

Modeling the Specific Glucose Consumption Rate for the Recombinant *E.coli* Bioprocesses Based on Aging-specific Growth Rate

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This study proposes the form of the physiological *E.coli* dependencies for the specific glucose consumption rate. It carries a two-fold purpose. The first one allows online estimation of the specific glucose consumption rate when the information on the cells' aging is accessible. The second relationship assesses that the glucose consumption is relative to the oxygen uptake rate content with no direct dependency on the biomass kinetics properties. Such portion of the oxygen uptake rate does not participate in biomass growth or maintenance-related state but instead increases the soluble by-product of metabolism. The latter can consist of extracellular metabolites, soluble proteins, or other by-products. The authors show that these solutes assumingly result in the corresponding boundary condition for the specific glucose consumption rate balance. The main novel idea of this work is the statement that the portion of oxygen uptake rate dedicated for soluble products does not depend on the biomass growth and maintenance but rather on average aging information. Moreover, the authors hypothesize that biomass yield from glucose can be treated as a function of the average aging information of the cell population by reusing the Monod term assumption. Finally, this study interconnects some of the co-authors' patent claims that directly link to the empirical law of the Luedeking-Piret.

1. Introduction

Historically the models of the specific glucose consumption rate of aerobic recombinant *E.coli* bioprocesses depended on the assumption that biopharmaceutical biosynthesis was a batch or fed-batch process (Varma et al., 1993; Galvanauskas et al., 2021a; Boecker et al., 2021). Therefore, the specific glucose consumption rate consisted of the maximal glucose consumption rate, biomass, glucose concentration, and target production expression. Recently, authors (Urniecezius et al., 2021) reiterated that the average age of the cells' population is as important as the specific growth rate when estimating the recombinant target product in the biopharmaceutical processes. This study combines the biomass cultivation knowledge using target product formation (Urniecezius, 2019a) and the novel idea that, instead of the biomass state variable, the average age of the cells' population and the specific growth rate (Survyla et al., 2021) must be reconsidered. Such development is essential for optimal harvesting technology in the design of continuous biosynthesis when the optimal average age of the cells' population, but not biomass concentration, is the crucial factor in the laboratory and pilot-scale industrial bioreactors. The relevant model of the specific glucose consumption rate can help manage microorganisms' cultivation conditions by selecting a rational supply of nutrient substrate to bioreactor to ensure interference-resistant and high-performance repeatable cultivations of recombinant *E.coli*.

2. Definition of Oxygen uptake rate

This study hypothesizes that the oxygen uptake rate *OUR* consists of a few parts that must be explicitly accounted for when dealing with the balance equations of biochemical conversions. Such treatment is essential because *OUR* could be used as a gain scheduling parameter in the adaptive control (Levisauskas et al., 2019). The suggest *OUR* portions entail

- the main *OUR* content dedicated for the biomass kinetics properties;
- the dilution effect content when *OUR* represents not the total oxygen uptake rate, but its concentration per volume unit;
- the secondary *OUR* content which does not occur in biomass kinetics but instead participates in the kinetics of soluble by-products. Specifically when glucose feed rate is not growth-limiting and particular glucose concentration is present in the nutrition medium.

The determination of the first two *OUR* items comes from the biomass kinetics, and the third one requires incorporating the glucose consumption rate's balance.

2.1 Biomass kinetics

This study uses two main fundamental models that disclose the states of the biomass growth and maintenance variables: the Luedeking-Piret relationship (Urnieszus, 2019b) and the biomass growth, biomass maintenance, and glucose kinetics related expressions from the patent publication in (Galvanauskas et al., 2021b). The former proposes the simplistic constitution of oxygen uptake rate appearing in two additive terms: biomass $X(t)$ growth $X'(t)$ and biomass maintenance

$$OUR(t) = \alpha \cdot X'(t) + \beta(t) \cdot X(t), \quad (1)$$

where stoichiometric coefficients (α for growth and β for maintenance) link biomass kinetics properties with *OUR*, and the specific growth rate $\mu(t)$ classically express the exponential growth model (Urnieszus et al. 2018)

