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Influence of cell death and metabolite inhibition on the performance of fed-batch and perfusion bioreactors: insights from mathematical modelling

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Abstract: Both fed-batch and perfusion bioreactors are used in biopharmaceutical production. This study applies mathematical modelling to investigate the influence of cell death, metabolite inhibition, bioreaction time and cell/metabolite control on bioreactor performance. The performance parameters are titre, productivity, product yield, wasted substrate and mean product residence time (t_{res}). Cell death and metabolite inhibition both impact on cell growth and the evolution of viable cell concentration. Major impacts are potential for significantly reducing productivity and increasing t_{res} . Higher values of substrate concentration in media added (S_m) tends to improve bioreactor performance, however higher S_m leads to higher metabolite concentrations, which may contribute to greater metabolite inhibition and reduced bioreactor performance. Consequently, there may exist an optimal S_m . Bioreaction time is very important. For perfusion, there exists a bioreaction time where productivity is maximised and t_{res} is minimised. For fed-batch, sufficient bioreaction time is required to increase titre, yield and productivity, however too long a time leads to major progressive reduction in productivity and possibly very long t_{res} . Overall, with inclusion of cell death and metabolite inhibition, perfusion still has an advantage over fed-batch in terms of higher productivities and shorter t_{res} , while obtaining comparable titres and yields.

Keywords: fed-batch; perfusion; bioreactor performance; biopharmaceutical

Nomenclature

B	cell bleed volumetric flowrate [perfusion] ($\text{m}^3 \text{h}^{-1}$)
D	fed-batch dilution rate (h^{-1})
F	media feed volumetric flowrate [perfusion] ($\text{m}^3 \text{h}^{-1}$)
F_{mean}	mean media feed volumetric flowrate [perfusion] ($\text{m}^3 \text{h}^{-1}$)
G	metabolite concentration (g L^{-1})
G_f	metabolite concentration at end of bioreaction (g L^{-1})
H	harvest volumetric flowrate [perfusion] ($\text{m}^3 \text{h}^{-1}$)
k_d	cell specific death rate (h^{-1})
$k_{d\text{max}}$	maximum metabolite related specific death rate (h^{-1})
k_{do}	cell specific death rate constant (h^{-1})
k_x	viable cell specific growth rate (h^{-1})
k_{xg}	intrinsic cell specific growth rate (h^{-1})
KD_G	metabolite cell death constant (g L^{-1})
KI_G	metabolite inhibition constant (g L^{-1})
K_s	Monod kinetic constant for substrate (g L^{-1})

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$\dot{m}_{pr}$	rate of recovered product produced during bioreaction time t_b (kg h ⁻¹)
m_s	specific rate constant for rate of substrate utilisation (h ⁻¹)
P	product concentration (g L ⁻¹)
P_f	product concentration at end of bioreaction (g L ⁻¹)
P_i	product concentration before media addition [fed-batch] (g L ⁻¹)
r_g	metabolite production rate (g L ⁻¹ h ⁻¹)
r_p	product production rate (g L ⁻¹ h ⁻¹)
r_s	substrate utilisation rate (g L ⁻¹ h ⁻¹)
r_x	viable cell growth rate (g L ⁻¹ h ⁻¹)
r_{xd}	cell death rate (g L ⁻¹ h ⁻¹)
r_{xg}	intrinsic cell growth rate (g L ⁻¹ h ⁻¹)
S	substrate concentration (g L ⁻¹)
S_L	lower limit substrate concentration in bioreactor [fed-batch] (g L ⁻¹)
S_m	substrate concentration in the media added (g L ⁻¹)
S_O	initial substrate concentration in the bioreactor (g L ⁻¹)
S_S	steady-state substrate concentration (g L ⁻¹)
S_U	upper limit substrate concentration in bioreactor [fed-batch] (g L ⁻¹)
t	time (h)
t_b	bioreaction time from the start to end of bioreaction process (h)
t_{res}	mean product residence time (h)
V	bioreactor working volume (m ³)
V_C	volume of cell concentrate after cell separation [fed-batch] (m ³)
V_f	final bioreactor working volume [fed-batch] (m ³)
V_i	working volume before media addition [fed-batch] (m ³)
V_m	volume of media added [fed-batch] (m ³)
V_O	initial bioreactor working volume [fed-batch] (m ³)
V_P	volume of recovered product solution [fed-batch] (m ³)
X	viable cell concentration (g L ⁻¹)
X_C	total cell concentration in cell concentrate after cell separation (g L ⁻¹)
X_d	dead cell concentration (g L ⁻¹)
X_f	cell concentration at end of bioreaction (g L ⁻¹)
X_m	physiological maximum cell concentration (g L ⁻¹)
X_t	total cell concentration (g L ⁻¹)
Yield2	yield of product recovered from substrate added (%)
Y_{XS}	yield coefficient for biomass
Y_{PS}	yield coefficient for product
%WS	percentage wasted substrate
β, β_g	specific rate constants for rate of product and metabolite production (h ⁻¹)
μ	specific growth rate (h ⁻¹)
μ_{max}	maximum specific growth rate (h ⁻¹)

1 Introduction

Both fed-batch and perfusion bioreactors are used in the industrial production of biopharmaceuticals [1, 2]. Fed-batch is the industry standard for the manufacture of stable proteins. Fed-batch bioreactors have dominated the biopharmaceutical industry over the past few decades and have exhibited steady increases in titre, productivity and performance [3]. Perfusion bioreactors are an established and industrially proven commercial manufacturing platform [4–6], however they have not displaced fed-batch as the dominant mode of biopharmaceutical production. Perfusion has been widely accepted by major pharmaceutical regulatory agencies, including the U.S. FDA [6]. Recent advances in tangential flow cell-retention systems, media design, and process control have enabled perfusion cultures to operate at cell densities exceeding 50–100 million cells/mL, resulting in markedly higher volumetric productivity [5–9]. High productivity coupled with the potential for longer operating duration contributes to reduced physical footprint and potentially lower cost [10, 11]. This makes perfusion an attractive alternative to fed-batch processes, even for stable proteins [2, 12]. Furthermore, perfusion tends to have much shorter product residence time, due to its continuous operation, making perfusion more advantageous for

unstable proteins. In the case of stable proteins, developing a perfusion process that can compete economically with fed-batch processes depends on significantly reducing the dilution rate while maintaining high cell density. Reducing dilution rate increases the hydraulic residence time (as hydraulic residence time is the inverse of dilution rate). This also increases the product residence time in the bioreactor, and this enables the achievement of higher titres, approaching that of fed-batch bioreactors. However, dilution rate can only be decreased so much as there is a lower limit below which poor growth and productivity lead to decreased performance [13]. Alternatively, the dilution rate can be reduced by concentrating the media, but there may also be limitations due to solubility and stability constraints of compounds in the media [2, 14].

Mathematical modelling of cell culture processes has the potential for being more widely used in the biopharmaceutical industry, where it can provide an enhanced understanding of the relationship between process variables which can assist in improving process performance [15]. Mathematical modelling can provide a more quantitative description of bioreaction processes, which could be helpful in providing better and more informed future process designs [16]. Monod type kinetics are commonly used to model the specific growth rate of mammalian cell cultures, where specific growth rate depends on a few growth limiting substrates and growth inhibitory metabolites [17–20]. Studies have been published on the optimisation of monoclonal antibody (mAb) production in fed-batch and perfusion bioreactors using mathematical modelling [3, 21–24]. These studies have investigated the effects of biomass, glucose, protein, lactate concentrations, temperature and the rate of feed addition, and how these can be optimised to maximise mAb productivity while trying to minimise nutrient requirements, wastage and reactor volume. Some studies include environmental and sustainability aspects [25–27], and other studies included economic analysis [22, 28]. In particular, kinetic modelling can be applied in the description and assessment of process performance and the optimisation of cell culture systems [29].

The overall objective and novelty of the study presented in this paper is the focus on mathematical modelling and the insights it can provide to investigating how various input parameters may influence the performance of fed-batch and perfusion bioreactors, and to compare their performances. The input parameters include cell death and metabolite inhibition, substrate concentrations, bioreaction time, and limiting cell and metabolite concentrations. The performance parameters used in this study to assess and compare the performance of both the fed-batch and perfusion bioreactors are product yield, productivity, titre, wasted substrate and mean product residence time in the bioreactor.

This paper builds on the work by the authors presented in the paper by Fitzpatrick et al. [30], which focussed on the influence of substrate concentration in the media added, operating substrate concentration in the bioreactor and bioreaction time on the performance of both bioreactors. The current paper is superimposing the effect of cell death and metabolite inhibition. In particular, the study investigates the influence of cell death and metabolite inhibition on the performance parameters and how this interacts with substrate concentrations, in particular the substrate concentration in the media added. This also includes the influence of bioreaction time, and the influence of limiting the concentrations of cells and metabolites to being within some predefined concentration limits.

Cell death in industrially relevant mammalian cell culture bioprocesses mainly occurs due to apoptosis, which is a form of programmed and controlled cell death [31]. Apoptosis in bioreactors is mainly caused by stresses imposed on the cell culture due to hypoxia, temperature, pH, osmolality, nutrient deprivation and metabolite toxicity. Cell death is an important consideration in bioreaction kinetic modelling because the cell death kinetics will influence the evolution of the viable cell concentration over time. This will impact on the evolution of substrate, product and metabolite concentrations over time because viable cells are the catalyst for the bioreaction. All this will impact on bioreactor performance. A commonly used kinetic model for cell death kinetics in mammalian culture bioreactors is a first order model, where the cell death rate is proportional to the viable cell concentration [16, 21, 24, 32].

Metabolites may impact bioreaction kinetics, mainly by contributing to cell apoptosis and/or by inhibiting cell growth. Cells produce metabolites as they grow and metabolise nutrients, which may become toxic and lead to apoptosis, with two important toxic metabolites being lactate and ammonia [18, 31]. This effect can be incorporated into the first order cell death model by incorporating a metabolite associated term [22, 24]. In addition, some metabolites may inhibit the bioreaction by slowing the specific growth rate of the cells. This is often modelled by

incorporating a non-competitive term associated with the metabolite into the specific growth rate model [21, 24, 29].

The previous paper by Fitzpatrick et al. [30] showed that the substrate concentration in the media added (S_m) had a major impact on the performance of both bioreactors. Higher S_m values resulted in more desirable values for most of the performance parameters for both bioreactors, although there will be practical limitations on how high S_m can be, such as solubility and stability constraints of compounds in the media. Higher values of S_m resulted in higher titre, yield and reduced wasted substrate for both bioreactors. Higher S_m resulted in higher productivity for fed-batch and had little effect on the productivity of perfusion. Higher S_m had little effect on the product residence time in fed-batch, however it resulted in longer product residence times for perfusion, which is undesirable. For example, for perfusion operating at a steady-state substrate concentration of 1 g L^{-1} , increasing S_m from 2 to 20 g L^{-1} resulted in a titre increase from 0.17 to 2.9 g L^{-1} , a product yield increase from 8.3 to 15.1 %, a reduction in wasted substrate from 50 to 5.4 %, and an increase in product residence time from 0.45 to 5.8 h. Overall, the previous study showed that perfusion displayed superior performance to fed-batch, with greater productivities and shorter mean product residence times. Furthermore, perfusion can obtain titres and yields comparable to fed-batch when operated at similar substrate concentrations. However, this study did not include the effects of cell death and metabolite inhibition which will impact performance of both bioreactors and their comparison. Consequently, the purpose of the current paper is to present work undertaken which superimposes the effects of cell death and metabolite inhibition on the previous study.

