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Thermodynamic Evaluation of Dual Substrate Growth

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ABSTRACT

Various C_1–C_2 compounds are increasingly available through electrochemical reduction of CO_2. Although not always suitable as a sole substrate, these compounds can supplement a primary substrate like glucose to enhance microbial growth. Yet, the mechanisms underlying the effects of dual substrate consumption on growth rate and growth yield remain poorly understood. We developed a generalized, species-agnostic thermodynamic framework to partition anabolic and catabolic fluxes for various glucose/secondary substrate combinations, predicting maximum growth rate and growth yield as a function of the substrate ratio. The optimal strategy is to use the secondary substrate as electron donor, conserving the most efficient carbon source, glucose, for assimilation. Because many substrates yield similar energy per electron, biomass yield remains constant until glucose becomes limiting for anabolism. When further lowering the glucose fraction, additional assimilation of the auxiliary carbon source reduces the yield. The growth rate follows similar trends. Dual substrate growth enables generalists to produce more biomass from the total resource pool than a combination of specialists, conferring a competitive edge under substrate-limiting conditions. These theoretical observations align with experimental observations of lower residual substrate concentrations and dominance of generalists in natural and engineered oligotrophic environments.

1 | Introduction

The electrochemical reduction of CO_2 to one- and two-carbon (C1, C2) compounds such as formate, methanol, acetate, and ethanol (Hu et al. 2019; Jhong et al. 2013) using green energy has been proposed to contribute to the mitigation of anthropogenic CO_2 emissions, providing means to store renewable energy. While most CO_2-derived C1 and C2 compounds are not directly applicable as fuels or chemical building blocks, they can readily serve as additional electron donors to support microbial growth (Claassens et al. 2018, 2019; Hill et al. 2025; Liu et al. 2020). Understanding how microbes utilize these CO_2-derived compounds alongside traditional carbon sources such as glucose requires insight into the principles of substrate co-feeding and dual substrate metabolism.

Substrate co-feeding is a strategy that aims to enhance the product yield on a primary substrate by supplying a secondary—auxiliary—substrate as an additional energy source (Babel 2009; Babel et al. 1993; Gommers et al. 1988). Yet in theory, each substrate could serve as a carbon source that is used for biomass formation, as an electron donor in catabolism to provide energy, or both. Microorganisms differ in how they exploit various available resources: *specialists* metabolize only one substrate, whereas *generalists* can utilize multiple substrates, either sequentially or simultaneously (Bell and Bell 2021; Kuenen 1983). For generalists, simultaneous growth on two substrates requires coordinated catabolic and/or anabolic activity for each substrate, resulting in variable biomass yields and growth rates. However, the auxiliary substrate concept has primarily been explored in pure culture conditions, where the machinery for anabolic conversion of the secondary substrate was not naturally present and studies focussed on its energetic contribution (Harris et al. 2007; Liu et al. 2017; van Winden et al. 2022).

This unharnessed potential can be explored using continuous cultivation of mixed cultures. Dual or mixed substrate

metabolism is common under natural oligotrophic conditions (Egli 2010; Upton and Nedwell 1989; Yin et al. 2024), which are approximated by chemostat bioreactors. The advantage of dual substrate consumption has been hypothesized to be in metabolic flexibility (Bay et al. 2021; Chen et al. 2021; Gottschal et al. 1981; Stoakes et al. 2022), which can eliminate lag phases that would otherwise occur when a substrate becomes unavailable. Competition in continuous bioreactors generally depends on the maximum growth rate (μ_{max}) and affinity constant (K_s) (Kuenen 2019). Furthermore, it has been proposed that the individual growth yields for each substrate have a significant effect on microbial competition in dual substrate regimes (Gottschal and Thingstad 1982; Taylor and Williams 1975). The substrate feeding ratio has also been shown to impact the physiological behavior of substrate co-consumers (Wood and Kelly 1981). Despite these insights, the quantitative impact of substrate combinations and their feeding ratios on microbial growth rate and yield remains poorly characterized.

Recently, Golan et al. 2025, proposed an effective black-box thermodynamics-based model for dual substrate growth to address this gap. To describe *Escherichia coli* growth in batch conditions, carbon substrates were differentiated into only assimilable substrates, without a catabolic role, and substrates that can be both directly anabolized and catabolized. The model uses phenomenologically derived parameters specifically for *E. coli* and thus does not directly extend to the broader metabolic potential present in microbial communities. Alternatively, flux balance analysis (FBA) has also been employed for dual substrate metabolism for specific species (van Pelt-KleinJan et al. 2021). However, FBA requires complete knowledge of the metabolic network of the species. This information is generally unavailable a priori, specifically when exploring natural communities. For that same reason, cybernetic modeling approaches—which include regulatory decisions influencing enzyme levels and activity, and have been successfully explored since the eighties (Kompala et al. 1984; Ramakrishna et al. 1996; Turner et al. 1988)—are also unsuitable in this case.

To overcome these limitations in predicting key parameters for dual substrate growth in generalists, we developed a generalized, "species-agnostic" framework that captures both anabolic and catabolic contributions when co-consuming two substrates. We apply this framework to evaluate suitable substrate combinations, to characterize the limits of microbial growth during co-consumption and to identify the most probable metabolic flux distributions. This enables qualitative assessment of potential improvements in growth yield or rate from co-substrate utilization, including CO_2-derived substrates, which could inform more sustainable biotechnological processes. The analysis is carried out using a coarse-grained theoretical framework based on a defined linear system of energy and mass balances governed by four independent anabolic and catabolic stoichiometries.