$$X'(t) = \mu(t) \cdot X(t). \quad (2)$$

Taking into account the fact that Eq(2) represents the biomass concentration $X(t)$ in the broth volume. The supply of base, acid, phosphate, or substrate solutions to the vessel of the bioreactor leads to the dilution of the broth, and the growth model of the biomass becomes (Galvanauskas et al., 2021b)

$$X'(t) = \mu(t) \cdot X(t) - \frac{F(t)}{W(t)} \cdot X(t), \quad (3)$$

where the total feed rate $F(t)$ dilutes current broth's volume $W(t)$. If there is zero growth or idle total growth condition, the biomass concentration will decrease due to dilution Eq(3). To connect the specific growth rate $\mu(t)$ with the maintenance of the biomass and the specific glucose consumption $\sigma(t)$, the yield of biomass concerning glucose consumed Y_{xs} is essential (Galvanauskas et al., 2021b)

$$\mu(t) = (\sigma(t) - m(t)) \cdot Y_{xs}(t), \quad (4)$$

where the specific glucose maintenance term $m(t)$ and its effect on the yield of biomass (Y_{xs}) implies that the glucose consumed contains two portions: a bigger one for the growth and the other for preserving the biomass viability. The yield coefficient ($Y_{xs}(t)$) has a form of a time function (Lyubenova et al., 2020) because such a yield tends to decline due to biomass increase or when the culture population ages in batch or fed-batch cultivation. Insertion of Eq(4) into Eq(3) yields

$$X'(t) = \left(\sigma(t) \cdot Y_{xs}(t) - \left(m(t) \cdot Y_{xs}(t) + \frac{F(t)}{W(t)} \right) \right) \cdot X(t). \quad (5)$$

Expressing the biomass growth from Luedeking-Piret Eq(1) gives

$$X'(t) = \frac{OUR(t)}{\alpha} - \frac{\beta(t)}{\alpha} \cdot X(t) \quad (6)$$

and its form with biomass as a multiplier is

$$X'(t) = \left(\frac{OUR(t)}{\alpha \cdot X(t)} - \frac{\beta(t)}{\alpha} \right) \cdot X(t) \quad (7)$$

The analogy between Eq(5) and Eq(6) implicitly means that the following two formulas hold

$$\begin{cases} \frac{OUR(t)}{\alpha \cdot X(t)} = \sigma(t) \cdot Y_{xs}(t); \\ \frac{\beta(t)}{\alpha} = m(t) \cdot Y_{xs}(t) + \frac{F(t)}{W(t)}. \end{cases} \quad (8)$$

Substituting $\alpha \cdot m(t) \cdot Y_{xs}(t)$ and reshaping Eq(8) results in

$$\begin{cases} \sigma(t) \cdot X(t) = \frac{OUR(t)}{\alpha \cdot Y_{xs}(t)}; \\ \beta^*(t) \equiv \alpha \cdot m(t) \cdot Y_{xs}(t); \\ \beta(t) = \beta^*(t) + \alpha \cdot \frac{F(t)}{W(t)}. \end{cases} \quad (9)$$

Replacing the maintenance in Eq(1) with the new form of Eq(9) produces a different form of the Luedeking-Piret model as follows

$$OUR(t) - \alpha \cdot \frac{F(t)}{W(t)} = \alpha \cdot X'(t) + \beta^*(t) \cdot X(t), \quad (10)$$

The physical meaning of the dilution term in Eq(10) justifies the statement that the left side of Eq(10) represents the *OUR* signal as oxygen uptake rate per liter of the bioreactor medium. Meanwhile, Eq(1) assumingly postulates that the left side of the equation is the total oxygen uptake rate in, e.g., oxygen grams per hour. In other words, when there is no growth term in Eq(10), the total oxygen uptake rate matches the total maintenance for the viability of the biomass. The *OUR* "concentration" per liter (as in Eq(10)) will decrease/increase since the volume accumulation or reduction occurs. The former is true when feeding specific substrates, and the latter might result from the evaporation effect or sampling for analysis. Therefore, Eq(10)'s left side discloses the compensated *OUR* for sustaining the biomass kinetic properties.

