Université de Montréal

Improving photofermentative hydrogen production through metabolic engineering and DOE (Design of Experiments)

Par

Yuan Liu

Département de microbiologie, infectiologie et immunologie

Faculté de Médecine

Thèse présentée à la Faculté des Études Supérieures et Postdoctorales

en vue de l'obtention du grade de Ph.D.

en Microbiologie et Immunologie

Mars, 2016

Université de Montréal

Faculté des Études Supérieures

Cette thèse intitulée:

Improving photofermentative hydrogen production through metabolic engineering and DOE (Design of Experiments)

Présentée par:

Yuan Liu

A été évaluée par un jury composé des personnes suivantes:

Dr. George Szatmari, Président-rapporteur

Dr. Patrick C. Hallenbeck, Directeur de recherché

Dr. Luke Masson, Membre du jury

Dr. Richard Villemur, Examinateur externe

RÉSUMÉ

A l'heure actuelle, les biocarburants renouvelables et qui ne nuit pas à l'environnement sont à l'étude intensive en raison de l'augmentation des problèmes de santé et de la diminution des combustibles fossiles. H_2 est l'un des candidats les plus prometteurs en raison de ses caractéristiques uniques, telles que la densité d'énergie élevée et la génération faible ou inexistante de polluants. Une façon attrayante pour produire la H_2 est par les bactéries photosynthétiques qui peuvent capter l'énergie lumineuse pour actionner la production H_2 avec leur système de nitrogénase. L'objectif principal de cette étude était d'améliorer le rendement de H_2 des bactéries photosynthétiques pourpres non sulfureuses utilisant une combinaison de génie métabolique et le plan des expériences.

Une hypothèse est que le rendement en H_2 pourrait être améliorée par la redirection de flux de cycle du Calvin-Benson-Bassham envers du système de nitrogénase qui catalyse la réduction des protons en H_2. Ainsi, un PRK, phosphoribulose kinase, mutant « knock-out » de *Rhodobacter capsulatus* JP91 a été créé. L'analyse de la croissance sur des différentes sources de carbone a montré que ce mutant ne peut croître qu'avec l'acétate, sans toutefois produire d' H_2. Un mutant spontané, YL1, a été récupéré qui a retenu l'*cbbP* (codant pour PRK) mutation d'origine, mais qui avait acquis la capacité de se développer sur le glucose et produire H_2. Une étude de la production H_2 sous différents niveaux d'éclairage a montré que le rendement d'YL1 était de 20-40% supérieure à la souche type sauvage JP91. Cependant, il n'y avait pas d'amélioration notable du taux de production de H_2. Une étude cinétique a montré que la croissance et la production d'hydrogène sont fortement liées avec des électrons à partir du glucose principalement dirigés vers la production de H_2 et la formation de la biomasse. Sous des intensités lumineuses faibles à intermédiaires, la production d'acides organiques est

importante, ce qui suggère une nouvelle amélioration additionnel du rendement H_2 pourrait être possible grâce à l'optimisation des processus.

Dans une série d'expériences associées, un autre mutant spontané, YL2, qui a un phénotype similaire à YL1, a été testé pour la croissance dans un milieu contenant de l'ammonium. Les résultats ont montré que YL2 ne peut croître que avec de l'acétate comme source de carbone, encore une fois, sans produire de H_2. Une incubation prolongée dans les milieux qui ne supportent pas la croissance de YL2 a permis l'isolement de deux mutants spontanés secondaires intéressants, YL3 et YL4. L'analyse par empreint du pied Western a montré que les deux souches ont, dans une gamme de concentrations d'ammonium, l'expression constitutive de la nitrogénase. Les génomes d'YL2, YL3 et YL4 ont été séquencés afin de trouver les mutations responsables de ce phénomène. Fait intéressant, les mutations de *nifA1* et *nifA2* ont été trouvés dans les deux YL3 et YL4. Il est probable qu'un changement conformationnel de NifA modifie l'interaction protéine-protéine entre NifA et PII protéines (telles que GlnB ou GlnK), lui permettant d'échapper à la régulation par l'ammonium, et donc d'être capable d'activer la transcription de la nitrogénase en présence d'ammonium. On ignore comment le nitrogénase synthétisé est capable de maintenir son activité parce qu'en théorie, il devrait également être soumis à une régulation post-traductionnelle par ammonium. Une autre preuve pourrait être obtenue par l'étude du transcriptome d'YL3 et YL4. Une première étude sur la production d' H_2 par YL3 et YL4 ont montré qu'ils sont capables d'une beaucoup plus grande production d'hydrogène que JP91 en milieu d'ammonium, qui ouvre la porte pour les études futures avec ces souches en utilisant des déchets contenant de l'ammonium en tant que substrats.

