33) and (30) show that $p_{33}$ and $p_{44}$ are also always negative. Therefore, the condition $a_1 > 0$ is always met. Thus, only the remaining conditions in eqs. (37)–(42) need to be imposed.

**Partial Washout Steady States (PW1, PW2, PW3)**

For this steady state $p_{11} = 0$ and $p_{33} = 0$, and the characteristic equation is

$$(\lambda - p_{22})(\lambda^3 + d_1\lambda^2 + d_2\lambda + d_3) = 0$$ (43)

where

$$d_1 = -(p_{33} + p_{44})$$

$$d_2 = p_{33}p_{44} - p_{14}p_{41} - p_{31}p_{53}$$ (44)

and

$$d_3 = p_{14}p_{31}p_{33} + p_{44}p_{31}p_{53}$$

For local stability

$$p_{22} < 0; d_1 > 0; d_2 > 0; \text{and} \quad d_1d_2 - d_3 > 0$$ (45)

The condition $p_{22} < 0$ physically means that when the withdrawal rate of $X_+$ cells is greater than their gen-
eration rate, $X_-$ cells wash out and therefore the partial washout is stable.

Bifurcation analysis for PW is done for two cases: one in which $p_{22} < 0$ and the other in which $p_{22} = 0$. For the first case, if condition (46) is met, one eigenvalue will become zero and static bifurcation occurs:

$$d_3 = 0; d_1 > 0; d_2 > 0$$  \hspace{1cm} (46)

When condition (47) is met, two eigenvalues become zero and if the derivative of each eigenvalue with respect to Da, the bifurcation parameter, has the same sign, then static bifurcation occurs also:

$$d_2 = d_3 = 0; d_1 > 0$$  \hspace{1cm} (47)

The necessary condition for Hopf bifurcation is

$$d_1 > 0; d_3 > 0; d_1d_2 - d_3 = 0$$  \hspace{1cm} (48)

For the second case, if condition (49), (50), or (51) is satisfied, static bifurcation from PW to CO can occur:

$$d_1 > 0; d_3 > 0; d_1d_2 - d_3 > 0$$  \hspace{1cm} (49)

$$d_3 = 0; d_1 > 0; d_2 > 0$$  \hspace{1cm} (50)

$$d_2 = d_3 = 0; d_1 > 0$$  \hspace{1cm} (51)

Condition (52) together with the condition $p_{22} = 0$ is the necessary condition for Hopf bifurcation and static bifurcation to occur at the same time:

$$d_1 > 0; d_3 > 0; d_1d_2 - d_3 = 0$$  \hspace{1cm} (52)

$R_3(x_{3b})$ and $R_4(x_{3a})$ are always negative and, thus, $p_{33}$ is always negative. $R_3(x_{3b})$ and $R_4(x_{3a})$ are always positive and, thus, $p_{44}$ is always negative. Therefore, the condition, $d_1 > 0$, is always met and the remaining conditions in eqs. (45)–(52) need to be met.

**Total Washout Steady State (TW)**

The characteristic equation for this steady state is

$$(\lambda + 1)(\lambda - p_{11})(\lambda - p_{22}) = 0$$  \hspace{1cm} (53)

For local stability, condition (54) must be met:

$$p_{11} < 0; p_{22} < 0$$  \hspace{1cm} (54)

Condition (54) physically means that the withdrawal rate must exceed the generation rate for both $X_-$ and $X_+$ cells, so that both type cells are washed out, thus making TW stable. Since all eigenvalues are real, no periodic solution is possible around this steady state. For static bifurcation, condition (55) or (56) must be satisfied:

$$p_{22} = 0; p_{11} < 0$$  \hspace{1cm} (55)

$$p_{22} < 0; p_{11} = 0$$  \hspace{1cm} (56)

If condition (55) is met, static bifurcation from TW to CO will occur and if condition (56) is met, static bifurcation from TW to PW will occur.