Two relationships carry practical meaning in Eq(9):

- Stoichiometry coefficient ratio $\frac{\beta(t)}{\alpha}$ serves as tuning for online estimation of the specific growth rate ($\mu(t) = f(OUR(t), t)$), Survyla et al., 2021.
- The maintenance coefficients $\beta^*(t) \sim m(t)$ are proportional (as in Galvanauskas et al., 2021b), and both carry identical purposes through a yields multiplier ($\alpha \cdot Y_{xs}(t)$), which represents the physical dimension of oxygen mass divided by the multiplication of time and volume units, and mass of glucose consumed. A similar relationship involving the respiratory quotient served for kinetics equations by Lyubenova et al., 2020, pages 7-8.

2.2 Glucose consumption rate

To better understand the secondary effect of *OUR* relative to the soluble contents that do not directly participate in biomass kinetics properties, the balance definition for glucose consumption rate is necessary. The generic form of such a balance (Lyubenova et al., 2020; Galvanauskas et al., 2021b) is

$$s'(t) = -\sigma(t) \cdot X(t) - \frac{F(t)}{W(t)} \cdot s(t) + \frac{F_s(t)}{W(t)} \cdot s_f(t), \quad (11)$$

where the total feed rate per volume without glucose ($F(t)$) dilutes the broth, thus with a minus sign, and the total feed rate per volume which contains glucose concentration ($s_f(t)$) upsurges glucose concentration ($s(t)$) inside the broth. Insertion of glucose consumption term ($\sigma(t) \cdot X(t)$, Eq(9)) into Eq(11) yields

$$s'(t) = -\frac{OUR_X(t)}{\alpha \cdot Y_{xs}(t)} - \frac{F(t)}{W(t)} \cdot s(t) + \frac{F_s(t)}{W(t)} \cdot s_f(t). \quad (12)$$

The main reason why Eq(12) holds $OUR_X(t)$, but not original $OUR(t)$, as in Eq(10), is the fact that hypothetically there exists a portion of *OUR* that does not participate in the biomass-related kinetics properties but transactions with the kinetics of extracellular metabolites, soluble proteins, permeases, or by-products. In other words, the cell membranes serve as carriers or transporters of solutes into the culture broth (Pinu et al., 2018). Such metabolic pathways might cause excess glucose consumption and the draw for excess *OUR*. Not rarely, this effect is stimulated by higher glucose concentrations (Vergara et al., 2018), resulting in cell growth inhibition.

As long as the *OUR*-based additive term (Eq(12)) represents the biomass-related (due to $Y_{xs}(t)$) glucose consumption rate for biomass kinetics properties, there is one more missing term (denoted as $\sigma_{solutes}(t)$) in the balance of glucose concentration rate. Consequently, the total specific glucose consumption rate is a sum of the glucose consumption rate linked with biomass ($\sigma_X(t)$) and the other item $\sigma_{solutes}(t)$, as follows

$$\begin{cases} \sigma_X(t) = \frac{OUR_X(t)}{\alpha \cdot Y_{xs}(t)}; \\ \sigma(t) = \sigma_X(t) + \sigma_{solutes}(t). \end{cases} \quad (13)$$

However, *OUR* requires further resolution due to the assumption that oxygen has a different yield variable associated when this specific oxygen participates in the production of soluble by-products, in comparison with $\alpha \cdot Y_{xs}(t)$. Therefore, similarly to the specific glucose consumption rate, the original *OUR* has also a split

$$OUR(t) = OUR_X(t) + OUR_{solutes}(t), \quad (14)$$

where the first term ($OUR_X(t)$) represents the Luedeking-Piret shape as in Eq(10), and the second one ($OUR_{solutes}(t)$) is still to be considered further. Prior to continuing, the OUR yield from the solutes produced ($Y_{solutes,os}$) is a constant parameter and links to its specific glucose consumption rate through

$$\sigma_{solutes}(t) = \frac{OUR_{solutes}(t)}{Y_{solutes,os}}, \quad (15)$$

As the glucose concentration of the medium affects the oxidative metabolic pathway and the production rate of soluble by-products (Vergara et al., 2018), a compensated OUR formulation becomes then necessary

$$\begin{cases} OUR_{solutes}(t) = OUR(t) \cdot (1 - factor(s)); \\ OUR_X(t) = OUR(t) \cdot factor(s), \end{cases} \quad (16)$$

where exponential ratio function ($factor(s)$) embodies the dependency of the production of by-products on the glucose concentration in the broth. The reasons for such an outcome might not necessarily depend on the properties of the biomass (cell membranes) but might also link to the hydrophobicity of the solutes.

