

# Metabolic Flux Balance Analysis for Biological Hydrogen Production by Purple Non-Sulfur Bacteria

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**Abstract.** A metabolic model was constructed in matrix formalism for the hydrogen production by the purple non-sulfur bacteria *Rhodobacter sphaeroides* involving 183 reactions and 153 metabolites. Assuming pseudo-steady state, mass balances were performed for each metabolite and the resulting equations were solved using optimization. The objective function was the maximization of hydrogen production. The comparison of experimental and computational results indicated the importance of light in hydrogen production.

**Keywords:** Hydrogen, purple non-sulfur bacteria, *Rhodobacter sphaeroides*, metabolic flux analysis.

## 1. Introduction

Due to the continuously increasing high energy demands of society and the finite nature of fossil fuels, alternative energy sources are becoming exceedingly important. Hydrogen is a promising alternative fuel because of its clean, renewable and high energy content of  $122 \text{ kJ g}^{-1}$  which is 2.75 times greater than the hydrocarbon fuels [1]. For hydrogen production, the biological process is the most attractive approach when compared to others such as water electrolysis, thermo-chemical processes and radiolytic processes. As it requires less energy, the overall  $\text{CO}_2$  emission can be almost neutral [2]. There are several microorganisms identified for hydrogen production and purple non-sulfur bacteria (PNB) such as *Rhodobacter sphaeroides* is a potential candidate. After decades of research however, there are still significant problems associated with bio-hydrogen production at industrial scale. These problems mainly involve low hydrogen production rates and the difficulty of provision of sufficient light to the culture. Metabolic flux balance analysis is a useful tool from metabolic engineering that helps with the development of novel strategies for hydrogen production.

In metabolic flux balance, the metabolic network reactions are presented in matrix formalism with the necessary stoichiometric, energy and redox balances. Assuming pseudo-steady state, a material balance equation is written for each metabolite. Since a metabolite normally participates in more than one metabolic reaction, the number of metabolite balances is less than the number of reactions, the specific rates of which are the unknown metabolic fluxes we want to calculate. The set of metabolite material balance equations are therefore solved via optimisation. In our case we used GAMS (General Algebraic Modelling System) with the objective functions of either specific growth rate or the specific hydrogen production rate with a few experimental and biological constraints such as the carbon-substrate uptake rate.

In the experiments, DL-malate was used as the carbon source. Using our model and the experimental results, we investigated the importance of light.

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## 2. Materials and methods

### 2.1. Microorganisms and culture conditions

In this study, we used *Rhodobacter sphaeroides* strain NCIMB 8253 from NCIMB LTD (Aberdeen, Scotland). The organism was grown photo-heterotrophically with 7.5mM DL-malate and 10mM sodium glutamate. For H<sub>2</sub> production, the carbon and nitrogen sources were 30mM DL-malate and 2mM sodium glutamate, respectively in modified Biebl & Pfennig media [3, 4] as indicated in Table 1.

Cells were grown anaerobically by using argon gas to create an anaerobic environment at 30 °C, pH 6.8, and under the illumination of a 3000 lux tungsten lamp.

**Table 1:** Composition of the modified Biel and Pfennig medium used for growth and maintenance [3, 4]

Compound	Amount (g l <sup>-1</sup> )
KH <sub>2</sub> PO <sub>4</sub>	0.500
MgSO <sub>4</sub> •7H <sub>2</sub> O	0.200
NaCl	0.400
CaCl <sub>2</sub> •2H <sub>2</sub> O	0.050
Yeast extract	0.200
Fe-citrate	0.005
Vitamin (B <sub>12</sub> )	0.001
*Trace element	0.001

\*1 ml l<sup>-1</sup> of the trace element solution was added containing the following chemicals (in mg/l): ZnCl<sub>2</sub>, 70; MnCl<sub>2</sub>•4H<sub>2</sub>O, 100; H<sub>3</sub>BO<sub>3</sub>, 60; CoCl<sub>2</sub>•6H<sub>2</sub>O, 200; CuCl<sub>2</sub>•2H<sub>2</sub>O, 20; NiCl<sub>2</sub> 6H<sub>2</sub>O, 20; NaMoO<sub>4</sub>•2H<sub>2</sub>O, 40; HCl (25%), 1 ml l<sup>-1</sup>.