**NUMERICAL ANALYSIS AND DISCUSSION**

The steady-state concentrations of the growth-limiting substrate, the plasmid-free cells, the plasmid-bearing cells, and the antibiotic were obtained from the solution of eqs. (16)–(19). Identification of three PW's was accomplished using IMSL ZRPOLY, a root finding routine for polynomials. System parameter values used for numerical analysis are listed in Table I. The values for $k_1$, $k_2$, and $k_3$ were picked from the values of $c_i (= k_1 = k_2)$ and $c_2/C_2 (= k_3)$ (Fig. 1). To reflect a decrease in the growth rate of the plasmid-bearing cells, a larger value was used for $K_2$ than $K_1$. Only the constant yield case was investigated. The yield coefficient for the plasmid-free and the plasmid-bearing cells were assumed to be 0.5 and 0.4, respectively. Preliminary calculations suggest that stability and bifurcation characteristics of the system will not change even for variable yield cases. Before presenting numerical results we consider some general results for the case in which $K_1 < K_2$.

**Coexistence Steady States at Various Antibiotic Feed Concentration Ranges, $K_1 < K_2$**

The coexistence steady state is characterized by eq. (20)

$$D = \mu_{+ .\text{net}} = (1 - \alpha)\mu_+ (S)$$

and, therefore, the mutual disposition of these two net specific growth rate curves, $\mu_{+/\text{net}}$ and $\mu_{-\text{net}}$, dictates the ranges of dilution rates over which CO can exist. Figures 2(a) and 2(b) show mutual dispositions of these two curves for the system under investigation, $K_1 < K_2$, for two extreme cases: 1) negligible total antibiotic consumption ($k_a = k_b = 0$) and 2) extremely significant total antibiotic consumption.

When the antibiotic consumption by $X_-$ cells and its decomposition are negligible, the antibiotic concentration in the fermentor is same as that in the feed ($B_F$) at all dilution rates and therefore the $\mu_{-\text{net}}$ curve is shifted down by the amount equal to $k_b B_F$ [Fig. 2(a)]. Hence, as $B_F$ is increased from zero the $\mu_{-\text{net}}$ curve will intersect the $\mu_{+/\text{net}}$ curve only once at low substrate concentration and therefore at low dilution rate. Further increases in $B_F$ will eventually lead to a situation in which these two curves intersect at two points.
Figure 2. Mutual disposition of the growth curves and possible ranges of dilution rates for coexistence steady state: (a) negligible total antibiotic consumption; (b) extremely significant total antibiotic consumption.

one at a low substrate concentration and the other at $S = S_F$. We denote this value of $B_F$ by $B_F^\ast$ that is at $B_F = B_F^\ast$, $\mu_{-\text{net}}(S_F,B_F^\ast) = \mu_{+\text{net}}(S_F)$. Thus, for $0 < B_F < B_F^\ast$ the net growth curves intersect at one point and therefore, CO can exist over a low range of dilution rate. As $B_F$ is further increased beyond $B_F^\ast$ the curves continue to intersect at two points and there comes a situation in which once again the curves intersect only at one point. We denote this point by $B_F^{**}$. Therefore, for $B_F^{**} \leq B_F < B_F^\ast$, the curves intersect at two points and thus CO can exist over two ranges of dilution rates, low and high [Fig. 2(a)]. Note that CO cannot exist over an intermediate dilution rate range. Finally, when $B_F > B_F^{**}$, $\mu_{+\text{net}} > \mu_{-\text{net}}$ for all values of $S$ and CO exists over all feasible values of $D$.

When antibiotic consumption is extremely large, especially due to decomposition, the antibiotic concentration in the fermentor decreases markedly as the dilution rate becomes smaller and the net growth rate of $X_-$ cells is very close to the base growth rate ($B_F =$
0) at low dilution rates while it is much lower than the base growth rate at high dilution rates [Fig. 2(b)]. Denoting by $B_F^*$ the value of $B_F$ at which the curves intersect at $S = S_F$, we note that if $0 < B_F < B_F^*$ the curves do not intersect and thus no CO exists. As $B_F$ is further increased beyond $B_F^*$ the curves continue to intersect only at one point and there comes a situation in which once again the curves do not intersect. We denote this point by $B_F^{**}$. When $B_F^* < B < B_F^{**}$, the curves intersect at one point and CO exists at high dilution rates. When $B_F > B_F^{**}$, $\mu_{+ - net} > \mu_{- - net}$ for all values of $S$ and CO exists for all feasible values of dilution rates. Thus the values of $B_F^*$ and $B_F^{**}$ can be used as indices for antibiotic sensitivity of a system.