2 | Computational Methods

The objective of the model created is to calculate the feasible stoichiometric space for aerobic microbial growth when using two carbon substrates simultaneously. In these conditions, it is assumed that the maximum growth rate is limited by the electron transfer capacity ($q_{e,CAT,max}$), which is set to 2 emol/Cmol X/h. This value is derived from the maximum respiration rate of 20 mmol O_2/gDW/h observed for various substrates during batch growth of *E. coli* (Andersen and von Meyenburg 1980). This maximum limit was proposed to emerge from the limited surface area of the microorganism, imposing a physical constraint on the number of electron transport chains that can be embedded in the membrane. This maximum respiration rate sets an upper limit on the number of electrons that can be transferred, even when substrate is in excess. Consequently, the total amount of substrate available is expressed in terms of electrons, and the two substrates are therefore referred to as electron donor 1 and 2 (eD_1 and eD_2). All possible substrate ratios are explored by varying f_{eD_1}, which is defined as the fraction of electrons derived from the primary substrate relative to the total. For each substrate composition the model returns (i) all feasible biomass yields; (ii) the corresponding substrate distribution among anabolism and catabolism; and (iii) the associated maximum growth rate. The required model inputs are the name, chemical composition, number of carbons, number of electrons, and degree of reduction of each substrate; the name and chemical composition of the nitrogen source for growth; and the pH.

To resolve all feasible metabolic stoichiometries capable of sustaining microbial growth, we define four variables that describe the distribution of substrates between anabolic and catabolic pathways in a dual substrate consumer. For both substrates eD_i we define a catabolic lambda ($\lambda^{CAT}_{eD_i}$, mol eD_i/mol X) and an anabolic one ($\lambda^{AN}_{eD_i}$, mol X/mol X). The catabolic lambdas specify the amount of substrate eD_i that is dissimilated through catabolism per mol biomass formed, whereas the anabolic lambdas indicate how much biomass is produced on each substrate eD_i.

It is assumed that all catabolic and anabolic pathways are independent of each other. To determine how the substrates are partitioned between these anabolic and catabolic processes for a given biomass yield and thus specific metabolic stoichiometry, we solve a linear system of four equations, calculating $\lambda^{CAT}_{eD_i}$ and $\lambda^{AN}_{eD_i}$ for each component i. These equations represent the overall mass and energy balances across a bioreactor system with variable substrate composition: (i) an energy balance for the overall metabolic activity, (ii) a mass balance for substrate eD_1, (iii) a mass balance for substrates eD_2, and (iv) a normalization constraint ensuring that one mole of biomass is produced. To set up this system of equations, first, all the catabolic and anabolic stoichiometries for both substrates are defined, followed by the calculation of their Gibbs free energy change to be used in the energy balance. Then, for each possible fraction of substrate available in the system (f_{eD_1} varying between 0 and 1 $e^-_{eD_1}/e^-_{total}$) the system of equations is solved for each biomass yield Y^{gen}_{X/e^-} in the range provided. This returns an array of solutions for each feed composition (f_{eD_1}), defining feasible flux distributions between anabolism and catabolism for both substrates ($\lambda^{CAT}_{eD_1}$, $\lambda^{AN}_{eD_1}$, $\lambda^{CAT}_{eD_2}$, and $\lambda^{AN}_{eD_2}$), each corresponding to a specific feasible biomass yield (Y^{gen}_{X/e^-}, mol X/mol $e_{tot}{}^-$). Only combinations of f_{eD_1} and Y^{gen}_{X/e^-} that return all non-negative lambdas are deemed feasible: the anabolic and catabolic reactions are assumed irreversible. This approach extends previous frameworks to predict single-substrate

stoichiometries (Heijnen 1991; Heijnen and Kleerebezem 2010). Finally, the maximum growth rate is calculated for each feasible stoichiometry found. Detailed explanation of the used equations and their solutions can be found in Supporting Information: S.I. A. The model itself is available online at https://github.com/Computational-Platform-IbM/Dual-substrate-thermo.

3 | Results

3.1 | Dual Substrate Use: From the Feasible Stoichiometric Solution Space to an Optimal Strategy

To exemplify the model results, we first calculated feasible metabolic activities and biomass yields (S.I. eq. 11) for two carbon substrates with distinct properties: glucose (eD1) and formate (eD2). We present the resulting biomass yields from all feasible stoichiometries obtained for different ratios of glucose and formate in Figure 1, ranging from $f_{eD1} = 0$ (no glucose) to $f_{eD1} = 1$ (only glucose). The obtained surface indicates that more than one biomass yield (y-axis, $Cmol_X/emol_{tot}$), and therefore more than one metabolic stoichiometry with different substrate distributions, is feasible for a specific feeding composition (x-axis, $emol_{eD1}/emol_{tot}$).