This study suggests a new limiting coefficient ($k_{OUR,s}$) to help resolve the OUR dilemma in the balance equations, which is

$$\begin{cases} factor(s) = \frac{k_{OUR,s}}{k_{OUR,s} + s(t) - s_{min}}, & s(t) \geq s_{min}; \\ factor(s) = 1, & 0 \leq s(t) < s_{min}, \end{cases} \quad (17)$$

where it is assumed that during the substrate feed, the glucose concentration of the medium could not fall below the minimum glucose concentration (s_{min}), i.e., $s(t) \geq s_{min}$. When there is no substrate feed negligible (for example, in batch stages of the bioprocess) or the feed's effect on solutes is negligible (for example, in the scenario of the growth limiting substrate feed, Urniezius et al., 2018), then the glucose concentration does not affect factoring function ($factor(s)$), and the expression $OUR_X(t) = OUR(t)$ is valid. Then both state equations Eq(10) and Eq(12) hold. Such a particular case of the Luedeking-Piret law explains why the precision of existing off-gas analysis equipment is acceptable when current biopharmaceutical manufacturers (Urniesius et al., 2019b) use them for growth-limiting substrate feeding profiles. Meanwhile, similar off-gas analysis hardly finds its application in control of batch bioprocesses or those that aim to express insoluble and soluble products. Based on Eq(16), the equation Eq(12) in a general scenario becomes

$$s'(t) = -\frac{OUR_X(t)}{\alpha \cdot Y_{xs}(t)} - \frac{OUR_{solutes}(t)}{Y_{solutes,os}} - \frac{F(t)}{W(t)} \cdot s(t) + \frac{F_s(t)}{W(t)} \cdot s_f(t). \quad (18)$$

Different researchers have stated that the biomass yield on the consumed glucose ($Y_{xs}(t)$) is time-dependent and tends to decrease over time in fed-batch processes (Lyubenova et al., 2020). This study presents the average age of the cell population ($Age(t)$) which serves as the "inhibition" term on the biomass yield from the glucose $Y_{xs}(t)$. Thus this yield can be treated as $Y_{xs}(Age) \equiv f(Age(t))$. Such assumption is more consistent to describe the upstream continuous or perfusion bioprocesses when the biomass does not change, but its age varies. Then the limiting term on the biomass yield is

$$Y_{xs}(Age) = \frac{k_{Age} \cdot Y_{xs}}{k_{Age} + Age(t)}, \quad (19)$$

where the constant yield term (Y_{xs}) can be treated as the initial or the logarithmic phase biomass yield, which later, as the average cell population ages, "inhibits" the biomass yield; and the average age has a candidate definition in Urniezius et al., 2021. Finally, the generic formulations, including the shape of more generic Luedeking-Piret model, to be used for soft sensors development that accounts for the production of solutes for bioprocesses with non-growth-limiting substrate feeding become

$$\begin{cases} s'(t) = -OUR(t) \cdot \left(\frac{factor(s)}{\alpha \cdot Y_{xs}(Age)} + \frac{1 - factor(s)}{Y_{solutes,os}} \right) - \frac{F(t)}{W(t)} \cdot s(t) + \frac{F_s(t)}{W(t)} \cdot s_f(t); \\ OUR(t) \cdot factor(s) - \alpha \cdot \frac{F(t)}{W(t)} = \alpha \cdot X'(t) + \beta^*(t) \cdot X(t). \end{cases} \quad (20)$$