**Steady-State Profiles and Bifurcation**

The cell concentration profiles for different steady states for the system parameters listed in Table 1 are shown in Figures 3(a)–3(c) as a function of the Damköhler number ($= \alpha \cdot (S_F)/D$). The characteristics of this system are closer to the case [Fig. 3(a), negligible antibiotic consumption] shown in Figure 2 than the case of Figure 3(b) and CO can exist over two ranges of dilution rates. Figure 3(a) represents a situation which corresponds to $0 < B_F < B_F^*$. Figure 3(b) shows $B_F^* \lesssim B_F < B_F^{**}$; and Figure 3(c) shows $B_F^* > B_F^{**}$. The values of $B_F^*$ and $B_F^{**}$ for this system are $9.0 \times 10^{-6}$ and $2.1 \times 10^{-5}$ g/L, respectively.

Theoretically, two coexistence steady states (CO1 and CO2), three partial washout steady states (PW1–PW3), and a total washout steady state (TW) are feasible for this system. But, only one CO, one PW, and one TW (CO1, PW2, and TW) were found. Thus, henceforth we shall refer CO and PW to CO1 and PW2, respectively. At point A in Figure 3(b) condition (55) is met and a static bifurcation from TW to CO occurs. At point B, a branching takes place from TW to PW. At points C (or D) and E (or F), $p_{22} = 0$ and condition (49) is met so that a static bifurcation from PW to CO occurs. No oscillatory state (Hopf bifurcation) was observed. The important thing to be noted is that whenever CO exists over one or two ranges of Da, PW over the same range(s) is unstable. This implies that within the range(s) of Da the system always goes to CO irrespective of perturbations and that the maintenance of plasmid-bearing cells is guaranteed.
Sensitivity Analysis

The important kinetic parameters of the system are that which dictates the rate at which \( X_+ \) cells revert to \( X_- \) cells, \( \alpha \), and those which dictate the rate at which \( X_- \) cells are destroyed: \( k_5, k_6, \) and \( k_7 \). Sensitivity analyses of these kinetic parameters were made using \( B_F \) and \( B_F^* \) as indices. With respect to \( k_5 \), which represents \( X_- \) cell susceptibility to antibiotic, the antibiotic consumption by \( X_- \) cells and its decomposition were neglected (i.e. \( k_6 = k_7 = 0 \)) in order to observe the effect of only \( k_5 \) on the system. This case is represented by Figure 2(a). Since the antibiotic concentration in the fermentor is the same as \( B_F \), \( B_F \) and \( B_F^* \) can be calculated from eqs. (58) and (59), respectively. \( B_F \) and \( B_F^* \) are inversely proportional to \( k_5 \):

\[
B_F = \frac{\mu_- (S_F) - (1 - \alpha) \mu_+ (S_F)}{k_5}
\]

(58)

\[
B_F^* = \frac{\mu_- (S^*) - (1 - \alpha) \mu_+ (S^*)}{k_5}
\]

(59)

where \( S^* \) satisfies

\[
\frac{d}{ds} [\mu_- (S) - (1 - \alpha) \mu_+ (S)]_{S=S^*} = 0
\]

(60)

Thus, increasing the \( k_5 \) value decreases \( B_F \) and \( B_F^* \). In other words, when the specific death rate of \( X_- \) cells due to antibiotic is large, a smaller antibiotic concentration in the feed can be used.

For the sensitivity analysis of \( k_6 \), the values of \( k_5 \) and \( k_7 \) were fixed at \( 10^4 \) L/g/h and 0 h\(^{-1}\), respectively and \( k_8 \) was varied from \( 1.0 \times 10^{-3} \) to 1.0 L/g/h (i.e. \( k_6/k_5 = 10^{-7} - 10^{-4} \)). For \( k_6 \) values greater than 0.1 L/g/h, the values of \( B_F \) and \( B_F^* \) increase rapidly [Fig. 4(a)]. This result is expected since higher antibiotic consumption rates would require higher antibiotic concentration in the feed.

For the sensitivity analysis of \( k_7 \), the values of \( k_5 \) and \( k_6 \) were fixed to be \( 10^4 \) and 0 L/g/h, respectively, and \( k_8 \) was varied from \( 1.0 \times 10^{-3} \) to 0.3 h\(^{-1}\). The results shown in Figure 4(b) show that \( B_F \) and \( B_F^* \) increase slightly with increasing \( k_7 \) values. The effect of antibiotic decomposition rate is not as significant as that of the consumption rate, at least in the parameter range investigated.

