Metabolic Flux Balance Analysis of the Primary Metabolism for Hydrogen Production in Purple Non-sulfur Bacteria

Pasika Chongcharoentaweesuk, Jiangdong Zhang, and Ferda Mavituna

***Abstract*—A metabolic model was constructed in matrix formalism for 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 the hydrogen production. The comparison of the experimental and computational results indicated the importance of light in hydrogen production.**

***Index Term*—Hydrogen, Metabolic Flux Balance Analysis, Purple Non-sulfur Bacteria, *Rhodobacter sphaeroides*.**

I. Introduction

Due to the continuously increasing high energy demands of the society and the finite nature of fossil fuels reserves, alternative energy sources are becoming exceedingly important. Hydrogen is a promising alternative fuel because it is clean and renewable. Furthermore, its energy content of 122 kJ/g is 2.75 times greater than that of the hydrocarbon fuels [1]. Production of hydrogen by biological routes is the most attractive approach when compared to other methods of production such as water electrolysis, thermo-chemical processes and radiolytic processes as it is less energy-intensive and the overall CO_2 emission is not harmful to the environment considering its life cycle analysis [2]. There are several microorganisms identified for hydrogen production and purple non-sulfur bacteria (PNB) such as *Rhodobacter sphaeroides* is a potential candidate. *Rhodobacter sphaeroides* is a gram-negative, anaerobic photoheterotrophic bacterium. Cells of *Rhodobacter sphaeroides* are ovoid, heart-shaped or short rods in defined media. Many occur in pairs, sometimes connected by a slender filament or tube [3]. After decades of research, there are still significant problems in hydrogen production that prevent industrial scale production from becoming a reality. These include low production levels and the difficulty of provision of sufficient light to the culture Metabolic flux analysis is a useful tool of metabolic engineering that can help with the development of novel strategies for hydrogen production. In our model, we investigated the importance of light in hydrogen production.

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II. Materials and Methods

A. 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 7.5 mM DL-malate as the carbon source and 12 mM sodium glutamate as the nitrogen source in modified Biebl & Pfennig medium [4], [5] as indicated in Table I. The liquid culture medium used for hydrogen production was exactly the same as the growth medium except the concentration of DL-malate and glutamate were 15mM and 2mM respectively.

TABLE I. Composition of the Modified Biebl and Pfennig Medium used for Growth and Maintenance of *R. sphaeroides*.

Compound	Amount (mg/l)
KH_2PO_4	500
$MgSO_4 \cdot 7H_2O$	200
NaCl	400
Yeast extract	200
$CaCl_2 \cdot 2H_2O$	50
Fe-citrate[a]	5 ml
Vitamin (B12)[b]	1 ml
Trace element[c]	1 ml

a) 0.1% wt./vol Fe-citrate, b) 1.0mg/100ml Vitamin B12, c)1 ml/l of the trace element solution was added containing the following chemicals (in mg/l): $ZnCl_2$, 70; $MnCl_2 \cdot 4H_2O$, 100; H_3BO_3, 60; $CoCl_2 \cdot 6H_2O$, 200; $CuCl_2 \cdot 2H_2O$, 20; $NiCl_2$ $6H_2O$, 20; $NaMoO_4 \cdot 2H_2O$, 40; HCl (25% v/v), 1 ml/l.

Cells were grown anaerobicly by using argon gas to create an anaerobic environment at 30 ºC, pH 6.8, and under the illumination of a 3000 lux tungsten lamp. The initial pH value of all the media was adjusted to around 6.8 using 1M NaOH solution.