2 Mathematical modelling

2.1 Bioreaction kinetics

The bioreaction kinetic models used by Fitzpatrick et al. [30] are used in this study and are presented below. In addition, modelling equations for cell death rate and metabolite inhibition are also included (equations (4)–(8)).

The intrinsic cell growth rate (r_{xg}) is modelled in equation (1),

$$r_{xg} = k_{xg}X \quad (1)$$

where X is viable cell concentration at time t and k_{xg} is the intrinsic cell specific growth rate. The Logistic kinetic model (equation (2)) is used to model k_{xg} ,

$$k_{xg} = \mu \left(1 - \frac{X_t}{X_m} \right) \quad (2)$$

where X_t is the total cell concentration (viable + dead cells) at time t and X_m is a physiological maximum cell concentration. The Monod model (equation (3)) is used to model the specific growth rate (μ),

$$\mu = \frac{\mu_{\max}S}{K_s + S} \quad (3)$$

where S is the substrate concentration in the bioreactor liquid, $\mu_{\max}$ and K_s are constants.

The cell death rate (r_{xd}) is modelled using the first-order model in equation (4),

$$r_{xd} = k_dX \quad (4)$$

where k_d is the specific cell death rate. k_d is modelled using metabolite and non-metabolite associated death (equation (5)),

$$k_d = k_{d\max} \frac{G}{KD_G + G} + k_{do} \quad (5)$$

where k_{do} is the non-metabolite associated constant, G is a metabolite concentration in the bioreactor liquid, $k_{d\max}$ and KD_G are constants.

Considering both intrinsic cell growth and cell death, the viable cell growth rate (r_x) is given in equation (6).

$$r_x = r_{xg} - r_{xd} \quad (6)$$

Alternatively, this can be written as presented in equation (7),

$$r_x = k_x X = (k_{xg} - k_d) X \quad (7)$$

where k_x is the viable cell specific growth rate.

To model the effect of metabolite inhibition, the specific growth rate model in equation (3) is modified to equation (8) by incorporating a non-competitive inhibition term,

$$\mu = \frac{\mu_{\max} S}{K_s + S} \left(\frac{K I_G}{K I_G + G} \right) \quad (8)$$

where G is metabolite concentration in the bioreactor liquid and $K I_G$ is a constant.

The rates of product production (r_p), metabolite production (r_g) and substrate utilisation (r_s) are modelled in equations (9)–(11), where β , β_g , m_s are specific rate constants.

$$r_p = \beta X \quad (9)$$

$$r_g = \beta_g X \quad (10)$$

$$r_s = -(m_s X) \quad (11)$$

Values for the bioreaction kinetic constants are provided in Table 1. The values presented in the first column of Table 1 are those used in the previous study [30]. The second column are the values for constants associated with cell death and metabolite inhibition. As highlighted in the previous study, the kinetic constant values presented in Table 1 are representative of mammalian cell culture systems data sourced from the literature [3, 21–24, 29, 33, 34]. The kinetic constant values reported in the literature vary considerably due to differences in the mammalian cell lines, recombinant products, and culture conditions investigated. The values presented in Table 1 were selected based on published data and are intended to be representative of mammalian cell culture systems. Furthermore, a sensitivity analysis is presented in the previous study [30] that provides an insight into how varying the values of $\mu_{\max}$, X_m , K_s , β and m_s influences the values of the performance parameters for both fed-batch and perfusion (noting that their values are the same values as those used in the current paper).

2.2 Bioreactor mass balances

2.2.1 Fed-batch

In fed-batch operation when there is no media addition, the component mass balance equation is the same as batch operation presented in equation (12),

$$\frac{dZ}{dt} = r_z \quad (12)$$

where Z is a component concentration, which is either cell (viable and dead), substrate, product or metabolite.

Table 1: Values for constants in bioreaction kinetic model.

X_m (g L ⁻¹)	50	k_{do} (h ⁻¹)	0–0.025
$\mu_{\max}$ (h ⁻¹)	0.04	$k_{d\max}$ (h ⁻¹)	0.025
K_s (g L ⁻¹)	0.1	KD_G (g L ⁻¹)	4.1
m_s (h ⁻¹)	0.06	KI_G (g L ⁻¹)	4.0
β (h ⁻¹)	0.01		
β_g (h ⁻¹)	0.03		

The substrate concentration (S) is controlled by intermittent media addition. When S decreases below a lower value (S_L), a volume of media (V_m) is added to restore S to an upper value (S_U). The volume of media added (V_m) is evaluated from a mass balance in equation (13),

$$V_m = \frac{V_i (S_U - S_L)}{S_m - S_U} \quad (13)$$

where V_i is the working volume before media addition and S_m is the concentration of substrate in the media.

For product, metabolite and cell (viable and dead) concentrations, media addition will dilute their concentrations. Their concentration (Z) after adding a volume of media is modelled by a mass balance in equation (14),

$$Z = \frac{V_i Z_i}{(V_m + V_i)} \quad (14)$$

where Z_i is the concentration of either cell, product or metabolite before media addition.

In this study, the substrate concentration is maintained close to a constant steady-state value S_S by implementing a pseudo-continuous addition of media. This is modelled by reducing the difference between S_U and S_L to a small difference of 0.01 g L^{-1} . The rationale for doing this is explained by Fitzpatrick et al. [30].

The viable cell mass balance equation for continuous addition of media in fed-batch operation is presented in equation (15),

$$\frac{dX}{dt} = r_x - DX = (k_x - D)X \quad (15)$$

where D is the fed-batch dilution rate.

2.2.2 Perfusion

A schematic of the perfusion bioreactor is presented in Figure 1. The viable cell, substrate, product and metabolite mass balance equations are presented for the perfusion bioreactor in equations (16)–(19), where V is the bioreactor working volume, F is the media flowrate containing substrate concentration (S_m), H is the harvest or perfusion flowrate, and B is the cell bleed flowrate.

$$V \frac{dX}{dt} = r_x V - BX \quad (16)$$

$$V \frac{dS}{dt} = F S_m + r_s V - (H + B)S \quad (17)$$

$$V \frac{dP}{dt} = r_p V - (H + B)P \quad (18)$$

$$V \frac{dG}{dt} = r_g V - (H + B)G \quad (19)$$

The media feed flowrate (F) is varied in perfusion to control the substrate concentration at a constant steady-state concentration (S_S). This also influences cell, product and metabolite concentrations. In perfusion, a cell bleed may also be applied to control the cell concentration if it starts to exceed a limiting value.

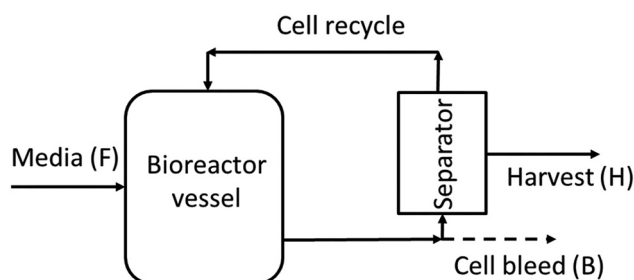


Figure 1: Schematic of perfusion bioreactor.

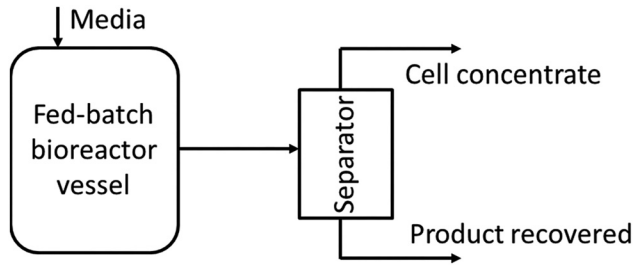


Figure 2: Schematic of product recovery from fed-batch bioreactor using a cell separator.

2.3 Product recovery

After the fed-batch bioreaction, a cell separator is used to recover product from the cell suspension, as illustrated in Figure 2. This will result in some product being lost to the separated cell suspension. The volume of recovered product solution (V_P) from the fed-batch bioreactor final working volume (V_f) is evaluated from the total and cell mass balances in equations (20) and (21).

$$V_f \rho_f = V_C \rho_C + V_P \rho_P \quad (20)$$

$$V_f X_{tf} = V_C X_{tc} \quad (21)$$

where X_{tf} is the total cell concentrations at the end of the bioreaction, V_C is the volume of the cell concentrate, X_{tc} is the total cell concentration in the separated cell suspension leaving the cell separator (100 g L^{-1}), and ρ is the density of the corresponding streams.

For the perfusion bioreactor, product is being continuously separated from the cells during perfusion operation, as shown in Figure 1. Furthermore, at the end of perfusion operation, additional product from the bioreactor is recovered from the bioreactor cell suspension and added to the harvest. This additional volume of product solution is estimated using mass balances similar to those in equations (20) and (21).

2.4 Bioreactor performance parameters

The bioreactor performance parameters used in this study are:

- **Product yield:** Mass of product recovered from the substrate added (Yield₂).
- **Productivity:** Mass of recovered product produced per unit bioreactor working volume per unit time.
- **Titre:** Product concentration in the final recovered volume.
- **Wasted substrate:** Percentage mass of substrate added that ends up being wasted (%WS) or is not used in the bioreaction.
- **Mean product residence time:** Mean time that product spends in the bioreactor.

Full details of the modelling equations for the bioreactor performance parameters are presented in Fitzpatrick et al. [30]. These performance parameters are used in assessing and comparing the performance of the fed-batch and perfusion bioreactors.

2.5 Initial conditions and model implementation

The initial cell concentration is given a value of 0.1 g L^{-1} , and product and metabolite concentrations are given initial values of 0 g L^{-1} . For the fed-batch simulations, the initial bioreactor working volume (V_0) is given a value of 0.05 m^3 and the initial substrate concentration in the bioreactor is the same value as the upper value, i.e. $S_0 = S_U$. For the perfusion simulations, the bioreactor working volume is given a constant value of 1 m^3 . The initial bioreactor substrate concentration is the same value as the constant steady-state concentration, i.e. $S_0 = S_S$.

Computations were conducted using a numerical method approach where the equations above were used to formulate algorithms for both fed-batch and perfusion. The differential equations were solved using a finite difference methodology. The computations were implemented in Matlab (MathWorks, USA), using a time step of 0.01 h in the running of the algorithms.

3 Results and discussion

As highlighted in the Introduction, this paper superimposes the effect of cell death and metabolite inhibition onto the previous work by the authors presented by Fitzpatrick et al. [30], to investigate how they impact on the performance of both bioreactors. This also includes the effect of bioreaction time, and the influence of limiting the concentrations of cells and metabolites to being within some predefined concentration limits.

3.1 Effect of cell death on the performance of the bioreactors

There are many factors that cause cell death, which includes toxicity effects of metabolites, such as lactate and ammonia, and many environmental factors in the bioreactor, such as stresses on the cells due to mixing and shear, hypoxia, temperature and pH. Considering this, the specific death rate (k_d) has two terms (equation (5)), the first term is metabolite associated and the second term (k_{do}) is environmental or non-metabolite associated. This section initially looks at how constant values of k_d influence cell growth and bioreactor performance, and then looks at the influence of metabolite associated cell death (metabolite toxicity).