Enfin, le reformage biologique de l'éthanol à H_2 avec la bactérie photosynthétique, *Rhodopseudomonas palustris* CGA009 a été examiné. La production d'éthanol avec fermentation utilisant des ressources renouvelables microbiennes a été traitée comme une technique mature. Cependant, la plupart des études du reformage de l'éthanol à H_2 se sont concentrés sur le reformage chimique à la vapeur, ce qui nécessite généralement une haute charge énergetique et résultats dans les émissions de gaz toxiques. Ainsi le reformage biologique de l'éthanol à H_2 avec des bactéries photosynthétiques, qui peuvent capturer la lumière pour répondre aux besoins énergétiques de cette réaction, semble d'être plus prometteuse. Une étude précédente a démontré la production d'hydrogène à partir d'éthanol, toutefois, le rendement ou la durée de cette réaction n'a pas été examiné. Une analyse RSM (méthode de surface de réponse) a été réalisée dans laquelle les concentrations de trois facteurs principaux, l'intensité lumineuse, de l'éthanol et du glutamate ont été variés. Nos résultats ont montré que près de 2 moles de H_2 peuvent être obtenus à partir d'une mole d'éthanol, 33% de ce qui est théoriquement possible.

Mots clés: bactéries photosynthétique pourpre non sulfureuse; cycle de Calvin-Benson-Bassham; génie métabolique; mutant PRK; YL1, YL2, YL3 et YL4; étude cinétique; rendement H_2 et le taux de production volumétrique; l'analyse de la répartition des électrons; transcription du nitrogénase; séquençage du génome; NifA1 et NifA2; Reformage biologique; la méthode de surface de réponse.

ABSTRACT

Currently, renewable and environmentally friendly biofuels are under intensive study due to increasing health concerns and diminishing fossil fuels. H_2 is one of the most promising candidates due to its unique characteristics, such as a high energy density and low to non-existent generation of pollutants. One attractive way to produce H_2 is through photosynthetic bacteria which can capture light energy to drive H_2 production with their nitrogenase system. The major aim of this study was to improve H_2 yield of the purple non-sulfur photosynthetic bacteria using a combination of metabolic engineering and design of experiments.

One hypothesis was that H_2 yield could be improved by redirection of Calvin-Benson-Bassham cycle flux to the nitrogenase system which catalyzes the reduction of protons to H_2. Thus, a PRK, phosphoribulose kinase, knock out mutant of *Rhodobacter capsulatus* JP91 was created. Analysis of growth with different carbon sources showed that this mutant could only grow in acetate medium without, however, producing any H_2. A spontaneous mutant, YL1, was recovered which retained the original *cbbP* (encoding PRK) mutation, but which had gained the ability to grow on glucose and produce H_2. A study of H_2 production under different illumination levels showed that the yield of YL1 was 20-40% greater than the wild type JP91 strain. However, there was no appreciable improvement of the H_2 production rate. A kinetic study showed that growth and hydrogen production are strongly linked with electrons from glucose being mostly directed to H_2 production and biomass formation. Under low to intermediate light intensities, the production of organic acids was significant, suggesting further improvement of H_2 yield is possible by process optimization.

In a related series of experiments, another spontaneous mutant, YL2, which has a similar phenotype to YL1, was tested for growth in ammonium-containing media. The results showed

that YL2 could only grow with acetate as carbon source, again, without producing any H_2. Prolonged incubation in media not supporting growth of YL2 enabled the isolation of two interesting secondary spontaneous mutants, YL3 and YL4. Western blot analysis showed that both strains had constitutive nitrogenase expression under a range of ammonium concentrations. The genomes of YL2, YL3 and YL4 were sequenced in order to find the mutations responsible for this phenomenon. Interestingly, mutations of *nifA1* and *nifA2* were found in both YL3 and YL4. It is likely that a conformational change of NifA alters the protein-protein interaction between NifA and PII proteins (such as GlnB or GlnK), enabling it to escape regulation by ammonium and thus to be capable of activating nitrogenase transcription in the presence of ammonium. It is not clear how the synthesized nitrogenase is able to maintain its activity since in theory it should also be subject to posttranslational regulation by ammonium. Further evidence could be obtained by studying the transcriptome of YL3 and YL4. An initial study of H_2 production by YL3 and YL4 showed that they are capable of much greater hydrogen production than JP91 in ammonium medium, which opens the door for future studies with these strains using ammonium-containing wastes as substrates.