The values of \( k_5, k_6, \) and \( k_7 \) were fixed at \( 10^4 \) L/g/h, 0 L/g/h, and 0 h\(^{-1}\), respectively, for the effect of \( \alpha \). For this case, \( B_F \) and \( B_F^* \) can be calculated from eqs. (58) and (59). The practical range of \( \alpha \) values is expected to be very limited. Within the range of \( \alpha \) values considered, 0.01–0.2, \( \alpha \) has a relatively large effect on \( B_F \) and \( B_F^* \) [Fig. 4(c)]. When the reversion rate is high, a higher antibiotic concentration in the feed is needed to kill \( X_- \) cells.

Optimization

One major drawback of using antibiotics as a selective pressure against \( X_+ \) cells is a high cost of the antibiotics. Use of large amounts of antibiotic can result in higher production of metabolic products, however at higher costs of antibiotics. Therefore, an optimization for maximum profit is necessary. The key factor in this optimization is the price ratio of the metabolic product to the antibiotic. The productivity of the metabolite can be expressed from eq. (8) as

\[
Pr = \pi X_+ = DM
\]

(61)

If the product is an intracellular metabolite and the yield coefficient for the metabolite is defined as \( Y_{MX_+} \), then,

\[
Pr = DM = Y_{MX_+} DX_+
\]

(62)

If the prices of antibiotic and the metabolite are defined as \( B_F \) and \( M_F \), respectively, then the profit from production of the metabolite will be expressed as

\[
PR = M_F Y_{MX_+} DX_+ - B_F DB_F
\]

(63)

A dimensionless profit, PI, was used as the performance index for optimization:

\[
PI = \frac{(x_2 - c\beta)}{Da}
\]

(64)

where

\[
C = \frac{B_F}{M_F Y_{MX_+}}
\]

(65)

Maximum values of the dimensionless performance index for the system whose parameter values are given in Table I are shown in Figure 5 at various dimensionless feed antibiotic concentrations, \( \beta \), with \( \epsilon \) as a parameter. For \( \epsilon \) values less than 1000 the performance index increases monotonically with \( \beta \). In other words, when the relative cost of antibiotic to metabolite is not too significant (small values of \( \epsilon \)), one should use maximum allowable antibiotic concentration in the feed with due consideration given to down stream purification. Only when the relative cost is very significant, there is an optimum value of antibiotic concentration in the feed. When \( \epsilon \) changes from \( 10^2 \) to \( 5 \times 10^4 \), the optimum value of \( B_F \) varies from \( 3.0 \times 10^{-5} \) to \( 8.0 \times 10^{-5} \) g/L [\( \beta = (3.0-8.0) \times 10^{-5} \)], and the antibiotic concentration in the fermentor, \( B \), varies from \( 2.8 \times 10^{-5} \) to \( 7.8 \times 10^{-5} \) g/L. This range of \( B \) value is much less than the values of \( C \) in eq. (3) and Figure 1. Therefore, the assumption that the specific death rate of the \( X_- \) cells by an antibiotic is linear to its concentration is valid around the optimum points in this range of \( \epsilon \) values. For most cases when the coexistence steady state exists over two ranges of dilution rates the maximum value of the performance index occurs within the higher range (or lower \( \epsilon \) in the range 3–4).
Figure 4. Effects of system parameters (other parameter values listed in Table I): (a) $k_7 = 0 \text{ h}^{-1}$, $\alpha = 0.05$; (b) $k_8 = 0 \text{ L/g/h}$, $\alpha = 0.05$; and (c) $k_9 = 0 \text{ L/g/h}$, $k_7 = 0 \text{ h}^{-1}$. 
CONCLUSIONS

A Monod form of specific death rate was suggested for the death of cells caused by antibiotics and found to simulate the actual bacterium–antibiotics system quite well. The static characteristics of continuous recombinant cultures fed with antibiotics have been investigated in detail in the present article using a specific death rate expression which is linear to antibiotics concentration. The local stability characteristics of the various steady states have been examined and situations in which static and Hopf bifurcations are possible have been identified. Appropriate numerical illustrations for the steady state characteristics have been provided.

Theoretically, two coexistence steady states, three partial washout steady states, and one total washout steady state are possible but only one coexistence steady state, one partial washout steady state, and one total washout steady state were realized for the system investigated numerically. No Hopf bifurcation was realized.