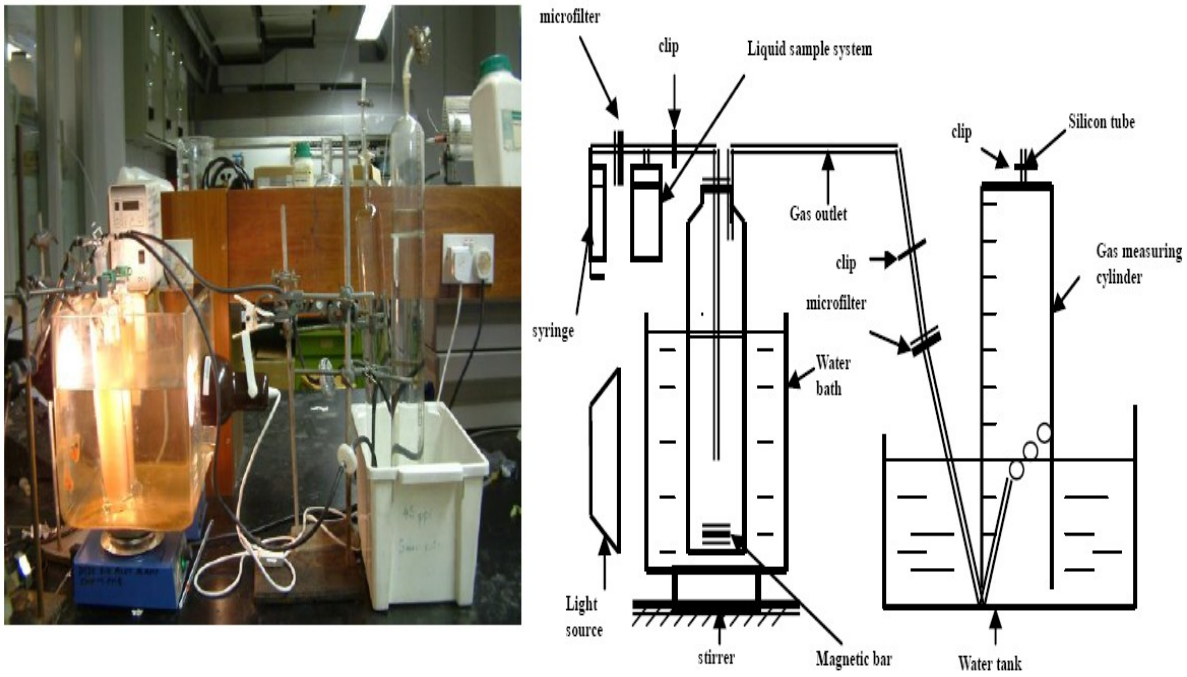

Fig. 1 The glass column photobioreactor used for hydrogen production.

B. Photobioreactor system

The experiments of hydrogen production were carried out in a 1 L glass column photobioreactor with a working volume of 600 ml. The inoculum was 10% v/v. The diameter of the vessel was 6 cm. The glass lid had three ports used for sampling, inoculation and hydrogen gas collection.

The temperature was controlled at 30 ºC using a water bath. The photobioreactor was operated with an agitation of 140 rpm using a magnetic stirrer.

C. Methods of analysis

Bacterial growth was measured at 660 nm 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 against optical density measurement at 660 nm). The biomass dry weights were determined using the same samples by centrifuging in the plastic tubes (10 ml) at 4000rpm for 15 minutes using IECCentra-3M centrifuge. The supernatants were discarded and the cells were dried in pre-weighed small vials for 24 hours. It was found that the optical density (OD) of 1 at 660 nm corresponded to the cell density of 0.5076 g dry weight per liter culture.

The evolved hydrogen was analyzed using a Gas Chromatograph (GC). HPLC analyses were used to monitor the concentration of DL malate and sodium glutamate. The supernatant samples (from IECCentra-3M centrifuge) were analyzed 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 0.7 ml/min. For the construction of the standard calibration curves, DL malate and sodium glutamate with different concentrations were analyzed first in order to obtain their retention times.

The pH of the culture medium was measured using Corning pH/ion meter 155.

D. In silico model construction and metabolic flux balance analysis (MFA)

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 textbooks. The main assumption of this model is that, the metabolic fluxes are at pseudo-steady state and the metabolite concentrations remain constant. This can be justified considering the homeostasis [6]. The steady-state balance equation is written [7] as

$$\mathbf{S}.\mathbf{v} = \mathbf{b} \quad (1)$$

where **S** is the stoichiometric matrix, **v** is the vector of fluxes, and **b** is the vector of net specific transport rates from the cell.