Finally, the biological reformation of ethanol to H_2 with the photosynthetic bacterium, *Rhodopseudomonas palustris* CGA009 was examined. Ethanol production with microbial fermentation using renewable resources has been treated as a mature technique. However, most studies of the reformation of ethanol to H_2 have focused on chemical steam reforming, which usually requires a high energy input and results in toxic gas emission. Thus biological reformation of ethanol to H_2 with photosynthetic bacteria, which can capture light to meet the energy requirement of this reaction, seems to be more promising. A previous study had demonstrated hydrogen production from ethanol, however, the yield or the duration of this

reaction were not examined. A RSM (response surface methodology) analysis was carried out in which three key factors, light intensity, ethanol and glutamate concentrations were varied. Our results showed that nearly 2 moles of H_2 could be obtained from one mole of ethanol, 33% of what is theoretically possible.

Key words: Purple non-sulfur photosynthetic bacterium; Calvin-Benson-Bassham cycle; metabolic engineering; PRK mutant; YL1, YL2, YL3 and YL4; kinetics study; H_2 yield and volumetric production rate; electron allocation analysis; nitrogenase transcription; genome sequencing; NifA1 and NifA2; biological reformation; response surface methodology.

TABLE OF CONTENTS

LISTS OF FIGURES

ABBREVIATIONS

CBB: Calvin-Benson-Bassham cycle

PRK: phosphoribulose kinase

Rubisco: ribulose-1,5-bisphosphate carboxylase/oxygenase

NifA: master transcriptional activator for nitrogenase genes

DraT: dinitrogenase reductase ADP-ribosyltransferase

DraG: dinitrogenase-reductase-activating glycohydrolase

AmtB: ammonium transporter

GlnK: signal transduction protein, belongs to PII group

GlnB: signal transduction protein, belongs to PII group

GlnJ: signal transduction protein, belongs to PII group

GlnD: uridylyltransferase/uridylyl removing enzyme

NtrB: sensor kinase

NtrC: nitrogen regulatory protein C

NifA: Transcriptional activator of *nif* genes

GlnA: glutamine synthetase

PHB: polyhydroxybutyrate

nif^{+}: mutants with nitrogenase expression

SMP: soluble microbial products

Bchl: bacteriochlorophyll

DOE: design of experiments

Uptake hydrogenase: hydrogenase which is more active in catalyzing H_2 oxidation

ANOVA: analysis of variance

N_2ase: nitrogenase

R. capsulatus: Rhodobacter capsulatus

Rho. rubrum: Rhodospirillum rubrum

Rps. palustris: Rhodopseudomonas palustris

R. sphaeroides: Rhodobacter sphaeroides

FOOTNOTES

Note on the text of chapter 1 and chapter 2

For chapter 1, all figures from other people's work were used with permission. For chapter 2, all contents were adapted from a joint review publication with permission from coauthor (Patrick C. Hallenbeck and Yuan Liu, 2016. Recent advances in hydrogen production by photosynthetic bacteria. **Int J Hydrogen Energy, 41:4446-4454**). It should be noted that this article was published by Elsevier, and it allows authors to use published material, in whole or part, without written permission or formal copyright transfer.

Note on the text of results and discussion

For chapter 3, all contents were adapted from a joint publication with permission (Yuan Liu and Patrick C. Hallenbeck, 2016. A Kinetic study of H_2 production by a Calvin-Benson-Bassham cycle mutant, PRK (phosphoribulose kinase), of the photosynthetic bacterium *Rhodobacter capsulatus*. **Int J Hydrogen Energy, 2016; 41, 11081-11089**). For chapter 5, all contents were adapted from a joint publication with permission (Yuan Liu, Dipankar Ghosh, Patrick C. Hallenbeck. Biological reformation of ethanol to hydrogen by *Rhodopseudomonas palustris* CGA009. **Bioresour. Technol. 2015; 176:189-95**). It should be noted that this article was published by Elsevier, and it allows authors to use published material, in whole or part, without written permission or formal copyright transfer.

ACKNOWLEDGEMENTS

I thank my supervisor Prof. Patrick Hallenbeck for helping me develop all required skills and knowledge for a PhD student, to be an independent, serious researcher. I also thank all my family members for their long term spiritual support and my government for their scholarship.

Prof. George Szatmari and Prof. France Daigle are thanked for their advice, patience, and encouragement during my first struggling two years of PhD.

I thank all my lab members, A. E. M. Abdelaziz, K. Paranjape, H. Meltzer, D. Ghosh, G. Leite, M. Abo-hashesh, Z. Choolaei, N. Boukharouba, undergraduate students and new comers for their accompany, helpful discussions and technical supports.

My thesis jury members, Dr. Luke Masson, Dr. George Szatmari and Dr. Richard Villemur are all thanked for spending their precious time to evaluate my thesis and make revisions.

I thank UdeM employees for providing me easily reachable library and gym resources and all kinds of direct and indirect help.