3.1.1 Effect of k_d on perfusion bioreactor performance

A constant k_d is an example of a constant environmental factor (k_{do}) that is causing cell death over time. The effect of a constant value of k_d on the evolution of viable cell concentration (X), dead cell concentration (X_d) and total cell concentration (X_t) over time in a perfusion bioreactor is illustrated in Figure 3a. For $k_d = 0$ (no cell death), X follows the Logistic model. For $k_d > 0$ (cell death), cell death is continuously occurring over time, thus the dead cell concentration increases and so does total cell concentration, but these concentrations are eventually limited over time by the Logistic model maximum (X_m), as illustrated in Figure 3a. The viable cell concentration (X) initially increases over time until it attains a maximum value at some time (t_{xmax}), after which it decreases. The viable cell growth rate (r_x) model (equation (6)) consists of two parts, intrinsic cell growth rate (r_{xg}), based on the Logistic model, and cell death rate (r_{xd}). Consequently, the reason for maximum X occurring at a particular time is due to the interplay between r_{xg} and r_{xd} (Figure 3b) as follows:

$$\begin{aligned} \text{For } t < t_{xmax}: r_{xg} > r_{xd}; \quad r_x > 0; \quad X \text{ increases} \\ \text{At } t = t_{xmax}: r_{xg} = r_{xd}; \quad r_x = 0; \quad X = \text{maximum} \\ \text{For } t > t_{xmax}: r_{xg} < r_{xd}; \quad r_x < 0; \quad X \text{ decreases} \end{aligned}$$

For a constant value of k_d shown in Figure 3b, the maximum X occurs because of the Logistic model, whereby r_{xg} initially increases faster than r_{xd} , but then it progressively decreases after the inflection point in the Logistic model and attains a value equal to r_{xd} at some point in time (t_{xmax}). Of course, if substrate concentration was to decrease or inhibiting metabolite concentration was to increase, then these could also cause r_{xg} to decrease over time, and thus contribute to the occurrence of maximum X .

The effect of different constant k_d values on the evolution of viable cell concentration (X) over time in a perfusion bioreactor is illustrated in Figure 4a. Increasing k_d causes a reduction in the viable cell growth rate (before maximum X) and a decrease in the maximum X attained. Consequently, cell death kinetics has the

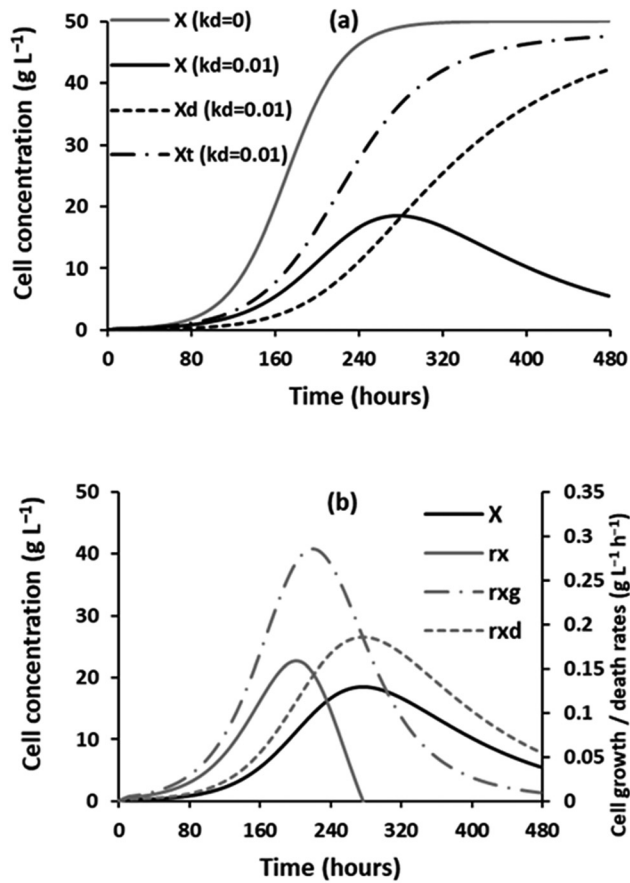


Figure 3: Effect of a constant value of k_d (0.01 h^{-1}) on the evolution of (a) cell concentrations and (b) cell growth and death rates over time in a perfusion bioreactor. $[S_m = 20 \text{ g L}^{-1}; S_s = 1 \text{ g L}^{-1}]$.

potential to have major impact on the viable cell concentration profile, which may in-turn influence bioreactor performance. Figure 4b shows that cell death slows down the evolution of the product concentration over time but this does eventually attain the same steady-state value as in the no cell death scenario. It is interesting to note that product (and metabolite) concentration attain a steady-state even though the viable cell concentration does not. Carrying out steady-state product and substrate mass balances show that the steady-state product concentration is independent of the viable cell concentration. Furthermore, the rate of product production (r_p) decreases as X decreases, but the rate of substrate utilisation (r_s) also decreases, which results in a decrease in F . Consequently, a decrease in r_p is being countered by a decrease in the rate of product removal from the bioreactor, which will eventually result in a constant steady-state product (and metabolite) concentration.

The effect of k_d values on the performance parameters for a perfusion bioreactor is presented in Table 2. These data are for a bioreaction time of 480 h, where the total cell concentration is approaching the Logistic model maximum. Increasing k_d (cell death) is causing a decrease in final product concentration, titre and yield (and an increase in %WS) but these are not major impacts, except for $k_d = 0.025 \text{ h}^{-1}$ where cell death is significantly impacting on the evolution of the product concentration (Figure 4b). The major impacts are on productivity and mean product residence time (t_{res}) with large undesirable changes in both. The ability of perfusion to attain high productivity is due to its ability to obtain high viable cell concentrations, consequently cell death results in much lower viable cell concentrations, which greatly reduces productivity, as seen in Table 2. Furthermore, a major reduction in productivity is associated with a major reduction in feed and harvest flowrates, as the titre has not majorly decreased. The reduction in these flowrates leads to a corresponding increase in hydraulic residence time, which increases the mean product residence time (t_{res}).

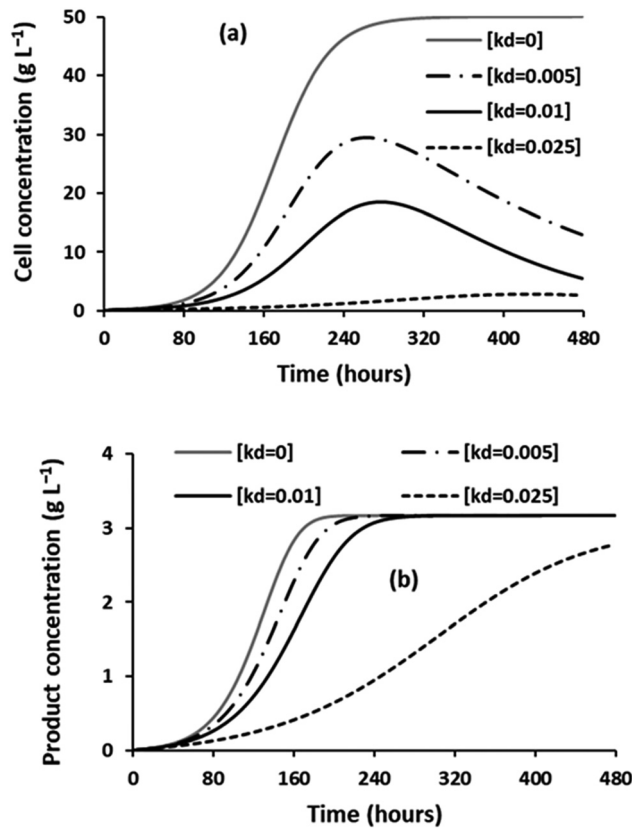


Figure 4: Effect of k_d on the evolution of (a) viable cell concentration and (b) product concentration over time in a perfusion bioreactor. [$S_m = 20 \text{ g L}^{-1}$; $S_s = 1 \text{ g L}^{-1}$].

Table 2: Effect of specific death rate (k_d) on perfusion performance. [$t_b = 480 \text{ h}$; $S_m = 20 \text{ g L}^{-1}$; $S_s = 1 \text{ g L}^{-1}$].

$k_d \text{ (h}^{-1}\text{)}$	Titre (g L^{-1})	Productivity ($\text{mg L}^{-1} \text{ h}^{-1}$)	$t_{\text{res}} \text{ (h)}$	Yield2 (%)	%WS (%)	$X_f \text{ (g L}^{-1}\text{)}$	$P_f \text{ (g L}^{-1}\text{)}$
0	3.10	318	3.8	15.7	5.1	50.0	3.2
0.005	3.03	150	7.7	15.5	5.2	12.8	3.2
0.01	2.94	85	13.1	15.2	5.4	5.4	3.2
0.025	2.10	12.8	71.3	14.2	7.2	2.6	2.8

3.1.2 Effect of k_d on fed-batch bioreactor performance

For $k_d = 0$ (no cell death), Figure 5a shows that the viable cell concentration (X) approaches a steady-state value like in perfusion. However, this is not due to μ approaching zero in the Logistic model (equation (2)) because the steady-state X is much lower than the Logistic model maximum (X_m). The main difference with fed-batch is that the dilution effect of added media is keeping the cell concentrations much lower than in perfusion, and the total cell concentration is well below X_m even at 480 h. What is happening can be understood from examining equation (15) (viable cell mass balance equation for continuous addition of media). At steady-state, $dX/dt = 0$, thus $k_x = D$, i.e. the cell growth effect (which increases cell concentration) is balanced by the dilution effect (which decreases cell concentration).