We can simplify the feasible stoichiometric space by depicting only those stoichiometries that will maximize growth, ignoring those that a priori result in suboptimal growth yields. The model uses a defined maximum capacity of electron transfer to constrain microbial growth in aerobic metabolic activities (Andersen and von Meyenburg 1980). Therefore, the stoichiometry that maximizes the growth yield at each feed composition, maximizes growth rate (μ_{max}) too (S.I. eq. 15). Figure 2 shows that until f_{eD1} is decreased to 0.70 $emol_{eD1}/emol_{tot}$, the maximum growth yield or growth rate calculated is seemingly independent of the fraction of carbon source fed. When taking a deeper look at the calculated lambda values, all formate is catabolized up until that point ($\lambda^{AN}_{eD_2} = 0$, $\lambda^{AN}_{eD_1} = 1$, Supporting Information: Figure S1, S.I. B). This increases the fraction of glucose that is assimilated compared to a glucose specialist, as less is required as an energy source in the catabolic activity. Comparing the ΔG^{01}_{CAT} of glucose and formate, it becomes clear why formate catabolism does not significantly aid the total biomass yield: the energy harvested per electron corresponds to −119.68 kJ/e^- for glucose versus −120.32 kJ/e^- for formate, a marginal difference. The increment in yield is therefore invisible in Figure 2. When lowering f_{eD1} further, glucose becomes limiting as a carbon source, and formate starts to be assimilated after that point. This decreases the yield and growth rate, as formate assimilation into new biomass requires significantly more energy than glucose: 335.26 kJ/e^- versus 8.79 kJ/e^-.

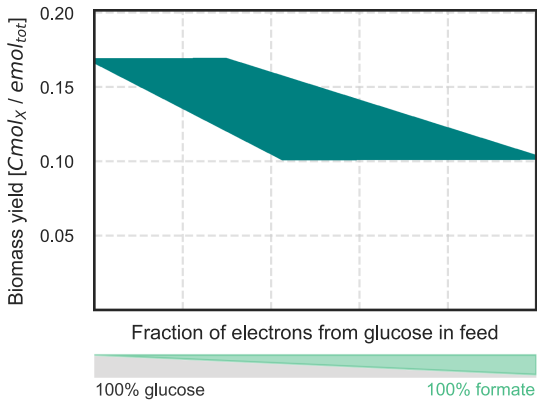

FIGURE 1 | The feasible stoichiometric space of growth on glucose and formate, showing the biomass yield as a function of the substrate ratio. Different feeding compositions (f_{eD1}) lead to various biomass yields ($Y_{X/S}$) and metabolic strategies. Only at $f_{eD1} = 0$ or $f_{eD1} = 1$ there is a single feasible biomass yield, as in the absence of either substrate the metabolism of the generalist is equal to that of a specialist.

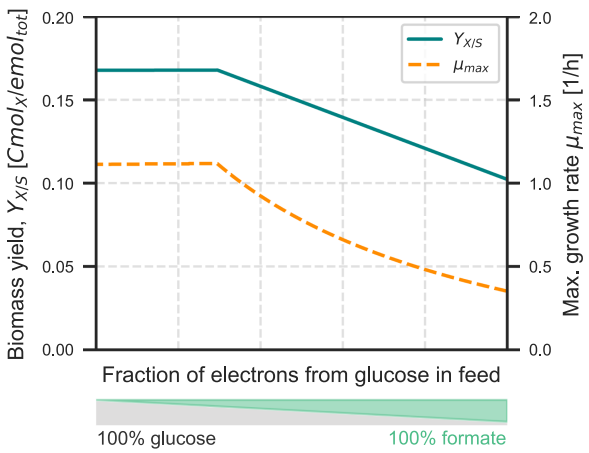

FIGURE 2 | The maximum feasible biomass yield ($Y_{X/S}$, teal) and corresponding maximum growth rate (μ_{max}, orange) at each total substrate composition (f_{eD1}). These values are derived from the growth rate maximization of all feasible stoichiometries as presented in Figure 1.

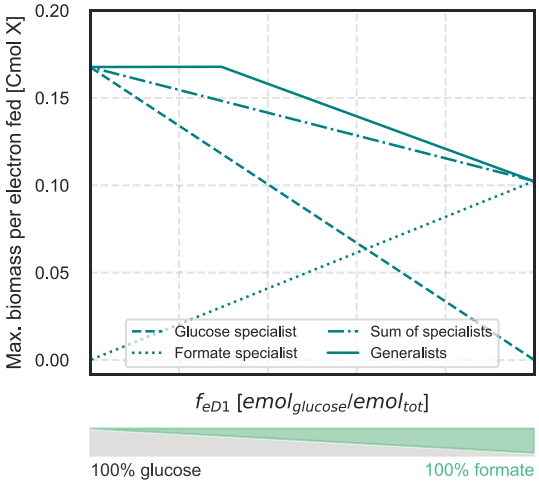

FIGURE 3 | The amount of biomass that can be formed per electron supplied for different functional groups at various mixtures (f_{eD1}) of glucose and formate. As f_{eD1} decreases from 1 to 0, the amount of biomass that can be produced solely on glucose (dashed line) decreases, and the amount of biomass produced on formate (dotted line) increases. The total amount of biomass produced by these two functional groups is indicated by the dash-dotted line, which follows a completely linear pattern. The biomass that a generalist can make on that same amount of total substrate (solid line) is always higher than for the two specialists combined.