LITERATURE REVIEW

Chapter 1: Research background

Increasing environmental concerns and diminishing fossil fuels are the major driving forces for the development of renewable bio-based fuels. At present, a number of biofuels have drawn a great deal of attention and are under intensive investigation, such as ethanol, butanol, biodiesel, and H_2. Here, four major biofuels will be discussed in terms of their general characteristics, microorganism producers, and current production status. An emphasis will be placed on various H_2 production methods.

1. Production of H_2 and some other biofuels

1.1 Bioethanol

Bioethanol is an attractive bio-fuel for many reasons. Firstly, from a practical point of view, it is compatible with conventional automobile engines when added to gasoline as a 10 to 22% supplement. For flexible fuel vehicles, ethanol can be used at a much higher proportion, for example, E85 is currently used in the United States and anhydrous or 95% ethanol in Brazil [1]. Secondly, ethanol combustion can be treated as free of net CO_2 emissions, since the substrates used to feed a ethanol producer, eg., yeast, are the result of plant photosynthesis which fixes CO_2 [2]. Thirdly, ethanol, as a volatile product, is relatively easy to separate from the fermentation broth (distillation point, STP, 78.15 ºC), thus the downstream processing cost could be less. Lastly, a variety of substrates for ethanol production, including corn, sugarcane, and lignocellulosic biomass, are readily available. Despite its many advantages, there are a few shortcomings that need to be mentioned. For example, it absorbs water, promoting corrosion in the infrastructure used to hadle it, it has a lower energy content than gasoline, and

its presence could encourage microbial growth in fuel (http://www.frost.co.uk/protect-your-vehicle-against-ethanol).

The most well-known ethanol producer is yeast, in particular *Saccharomyces cerevisiae* which has a long history of being used in the wine industry. It can produce ethanol at high yield, as high as 90 percent of theoretical yield has been reported [3]. It is relatively insensitive to changes in pH, temperature and osmotic conditions. However, very high concentrations of ethanol can be toxic [3]. One major issue with this species is that it lacks enzyme systems for the degradation of pentose sugars, thus it cannot use the hemicellulose fraction of lignocellulose biomass as substrate. Another important yeast species is *Pichia stipitis*, which is superior to *S. cerevisiae* in that it can ferment nearly all of the major sugars, including xylose, found in wood [4]. This is an important characteristic since xylose exists in such a high abundance, making up to 15 to 25% of all hard woods and agricultural residues [5]. Genetic approaches are expected to play a future role in integrating all good features from different microorganisms into a super microorganism that can produce biofuel from lignocellulosic substrates [6].

Currently, bioethanol has already been commercialized and produced in large quantity. However, greater production is expected by both the government and the market. According to RFA (the Renewable Fuel Association), the number of ethanol plants increased from 50 to 198 from 2000 to 2011, a remarkable 4-fold increase [2].

1.2 Biobutanol

Compared to ethanol, butanol is a superior fuel in a number of ways. It has a lower volatility and hydrophilicity, higher miscibility with other hydrocarbons, and higher energy content per mass unit, 36 MJ/kg versus 23.4-26.8 MJ/kg for ethanol. There are a few negative aspects for butanol, for example, it stinks. As well, it could be oxidized to the more toxic and odorous butyraldehyde and butyric acid during transportation (http://cen.acs.org/articles/84/i46/Biobutanol-Stinks.html).

Clostridia are the most widely used microorganisms for butanol (1-butanol or n-butanol) production, which catalyze a traditional ABE (acetone, butanol and ethanol) process. An interesting fact is that bio-butanol was commercialized as early as 1918, and successfully served the market for nearly 40 years before it eventually was replaced by a more cost-competitive petrochemical method [7]. Currently, final concentrations of butanol produced by ABE fermentations are low, ranging from a few grams per liter to about 20 grams per liter [7]. It is expected that bio-butanol can be blended into gasoline and act as bio-fuel in the near future (http://www.grandviewresearch.com/industry-analysis/bio-butanol-industry).

1.3 Biodiesel

Biodiesel has a few characteristics of special interest, in particular it has a much better lubricating and higher cetane rating than today's lower sulfur diesel fuels. Its calorific value is about 37.27 MJ/L which is 9% lower than regular petrodiesel (www.berkeleybiodiesel.org/useful-guide-understand.html). However, in many cases, biodiesel produced failed to meet ASTM 6751 quality due to the difficulty in removing all