### 2.2. Methods of analysis

Bacterial growth was measured at 600nm using JENWAY 6305 UV/VIS Spectrophotometer. The biomass dry weights were determined by measuring cell dry weight using a standard calibration curve (cell dry weight, g l<sup>-1</sup> against optical density measurement at 660nm). It was found that the optical density (OD) of 1 at 660nm corresponded to the cell density of 0.5076 g dry weight per liter culture.

The evolved hydrogen was analysed using a Gas Chromatograph (GC). HPLC analyses were used to monitor the concentration of DL-malate and sodium glutamate. The supernatant samples were analysed by HYPERSIL (250×4.6mm, 5μ) BDS C8 column at a pressure of 94 atm with 0.05M phosphoric acid as a mobile phase at a flowrate of 0.7ml min<sup>-1</sup>. For the construction of the standard calibration curves, DL-malate and sodium glutamate with different concentrations were analysed first in order to obtain their retention times. The pH of the culture medium was measured using Corning pH/ion meter 155.

### 2.3. *In silico* model construction and metabolic flux balance analysis (MBA)

The anaerobic metabolic network for purple non-sulfur bacteria (PNB) was reconstructed in matrix formalism from the information available on *Rhodobacter sphaeroides* in the literature, the KEGG metabolic pathways database and standard biochemistry text books. The main assumption of this model is that, the metabolic flux is at pseudo-steady state and therefore the metabolite concentrations remain constant [5]. This is an acceptable assumption when cell homeostasis is considered. The computational approach then involves making material balances around each metabolite and solving the resulting equations for the unknown fluxes using optimisation with the experimental and biological constraints. The summary of the overall metabolic pathway is shown in Fig. 1. The metabolic network of *Rhodobacter sphaeroides* was constructed to contain 183 reactions and 153 metabolites.

The list of simulated metabolic pathways include glycolysis/gluconeogenesis, pentose phosphate pathway, pyruvate metabolism, tricarboxylic acid (TCA) cycle, electron transport, amino acid biosynthesis, nucleic acid biosynthesis, fatty acid biosynthesis, phospholipid biosynthesis, carbohydrate biosynthesis,

transhydrogenase reactions, DNA and RNA polymerisation, protein biosynthesis, transport reactions and biomass formation.

Biomass is assumed to consist mainly of proteins, carbohydrates, RNA, DNA and phospholipids. The percentages of proteins, carbohydrates, RNA, DNA and phospholipids as well as the polymerisation energies required for their synthesis are assumed to be the same as those for *E. coli* [6].