The effect of a constant value of k_d ($k_d = 0.01 \text{ h}^{-1}$) on the evolution of viable cell concentration, dead cell concentration (X_d) and total cell concentration (X_t) over time in a fed-batch bioreactor is illustrated in Figure 5a. These cell concentrations also attain steady-state values but after a longer time duration (around 400 h) than for no cell death (around 260 h). Interestingly, the steady-state total cell concentration for $k_d = 0.01 \text{ h}^{-1}$ is the same as that for no cell death. Figure 5b shows the evolution of the rate parameters over time, which also attain steady-

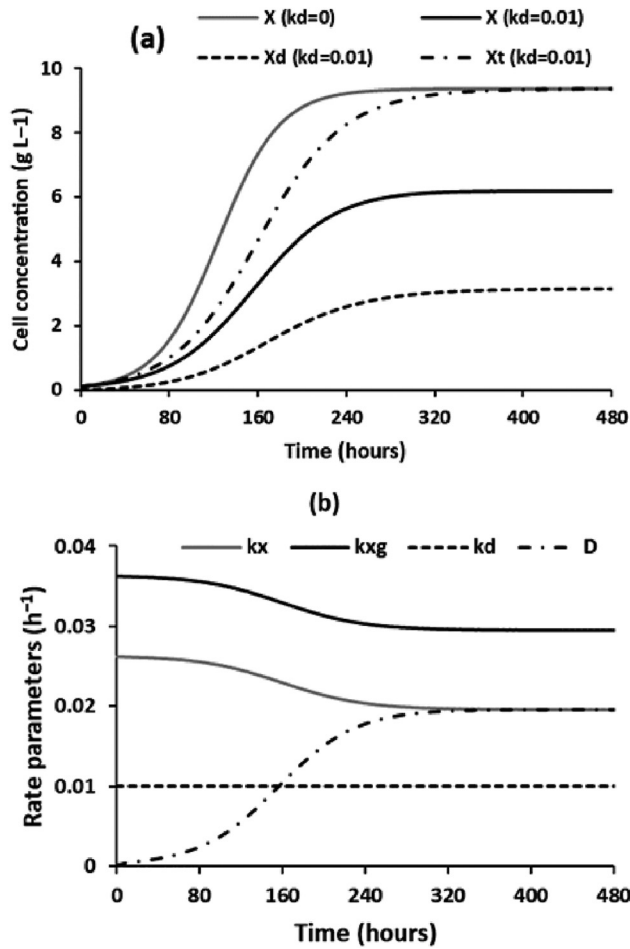


Figure 5: Effect of a constant value of k_d (0.01 h^{-1}) on the evolution of (a) cell concentrations and (b) cell specific growth/death rates and dilution rate over time in a fed-batch bioreactor. $[S_m = 20 \text{ g L}^{-1}$; $S_U = 1 \text{ g L}^{-1}$; $S_L = 0.99 \text{ g L}^{-1}$].

state values. k_{xg} attains a constant steady-state value when the total cell concentration attains a steady-state (from considering equation (2)). k_x attains a constant value as $k_x = k_{xg} - k_d$, and the steady-state D can be evaluated from $k_x = D$. Before the steady-state, X increases because $k_x > D$. In fact, at $t = 0$, k_x has its largest value and D has its lowest value of zero, as illustrated in Figure 5b. Consequently, k_x decreases over time and D increases over time until they equilibrate. This is similar to what happens for no cell death except there is no k_d and $k_x = k_{xg}$ for no cell death.

The effect of different constant k_d values on the evolution of viable cell concentration (X) over time in a fed-batch bioreactor is illustrated in Figure 6a. This is somewhat similar to perfusion. Like in perfusion, higher k_d values reduce the viable cell concentration profile obtained, however cell concentration attains a constant value over time for $k_d = 0.005$ and 0.01 h^{-1} similar to the no cell death scenario. Furthermore, Figure 5b shows that higher k_d values slow down the evolution of the product concentration, however the product concentration eventually attains the same value as the no cell death scenario, given enough time, which is very similar to perfusion.

For the case of $k_d = 0.025 \text{ h}^{-1}$, the viable cell concentration attains a maximum value at 512 h and then decreases over time but does eventually attain a steady-state concentration at around 2000 h. At higher k_d values, the decrease in viable cell concentration becomes more pronounced. Figure 7 illustrates the effect for a higher k_d value ($k_d = 0.03 \text{ h}^{-1}$) on the cell concentrations and rate parameters. Figure 7a shows the dead cell concentration becomes much higher than the viable cell concentration, and the viable concentration attains a maximum at around 592 h. It also shows that the total cell concentration does not attain a steady-state like that seen in Figure 5a. Figure 7b shows k_{xg} and k_x continuously decreasing over time and also not attaining steady-state values. It also shows the fed-batch dilution rate (D) increasing over time until it equals k_x at the viable cell concentration maximum at 592 h. However, they do not attain a steady-state value. Instead, k_x undershoots D , causing X to

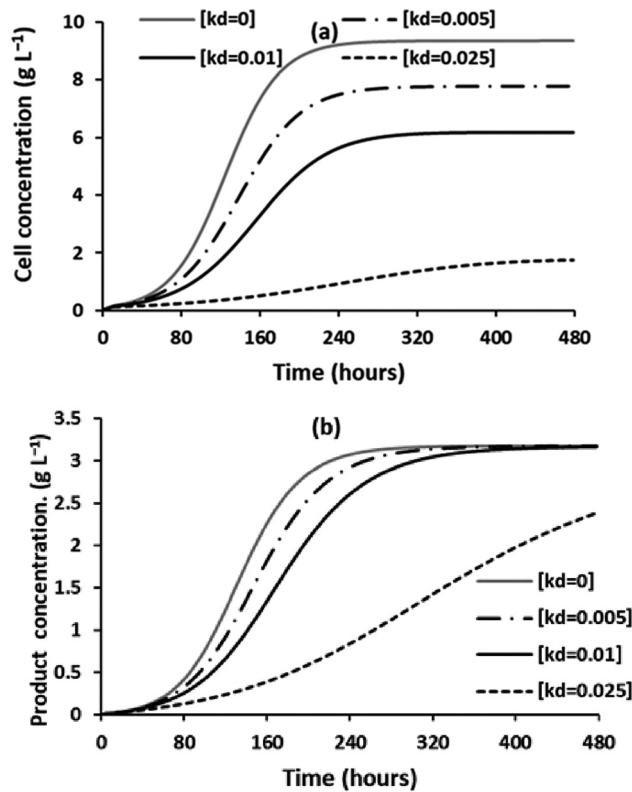


Figure 6: Effect of k_d on the evolution of (a) viable cell concentration and (b) product concentration over time in a fed-batch bioreactor. $[S_m = 20 \text{ g L}^{-1}$; $S_U = 1 \text{ g L}^{-1}$; $S_L = 0.99 \text{ g L}^{-1}$].

decrease. D is influenced by the rate of substrate utilisation (r_s), with lower D required to satisfy a lower r_s . As X decreases after the maximum, r_s also decreases (from equation (11)). This results in D decreasing over time after the maximum, as illustrated in Figure 7b, however it does not equilibrate with k_x . Consequently, the viable cell concentration continues to decrease over time due to $k_x < D$.

Overall, k_d influences the evolution of the viable cell concentration over time. Three scenarios have been observed as follows:

- Scenario 1: X increases over time and levels off at a steady-state X . This occurs for no cell death and relatively lower values of k_d , as illustrated in Figure 5.
- Scenario 2: A maximum viable cell concentration ($X_{\max}$) is observed, and a steady-state X is attained during the bioreaction time. This occurs at intermediate values of k_d .
- Scenario 3: A maximum viable cell concentration ($X_{\max}$) is observed, but a steady-state is not attained during the bioreaction time, as illustrated in Figure 7. This is associated with higher values of k_d .

If a steady-state is attained this is associated with the total cell concentration attaining the no cell death steady-state cell concentration.

Table 3 shows the effect of k_d on fed-batch performance. k_d does not have a major impact on titre, productivity, yield and wasted substrate. At 480 h, the titres are very similar except for $k_d = 0.025 \text{ h}^{-1}$, however this will increase given more time (Figure 6b). The productivity trend follows the same trend as titre in fed-batch. Like in perfusion, increasing k_d increases t_{res} , which may be undesirable depending on the product.

The standout impact of k_d is on the final working volume (V_f), where increasing k_d causes a major reduction in V_f . This suggests that lower media addition has taken place during the 480 h bioreaction time, due to lower substrate utilisation rate and thus there is a lower product production rate. Even though k_d is having only a small impact on productivity, this is somewhat misleading. This is because increasing k_d is having a major impact on rate of recovered product production ($\dot{m}_{pr}$), which is defined in equation (22).

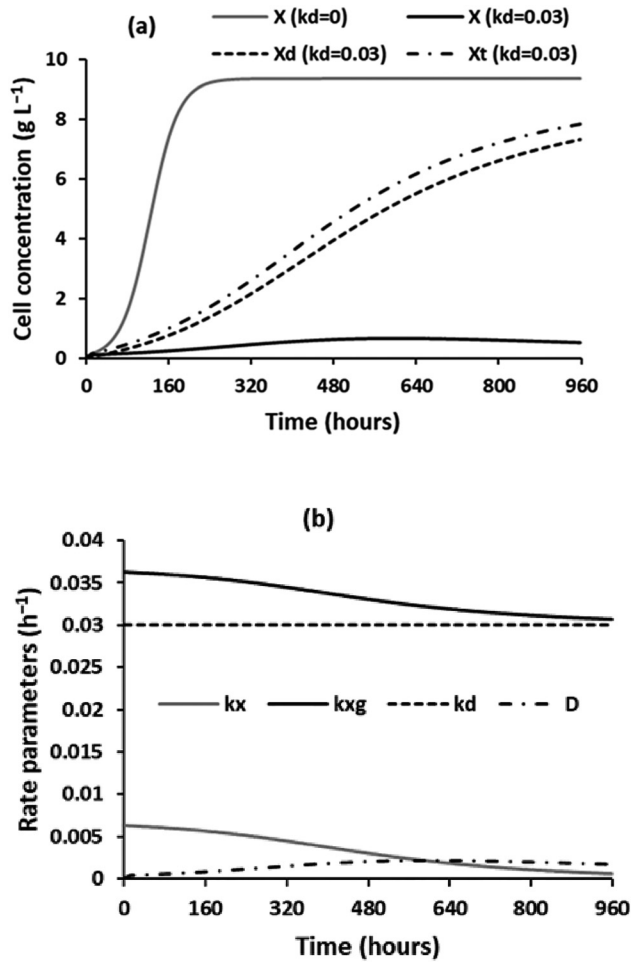


Figure 7: Effect of a higher k_d ($k_d = 0.03 \text{ h}^{-1}$) on the evolution of (a) cell concentrations and (b) cell specific growth/death rates and dilution rate over time in a fed-batch bioreactor. [$S_m = 20 \text{ g L}^{-1}$; $S_U = 1 \text{ g L}^{-1}$; $S_L = 0.99 \text{ g L}^{-1}$].

Table 3: Effect of specific death rate (k_d) on fed-batch performance. [$t_b = 480 \text{ h}$; $S_m = 20 \text{ g L}^{-1}$; $S_U = 1 \text{ g L}^{-1}$; $S_L = 0.99 \text{ g L}^{-1}$; $V_o = 0.05 \text{ m}^3$].

$k_d (\text{h}^{-1})$	Titre (g L^{-1})	Productivity ($\text{mg L}^{-1} \text{ h}^{-1}$)	$t_{\text{res}} (\text{h})$	Yield2 (%)	%WS (%)	$X_f (\text{g L}^{-1})$	$V_f (\text{m}^3)$	$\dot{m}_{pr} (\text{mg h}^{-1})$
0	3.17	5.98	33.9	14.36	4.97	9.36	1,799	10,760
0.005	3.17	5.98	40.7	14.35	4.98	7.77	219	1,309
0.01	3.16	5.97	50.7	14.35	4.99	6.19	29	173
0.025	2.39	4.60	114	14.39	6.46	1.74	0.20	0.94

$$\dot{m}_{pr} = \frac{\text{mass of product recovered}}{t_b} \quad (22)$$

$\dot{m}_{pr}$ can also be expressed in equation (23) from the definition of productivity [30].

$$\dot{m}_{pr} = (\text{productivity}) V_f \quad (23)$$

Table 3 shows the major reduction in $\dot{m}_{pr}$ as k_d increases. This is being caused by lower viable cell growth/concentrations at higher k_d , which is leading to lower rates of substrate utilisation, requiring less substrate addition (and thus lower V_f values).