3.2 | Biomass Formed per Substrate Available

The highest maximum growth rate and yield calculated for a generalist capable of simultaneously consuming glucose and formate are equal to the maximum growth rate and yield of a glucose specialist. Co-consuming both substrates, therefore, does not appear to provide an advantage. Still, the generalist does have higher substrate availability and might therefore be able to produce more biomass than two separate substrate specialists could produce in that same system. This was explored for a generalist with its stoichiometry dependent on f_{eD1} as shown in Figure 2. For the total range of possible feed compositions ($0 \leq f_{eD1} \leq 1$), the maximum biomass formed per electron fed to the system is presented in Figure 3. The dashed and dotted lines show the amount of biomass produced by a microorganism capable of only consuming one of the substrates (glucose or formate, respectively). The total amount of biomass generated by the two specialists would be as indicated by the dash-dotted line. A microorganism capable of simultaneously consuming both carbon sources would be able to form more biomass on the total substrate available than would be formed in total by the two specialist organisms (solid line, Figure 3). The feed composition at which the difference between the biomass produced by an optimized generalist and two specialists is at its maximum, is calculated to be $f_{eD1} = 0.705\ emol_{eD1}/emol_{tot}$, which was identified previously as the tipping point for an emerging disadvantage in biomass yield and growth rate resulting from dual substrate metabolism.

3.3 | Comparing Different Auxiliary Substrate Combinations

To explore which carbon substrates will act as a good secondary electron donor when co-fed with glucose, the ΔG^{01}_{CAT} values are presented per mole of electrons in Figure 4. This shows that many compounds release a ΔG^{01}_{CAT} of around 110 kJ/e$^-$ upon full oxidation to CO_2, like methanol, acetate, and ethanol (diagonally hatched red in Figure 4). Glucose itself (dotted yellow) and formate (diagonally hatched red) are already on the higher end of Gibbs free energy released in catabolism. The two compounds that release the most energy upon full oxidation in catabolism are CO and oxalate (cross-hatched gray), indicating that both are promising candidates as auxiliary substrates supplied alongside glucose. The addition of either substrate would, in theory, increase the yield and growth rate compared to glucose alone. The total energy input required for growth shows that all compounds except mannitol showed higher energy demands than glucose (Supporting Information: Figure S2), indicating they are not better carbon compounds than glucose for biomass formation.

The linear systems of equations for the flux distributions and corresponding biomass yield (S.I. eq. 11), and maximum growth rate (S.I. eq. 20) were solved for the combinations of glucose with the commonly CO_2-derived C1- and C2-substrates methanol, acetate, and ethanol. In addition, CO and oxalate were included, as they exhibited the two highest ΔG^{01}_{CAT} values among the substrates analyzed Figure 4. CO is an atmospheric trace gas and a common industrial waste gas. However, it is also

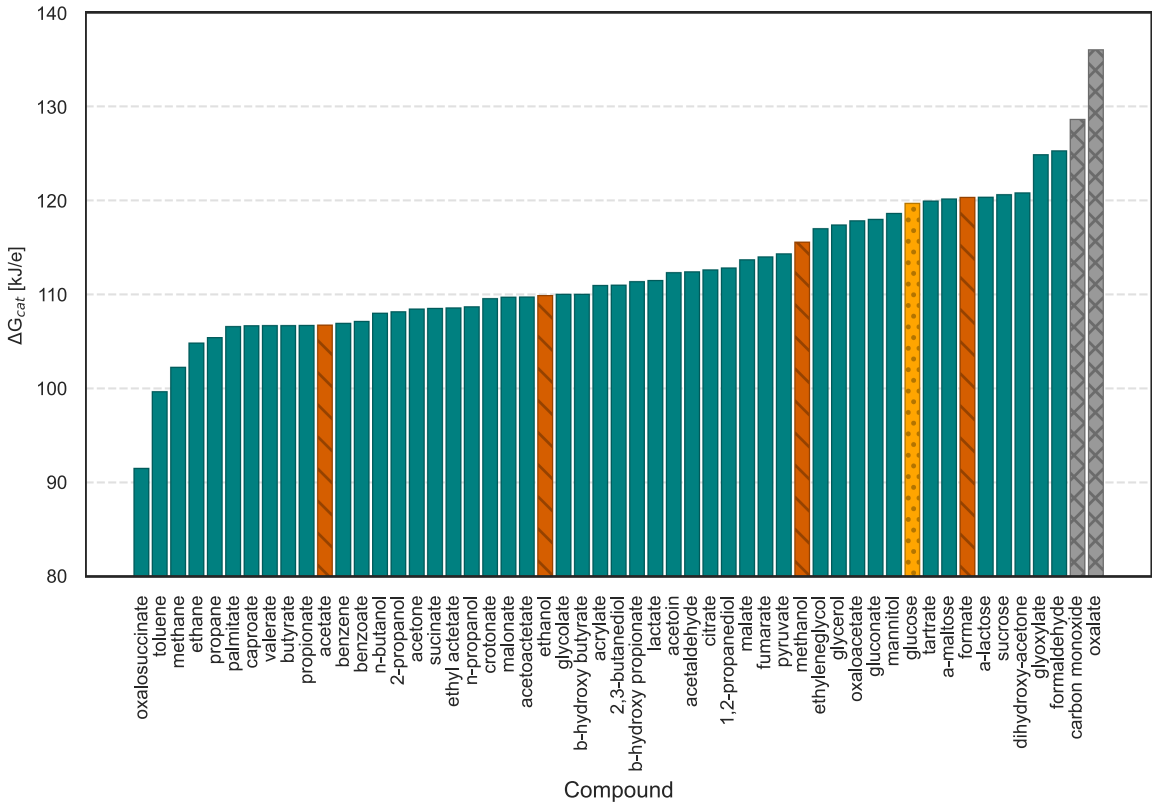

FIGURE 4 | The Gibbs free energy change in catabolism (ΔG^{01}_{CAT}, kJ/e) of different gaseous or liquid carbon substrates. Glucose is highlighted in dotted yellow. The common C1–C2 substrates formate, methanol, acetate and ethanol are highlighted in diagonally hatched red bars. The two substrates that show the highest ΔG^{01}_{CAT} of all compounds analyzed—carbon monoxide (CO) and oxalate—are highlighted in gray cross-hatched bars.