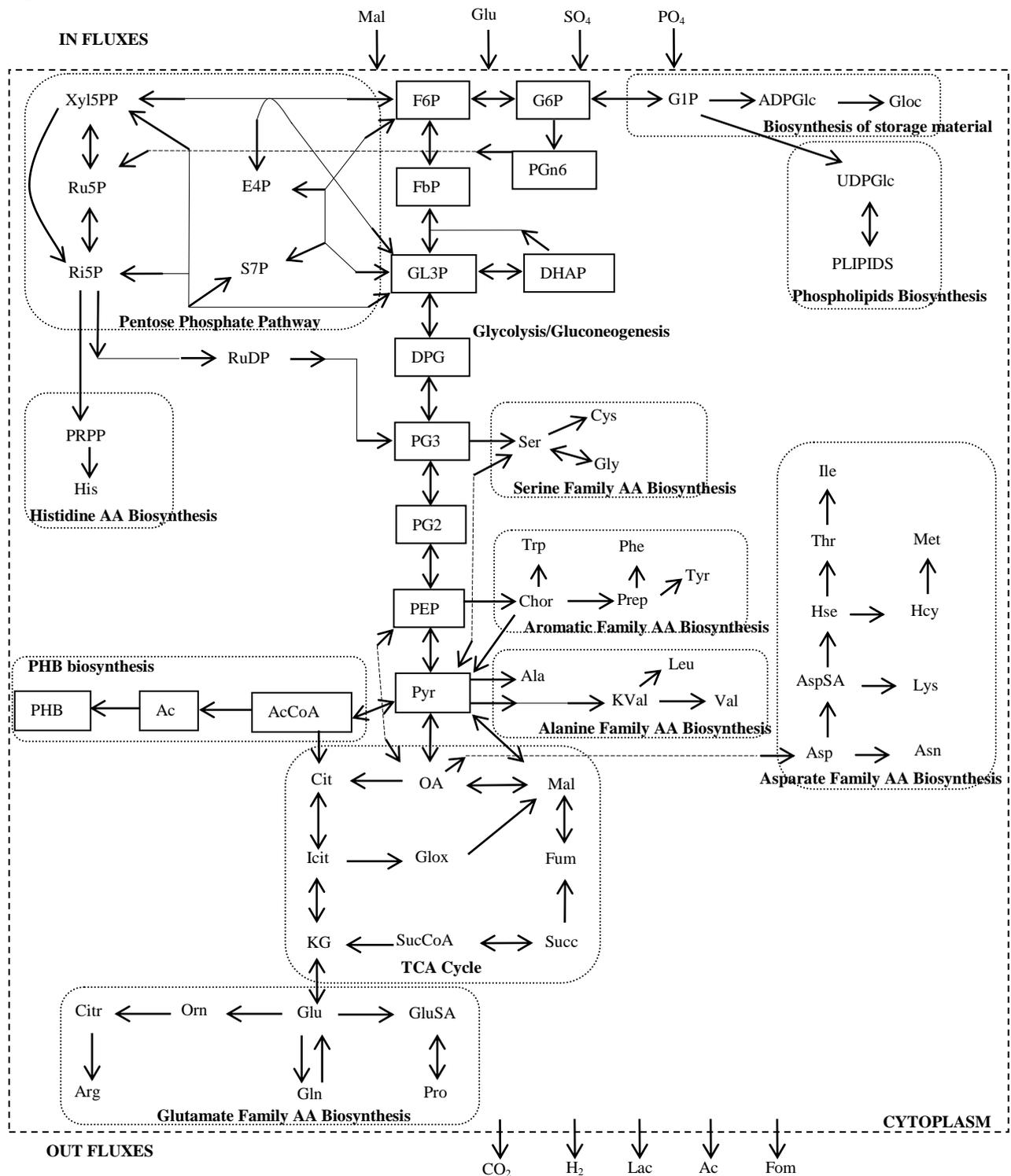


Fig. 1: Summary of the overall metabolic network of *Rhodobacter sphaeroides* used for the hydrogen production model.

In this study, the metabolic model was constructed for the dark anaerobic condition. Therefore, no photosynthetic reactions were involved in this metabolic flux balance analysis. This was to test whether the purple non-sulfur bacteria (PNB) such as *Rhodobacter sphaeroides* could grow and produce hydrogen gas under the anaerobic dark conditions *in silico*.

## 2.4. Objective functions of the model

The main input parameters of this model were the specific consumption rates of DL-malate and sodium glutamate. The objective function for the optimisation was either the maximisation of the specific cell growth rate or the specific hydrogen production rate

## 2.5. Software

The linear programming problem was solved using General Algebraic Model System (GAMS) software (<http://www.gams.com/>). The solution not only gave the value of the objective function but also the values of all the metabolic fluxes.

## 3. Results and discussion

Although the hydrogen production experiment was carried out under the light intensity of 3000 lux, provided by a tungsten lamp, the metabolic model was constructed for the anaerobic dark condition. Fig. 2 shows the comparison of hydrogen production obtained from experimental and computational results where the hydrogen produced in both cases increased until the cell growth reached the stationary stage.

