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Multi-Objective Optimization of Biomass and Riboflavin Production Using Dynamic Flux Balance Analysis: A Study of *Methylocystis Hirsuta*

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Abstract **-** In this study, we investigate the discrepancies between the dynamic flux balance analysis model and experimental data regarding the growth of the microorganism *Methylocystis hirsuta*. While the model accurately predicts substrate uptakes, it tends to overestimate biomass production, resulting in significant deviations from observed growth outcomes. This comparison highlights the metabolic potential of this microorganism to produce other metabolites, particularly riboflavin during its growth phase, which may explain these discrepancies. The dynamic flux balance analysis model depends on genome-scale metabolic models, highlighting the need for careful selection of objective functions for calculating reaction fluxes and defining metabolic pathways. By focusing on maximizing both biomass and riboflavin production, we advocate for a multi-objective optimization approach. To address this, we employed Pareto analysis to assess the trade-offs between these two objectives, providing valuable insights into the optimal conditions for enhancing both biomass yield and riboflavin synthesis. Our findings emphasize the importance of developing refined modelling techniques that closely align with experimental results, ultimately aiding in the metabolic engineering studies and the design of more effective bioprocesses for microbial production systems.

Keywords**:** Dynamic Flux Balance Analysis - *Methylocystis hirsuta* - Multi-Objective Optimization - Pareto analysis

1. Introduction

Secondary metabolites are specialized chemical compounds synthesized by plants and microorganisms. These compounds are produced during the stationary phase of an organism's life cycle, which occurs after the growth phase. While secondary metabolites are not crucial for the growth of microbes, they provide significant advantages in their respective environments and fulfill various essential functions. Notably, these metabolites play roles in bacterial survival and ecological interactions. Additionally, these secondary metabolites hold significant economic and medicinal value [1].

The processes of uptake, secretion, and intracellular fluxes in microorganisms can be effectively modelled through computational approaches, predicated on the assumption that cellular systems operate at optimal efficiency to fulfil specific biological objectives via biochemical networks. This computational framework enables researchers to simulate and analyse microbial behaviour. Utilizing Flux Balance Analysis (FBA), it becomes possible to predict a metabolic network's capabilities and the distribution of fluxes within it. This method relies solely on the reaction stoichiometry of the network. However, since the linear system of mass balance equations in a steady state is under-determined, it is essential to establish suitable cellular functions (objectives) and apply additional constraints. These elements are crucial for obtaining a viable solution, ensuring a more accurate representation of microbial metabolic pathways [2]. FBA focuses on optimizing an objective (e.g. growth) by analysing primary metabolism but tends to neglect secondary metabolism and the production of other compounds, which can be significant for various applications. To address this limitation, researchers have developed multi objective optimization methods [1].

This research focuses on the conversion of methane into biodegradable polyhydroxybutyrate (PHB) using methanotrophic microorganisms. PHB as a bioplastic has the potential to significantly address two pressing environmental issues: global warming and plastic pollution. By preventing methane emissions, production of PHB could play a crucial role in mitigating climate change, as methane is a potent greenhouse gas. Additionally, its biodegradable properties mean that it can break down naturally over time, and by replacing traditional fossil fuel-based plastics with a biodegradable alternative, the accumulation of plastics in landfills and oceans will be reduced [3], [4]. This dual benefit not only supports a healthier planet but also paves the way for more sustainable practices in manufacturing and waste management which shows the importance of investigating this process.

The process of methane conversion to PHB unfolds in two main phases: the growth phase under nutrient-rich conditions and the PHB production phase under nutrient-deprivation conditions [3]. We specifically investigate the microbial metabolism of *Methylocystis hirsuta* (*M. hirsuta*) during the growth phase, aiming to convert methane into biomass in the first phase of conversion.

Our primary objective is to evaluate and compare two modelling approaches: the Monod model, which relies on existing experimental data, and the dynamic flux balance analysis (dFBA), a constrained-based model which offer a structured methodology to predict metabolic fluxes and understand how *M. hirsuta* utilizes methane for growth. This comparison is due to evaluate the accuracy of considering only maximization of growth objective in dFBA calculations.

Additionally, we enhance our analysis by employing a multi-objective optimization framework using FBA and create a Pareto chart, which represents the trade-offs between various objectives.