3.1.3 Effect of metabolite-associated cell death (metabolite toxicity)

This effect is very similar to the previous two sections, as metabolite toxicity is also contributing to k_d , which influences the viable cell concentration and its impact on bioreactor performance. The difference is that k_d is not constant over the bioreaction time; it varies with the variation in the toxic metabolite concentration (G). Consequently, k_d increases as G increases and it attains a constant value if G attains a steady-state value during the bioreaction time. The substrate concentration in the media added (S_m) has a major impact on metabolite toxicity and the evolution of the viable cell concentration over time. This is because higher S_m results in higher values of G which produce higher k_d values resulting in greater cell death due to metabolite toxicity. Figure 8 shows the influence of metabolite toxicity and S_m on the evolution of the viable cell concentration for both perfusion and fed-batch.

For perfusion (Figure 8a), metabolite toxicity has the effect of X increasing to a maximum and then decreasing, which is the same as seen previously when including cell death. It also shows that the higher S_m value results in lower X values due to higher k_d values caused by higher G . For fed-batch (Figure 8b), metabolite toxicity is displaying the same type of viable cell concentration profiles as seen previously when including cell death. Unlike perfusion, S_m impacts on X for the case of no cell death, with higher S_m resulting in higher X . The important aspect to note in Figure 8b for fed-batch is that metabolite toxicity at the higher S_m has a much greater effect on the viable cell concentration profile. For $S_m = 20 \text{ g L}^{-1}$, metabolite toxicity causes a large reduction in X , while there is a much lower reduction for $S_m = 2 \text{ g L}^{-1}$. This is because G is much greater at the higher S_m (as highlighted above), which results in higher k_d values, more cell death and a greater reduction in the viable cell concentration profile. Finally, the effect of metabolite toxicity on bioreactor performance is similar to that presented in the previous sections on the effect of cell death.

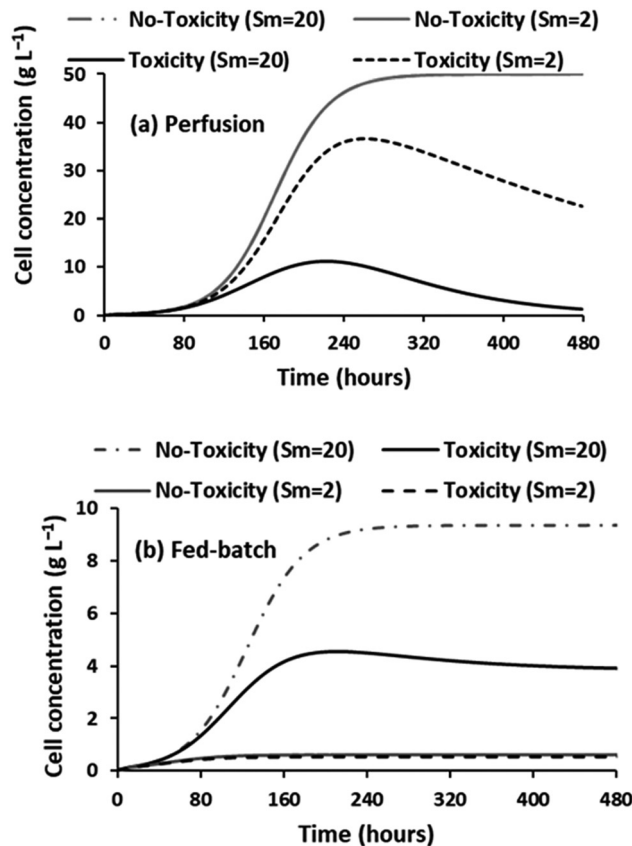


Figure 8: Effect of cell death caused by metabolite toxicity [$k_{dmax} = 0.025 \text{ h}^{-1}$; $KD_G = 4.1 \text{ g L}^{-1}$] on the evolution of the viable cell concentrations in (a) perfusion ($S_U = 1 \text{ g L}^{-1}$) and (b) fed-batch ($S_U = 1 \text{ g L}^{-1}$; $S_L = 0.99 \text{ g L}^{-1}$). [Note: In (a) the two No-toxicity curves overlap; In (b) the two ($S_m = 2$) curves are very close to each other and appear to be overlapping].

Overall, the effect of the specific cell death rate (k_d) on bioreactor performance is similar for both perfusion and fed-batch. Varying k_d has no major impact on yield, % wasted substrate and titre, although it does slow their evolution over the bioreaction time. Increasing k_d increases t_{res} for both perfusion and fed-batch, and reduces productivity for perfusion only. Increasing k_d has no major impact on the productivity of fed-batch, however this is deceptive, as it does cause a major reduction in the rate of recovered product production.

3.2 Effect of metabolite inhibition on the performance of the bioreactors

The effect of the concentration of an inhibiting metabolite, such as lactate or ammonia, on cell growth is modelled using equation (8). This has the effect of reducing the specific growth rate and slowing cell growth. This does depend on the concentration of the inhibiting metabolite (G); the higher the concentration the greater the effect.

Earlier it is highlighted that there are advantages to operating at higher concentrations of substrate concentration in the media added (S_m). However, this does interact with the potential for metabolite inhibition. Product and metabolite concentrations are higher at higher values of S_m for both perfusion and fed-batch bioreactors. Consequently, G increases as S_m increases, and this will result in greater metabolite inhibition and slower cell growth.

3.2.1 Effect of metabolite inhibition on perfusion bioreactor performance

Figure 9a illustrates the effect of metabolite inhibition and S_m on cell growth in a perfusion bioreactor. In the case of no metabolite inhibition, S_m has no effect on cell growth, i.e. the cell concentration curves are the same [30]. As expected, Figure 9a shows that metabolite inhibition slows cell growth. More importantly, Figure 9a shows that S_m does influence cell growth when there is metabolic inhibition. The higher value of S_m ($=20 \text{ g L}^{-1}$) results in slower cell growth, and this is because G is higher at higher values of S_m , as shown in Figure 9b. Figure 9b also shows that metabolite inhibition slows the evolution of metabolite (and product) concentration over time.

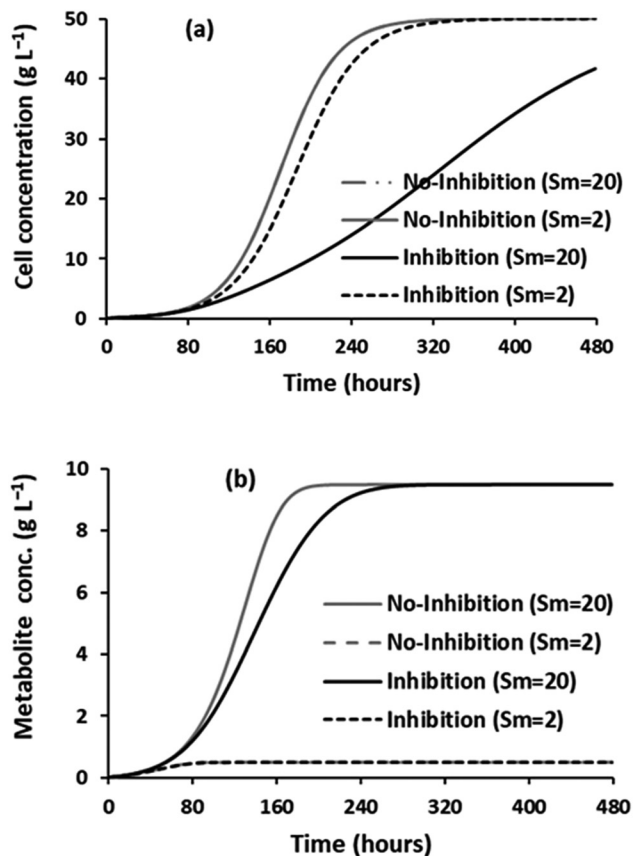


Figure 9: Effect of metabolite inhibition and S_m on the evolution of (a) viable cell concentration and (b) metabolite concentration over time in a perfusion bioreactor. [$K_G = 4 \text{ g L}^{-1}$; $S_S = 1 \text{ g L}^{-1}$] [Note: In (a) the two No-inhibition curves overlap; In (b) the two ($S_m = 2$) curves are very close to each other and appear to be overlapping].

Table 4: Effect of metabolite inhibition and S_m on perfusion performance. [$t_b = 480$ h; $S_5 = 1$ g L⁻¹].

S_m (g L ⁻¹)	Titre (g L ⁻¹)	Productivity (mg L ⁻¹ h ⁻¹)	t_{res} (h)	Yield2 (%)	%WS (%)	X_f (g L ⁻¹)	P_f (g L ⁻¹)	G_f (g L ⁻¹)
No-inhibition								
2	0.17	322	0.23	8.3	50.0	50.0	0.17	0.50
20	3.10	319	3.8	15.7	5.1	50.0	3.17	9.50
Inhibition ($KI_G = 4$ g L⁻¹)								
2	0.17	305	0.25	8.3	50.0	50.0	0.17	0.50
20	3.04	165	6.95	15.5	5.2	41.9	3.17	9.5

As highlighted earlier, S_m has a major impact on bioreactor performance. However, in the case of metabolite inhibition, S_m also impacts on G , which in-turn influences cell growth and thus bioreactor performance. This may increase the potential for performance trade-offs. For example, Table 4 shows the effect of metabolite inhibition on the perfusion bioreactor performance parameters for two values of S_m . In the case of no inhibition, higher S_m results in more desirable values for titre, yield, wasted substrate but has less desirable t_{res} , while the productivities obtained are very similar. For the case of metabolite inhibition, there is little effect on the performance parameters for the low S_m value of 2 g L⁻¹. However, for the high S_m of 20 g L⁻¹, t_{res} increases further, which is undesirable. Furthermore, the evolution of the cell concentration is slower at the higher S_m . This is due to the much higher G , and this significantly reduces the productivity, which makes higher S_m less desirable. Productivity is an important bioreactor performance parameter, and significantly lower productivity is undesirable, and may impact on any performance trade-off decisions. Consequently, metabolite induced lower productivity and longer t_{res} at higher S_m trades off against better performance values for titre, yield and wasted substrate, which may influence the selection of the operating S_m value.

3.2.2 Effect of metabolite inhibition on fed-batch bioreactor performance

Figure 10a illustrates the effect of metabolite inhibition and S_m on cell growth in a fed-batch bioreactor. This is somewhat different to perfusion because in the case of no metabolite inhibition, S_m does affect cell growth for fed-batch, while it doesn't for perfusion (Figure 9). For fed-batch, higher S_m results in higher cell concentration as a result of lower dilution effect when adding media (as outlined earlier). In the case of metabolite inhibition, cell growth is slowed especially as metabolite concentration increases, and the viable cell concentrations are lower than that for the case of no-inhibition. This effect is more pronounced at higher S_m because the inhibiting metabolite has a higher concentration (Figure 10b). The reason for the lower viable cell concentrations and slower progression is because increasing metabolite concentration reduces the specific growth rate (equation (8)).