a common product from electrochemical CO_2 reduction that can be produced with high Faradaic efficiency (FE), whereas most of its other carbon products are generated with lower efficiency and selectivity (Birdja et al. 2019; Jiang et al. 2021). Although CO is considered a toxic gas for many microbes, it can efficiently be oxidized to CO_2 by the enzyme CO dehydrogenase (CODH), which is in fact considered a widespread "survival" strategy among aerobic heterotrophic bacteria (Bährle et al. 2023; Bay et al. 2021; Cordero et al. 2019; Dent et al. 2023). Oxalate—or its conjugate acid—on the other hand is regarded as a valuable platform chemical serving many industrial applications (Kumar et al. 2024; Mohammadi et al. 2024). It is generally produced through oxidation of carbohydrates or CO, but is receiving increasing interest as another potential product from electrochemical reduction of CO_2 (Boor et al. 2022; Brower et al. 2025; Sale et al. 2023). Oxalotrophy is—like CO oxidation—now believed to be a widespread and influential metabolic strategy among bacteria and fungi (Cowan et al. 2024; Grąz 2024; Hervé et al. 2016).

The analysis performed showed that supplying CO or oxalate as auxiliary substrate to glucose can indeed sustain higher biomass yield when a microorganism is able to co-utilize both substrates, compared to two microorganisms growing only on a single substrate at a time (Figure 5a,b). The solid lines in Figure 5 correspond to the maximum biomass calculated per electron supplied for a generalist growing on specific substrate mixtures of either carbon monoxide or oxaloacetate and glucose. The optimum CO-glucose mixture returning the highest yield is predicted at $f_{eD1} = 0.719$ $emol_{eD1}/emol_{tot}$ and at $f_{eD1} = 0.778$ $emol_{eD1}/emol_{tot}$ for glucose-oxalate. Contrary to formate (Figure 3), which has a similar ΔG^{01}_{CAT} per electron as glucose, these substrates might be able to increase the biomass yield per electron supplied compared to the yield on glucose alone, depending on the feed composition f_{eD1}. The feeding composition after which the yield per electron starts to decrease for each substrate ($f_{eD1,opt}$) is the point at which the generalist has the highest competitive advantage for growth, as it can maximize glucose assimilation. This relative competitive advantage varies between the considered auxiliary substrates (Figure 6).

4 | Discussion

The results presented show that the preferred metabolic strategy for substrate co-consumption is to use an alternative carbon source as an electron donor to save the anabolically most efficient carbon source—glucose—for assimilation. Initially, this strategy keeps the biomass yield approximately constant as many carbon substrates release similar amounts of energy per electron; 55% of the substrates analyzed release between −105 and −115 kJ e^{-1}. This explains why the graphs show little

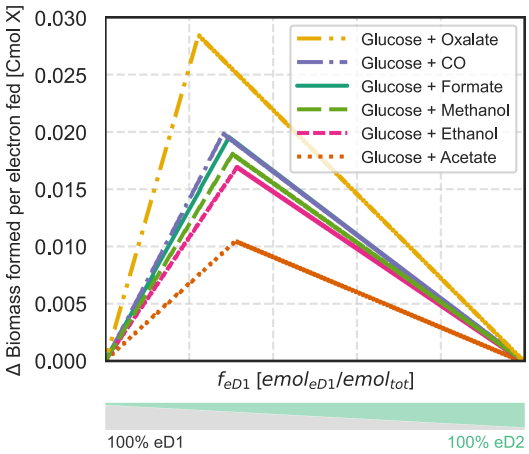

FIGURE 6 | The difference in the maximum amount of biomass per electron fed that can be produced in a system by a dual substrate generalist compared to the two substrate specialists, for various substrates combined with glucose. From the highest to lowest amount of additional biomass that a generalist can produce compared to two substrate specialists combined: oxalate, carbon monoxide (CO), formate, methanol, ethanol, and acetate.