The coexistence steady state is possible over one or two ranges of dilution rates and the number of ranges depends on the feed antibiotic concentration. Two critical values of the feed antibiotic concentrations are $B_F^*$ and $B_F^{**}$. When the total antibiotic consumption rate is insignificant, which is the case studied numerically here, the coexistence steady state is possible over a range of high Damköhler numbers (or low dilution rates) when the antibiotic feed concentration is low, $0 < B_F < B_F^{**}$; over two ranges of Damköhler numbers, high and low, when the feed concentration is moderate, $B_F^* < B_F < B_F^{**}$; and over an entire range of Damköhler numbers when the feed concentration is high, $B_F > B_F^*$. The coexistence steady state is globally stable. If the partial washout steady state and/or total washout steady state exists together with the coexistence steady state, they are unstable.

Some simplified optimization calculations for maximum profit have been carried out with the relative price of the metabolite product to the antibiotics as a parameter. Only when the relative cost of antibiotic to metabolite is high ($\epsilon > 1000$) there is an optimum level of antibiotic in the feed. With a fixed antibiotics concentration in the feed the maximum profits occur at high dilution rates.
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NOMENCLATURE

- \( a_1, a_2, a_3, a_4 \) parameters defined in eq. (36)
- \( B \) concentration of antibiotic (g/L)
- \( B_s \) unit price of antibiotic ($/g)
- \( C_0, C_{O1}, C_{O2} \) coexistence steady states
- \( c_1, c_2 \) kinetic parameters (h\(^{-1}\))
- \( C_2 \) kinetic parameter (g/L)
- \( D \) dilution rate (h\(^{-1}\))
- \( D_a \) Damkohler number defined in eq. (30)
- \( d_1, d_2, d_3 \) parameters defined in eq. (44)
- \( k_1, k_2, k_3 \) kinetic parameters, \( k_i \) (h\(^{-1}\)), \( k_s \), \( k_a \) (L/g/h)
- \( K_1, K_2, K_3, K_4 \) kinetic parameters (g/L)
- \( M \) concentration of metabolite (g/L)
- \( M_s \) unit price of metabolite ($/g)
- \( P \) linearized stability matrix defined in eq. (33)
- \( \rho_{lj} \), \( l, j = 1, 2, 3, 4 \) elements of matrix \( P \)
- \( P_l \) dimensionless performance index, \( PR/M_Y_s, \) \( S_{pr}, (S_p) \), defined in eq. (64)
- \( P_r \) productivity defined in eqs. (61) and (62)
- \( P' \) profit defined in eq. (63)
- \( PW, PW_{W1}, PW_{W2}, PW_{W3} \) partial washout steady states
- \( R_{W1}, R_{W2} \) dimensionless specific rates defined in eq. (30)
- \( R_1, R_2 \) dimensionless antibiotic decomposition rate defined in eq. (30)
- \( S \) concentration of limiting substrate (g/L)
- \( s \) time (h)
- \( T_W \) total washout steady state
- \( N \) concentration of cells (g/L)
- \( x_1, x_2, x_3, x_4 \) dimensionsless concentrations defined in eq. (30)
- \( Y_{M/X} \) yield of metabolite from plasmid-bearing cells (g/g)

Greek letters

- \( \alpha \) fraction of plasmid-bearing cells that revert dimensionless feed antibiotic concentration defined in eq. (30)
- \( \beta \) cost parameter defined in eq. (65)
- \( \eta \) antibiotic decomposition rate defined in eq. (15) (g/L/h)
- \( \lambda_1, \lambda_2, \lambda_4 \) eigenvalues of linearized stability matrix \( P \)
- \( \mu \) specific growth rate defined in eqs. (2), (9), and (10) (h\(^{-1}\))
- \( \pi \) specific product formation rate (h\(^{-1}\))
- \( \sigma \) specific substrate consumption rate defined in eqs. (11) and (12) (h\(^{-1}\))
- \( \tau \) time defined in eq. (30) (dimensionless)
- \( \phi \) specific death rate of plasmid-free cells by antibiotic defined in eqs. (3) and (13) (h\(^{-1}\))
- \( \psi \) specific antibiotic consumption rate defined in eq. (14) (h\(^{-1}\))

Subscripts

- \( \text{dead} \) dead cell
- \( F \) feed
- \( s \) steady state
- \( + \) plasmid-bearing cells
- \( - \) plasmid-free cells

Superscripts

- \( *, ** \) critical values

References