2. Materials and Methods

M. hirsuta is a type II methanotroph with remarkable capability of PHB accumulation (43-45% w/w). This aerobic microorganism utilizes methane as carbon and energy source and produces biomass under nutrient-rich condition. The study of *M. hirsuta* in this reseach is divided into two sections: the first section employs the dFBA modeling approach to analyze the conversion of methane into biomass, while also comparing the outcomes with a Monod-based model and experimental data [3]. The second section applies a multi-objective approach for FBA calculations, resulting in a Pareto chart to as visualization of the optimality trade-offs.

2.1. dFBA model

In a recent study, researchers developed a Monod model for converting methane to biomass using *M. hirsuta* in a controlled environment [3]. In the present study, for this setup and experimental data we employed a dFBA model to investigate the metabolic pathways within *M. hirsuta* during growth phase.

The dFBA model operates by formulating a linear programming problem (LP) that represents the steady-state stoichiometric relationships inherent in the metabolism of the microorganism. By doing so, we can calculate the intracellular fluxes that dictate metabolic activity. This LP problem due to its under-determined nature needs an objective function to reach a suitable solution. Furthermore, these exchange fluxes are integrated with the phenomenon of extracellular mass transfer, enabling us to assess the dynamic behaviour of the entire system [4].

Through this methodology, we aim to elucidate the differences between results of Monod and dFBA model to enhance our understanding of *M. hirsuta* metabolism, and to check the accuracy of using a growth objective in the LP problem.

The Monod model and experiments in [3] were conducted in a serum bottle reactor under aerobic conditions. The reactor had a total volume of 2.2 liters, with a liquid phase of 0.4 liters. The experiments were conducted at a constant temperature of 25°C, providing an ideal environment for microbial growth and metabolic activity. This study involved varying the headspace gas composition within the reactor. The researchers tested different molar ratios of oxygen (O_2) to methane (CH_4) (1:1, 1.5:1, and 2:1) [3].

The details of the implemented model will be explained in the following.

2.1.1. Governing Equations

The mass balance equations for biomass, gas phase compounds, and liquid phase compounds were described using ordinary differential equations (ODEs). The biomass equation is represented as Eq (1).

$$\frac{dX}{dt} = \nu_{s,g} X - k_{dec} X,\ X(0) = X_0 \tag{1}$$

|In this equation, $X\left(g\ DW/L\right)$ represents the concentration of biomass at any given time. The specific growth rate, denoted as $\nu_{s,g}\left(1/d\right)$ is the specific growth rate, $k_{dec}\,(d^{-1})$ [3] is the biomass decay rate, and $X_0\,(g\,DW/L)$ is initial biomass concentration for each experiment.

The equations for the gas phase compounds were formulated for $CH_4, O_2,$ and CO_2 as represented by Eq (2).

$$\varepsilon_G \frac{dC_{G,i}}{dt} = -\left(k_{L,i}a\left(C_{G,i}H_i^{cc} - C_{L,i}\right)\right), \qquad C_{G,i}(0) = C_{G,i,0} \tag{2}$$

In equation (2), ε_G is the gas fraction in the reactor, $C_{G,i}\left(mmol/L\right)$ is the concentration of the i-th gaseous component, $k_{L,i}a\left(d^{-1}\right)$ is the liquid mass transfer coefficient of the i-th component [3], H_i^{cc} is Henry's law constant [5], $C_{L,i}\left(mmol/L\right)$ is the concentration of the i-th dissolved component, $C_{G,i,0}\left(mmol/L\right)$ is the initial gas concentration in the serum bottle.

The equations of liquid phase compounds were formulated for CH_4, O_2 and CO_2 as represented by Eq (3).

$$\varepsilon_L \frac{dC_{L,i}}{dt} = \varepsilon_L \nu_i X + \left(k_{L,i}a\left(C_{G,i}H_i^{cc} - C_{L,i}\right)\right),\ C_{L,i}(0) = C_{L,i,0}, \tag{3}$$

In equation (3), ε_L is the liquid fraction in the reactor, ν_i is the uptake or production flux of dissolved components, and $C_{L,i,0}\left(mmol/L\right)$ is the initial concentration of the i-th dissolved component. In the dFBA modelling, ν_i and $\nu_{s,g}$ are calculated based on LP optimization of FBA.