For the case of metabolite inhibition at higher S_m , the viable cell concentration attains a maximum value at 218 h but does eventually attain a steady-state value at around 650 h. For the lower S_m , the viable cell concentration attains a steady-state value at around 300 h. This steady-state is only attained when the metabolite concentration attains a steady-state. This is because the metabolite concentration influences the specific growth rate in equation (8), whereby an increasing metabolite concentration results in a decreasing specific growth rate. After the cell concentration maximum, k_x undershoots D because steady-state has not been attained (similar to higher k_d in Figure 7b). This leads to the decrease in the cell concentration until a steady-state is attained when the metabolite attains a steady-state concentration. Overall, the evolution of the metabolite concentration over time influences the evolution of the viable cell concentration, which leads to three scenarios for metabolite inhibition being observed as follows (which are similar to those for cell death):

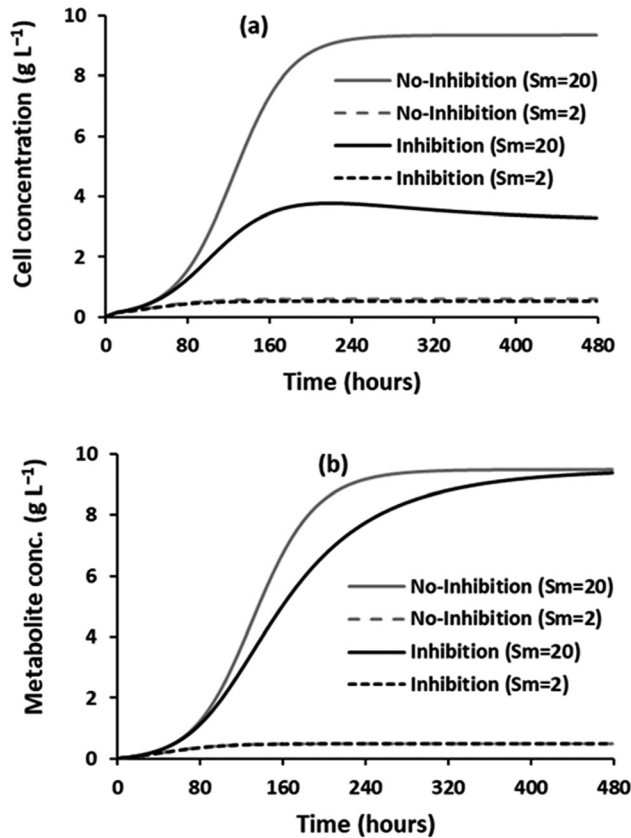


Figure 10: Effect of metabolite inhibition and S_m on the evolution of (a) viable cell concentration and (b) metabolite concentration over time in a fed-batch bioreactor.

[$KI_G = 4 \text{ g L}^{-1}$; $S_U = 1 \text{ g L}^{-1}$; $S_L = 0.99 \text{ g L}^{-1}$] [Note: In (a) & (b) the two ($S_m = 2$) curves are very close to each other and appear to be overlapping].

- Scenario 1: X increases over time and levels off at a steady-state X . This occurs for no metabolite inhibition and relatively lower levels of metabolite inhibition, which are influenced by the values of S_m and KI_G .
- Scenario 2: A maximum viable cell concentration ($X_{\max}$) is observed, and a steady-state X is attained during the bioreaction time. This occurs at intermediate levels of metabolite inhibition.
- Scenario 3: A maximum viable cell concentration ($X_{\max}$) is observed, but a steady-state is not attained during the bioreaction time. This occurs at higher levels of metabolite inhibition.

If a steady-state X is attained this is associated with the metabolite concentration attaining a steady-state during the bioreaction time.

Like in perfusion, the effect of metabolite inhibition depends on S_m because S_m influences the concentration of the inhibiting metabolite (G). Table 5 shows the effect of metabolite inhibition on the fed-batch bioreactor performance parameters for two values of S_m . In the case of no inhibition, higher S_m results in more desirable values for titre, productivity, yield, wasted substrate, and the t_{res} is only a little longer. Metabolite inhibition is having little effect on the performance parameters at the low S_m value due to the low value of G . For the high value ($S_m = 20 \text{ g L}^{-1}$), productivity is actually higher. However, as seen with cell death in fed-batch, this higher productivity is somewhat misleading because Table 5 shows that metabolite inhibition is having a significant impact on V_f , especially at the higher value of S_m . The decrease in V_f values means that the bioreaction is progressing more slowly, thus metabolite inhibition reduces the rate of recovered product production, and this effect is much greater at higher S_m . Furthermore, t_{res} has greatly increased at $S_m = 20 \text{ g L}^{-1}$, which may be very undesirable. Consequently, metabolite induced lower product production rate and longer t_{res} at higher S_m trades off against better performance values for titre, yield and wasted substrate, which may influence the selection of the operating S_m value in fed-batch operation. This is a somewhat similar outcome to that in perfusion.

Table 5: Effect of metabolite inhibition and S_m on fed-batch performance. [$t_b = 480$ h; $S_U = 1$ g L⁻¹; $S_L = 0.99$ g L⁻¹; $V_o = 0.05$ m³].

S_m (g L ⁻¹)	Titre (g L ⁻¹)	Productivity (mg L ⁻¹ h ⁻¹)	t_{res} (h)	Yield2 (%)	%WS (%)	X_f (g L ⁻¹)	G_f (g L ⁻¹)	V_f (m ³)
No-inhibition								
2	0.17	0.35	27.9	8.4	49.6	0.60	0.50	258,333
20	3.17	5.98	33.9	14.4	5.0	9.36	9.50	1,799
Inhibition ($KI_G = 4$ g L⁻¹)								
2	0.17	0.35	31.3	8.4	49.6	0.54	0.50	54,531
20	3.13	6.30	87.6	15.3	5.05	3.30	9.38	3.83

3.2.3 Controlling a metabolite concentration

As highlighted above, a metabolite produced by the bioreaction may inhibit cell growth and negatively impact on bioreactor performance, thus it may be desirable to control the metabolite concentration (G) so that it does not exceed a certain maximum value (G_{max}) that significantly inhibits the cell culture.

The substrate concentrations have a major influence on whether or not G exceeds G_{max} during the bioreaction time t_b . As can be seen in Figure 9b (perfusion) and Figure 10b (fed-batch), it can be seen that higher S_m values result in higher values of G . The operating substrate concentration inside the bioreactor (S) will also influence G , because $(S_m - S)$ influences the evolution of G over time. Consequently, for a fixed S , there exists a limiting value of S_m below which G will not exceed G_{max} during the bioreaction time t_b . Considering the above, S_m may be manipulated to constrain G below a specified G_{max} during the bioreaction time t_b .

Overall, for both fed-batch and perfusion operation, there exist limiting values of S_m (for a given substrate operating concentration inside the bioreactor) that can be applied in bioreactor operation to prevent a metabolite concentration exceeding a specified maximum value. However, as highlighted earlier, S_m has a major influence on bioreactor performance. As it also influences G , which also influences bioreactor performance, there are potential trade-offs, consequently S_m is a key optimisation variable.

3.3 Effect of bioreaction time on bioreactor performance

The influence of cell death and metabolite inhibition on cell, product and metabolite concentration profiles over time has been illustrated in a number of Figures in the previous sections. This section focusses on the effect of bioreaction time on the performance parameters for both fed-batch and perfusion bioreactors. This is presented for the case where there is no cell death and no metabolite inhibition and for the case where there is both cell death ($k_d = 0.01$ h⁻¹) and metabolite inhibition ($KI_G = 4$ g L⁻¹).

3.3.1 Perfusion

For perfusion operation, the effect of bioreaction time on the performance parameters is presented in Table 6 for the cases with and without cell death and metabolite inhibition. To begin with, the effect of the combination of cell death and metabolite inhibition on the evolution of the viable cell concentration is similar to that of cell-death except that the evolution is slower and the maximum viable cell concentration is lower. Furthermore, the combination slows the evolution of product and metabolite concentrations over time but they eventually attain steady-state values if the bioreaction time is long enough.

For the case of no cell death and no metabolite inhibition, the main influences of bioreaction time are as follows:

- *Titre, yield and %WS:* Titre and yield increase over time until they attain a constant value, and %WS decreases over time to a constant value.

Table 6: Effect of bioreaction time on perfusion performance. [$S_m = 20 \text{ g L}^{-1}$; $S_S = 1 \text{ g L}^{-1}$].

Time (h)	Titre (g L^{-1})	Productivity ($\text{mg L}^{-1} \text{ h}^{-1}$)	t_{res} (h)	Yield2 (%)	%WS (%)	X_f (g L^{-1})	G_f (g L^{-1})
No cell death and no metabolite inhibition							
48	0.1	2.7	48	7.1	57.3	0.57	0.38
120	1.2	16	43	13.9	12.0	6.8	4.42
240	2.9	142	5.8	15.1	5.4	46.3	9.50
480	3.1	319	3.8	15.7	5.1	50.0	9.50
960	3.1	409	3.4	15.8	5.0	50.0	9.50
Cell death and metabolite inhibition [$k_d = 0.01 \text{ h}^{-1}$; $KI_G = 4 \text{ g L}^{-1}$]							
48	0.1	2.0	48	5.9	64.5	0.34	0.28
120	0.5	5.3	120	12.5	23.6	1.36	1.77
240	1.7	14.1	66.1	14.5	9.1	3.14	6.37
480	2.4	22.2	53.2	15.0	6.3	2.70	9.21
960	2.7	18.6	77.0	15.2	5.8	0.68	9.47

- Mean product residence time: This decreases over time, with residence time being much higher early on, after which it approaches a constant value. Bioreaction rates are very slow initially and increase over time. This leads to higher feed/harvest flowrates over time, which reduces residence time. These flowrates eventually attain steady-state values, which results in mean product residence time approaching a constant value.
- Productivity: This increases significantly over time, due to the attainment of higher titres and also higher harvest flowrates over time.

For the case of cell death and metabolite inhibition, cell growth is slowed and it is very important to note that there is a major reduction in the viable cell concentrations. Table 6 shows that viable cell concentration attains a maximum value that is much lower than the X_m value attained with no cell death, and the viable cell concentration decreases to a very low value by 960 h. Product and metabolite concentrations are slower to evolve over time. The major impacts of this on the performance parameters in comparison to the no cell death/no inhibition case are as follows:

- *Titre, yield and %WS*: These values are somewhat similar to those for the no cell death/no inhibition case, except that they are developing more slowly.
- Mean product residence time: This decreases more slowly over time and is much greater than for the case of no cell death/no inhibition case. For example, Table 6 shows that mean product residence times are around 10 to 20 times greater during bioreaction times between 240 and 960 h. Furthermore, product residence time attains a minimum value at a certain bioreaction time, after which it increases. This is due to the cell death and the viable cell concentration profile, as illustrated in Figure 4a.
- *Productivity*: There is a major reduction in productivity in comparison to no cell death/no inhibition. For example, Table 6 shows that the productivity is around 5–10 % of the no cell death/no inhibition values during bioreaction times between 240 and 960 h. Furthermore, productivity attains a maximum value over time and then declines. This is due in particular to the effect of cell death and how this influences the viable cell concentration profile over time (Figure 4a).

Considering the above, the analysis suggests that for the case of no cell death/no inhibition, it advantageous to operate the perfusion bioreactor over a longer period of time. However, for the case of cell death and metabolite inhibition, there exists a bioreaction time where productivity is maximised and mean product residence time is minimised.