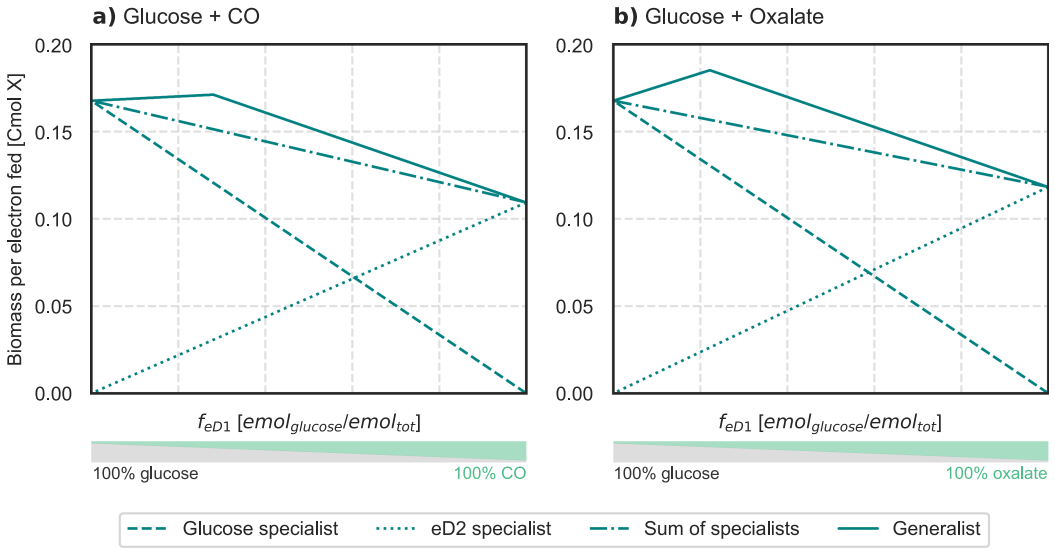

FIGURE 5 | The maximum biomass production on mixtures of glucose (eD1) with (a) carbon monoxide (CO) or (b) oxalate. It compares a generalist that co-utilizes both substrates (solid lines) with two specialist populations consuming one substrate each (dash-dotted lines), indicating the generalist can always form more biomass on the total substrate than the two specialists combined for these substrate combinations.

change in yield when first decreasing f_{eD1}. Only when glucose becomes a limiting carbon source for anabolism is assimilation of the auxiliary substrate predicted. At that point, the biomass yield decreases due to the higher energetic cost associated with assimilating the auxiliary substrate. Yet, from a biotechnological perspective, even partial substitution of glucose is valuable: the same amount of biomass could be generated on less glucose, reducing dependence on this water- and land-intensive substrate (Hanaki and Portugal-Pereira 2018), which lessens competition with food supply (Clomburg et al. 2017) and decreases environmental impact (Lovat et al. 2025).

From a thermodynamic perspective, dual substrate growth offers no direct advantage when both substrates have similar ΔG^{01}_{CAT} values. This is visible in our simulations: the biomass yield does not increase unless the auxiliary substrate provides significantly more energy per electron than glucose—such as oxalate, with its > 10% higher ΔG^{01}_{CAT} compared to glucose. Following the relationship between the biomass yield and the electron transfer capacity, the maximum growth rate is similarly affected by the difference in ΔG^{01}_{CAT} between the substrates. Indeed, previous studies have reported that the growth rate of an organism does not reach or increase beyond its μ_{max} on glucose when mixing glucose with various carbon substrates with similar or lower ΔG^{01}_{CAT} values (Hermsen et al. 2015; Narang et al. 1997a). Unfortunately, data on the effect of supplying CO or oxalate combined with glucose on the maximum growth rate is unavailable.

Despite the lack of a direct advantage in conventional growth parameters, the generalist strategy still confers a distinct benefit. This benefit has been hypothesized to be in metabolic flexibility, serving as a survival strategy in changing environments, removing any lag phase in the case that a preferred substrate disappears (Bay et al. 2021; Chen et al. 2021; Gottschal et al. 1981; Stoakes et al. 2022). However, our results point to a clear quantitative benefit: while the generalist's biomass yield per *total* substrate may be lower than for a glucose specialist, the generalist can access a broader combined resource pool and therefore generate more overall biomass under dual substrate conditions than two single substrate specialists could. When the yields are evaluated on a per-substrate basis rather than per total substrate, the generalist exhibits higher yields on each individual substrate compared to the corresponding specialists. Consequently, the generalist requires less of each substrate to sustain a given rate of biomass formation. While the yield per electron fed for a generalist growing on glucose and oxalate is only around 0.027 Cmol X higher than for the total yield for the sum of specialists, it does come to ~15% more biomass. This effect is not as large for the other substrates discussed, but the amount of biomass produced per glucose (or the secondary substrate) is much higher than for the specialist (Supporting Information: Figure S3). Gottschal and Thingstad already proposed in 1982 that these yields do have a significant effect on microbial competition (Gottschal and Thingstad 1982).

If this reduced substrate requirement can be translated into increased substrate affinity to sustain higher substrate uptake rates than specialists, then the generalist would be expected to outcompete specialists under low-substrate conditions. The latter has been confirmed by both field and laboratory evidence: dual or mixed substrate metabolism is common under natural oligotrophic conditions (Egli 2010; Upton and Nedwell 1989; Yin et al. 2024), and a specific case highlighting that a generalist strategy can prevail over two specialist species was published by Gottschal and Kuenen (1980b). They established enrichment cultures of generalists metabolizing acetate and thiosulfate in continuous cultures under dual substrate limitation, starting from freshwater samples. Importantly, various controlled experiments have shown lower residual substrate concentrations for dual substrate consumption, compared to single substrate growth (Brinkmann and Babel 1996; Kovárová-Kovar and Egli 1998; Lendenmann et al. 1996; Sjöstedt et al. 2022), and additional studies have indicated that combining various substrates lowers the utilization threshold concentrations (Alexander 1999; Egli 2010). These residual concentrations can become so low that single-substrate consumers are no longer able to sustain themselves, thereby conferring a decisive competitive advantage to generalists under mixed-substrate limitation. However, it remains unclear whether this is a result of an increased μ_{max}, decreased K_s, or other mechanisms, because the kinetic basis of dual substrate competition is still poorly understood. Numerous formulations of dual substrate Monod kinetics have been proposed, ranging from purely additive to multiplicative and interaction-based forms (Bell 1980; Gottschal and Thingstad 1982; Lee and Zhang 2016; Lendenmann and Egli 1998; Liu et al. 2020; Megee III et al. 1972, among others). Several of these models implicitly grant generalists a direct kinetic advantage, but the mechanistic origin of such advantages remains unresolved. As a result, it remains challenging to translate observations of competitive dominance of generalists under dual substrate limitation into mechanistically grounded and predictive kinetic models.