impurities and water during washing and refining processes, and biodiesel can gel in colder weather (http://greenthefuture.com/BIODIESEL_PROSCONS.html). It is generally produced in a transesterification process in which oil or fat are reacted with methanol under acidic conditions. There are a variety of sources for oil or fat including extracts from plants, animals, or algae. Technologies for producing biodiesel from vegetable oils are well developed. However, vegetable oil is not the best option as culturing plants takes a long time. In addition, since their cultivation largely requires arable land, the large scale production of oil-bearing plants might threaten food security given the already large, and still increasing, global population. Hence, lipid production by microalgae may be a better solution as these organisms have faster growth rates (doubling time about 24 hours) and their nutrient requirements can be met with wastewater only [8]. In order to estimate the potential of microalgae-based biodiesel production in the most accurate way, a model has been developed which takes into account nutrient uptake, respiration, temperature and other effects to predict growth and lipid production parameters [9]. This model suggests that maximum annual average lipid yields could reach 24 to 27 $m^3\ ha^{-1}.y^{-1}$ in some countries close to the equator, such as Australia, Brazil, and India, with, obviously, sometimes appreciably lower yields in other countries with different geographical locations [9]. Other estimates were based mainly on laboratory scale data which probably have overestimated the potential use of microalgae for fuel production [10-12].

Currently, biodiesel has already been brought to the market. For example, in the US, the production of biodiesel was 116 million gallons in May 2015 (www. eia.gov/biofuels/biodiesel/production/).

1.4 Biohydrogen

H_2 has many advantages as an energy carrier, including its high gravimetric energy density, clean combustion waste (H_2O), and high conversion efficiency in fuel cell systems. It also have a few disadvantages, for example, it is hard to transport in a reasonable fashion and it is highly flammable (http://www.conserve-energy-future.com/Advantages_Disadvantages_HydrogenEnergy.php). There are three major types of biological H_2 production and each of them will be discussed below. I will put special emphasis on photofermentative H_2 production since this is one of the subjects of this thesis. Currently, hydrogen produced biologically is in preliminary research stage.

1.4.1 Dark fermentation.

There are two well-known H_2 producers for dark fermentation, *Escherchia coli* and *Clostridium*. Both use glycolysis to break down glucose to pyruvate and ATP with the concomitant reduction of NAD to NADH. The oxidization of NADH will lead to several other products depending on the pH conditions and the organism. These final byproducts can include butanol, butyrate, ethanol and acetone. One of the distinguishing factors between the two metabolic types is the breakdown of pyruvate leading eventually to the production of hydrogen. Facultative anaerobes (*E. coli*) will metabolize pyruvate to acetyl-CoA and formate by pyruvate formate lyase. Formate can then be used to produce hydrogen by a [NiFe] hydrogenase activity. Strict anaerobes (*Clostridium*) use pyruvate: ferredoxin oxidoreductase to break down pyruvate into acetyl-CoA, CO_2, and reduced ferredoxin. Hydrogen is then produced by a [FeFe] hydrogenase driven by the reduced ferredoxin. In either microorganism,

the theoretical maximum H_2 yield through this metabolic pathway (pyruvate to H_2) is 2 mol H_2/mol glucose. However, many strict anaerobes have additional enzyme systems capable of generating hydrogen directly from NADH, such as the newly described bifurcating hydrogenases [13]. Metabolic pathways for H_2 production are shown in Fig. 1 (Clostridia-left, *E. coli*-right).

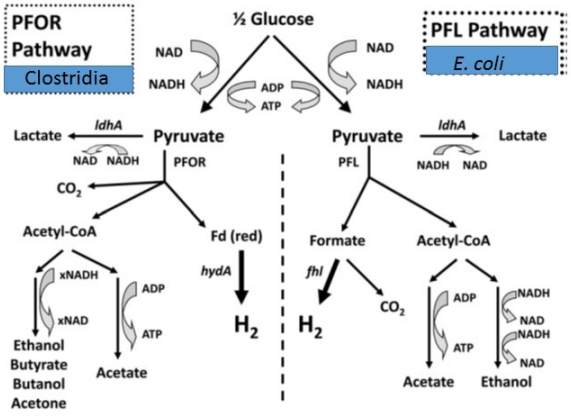


Figure 1. H_2 Metabolic pathways of *E. coli* (right) and *Clostridium* (left). PFOR: pyruvate ferredoxin oxidoreductase. PFL: pyruvate formate lyase (adapted from Hallenbeck and Ghosh, 2012, with permission [13])**.**