3.3.2 Fed-batch

Before comparing the performance parameter values for fed-batch operation, it is important to know the effect of the cell death/metabolite inhibition on the evolution of the viable cell concentration and whether it attains a steady-state value or attains a maximum value and then decreases over time. This is illustrated in Figure 11 along with the evolution of the rate parameters for the case of cell death and metabolite inhibition [$k_d = 0.01 \text{ h}^{-1}$; $KI_G = 4 \text{ g L}^{-1}$]. Here, the evolution of the viable cell concentration attains a maximum value at around 218 h and then decreases over time. As seen previously (Figure 7b), Figure 11b shows k_x undershooting D around this time and even though D also decreases after this time, it never equilibrates with k_x . Consequently, a steady-state is not attained during the 960 h bioreaction time. Considering this, the effect of bioreaction time on the performance parameters is presented in Table 7 for both the case where there is no cell death and no metabolite inhibition and for the case where there is cell death ($k_d = 0.01 \text{ h}^{-1}$) and metabolite inhibition ($KI_G = 4 \text{ g L}^{-1}$).

For the case of no cell death and no metabolite inhibition, the main influences of bioreaction time are as follows:

- *Titre, yield and %WS*: Titre and yield increase over time until they attain a constant value, and %WS decreases over time to a constant value.
- *Bioreactor working volume*: The final bioreactor working volume (V_p) essentially increases in an exponential manner over time due to greater media volumes being required as the working volume increases over time.

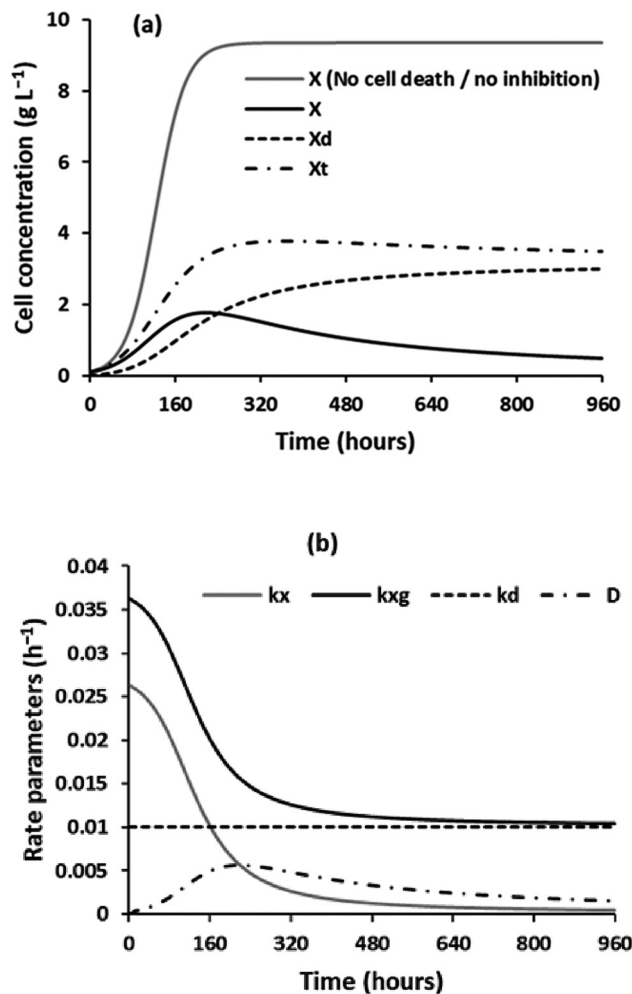


Figure 11: Case with cell death ($k_d = 0.01 \text{ h}^{-1}$) and metabolite inhibition ($KI_G = 4 \text{ g L}^{-1}$): Evolution of (a) cell concentrations and (b) cell specific growth/death rates and dilution rate over time in a fed-batch bioreactor. [$S_m = 20 \text{ g L}^{-1}$; $S_U = 1 \text{ g L}^{-1}$; $S_L = 0.99 \text{ g L}^{-1}$].

Table 7: Effect of bioreaction time on fed-batch performance. [$S_m = 20 \text{ g L}^{-1}$; $S_U = 1 \text{ g L}^{-1}$; $S_L = 0.99 \text{ g L}^{-1}$].

Time (h)	Titre (g L^{-1})	Productivity ($\text{mg L}^{-1} \text{h}^{-1}$)	t_{res} (h)	Yield2 (%)	%WS (%)	X_f (g L^{-1})	G_f (g L^{-1})	V_f (m^3)
No cell death and no metabolite inhibition								
48	0.1	2.57	17.4	7.1	57.2	0.54	0.37	0.05
120	1.2	9.78	26.7	14.1	11.9	4.29	3.68	0.08
240	3.1	11.6	32.3	14.4	5.1	9.22	9.19	1.52
480	3.2	5.98	33.9	14.4	5.0	9.36	9.50	1,799
960	3.2	2.99	33.9	14.4	5.0	9.36	9.50	$2.6 (10^9)$
Cell death and metabolite inhibition [$k_d = 0.01 \text{ h}^{-1}$; $K_G = 4 \text{ g L}^{-1}$]								
48	0.1	1.90	19.3	5.9	64.4	0.33	0.27	0.05
120	0.5	4.45	37.0	12.6	23.4	1.15	1.63	0.06
240	1.7	7.02	68.3	14.7	8.7	1.77	5.24	0.11
480	2.7	5.37	154	15.1	5.9	1.06	8.03	0.32
960	3.0	3.02	351	15.2	5.3	0.49	9.00	0.95

- *Mean product residence time:* This increases over the bioreaction time, however it approaches a constant value after sufficient bioreaction time. This is because most of the product is being produced later on due to the exponential increase in the bioreactor working volume.
- *Productivity:* This initially increases due to the titre increasing over time and achieves a maximum value. Then, productivity decreases progressively over time, reducing roughly in half as the bioreaction time doubles, because the titre is displaying little or no change.

For the case of cell death and metabolite inhibition, cell growth is slowed and viable cell concentrations are much lower, while product and metabolite concentrations are slower to evolve over time, as shown in Table 7. The major impacts of this on the performance parameters in comparison to the no cell death/no inhibition case are as follows:

- *Titre, yield and %WS:* These values are somewhat similar to those for the no cell death/no inhibition case, except that they are slower to develop over time.
- *Bioreactor working volume:* This is developing much more slowly, which is due to the bioreaction progressing more slowly over time.
- *Mean product residence time:* These values are much greater especially at longer bioreaction times, being around 10 times greater at 960 h. Furthermore, t_{res} does not attain a constant value during the 960 h. The reason for this is because the increase in the bioreactor working volume over time is much slower than the case of no cell death and no metabolite inhibition.
- *Productivity and rate of recovered product production:* Productivity values are lower for the case of cell death/metabolite inhibition, however this may be somewhat misleading and the impact may be much greater. As highlighted earlier, cell death and metabolite inhibition may have a major impact on slowing the bioreaction. This slows the evolution of V_f over time which can greatly reduce rate of recovered product production ($\dot{m}_{pr}$) obtained at a specific bioreaction time, as shown in Table 3.

More generally, an unusual and complicating aspect of fed-batch is that the bioreactor working volume is increasing over time. This can lead to a situation where two bioreactions have similar productivities but have very different recovered product production rates, and this is occurring due to the bioreactions progressing at different rates. Thus, care needs to be taken in comparing the productivities of bioreactions in fed-batch bioreactors.

Considering the complicating effect of the changing bioreactor working volume in fed-batch operation, it might be more appropriate to compare the effect of a constant final bioreactor working volume (V_f) or constant

Table 8: Effect of ratio of final to initial bioreactor working volume (V_f/V_o) and bioreaction time on fed-batch performance. [$V_o = 0.05 \text{ m}^3$; $S_m = 20 \text{ g L}^{-1}$; $S_u = 1 \text{ g L}^{-1}$; $S_L = 0.99 \text{ g L}^{-1}$].

V_f/V_o	$V_f (\text{m}^3)$	Time (h)	Titre (g L^{-1})	Productivity ($\text{mg L}^{-1} \text{ h}^{-1}$)	t_{res} (h)	Yield2 (%)	%WS (%)	$X_f (\text{g L}^{-1})$	$G_f (\text{g L}^{-1})$
No cell death and no metabolite inhibition									
4	0.2	166	2.38	13.2	29.4	14.4	6.6	7.68	7.13
16	0.8	218	2.97	12.4	31.6	14.4	5.3	9.05	8.91
64	3.2	266	3.12	10.6	32.9	14.3	5.1	9.30	9.35
Cell death and metabolite inhibition [$k_d = 0.01 \text{ h}^{-1}$; $KI_G = 4 \text{ g L}^{-1}$]									
4	0.2	356	2.38	6.43	107	15.0	6.6	1.41	7.13
16	0.8	856	2.97	3.34	307	15.2	5.3	0.56	8.91
64	3.2	2,230	3.12	1.35	894	15.3	5.1	0.20	9.35

(V_f/V_o) ratio on the performance parameters for the cases with and without cell death and metabolite inhibition. This is possibly a more realistic means of comparison because V_f will be a fixed value in practice. This is presented in Table 8. Here, it can more clearly be seen that for a constant value of V_f (or V_f/V_o), cell death/metabolite inhibition is slowing the bioreaction resulting in much longer bioreaction times and correspondingly much lower productivities. It also highlights that cell death or metabolite inhibition may cause the viable cell concentration to decrease towards zero over prolonged time (Table 8).

Considering the above, operating the fed-batch bioreactor for time-periods beyond the productivity maximum is not advantageous, because productivity progressively reduces over time. For the case where cell death and metabolite inhibition is not having a significant effect, the exponential increase in working volume may limit the bioreaction time. For the case of cell death and metabolite inhibition, the evolution of the bioreactor working volume over time is greatly slowed, and this results in lower productivities and may also limit the evolution of the bioreactor working volume. Furthermore, the product residence time increases significantly with greater bioreaction time, which may also limit bioreaction time. Overall, bioreaction time (and V_f/V_o ratio) presents a trade-off. On one hand, longer bioreaction time provides for higher titre and greater final working volume, while on the other hand this may result in lower productivity and possibly longer product residence time.

3.4 Controlling cell concentration and its effect on bioreactor performance

In the logistic model, there is a maximum cell concentration that the cells don't naturally grow above (X_m). A value of 50 g L^{-1} is applied in the modelling [30]. In practice, it may be desirable to control the cell concentration below this concentration. This section investigates the effects of controlling the cell concentration below X_m . From the results presented in previous sections, the maximum cell concentration obtained for fed-batch operation was well below this at about 10 g L^{-1} , while the maximum cell concentration obtained in perfusion was close to 50 g L^{-1} . Consequently, for fed-batch operation it may not be required to control the cell concentration below a certain value, thus this section mainly looks at the influence of controlling the cell concentration in the perfusion bioreactor.

As shown previously, S_m may be used for controlling the concentration of metabolites. However, in perfusion, S_m cannot be used for controlling cell concentration because the cells are totally retained and not perfused. Previous sections have shown that S_m has no effect on the cell concentration profile, except if there is metabolite inhibition. In this case, higher S_m leads to higher G , which slows down cell growth but the cell concentration will attain over time a similar concentration as in no-inhibition.