2.1.2. FBA calculations

Metabolic modelling serves as a powerful tool for understanding the biochemical pathways and metabolic capabilities of organisms. In this study, a genome-scale metabolic model (GSMM) of *M. hirsuta* [6] is used for FBA calculations. To ensure the accuracy of these calculations, certain constraints are necessary to guarantee the reliability of the results [2]. In this model, the Monod equation is employed to calculate the constraint on the methane uptake flux [3]. This constraint is calculated using the Eq (4).

$$v_{CH_4-growth} = \left[\left(\frac{-1}{Y_{g,CH_4}}\right) \times \mu_{g,\,CH_4} \frac{C_{L,\,CH_4}}{K_{CH_4} + C_{L,\,CH_4}} \frac{C_{L,O_2}}{K_{O_2} + C_{L,O_2}}\right] \times \frac{10^3\ mmol\ CH_4}{64\ g\ COD} \times \frac{160\ g\ COD}{113\ g\ biomass} \tag{4}$$

In equation (4) $v_{CH_4-growth}\left(mmol/(g\,DW\,d)\right)$ is the constraint on methane uptake, $Y_{g,CH_4} = 0.14\ g\ COD/g\ COD$ is the yield of biomass on CH_4, $\mu_{g,\,CH_4} = 1.17 g\ COD/(g\ COD\ d)$ is the maximum growth rate on CH_4, $C_{L,\,CH_4}\left(mmol/L\right)$ is the dissolved CH_4 concentration, $C_{L,O_2}\left(mmol/L\right)$ is the dissolved O_2 concentration, $K_{CH_4}\left(0.079\ mmol/L\right)$ is the CH_4 affinity constant, and $K_{O_2} = 0.128\ mmol/L$ is the O_2 affinity constant [3] For the calculations of chemical oxygen demand (COD) biomass is assumed to have empirical formulas of $C_5H_7O_2N$ [3].

2.1.3. Computational Solution

The DFBAlab software [7], which is based on MATLAB, was used for solving the dFBA model. This software employs CPLEX, a high-performance optimization tool, to efficiently and accurately solve the LP problems derived from FBA. In DFBAlab, lexicographic optimization is applied to determine a unique optimal solution through a prioritized order of objectives. For the present model, the objectives are chosen as (1) maximizing biomass growth, (2) maximizing CO2 production flux, (3) maximizing methane uptake flux, and (4) maximizing oxygen uptake flux. The ordinary differential

equations (ODEs) derived from extracellular mass balance equations were integrated with the ode15s solver in MATLAB. For initial conditions, gas phase and biomass concentration data were taken from experimental data [3], while the liquid phase was assumed to be saturated with the gas components according to Henry's law.

2.2. Multi Objective Approach for FBA Calculations

According to the literature [8], certain species from the genera Methylocystis, Methylosinus, and Methylococcus have the capability to secrete flavins (riboflavin and flavin mononucleotide (FMN)) during their growth. This suggests that flavin secretion may be a common characteristic among methanotrophic bacteria. Considering this, we conducted an evaluation of biomass production and the trade-off with riboflavin secretion. To facilitate this assessment, we created a Pareto chart to analyze the relationship between biomass and flavin production in *M. hirsuta*.

To systematically assess this relationship, we employed a Pareto chart to analyze the optimality trade-offs between biomass and riboflavin production. For this purpose, we have conducted a bilevel optimization. The primary focus of this optimization was to maximize the biomass production flux, while the secondary objective targeted the optimization of riboflavin production flux. The optimization process begins with the calculation of the maximum (ω_1^{max}) and minimum (ω_1^{min}) values of the biomass for each methane uptake fluxes using GSMM. Subsequently, a series of P point solutions are derived by constraining the GSMM P-1 times, using Eqs (5) – (7) that govern the first objective. For each of $\omega_{1,n}$ values, a corresponding optimal value for $\omega_{2,n}$ (second objective) is calculated [1]. All calculations related to Flux Balance Analysis (FBA) in this study were done with CPLEX.

$$\omega_{1,n} = \omega_1^{max} - \frac{n \times \Delta}{P-1} \tag{5}$$

$$n = \{1,\ldots,P-1\} \tag{6}$$

$$\Delta = \omega_1^{max} - \omega_1^{min} \tag{7}$$

3. Results

In this study the performance of the dynamic Flux Balance Analysis (dFBA) model has been compared with the reimplemented Monod model [3] and experimental data [3], particularly focusing on the varying molar ratios of oxygen (O2) to methane (CH4). The specific ratios examined were 1:1, 1.5:1, and 2:1. The results of this comparative analysis are illustrated in Fig. 1, showcasing the distinct behaviours of these models under the specified conditions.