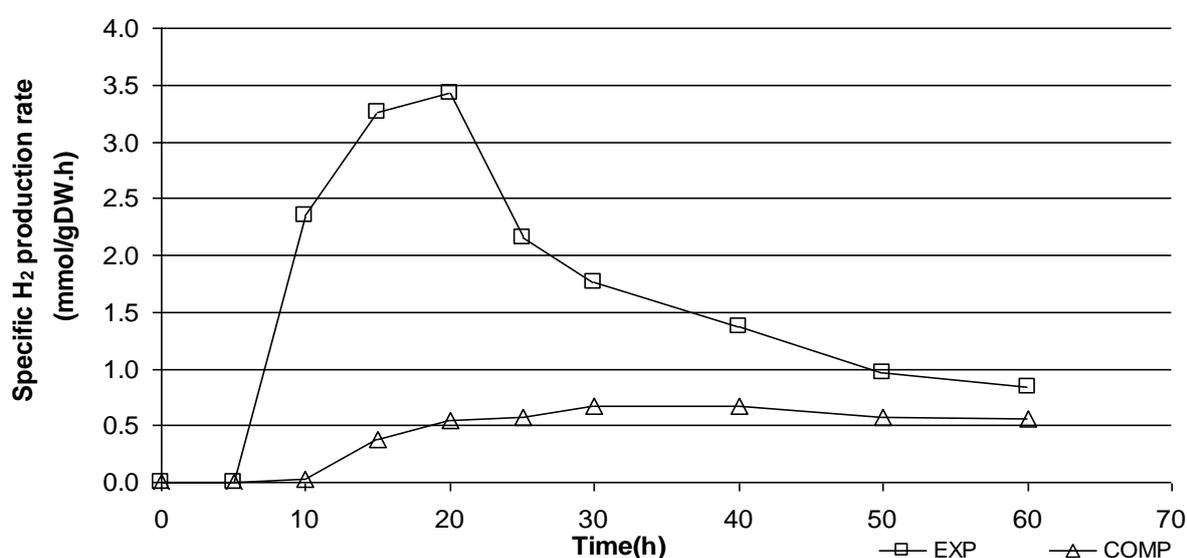


Fig. 2: Comparison of experimental and computational specific hydrogen production rates during the batch culture.

Hydrogen production is associated with hydrogenase and nitrogenase enzymes however, hydrogen production activity of nitrogenase is provided by the photosynthetic apparatus, which converts light energy into chemical bond energy (ATP). In the dark, hydrogen is generated via formate-hydrogen lyase (FHL) complex where formate is oxidised to carbon dioxide releasing hydrogen. This is accompanied with the regeneration of  $\text{NAD}^+$  required for the redox balance during the fermentative growth [7]. As indicated in Fig 2, the computational hydrogen production rate was lower than the experimental result. This has proven that hydrogen production of purple non-sulfur bacteria is mainly correlated with the activity of nitrogenase.

When the objective function was the maximization of the specific growth rate, similar results were obtained. The comparison of experimental and computational results in Fig 3 indicates that the metabolic model predicts lower specific growth rates in the absence of light, except for the late stages of the fermentation.

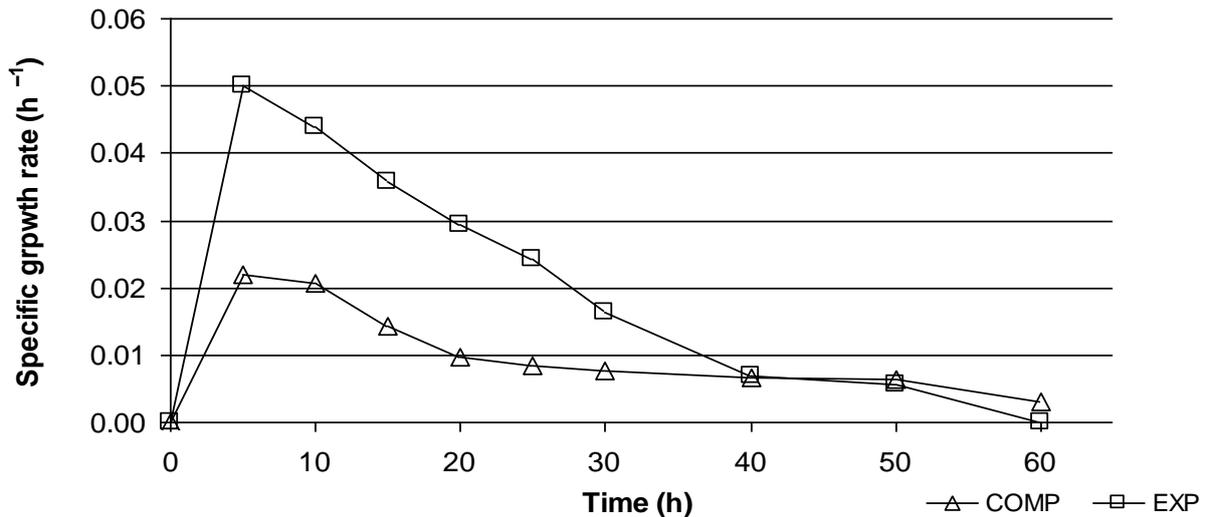


Fig. 3: Comparison of the experimental and computational growth rates during the batch culture.

## 4. Conclusion

The comparison of experimental and computational results indicates that the metabolic model predicts lower specific rates of hydrogen production in the absence of light. We can conclude that the nitrogenase synthesis, which plays a significant role in hydrogen formation, is strongly stimulated by light and therefore the *in silico* production of hydrogen in the dark would be inferior to production in the light. The metabolic model can further be developed to include the light influenced reactions and subsequently to identify the metabolic bottlenecks in biological hydrogen production so that process and genetic engineering strategies can be developed.

## 5. References

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