Considering the above, the cell concentration is controlled by a cell bleed (Figure 1), which prevents a total cell concentration from exceeding a specified maximum. The cell bleed removes some of the content of the bioreactor, and the working volume inside the bioreactor is maintained constant by correspondingly reducing the harvest

Table 9: Effect of cell concentration control (max. total cell conc.: 20 g L^{-1}) on the performance parameters for the perfusion bioreactor [$t_b = 480 \text{ h}$; $S_m = 20 \text{ g L}^{-1}$; $S_s = 1 \text{ g L}^{-1}$].

	Titre (g L^{-1})	Productivity ($\text{mg L}^{-1} \text{ h}^{-1}$)	t_{res} (h)	Yield2 (%)	%WS (%)	X_f (g L^{-1})	P_f (g L^{-1})
No cell death ($k_d = 0$)							
No-cell-control	3.10	319	3.8	15.7	5.10	50.0	3.17
Cell-control	2.98	101	8.4	10.8	5.21	20.0	3.17
Cell death ($k_d = 0.01 \text{ h}^{-1}$)							
No-cell-control	2.94	85	13.1	15.2	5.35	5.4	3.17
Cell-control	2.84	55	14.9	11.1	5.40	10.9	3.17

flowrate. Simulations were performed at $S_m = 20 \text{ g L}^{-1}$ for no cell death and cell death ($k_d = 0.01 \text{ h}^{-1}$). The effect on the performance parameters is presented in Table 9 for the case of no cell concentration control and where the total cell concentration is controlled so that it does not exceed a maximum of 20 g L^{-1} .

Controlling the cell concentration has the effect of reducing the recovered yield (Yield2) because less product is harvested due to product loss to the cell bleed. There was little or no change in %WS because the substrate loss in the cell bleed was counteracted by a corresponding reduction of substrate loss in the reduced harvest. For the case of no cell death, productivity decreases due to product loss in the bleed and also due to lower rate of product production because of lower average cell concentrations. This also has the effect of increasing mean product residence time (t_{res}).

Somewhat surprisingly, in the case of cell death, the productivity does not decrease so much with cell control. The reason for this is due to the effect of cell removal, including dead cell removal, which influences the viable cell concentration profile, leading to a greater final viable cell concentration, as illustrated in Figure 12. This results in a higher rate of product production from about 400 h onwards, which partly counters the loss of product in the cell bleed. In fact at a bioreaction time of 960 h for the cell death case, the productivity of cell control is higher than that of no cell control, and t_{res} is much lower. This is because in cell control, the viable concentration achieves a steady-state concentration of 10.8 g L^{-1} at 960 h, while the viable cell concentration for no cell control continues to fall to a very low value of 0.1 g L^{-1} . Consequently, the simulations show that it may be more beneficial to operate with a cell bleed if there is significant cell death and a longer bioreaction time, so as to allow removal of dead cells and to attain a higher steady-state viable cell concentration, resulting in higher productivity and lower t_{res} .

Some simulations were performed on the fed-batch bioreactor where the cell concentration was controlled at a value below the maximum attained without control. This was achieved by adding media to reduce the cell concentration by dilution when it exceeded the control maximum. This had the effect of also diluting the product, which reduced the titre. However, this extra addition of media may also result in a loss of control of the substrate

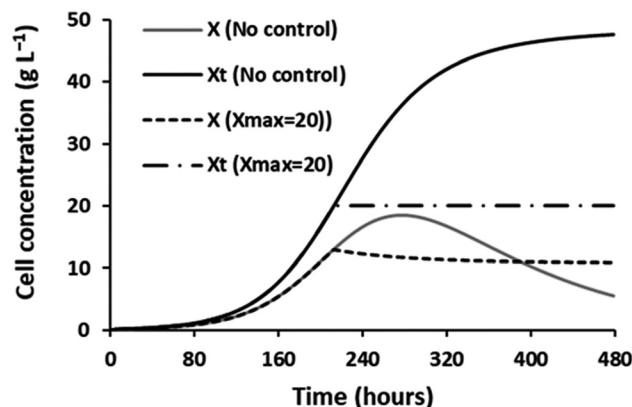


Figure 12: Effect of cell control ($X_{\text{max}} = 20 \text{ g L}^{-1}$) on the evolution of the viable (X) and total (X_t) cell concentrations over time in a perfusion bioreactor for $k_d = 0.01 \text{ h}^{-1}$ [$S_m = 20 \text{ g L}^{-1}$; $S_s = 1 \text{ g L}^{-1}$].

concentration inside the bioreactor, whereby the substrate concentration would exceed its upper control limit. This results in more wasted substrate and a reduction in yield.

3.5 Comparison of fed-batch and perfusion bioreactor performance

In comparing the performance of fed-batch and perfusion bioreactors, it is important that values of S_m and the substrate concentration inside the bioreactor are the same. Considering this, Tables 6 and 7 are used to compare the performance of the bioreactors for the cases with and without cell death and metabolite inhibition.

For the case of no cell death and no metabolite inhibition, comparing Tables 6 and 7 shows that fed-batch and perfusion bioreactors systems achieve similar product titres, yields, and % wasted substrate throughout the bioreaction time. While fed-batch is known for producing high titres, perfusion can achieve comparable titres when operated under the same conditions. A key advantage of perfusion is its significantly higher productivity, especially as the bioreaction time increases. Comparing Tables 6 and 7 shows that the productivities are similar at 48 h, however perfusion attains productivities that are 12 times and 137 times that of fed-batch at 240 h and 960 h, respectively. This is achieved through the higher cell concentrations enabled by cell retention and continuous operation, and also due to available working volume not being in use all of the time in fed-batch mode. This increased productivity reduces the required bioreactor volume, physical footprint, and capital costs. However, excessively high cell concentrations in perfusion may require cell bleeding, which can reduce productivity and yield. Perfusion also offers much shorter product residence times but only after a bioreaction time at around 150 h, with perfusion residence times being about 18 % and 10 % of fed-batch at 240 h and 960 h, respectively, which can benefit the production of unstable products. Furthermore, Fitzpatrick et al. [30] showed that residence time in perfusion can be further reduced by lowering S_m , although this comes at the expense of lower titre, yield, and increased substrate wastage.

For the case of cell death and metabolite inhibition, comparing Tables 6 and 7 shows that fed-batch and perfusion bioreactors systems achieve similar product titres, yields, and % wasted substrate throughout the bioreaction time. These values evolve more slowly but will eventually attain values similar to with no cell death and no metabolite inhibition. The major impact of cell death and metabolite inhibition is on the cell concentrations which are much lower for both systems in comparison to the case of no cell death and no metabolite inhibition. This is mainly due to the effect of cell death, but metabolite inhibition slows the evolution of cell concentration over time. This has a greater impact on perfusion than fed-batch, where the ratio of cell concentration in perfusion to fed-batch is lower for the case of cell death and metabolite inhibition throughout the bioreaction time (e.g. ratio of 5.3 vs 2.6 at 480 h). This major impact on cell concentration has a consequential impact on reducing the productivity for both systems, as shown in Tables 6 and 7. Perfusion still has a major productivity advantage over fed-batch, being 2 and 6 times greater at 240 h and 960 h, respectively, but this advantage is a lot less than for the case of no cell death and no metabolite inhibition presented above. Likewise, the much-reduced cell concentrations also impact on product residence times, with much higher product residence times for both systems, as seen in Tables 6 and 7. Here the shorter product residence times associated with perfusion occur after a later bioreaction time of 240 h, with perfusion residence times being about 30 % and 20 % of fed-batch at 480 h and 960 h, respectively. Overall, the comparative advantages of perfusion over fed-batch in terms of increased productivity and reduced product residence times still remains for the case of cell death and metabolite inhibition, however these advantages are reduced.

4 Conclusions

This study shows the effects of cell death and metabolite inhibition are very important and need to be included in the comparison of the bioreactor performances. Cell death and metabolite inhibition both impact on cell growth and the evolution of the viable cell concentration over time with three scenarios being observed for both cell death and metabolite inhibition. The major impacts of cell death and metabolite inhibition are the potential for

significantly reducing productivity and increasing t_{res} , as can be seen from comparing Tables 2 and 3 for the effect of cell death for perfusion and fed-batch, respectively. Cell death can have a major impact on reducing the viable cell concentration, with a consequential major reduction in productivity of perfusion bioreactors. The effect on the productivity of fed-batch bioreactors appears to be much smaller than on perfusion, however this is misleading (as mentioned earlier), because cell death slows the bioreaction which can majorly reduce the rate of product production for fed-batch. The effect of cell death on productivity is possibly more impactful on the perfusion bioreactor because of its inherent ability to operate at higher viable cell concentrations leading to a greater reduction in viable cell concentration. Even with the inclusion of cell death and metabolite inhibition, perfusion still has an advantage over fed-batch in terms of higher productivities and shorter t_{res} . For example, from comparing Tables 2 and 3, the productivity of perfusion for $k_d = 0.01 \text{ h}^{-1}$ is still 16 times greater than that for fed-batch.

Higher values of S_m results in higher metabolite concentrations, which increases the potential for metabolite toxicity and metabolite inhibition, which impact negatively on bioreactor performance. Consequently, there may exist an optimal S_m value that balances the benefits of higher S_m and disadvantages of metabolite toxicity and inhibition so as to provide a superior overall bioreactor performance. In some bioreactions, it may be required to prevent the concentration of some metabolite exceeding a certain concentration. S_m can be used to achieve this as S_m influences the evolution of metabolite concentration over time. For a given operating substrate concentration in the bioreactor, there exists a limiting or upper value of S_m for both fed-batch and perfusion that can be applied to prevent the metabolite concentration exceeding a pre-defined value.

Bioreaction time is a key variable for perfusion and fed-batch operation. For perfusion, it is advantageous to operate the perfusion bioreactor over a longer period of time for the case of no cell death/no inhibition. However, for the case of cell death and metabolite inhibition, there exists a bioreaction time where productivity is maximised and mean product residence time is minimised. Furthermore, the implementation of cell control using a cell bleed allows the removal of cells, including dead cells, which allows the attainment of a steady-state viable cell concentration, which may improve bioreactor performance, especially at longer bioreaction times. For fed-batch, sufficient bioreaction time is required to increase titre, yield and productivity, however too long a time leads to a major progressive reduction in productivity, and possibly very long product residence times if there is significant cell death. The varying volume in fed-batch adds complication to the analysis that may result in misleading interpretations, in particular for the effects of cell death and metabolite inhibition on productivity. Consequently, it is advisable to include studies where the final bioreactor volume is kept constant. Overall for fed-batch, increased bioreaction time (or increased V_f/V_o ratio) results in a trade-off between having higher titre and larger final working volume on one hand and lower productivity and potentially longer product residence time on the other hand.

Overall, the study shows that perfusion tends to outperform fed-batch for the performance parameters considered in this study, achieving higher productivities and shorter mean product residence times. Furthermore, perfusion can obtain titres and yields comparable to fed-batch when operated at similar substrate concentrations. In the perfusion modelling in this study, cell separation is assumed to be occurring continuously throughout the bioreaction time, while cell separation occurs only after the end of the fed-batch operation. On one hand, it could be argued that this could be an advantage for perfusion because there is little or no time required for cell separation after the end of the bioreaction time. On the other hand, this cell separation could also be a major disadvantage for perfusion due to the potential for higher levels of shear stresses and turbulence associated with cell separation that may increase the specific cell death rate for perfusion and thus influence the bioreactor performance comparison.

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