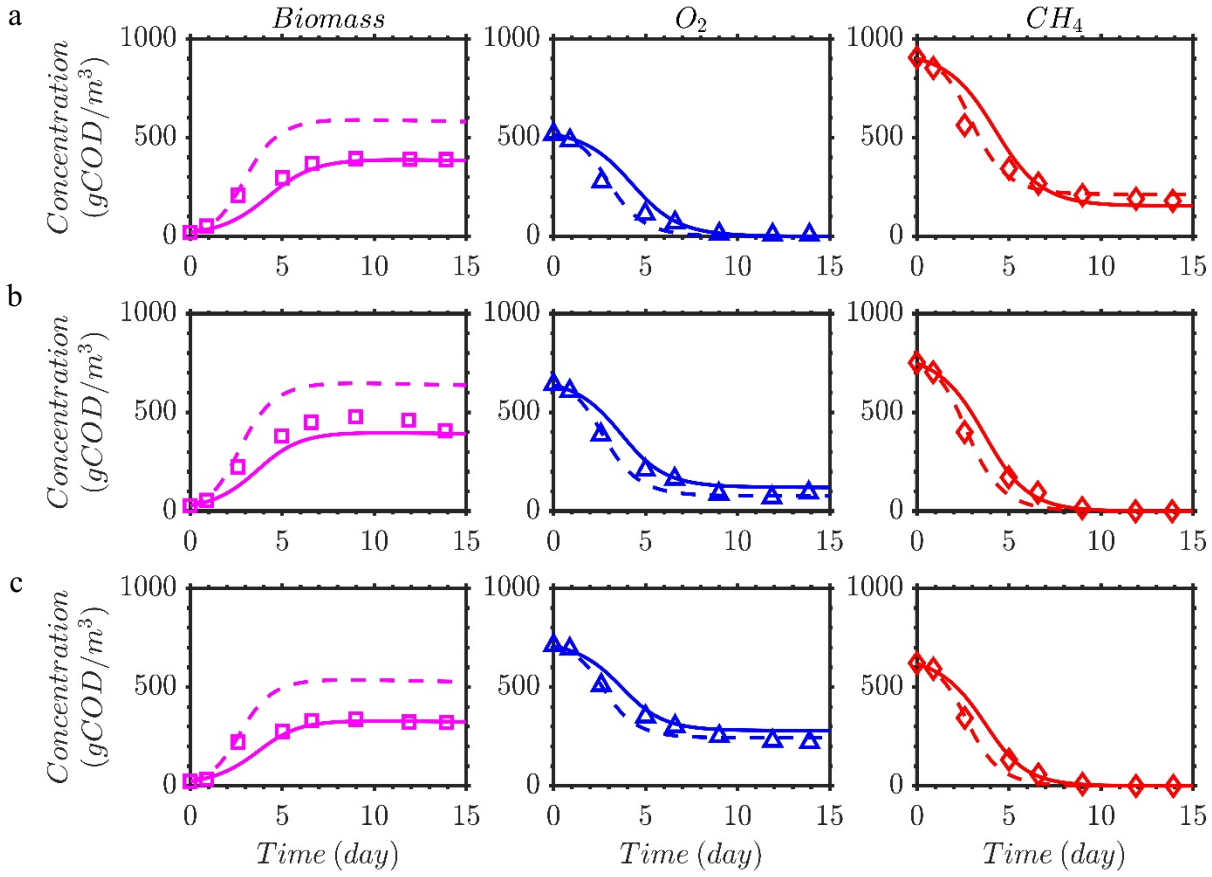

Fig. 1: The comparison of results of dFBA model (dashed), Monod model (line), and experimental data (biomass (□), O_2(△) , and CH_4(◊)): O_2 to CH_4 ratio of a) 1:1, b) 1.5:1, and c) 2:1

The findings indicate that the dFBA model predicts a significantly higher biomass production compared to both the experimental data and the results derived from the Monod model, despite showing almost similar uptake fluxes for CH4 and O2. Interestingly, while the uptake fluxes for CH4 and O2 were nearly similar across the models, the dFBA model displayed slightly lower concentrations of these substrates during the initial five days of the experiment. This can be attributed to the dFBA model's ability to yield more biomass and consequently its increased consumption rates of CH4 and O2 when nutrients are available. The reported yield of biomass on CH4 for the Monod model is 0.14 g COD/g COD[3], whereas the dFBA model achieved a higher yield of 0.18 g COD/g COD.