4.1 | Qualitative Accordance With Literature Reported Yields

The predicted biomass yields for single-substrate growth were compared with various literature experiments to evaluate the accuracy of the predictions. Although a subset of values deviates by more than 20% from the predictions (Supporting Information: Table S1), most data is in good agreement with the predictions. When the equation to determine the energy dissipation of any carbon source was first proposed by Heijnen, an average of 19% deviation between the predicted yield on a substrate and the measured yields of various species was already stated (Heijnen 1991). Additional data follows the same trends, confirming that this empirical approximation still serves as a relatively reliable parameter to obtain a first estimate for the biomass yield.

The yield predictions for dual substrate metabolism were also compared with literature values, which are limited to studies involving glucose and formate. Bruinenberg et al. reported a maximum yield increase from 0.5 $gDW/g_{glucose}$ to 0.69 $g_X/g_{glucose}$ in *Candida utilis* at a formate-to-glucose ratio of 3.3 mol/mol followed by a plateau before decreasing at higher ratios: while additional formate was still consumed, the biomass produced remained initially constant and then dipped (Bruinenberg et al. 1985). Van Winden et al. reported a linear increase from 0.50 ± 0.02 to 0.60 ± 0.01 $g_X/g_{glucose}$ until a formate-to-glucose ratio of 5 for *Yarrowia lipolytica,* and then no further effect on the yield was observed(van Winden et al. 2022). These values translate to a reported yield of 0.15 $Cmol_X/emol_{tot}$ on glucose

alone, which slightly decreased to 0.143 $Cmol_X/emol_{tot}$ at f_{eD1} = 0.78 $emol_{eD1}/emol_{tot}$ for *C. utilis* and to 0.148 $Cmol_X/emol_{tot}$ at f_{eD1} = 0.71 $emol_{eD1}/emol_{tot}$ for *Y. lipolytica*. Considering the reported plateau and decrease in the biomass yield on glucose (disregarding the formate consumed), this translates to a steep drop in the biomass per total electrons consumed. Figure 2 showed the model predicting a stable biomass yield of ~0.17 $Cmol_X/emol_{tot}$ on glucose and formate for $f_{eD1} > 0.70$ $emol_{eD1}/emol_{tot}$, indicating a similar trend as reported for *Y. lipolytica*. In contrast, *C. utilis* reportedly shows stable yields on glucose and formate for $f_{eD1} > 0.78$ $emol_{eD1}/emol_{tot}$—a difference that could reflect interspecies variations in formate dehydrogenase (FDH) localization, enzyme cofactor specificity, or the efficiency of mitochondrial nicotinamide adenine dinucleotide shuttles, all of which can influence overall oxidation efficiency.

4.2 | Limitations to Energetic Quantifications and Growth Predictions

An underlying assumption of the model is that anabolic and catabolic pathways of each substrate operate independently, reflected in the separate calculations of each ΔG^{01}_{CAT}, ΔG^{01}_{AN}, and ΔG_{Dis}. This assumption can be violated by regulatory interactions such as carbon catabolite repression (CCR), where the presence of a particular carbon source inhibits the expression of the enzymes required to consume an alternative substrate (Brückner 2002; Deutscher 2008). This may be specifically relevant when co-feeding formate or methanol. FDH, with a catabolic role for both substrates, has been reported to be increasingly repressed for pure cultures in chemostats at higher dilution rates, likely due to higher residual glucose levels (Egli et al. 1980, 1982), resulting in incomplete substrate oxidation. Likewise, ribulosebisphosphate carboxylase/oxygenase (RuBisCO), which assimilates CO_2 derived from methanol and formate in the Calvin cycle, is often, but not always (Dijkhuizen and Harder 1984), reported to exhibit partial to full repression in the presence of a secondary substrate under varying specific experimental conditions (Dijkhuizen and Harder 1979a, 1979b; Gottschal and Kuenen 1980a). In these cases, the metabolism predicted by the model may not occur. In addition, incomplete substrate oxidation due to overflow metabolism (Chowdhury et al. 2025) is also expected to lead to deviations between predicted and observed energetics. Alternatively, synergetic effects can arise due to co-feeding, such as an increased μ_{max} (Dijkhuizen and Harder 1979a; Egli et al. 1986; Hermsen et al. 2015; Joshua et al. 2011; Lameiras et al. 2018; Lendenmann et al. 1996; Narang et al. 1997b) or a reduced ATP demand leading to an increased yield (Aon and Cortassa 1991; Páez-Watson et al. 2025; Park et al. 2019). These effects are generally hypothesized to be a result of the omission of rate-limiting steps through a bypass generated by co-substrate assimilation (Liu et al. 2020; Ramakrishna et al. 1996; Wang et al. 2019).