1.4.1.1 E. coli type dark fermentation

E. coli catalyzes mixed acid fermentation under anaerobic conditions, thus the H_2 yield is usually low. Naturally, H_2 is produced by the activity of formate hydrogenlyase (FHL). Several attempts have been made in order to increase the yield of hydrogen in *E. coli*. One modification that has been done is to inactivate *hyd1* and *hyd2* encoding hydrogenases which are more active in catalyzing H_2 oxidation. This double mutation resulted in a 37% increase in net H_2 production [14]. Another potential mutation site is *ldhA* which encodes lactate dehydrogenase, since it could cause an undesirable drain in the pyruvate pool. Inactivation of this gene led to an increase of 20-40% in net H_2 production [14-17]. However, these effects will be manifested only under acidic conditions when *ldhA* is expressed at high levels. Fumarate reductase (*frdBC*) also could give a similar effect as lactate dehydrogenase. With the inactivation of this gene, the hydrogen yield was moderately increased by 10-15% [18-20]. H_2 production from a synthetic pathway has also been proposed and demonstrated [21, 22]. In theory, 2 additional moles H_2 per mole of glucose could be obtained from the oxidation of NADH generated from the glyceraldehyde-3-phosphate node of glycolysis. In practice, three proteins, NAD(P)H:ferredoxin oxidoreductase, ferredoxin, and hydrogenase were expressed in a wild type strain *E. coli* BL21 (DE3) which doesn't produce H_2 at detectable levels, and interestingly, small amounts of H_2 were produced with a yield of 5.2 mmol H_2/mol glucose. In a similar study where the *Ralstonia eutropha* SH hydrogenase (NAD-reducing [NiFe] hydrogenase), which can use NADH as direct substrate, was introduced into *E. coli* FTD147, the hydrogen yield reached 2.11 mol H_2/mol glucose, exceeding the theoretical maximum by using FHL alone [23]. Relatively high levels of hydrogen production, 1.46 mol H_2/mol

glucose, were observed when an additional endogenous gene, *ydbK* (encoding pyruvate-ferredoxin oxidoreductase), was co-expressed with hydrogenase and ferredoxin genes [24].

1.4.1.2 Clostridium type dark fermentation

The Clostridia are strict anaerobes and can effectively metabolize a variety of substrates, including, cellulose, organic wastes, glycerol and many others [25]. They are fast growers and produce hydrogen in a growth-linked manner, thus their hydrogen productivity is high. However, like *E. coli*, one major problem with *Clostridum* type hydrogen production is that the H_2 yield is usually low due to the production of several other metabolites. Metabolic pathways and approaches toward high level H_2 production are discussed in the following sections.

1.4.1.2.1 Pathways involved in H_2 production

In Clostridia, as in many other organisms, sugars are degraded using the classical glycolytic pathway, in which two net NADH are produced through glucose degradation to pyruvate. Under low H_2 partial pressures, oxidation of these two NADH could be coupled to proton reduction to make two H_2. So far the exact mechanism of this process is not completely understood [26, 27]. Pyruvate is then converted to Acetyl-CoA by pyruvate:ferredoxin oxidoreductase with the production of a reduced ferredoxin (Fd (red)). The reduced form of ferredoxin then donates electrons to a [FeFe] hydrogenase to make another two H_2 (see Fig. 1). Thus, in theory, four mol of H_2 can be produced from 1 mol of glucose using the existing metabolic pathways. However, even 4 mol H_2/mol glucose only represents 33% of the

theoretical maximum (12 mol H_2/mol glucose). Thus, it would be desirable to introduce new metabolic pathways with the aid of synthetic biology to obtain stoichiometric conversion.

1.4.1.2.2 Metabolic engineering strategies for improving H_2 production

Klein *et al*., attempted to overexpress two different types of hydrogenases in *Clostridium acetobutylicum* DSM 792 for improving H_2 production [28]; however, the hydrogen yields and volumetric productivities of the recombinant strains were similar to the wild type. This suggests that overexpression of an enzyme would only have an effect when it catalyzes a rate limiting step. Another study, which used the same approach with a different strain, *C. tyrobutyricum* JM1, resulted in a significant improvement, 1.5-fold [29]. This indicates that, depending on the organisms and their unique metabolic pathways, overexpression of a key enzyme might bring fruitful results. Even so, thus far neither the yield nor the volumetric productivity are anywhere near the levels required for a practical system.

1.4.2.2.3 Elimination of competing pathways

As shown in Fig. 1, several products such as ethanol, lactate and butyrate are co-produced with H_2. Thus, inactivation of those pathways which lead to production of undesirable products could in theory improve H_2 production. Some examples of such a practice have already been published. In one study, the *aad* gene (encoding aldehyde-alcohol dehydrogenase) of *C. butyricum* was disrupted, however, the results showed that the hydrogen production of the mutated strain was equivalent to that of the wild type [30]. There are at least two explanations for this result. For example, ethanol was not a major metabolite under the

culture conditions used, or other existing pathways were more favorable. In another study, the *ldh*A gene (encoding lactate dehydrogenase) of *C. perfringens* W11 was disrupted, which resulted in a 44% improvement in hydrogen yield [31]. A third report, which studied the effects of the inactivation of the *atoB* (encoding acetyl-CoA acetyltranferase, the first enzyme in the butyrate formation pathway) on H_2 production showed decreased hydrogen yields with a corresponding increase in ethanol production [32]. Thus, it seems that inactivation of supposedly competing pathways does not always work in the expected way.