There are still two additional terms to consider when considering the biosynthesis of such soluble products as acetates or biomass growth inhibition due to high glucose concentrations that make glucose concentration practically serve as a preservative. Then the expression of both soluble and insoluble products (e.g., in the

inclusion bodies in the case of the recombinant protein, Urniezius et al., 2019b) might cause excess oxygen uptake rate, resulting in additional glucose consumption. For this, the maintenance component was designed as a function of glucose concentration

$$m(s) = \left(k_m + \frac{k_{ms} \cdot s(t)}{k_s + s(t)} \right) \cdot \frac{k_{ys}}{k_{ys} + s(t)} \quad (21)$$

where the maximum specific glucose consumption rate (per biomass unit) for insoluble and soluble by-products is k_{ms} and its expression factor is k_s ; the limiting factor (k_{ys}) shows at what concentration the glucose starts acting as a preservative. Therefore, the inequality ($k_s \ll k_{ys}$) holds by definition.

3. Experimental verification

There were three main hypotheses verified with hybrid (empirical and gradient-based) fitting experimental data to the minimization of the empirically selected criterion on $n=12$ experiments from two R&D sites

$$S = \sum_{i=0}^{n-1} \frac{\sum_{j=0}^{n_i-1} \left(MAE_{x,i} \cdot \frac{t_i - t_0}{2} \cdot 30 + MAE_{s,i} \right)}{n_i}, \quad (22)$$

where the total number of samples (n_i) for each site was 25 instances on average; each site had three limited growth and three non-growth-limiting substrate feed experiments; and MAE for both biomass and glucose concentrations had the same definitions as in Urniezius et al., 2021. The two sites had the strains *E. coli* BL21 (DE3) pET21-IFN-alfa-5, as in Survyla et al., 2021; and *Escherichia coli* BL21(DE3) pETM-11+EGFP, as in Dümmler et al., 2005. The three competing hypotheses were:

1. there is no excess OUR for soluble products, i.e. $OUR_x(t) = OUR(t)$ holds for all experiments;
2. the biomass yield $Y_{xs}(t)$ is a time-dependent function with a maximum scaler (among subsequent estimation steps) of 1.3 or 1.0 to mimic a scenario similar to assumptions in Lyubenova et al., 2020;
3. the biomass yield ($Y_{xs}(Age)$) is age-dependent function.

The upper row in a cell of Table 1 represents the first site's estimated parameter value, and the lower entry displays the second site's information. A single entry carries identical values for both sites' strains.

Table 1: The results of model fitting on experimental data for three hypotheses

Hypothesis	S, as in Eq(22)	Sum of $MAE_{x,i}$ averages	α	k_m	k_{ms}	k_s	k_{ys}	$Y_{solute,os}$	k_{Age}	Y_{xs}
3.1	97.6	2.781	1.15	0.00143	0.3044 0.1293	0.1969 0.2851	39.7 3.96	-	3.586 301.7	0.915 0.447
3.2	85.49	2.407	1.168	0.00132	0.1196 0.1037	0.2351 0.4497	33.66 3.43	4.86 9.32	-	0.931 0.44
3.3	84.69	2.367	1.137	0.00158	0.1384 0.1385	0.1663 0.1764	38.15 2.64	4.91 9.34	1.711 300.2	0.9 0.44

The table shows that the emphasis of the optimization was put on verifying the claims on OUR and biomass kinetics properties, less on glucose concentration. It shows no significant difference whether biomass yield (Y_{xs}) is a function of time or cell population age. The assumption on oxygen uptake rate ($OUR_x(t) < OUR(t)$) allows improving the estimation by 17%.

4. Conclusions

This study introduces a novel improvement in the understanding of the Luedeking-Piret dependencies. The oxygen uptake rate portion dedicated for soluble by-products that cause excess glucose consumption allows improving the biomass model fitting results by 17%. Moreover, the authors show no significant difference in whether biomass yield is assumed to be a dynamic function of time or a direct dependency on cell population age, which potentially adds more observability to an upstream developer. This work's findings might illuminate why off-gas tools and parametric models slowly gain momentum in industrial production. Batch bioprocesses usually depend on excess glucose concentrations for soluble metabolic products. Therefore, to use available equipment, the Luedeking-Piret form should be generalized when taking benefit of kinetics balances.

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