4.3 | Process Yield Considering Co-Substrate Production By Electrochemical Reduction of CO_2

Beyond the thermodynamic limits that the model sets on the biological yield, the overall yield of the process is further constrained by the efficiency with which these auxiliary substrates are produced electrochemically. Although electrochemical production of formate, methanol, acetate, ethanol, CO, and oxalate can occur under milder conditions than the alternative hydrogenation processes, which typically require both high temperature and high pressure (Artz et al. 2018), it still requires substantial energetic input (Leonzio et al. 2024; Vega et al. 2024). Therefore, the FE, % with which the auxiliary substrate is generated can be considered to calculate a true yield per electron to account for energetic losses prior to cultivation. Birdja et al. composed an overview of the Faradaic efficiencies of

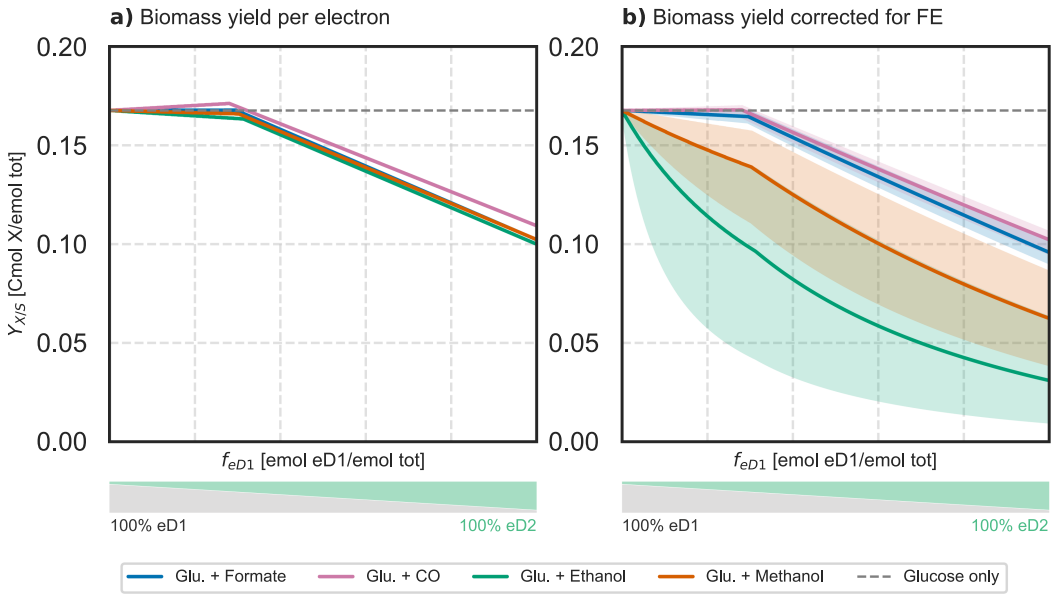

FIGURE 7 | Biomass yield per electron in dual substrate growth when unaccounted (left) and accounted (right) for the Faradaic efficiency (FE) of the electrochemical production of the auxiliary substrate. The lines on the right indicate the yield corrected with the average reported FE value by Birdja et al. (2019), while the shaded areas around this indicate the potential yields considering the minimum and maximum FE values reported. Ethanol and methanol show large ranges of biomass yield, as they contain a higher number of electrons. As formate and carbon monoxide (CO) have high FE (> 90%) and both contain two electrons, there is limited deviation between the biomass yield when accounting and not accounting for the FE.

electrochemical conversion of CO_2 into methanol, ethanol, formate, and carbon monoxide using highly selective metal(-derived) catalysts in 2019, which was used to recalculate the yields for dual substrate growth (S.I. C). More recent FE values are within similar range (Fan et al. 2020; Kaliyaperumal et al. 2023; Leonzio et al. 2024; Sajna et al. 2023; Wiranarongkorn et al. 2023).

Considering the low FE of ethanol and methanol production, the biomass yield of the process significantly decreases when taking this into account due to their high electron density (Figure 7). Only formate and CO show similar yields compared to the growth on glucose alone (dashed line), as their FE are reported to reach ~95%. Nevertheless, it should be noted that bioprocess design is best guided not only by microbial or process yield, but also by a range of additional factors that shape the environmental and techno-economic impact of the system (Artz et al. 2018; Birdja et al. 2019; Lai et al. 2022; Leonzio et al. 2024; Memon et al. 2025; Sajna et al. 2023; Somoza-Tornos et al. 2021; Tang et al. 2025; Vega et al. 2024).

5 | Conclusions

The coarse-grained thermodynamic design of the model presented enables analysis of co-substrate utilization across a wide range of different carbon components, providing a generalized, species-agnostic framework for predicting metabolic behavior as a function of substrate ratio. By focusing on fundamental thermodynamic and stoichiometric constraints, it establishes clear upper boundaries on growth rate and biomass yield. This offers a baseline for interpreting experimental deviations, arising from kinetic regulations or synergistic co-feeding. The strategy that maximizes microbial biomass yield per electron prioritizes the secondary substrate as electron donor, thereby conserving the most efficient anabolic substrate, glucose, for assimilation. This approach maintains optimal growth rate and growth yield when the secondary substrate provides at least as much Gibbs free energy per electron as glucose oxidation, such as for formate, carbon monoxide, and oxalate. For most substrate combinations with glucose, there seems to be no direct advantage in growth rate or overall growth yield. However, dual substrate growth allows generalists to produce more total biomass from a given resource pool than two specialists could generate together. This suggests that any competitive advantage arises from differences in substrate consumption rates, highlighting the need for improved, mechanistically grounded kinetic descriptions of dual substrate consumption.

Author Contributions

Marit A. Verheijen: conceptualization, methodology, investigation, writing – original draft, review and editing. **Tim Meyboom:** methodology, investigation. **Mark C.M. van Loosdrecht:** supervision, writing – review and editing. **Rebeca Gonzalez-Cabaleiro:** conceptualization (lead), methodology, supervision, funding, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in GitHub at https://github.com/Computational-Platform-IbM/Dual-substrate-thermo.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File: bit70217-sup-0001-Supplementary_Information_revised.docx.