The computational approach then involves making material balances around each metabolite and solving the resulting equations for the unknown fluxes using optimization with the experimental and biological constraints. The optimization is needed because since each metabolite usually takes part in more than one reaction and therefore the number of unknown (metabolic fluxes) are greater than the number of equations (the mass balance equations for each metabolite). The metabolic network of *Rhodobacter sphaeroides* was constructed to conatin 183 reactions and 153 metabolites. The summary of the overall metabolic pathway is shown in Fig. 2.

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

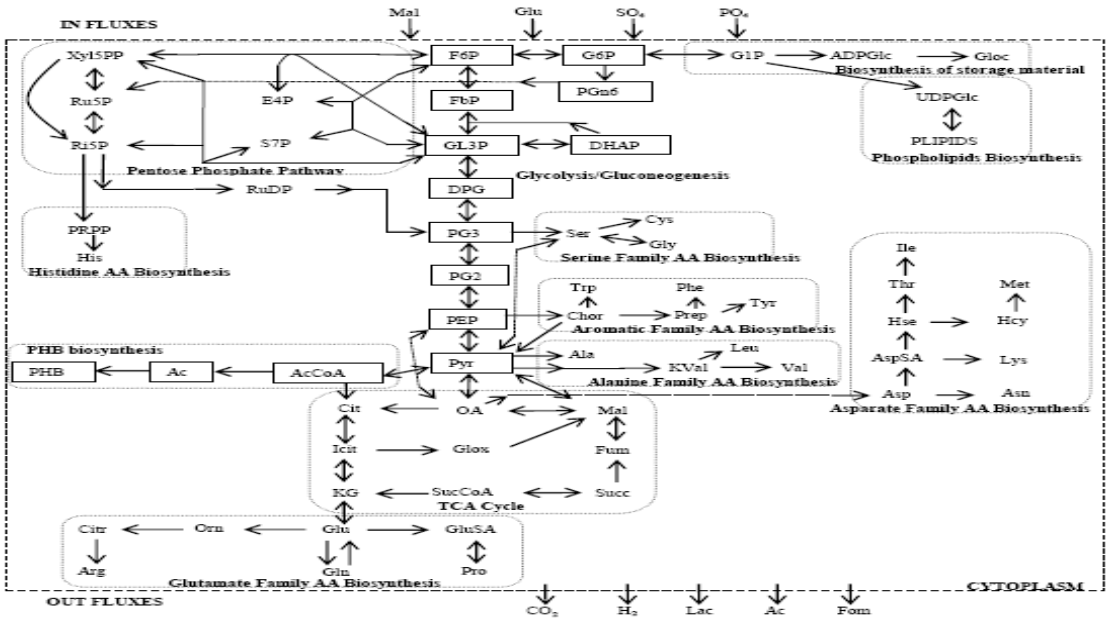

Fig. 2 The overall metabolic network of *Rhodobacter sphaeroides* used for the hydrogen production model

Biomass is assumed to consist mainly of proteins, carbohydrates, RNA, DNA and phospholipids. The percentages of protein, carbohydrates, RNA, DNA and phospholipids are assumed to be the same as *E. coli* [8]. The polymerisation energies required for protein, RNA and DNA are also taken from *E. coli* [8].

In this study, the metabolic model was constructed for the dark anaerobic condition. Therefore, no photosynthetic reactions were involved in this metabolic flux 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*.

E. Objective functions of the model

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

F. Software

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

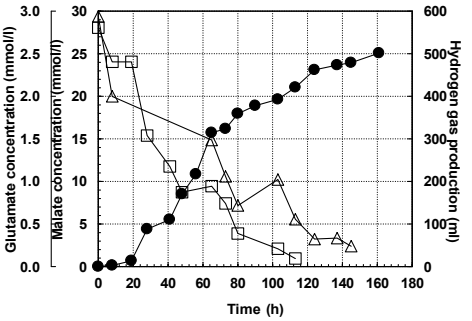

Fig. 3 Malate consumption (□), Glutamate consumtion (Δ) and hydrogen gas production (●) by *Rhodobacter sphaeroide* in the 1L bioreactor.