1.4.2 Photofermentation

1.4.2.1 A brief history of photofermentative hydrogen production

In 1949, Gest and Kamen observed vigorous hydrogen production when they grew *Rhodospirillum rubrum* (*Rho. rubrum*) photosynthetically with certain oxidized substrates as carbon source and glutamate or aspartate as a nitrogen source [33, 34]. Through careful comparison of conditions that repressed H_2 production to those that derepressed H_2 production, they were able to find evidence for the existence of a functioning nitrogenase system in *Rho. rubrum* [35]. However, the technique for purification of the nitrogenase complex wasn't developed until 1966 when Bulen and LeComte successfully purified nitrogenase from *Azotobacter* [36]. In 1978, the isolation and purification of nitrogenase from the photosynthetic bacterium *Rho. rubrum* in an active form was separately done by two groups [37, 38], after which it became possible to study the general characteristics of nitrogenase *in vitro*.

1.4.2.2 Nitrogenase composition and catalytic characteristics

Nitrogenase consists of two proteins, MoFe protein (also called dinitrogenase), a molybdenum and iron-containing protein, and Fe protein (also called dinitrogenase reductase), an iron-containing protein. Neither of these two proteins show nitrogenase activity separately. Both proteins function as a system in such a way that at the first step, the Fe protein receives electrons from a low redox potential electron donor, such as ferredoxin or flavodoxin, then electrons are passed to the MoFe protein where electrons are used to reduce N_2. When N_2 is not available, nitrogenase can reduce protons to produce H_2. This is why photofermentative hydrogen production has been performed under argon or helium atmospheres. The main physiological reaction catalyzed by nitrogenase can be represented by equation 1:

$$N_2 + 8H^+ + 8e^- + 4MgATP \longrightarrow 2NH_3 + H_2 + 4MgADP + 4Pi$$

Besides N_2 and H^+, a number of other substances serve as substrates of nitrogenase, such as HCN, N_2O and C_2H_2 [39]. The reduction of acetylene to ethylene, discovered by Dilworth in 1966, forms the basis of a highly sensitive assay for nitrogenase [40]. The reaction catalyzed by nitrogenase is ATP dependent, thus it is irreversible. It was found that nitrogenase keeps catalyzing H_2 production even under 100% H_2 [34]. More surprisingly, nitrogenase can still produce H_2 at barometric pressures of up to 50 atm [41]. However, nitrogenase is slow with a turnover number (also called K_{cat}) of 6.4 s^{-1} [42]. A typical TCA cycle enzyme, such as fumarase, has a turnover number of 800-900 s^{-1}. Thus the catalytic efficiency of nitrogenase is about a hundred-fold lower [43]. In order to couple with other biological processes, it is not uncommon to observe cells synthesizing large quantities of nitrogenase. Both components of

nitrogenase are highly O_2 labile, especially the Fe-protein, with a half-life about 30 seconds when exposed to atmospheric O_2 [44].

1.4.2.3 Energy sources for nitrogenase activity

In photosynthetic bacteria, ATP can be generated in at least three ways, photophosphorylation, substrate level phosphorylation and oxidative phosphorylation. Of these, photophosphorylation is the most efficient, as it was reported that the rate of photophosphorylation at 5000 lux illumination was about 10-fold higher than oxidative phosphorylation with H_2 as substrate [45] Under microaerobic conditions, for example, O_2 represents 0.5% percent of the gas phase, trace amounts of O_2 were found to support nitrogenase activity by providing energy [46]. It should be noted that the O_2 percentages of permissive microaerobic conditions are species-dependent. It has been demonstrated by Madigan *et al*. that *Rhodobacter capsulatus* can grow and fix N_2 under dark anaerobic conditions with fructose as a fermentable substrate. Thus it can be inferred that the ATP supply for nitrogenase can be met by substrate level phosphorylation [47].

1.4.2.4 Natural electron donors to nitrogenase

Although it is generally accepted that organic substrates serve as the ultimate electron donor to nitrogenase for the photoheterotrophically grown purple non-sulfur photosynthetic bacteria, the entire electron transport pathway from organic substrates to nitrogenase has not been completely explored. One of the biggest difficulties is that the reduction of low redox potential ferredoxin by illuminated chromatophores (analogous to plant chloroplasts) of non-