It is essential to acknowledge that the objectives of microorganisms extend beyond mere biomass production; methane can be converted into other compounds during metabolic processes. Supporting this notion, Balasubramanian et al. [8] highlighted a species from the Methylocystis genus that produces flavins throughout its growth phase. This study has revealed that cells secrete approximately twice the amount of flavins when they reach the stationary phase of growth. This study indicates a significant relationship between flavin secretion and the growth stage of the cells. Therefore, it can be concluded

from these results that the LP optimization of the FBA solution for this microorganism requires a multi-objective optimization approach.

For the analysis of multi-objective optimization concerning methane consumption, the study referenced [8] suggests that biomass and riboflavin are the primary objectives. Fig. 2 shows the results of this bi-level optimization for different methane uptake fluxes. Upon careful examination of the results from our comparative analysis, a significant trade-off emerges between the two objectives. The data clearly indicates that an increase in biomass production correlates with a decrease in riboflavin yield, and the production of riboflavin could explain the reduced yield of biomass in the Monod model.

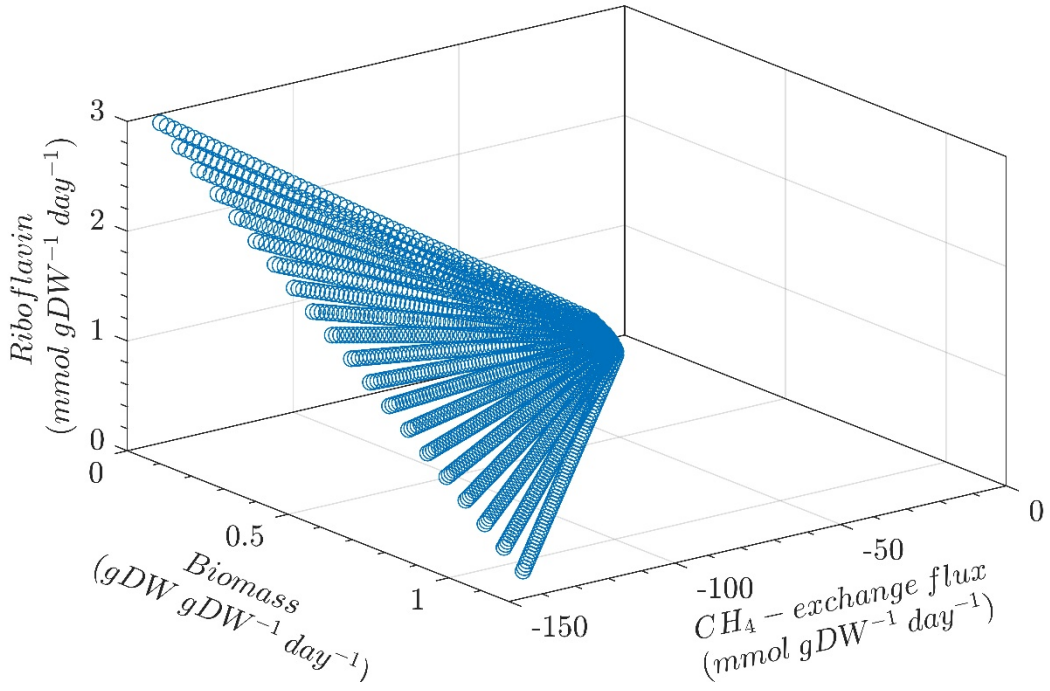

Fig. 2: Pareto chart of bi-level optimization for different methane exchange flux.

4. Conclusion

In this study, the results of the dFBA model simulation for conversion of methane to PHB were examined and compared with experimental data and the Monod model. The results indicated a higher predicted biomass production in the dFBA model compared to the Monod model and the experimental data. Based on this, it was inferred that the sole objective of the utilized microorganism is not merely the complete conversion of methane to biomass; riboflavin could also be a candidate for a secondary objective. This was investigated through multi-objective optimization, and the results showed that riboflavin and biomass can be produced competitively.

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