III. RESULTS AND DISCUSSION

A uniform light intensity of 4-9 W/m^2 for cell growth and the light intensity of 9-15 W/m^2 for optimal hydrogen production in anoxic environment has been reported [9], [10]. The experiment of the hydrogen production was performed under the light intensity of 4 W/m^2, provided by a tungsten lamp. DL malate, glutamate concentration and hydrogen production during batch culture of R. sphaeroides are presented in Fig 3. From the experiment results, specific rates were calculated and were then used as input data such as DL malate and glutamate consumption and for comparison, such as the specific hydrogen production rate.

The metabolic model was constructed for the anaerobic dark condition. Fig. 4 shows the comparison of hydrogen production obtained from the experimental and computational results where the hydrogen produced in both cases increased until cell growth reached the stationary stage.

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 gas is generated via formate-hydrogen lyase (FHL) complex where formate is oxidized to carbon dioxide as well as releasing hydrogen with the regeneration of NAD^+ required for the redox balance during fermentative growth [11].

As indicated in Fig 4, the computational hydrogen production rate was lower than the experimental result.

This has proven that hydrogen production by purple non-sulfur bacteria is mainly correlated with the activity of nitrogenase.

The comparison of the experimental and computational results in Fig 5 indicates that the metabolic model predicts lower specific growth rates except for the late stages of the fermentation.

Hydrogen evolution started at 10 which shows that

hydrogen is produced from mid to late exponential growth phase.

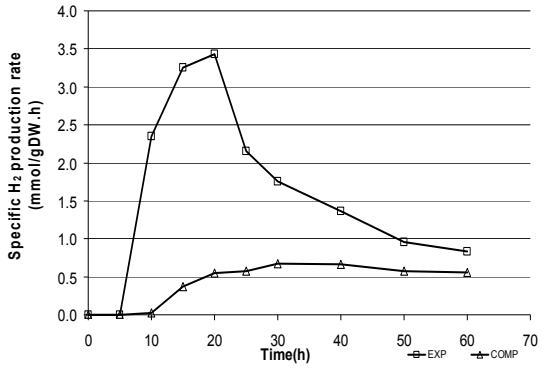

Fig. 4 Comparison of experimental and computational specific hydrogen production rates during the batch culture.

During the exponential growth (5-20 h), DL-malate (organic carbon) was used for the generation of NADH via oxidation into carbon dioxide in the tricarboxylic acid (TCA) cycle and also for the biosynthesis of biosynthetic pathways (amino acid, nucleic acid, fatty acid, PHB, phospholipid, carbohydrate and protein synthesis). The electron extracted from the oxidation of malate is directed towards hydrogen. In the stationary phase (20-40 h), most of the DL malate is oxidized into carbon dioxide which means that biosynthesis is minimal. Therefore most of the extracted electrons are directed towards hydrogen.

Although the model included the biosynthesis of poly-3-hydroxybutyric acid (PHB), there was no flux towards this by-product formation in the computed results. This was because there was not enough NADH which was generated from the carbon source (malate) to synthesise PHB and no abundant energy from the nitrogenase complex.

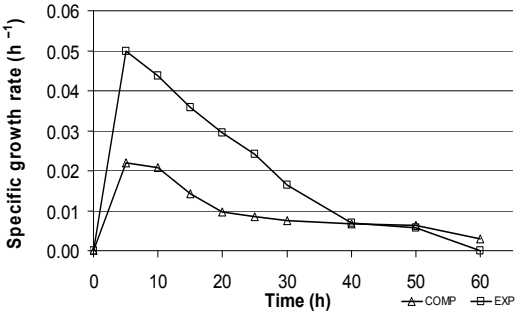

Fig. 5 Comparison of experimental and computational growth rates during the batch culture.

IV. Conclusions

The comparison of experimental and computational results indicates that the metabolic model predicts lower specific rates of hydrogen production. 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 the 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.

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