sulfur purple bacteria has never been achieved, whereas ferredoxin can be reduced by the illuminated chloroplasts of plants. This raises the question of how the direct electron donor to nitrogenase is reduced. One hypothesis proposed many years ago is that generation of reductant by *Rho. rubrum* (one of the best known purple non-sulfur phtotosynthetic bacteria) during photosynthesis may occur by reverse electron flow powered by ATP hydrolysis to NAD rather than to ferredoxin [48], then the NAD/NADH pool would form an equilibrium with the ferredoxin (red)/ferredoxin (oxd) pool to supply electrons to nitrogenase. Until now, this theory still serves as the basis for an understanding of photofermentative H_2 production by the nitrogenase system. Despite the ambiguity of the entire electron transport chain towards nitrogenase, ferredoxin in its reduced form, a small molecular weight protein, has been assumed to be the direct electron donor to nitrogenase. The reaction center can be 4Fe-4S, 2Fe-2S or other combinations, depending on the types of ferredoxin [49]. In *R. capsulatus*, Ferredoxin I (FdI) encoded by *fdxN* is believed to be the natural electron donor to nitrogenase [50]. This is primarily based on the following discoveries: (I) the *fdxN* gene is located upstream of *nif* gene clusters and its synthesis is under the control of the *nif* regulatory gene *nifR1* [51], (II) *In vitro* nitrogenase assays showed that FdI is an efficient electron donor to nitrogenase [52]. In *Rho. rubrum* it was once believed that FdI (encoded by *fdxN*) was the primary electron donor to nitrogenase, since (I) during diazotrophic growth condtions, the *fdxN* gene was found to be up regulated two-fold; (II) *In vitro* nitrogenase assays showed FdI is about three times more efficient than FdII [53]. However, a recent study suggests FdN, instead of FdI, is the primary electron donor to nitrogenase [54].

Another electron donor of the intermediate electron transport chain that has been identified so far is the Rnf complex, a putative membrane bound complex. Physiological growth studies

have shown that the Rnf complex was necessary for nitrogenase activity under photoheterotrophic conditions since insertion and deletion mutants lost the ability to grow diazotrophically [55]. Another piece of evidence is from a study of the effect of overexpression of *rnf* on nitrogenase activity. The results of this study showed that *in vivo* nitrogenase activity was significantly improved. [56].

1.4.2.5 Calvin-Benson-Bassham cycle- A major competitor of nitrogenase

Under photofermentative conditions, the excess reductants generated from the central metabolism are consumed by Calvin-Benson-Bassham cycle (Calvin cycle), PHB synthesis, and nitrogenase system in order to maintain redox balance. Several studies have shown that the Calvin cycle is the preferred pathway to serve this purpose [57, 58]. Thus, elimination of Calvin cycle flux should result in improved H_2 production catalyzed by nitrogenase. In *R. capsulatus*, genes encoding Calvin cycle enzymes are located in two operons [59], namely cbb_I and cbb_{II}, shown in Figure 2.

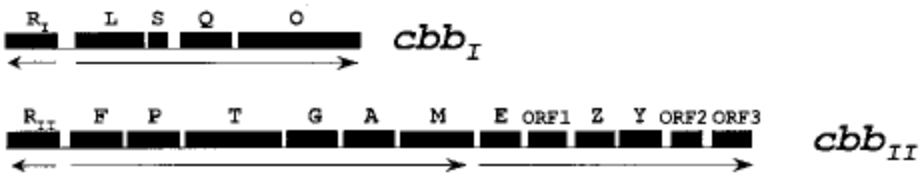


Figure 2. Gene organization of *Rhodobacter capsulatus* cbb_I and cbb_{II} operons. The cbb_I operon contains *cbbL* and *cbbS*, encoding the large and small subunits, respectively of form I RubisCO. It is not established if *cbbQ* and *cbbO* are definitely part of the same operon and their functions are unknown. $cbbR_I$ is located upstream and is transcribed from the oposite direction to *cbbLS*. The cbb_{II} operon contains *cbbM*, which encodes form II RubisCO (encoded by *cbbM*), and other *cbb* structural genes. The $cbbR_{II}$ is located upstream and divergently transcribed from cbb_{II} operon. Arrows indicate the direction of transcription. Gene designations are as follows: *cbbF*, fructose-1,6-bisphosphate/sedoheptulose-1,7-bisphosphatase (FBP); *cbbP*, phosphoribulokinase (PRK); *cbbT*, transketolase (TKL); *cbbG*, glyceraldehye-3-phosphate dehydrogenase (GAP); *cbbA*, fructose-1,6-bisphosphate/sedoheptulose 1,7-bisphosphate aldolase (FBA);cbbE, ribulose-5-phosphate-3-epimerase (EPI); *cbbZ*, phosphoglycolate phosphatase; *cbbY*, ORF1, ORF2 and ORF3 encode genes of unknown function (adapted from Vichivanives *et al*., with permission [59]) .

The complete Calvin-Benson-Bassham cycle pathways are shown in Figure 3. The metabolites and enzymes are annotated in the figure legend [60]. It should be noted here that this figure is generalized from many autotrophic bacteria, thus it also fits *Rhodobacter capsulatus*.

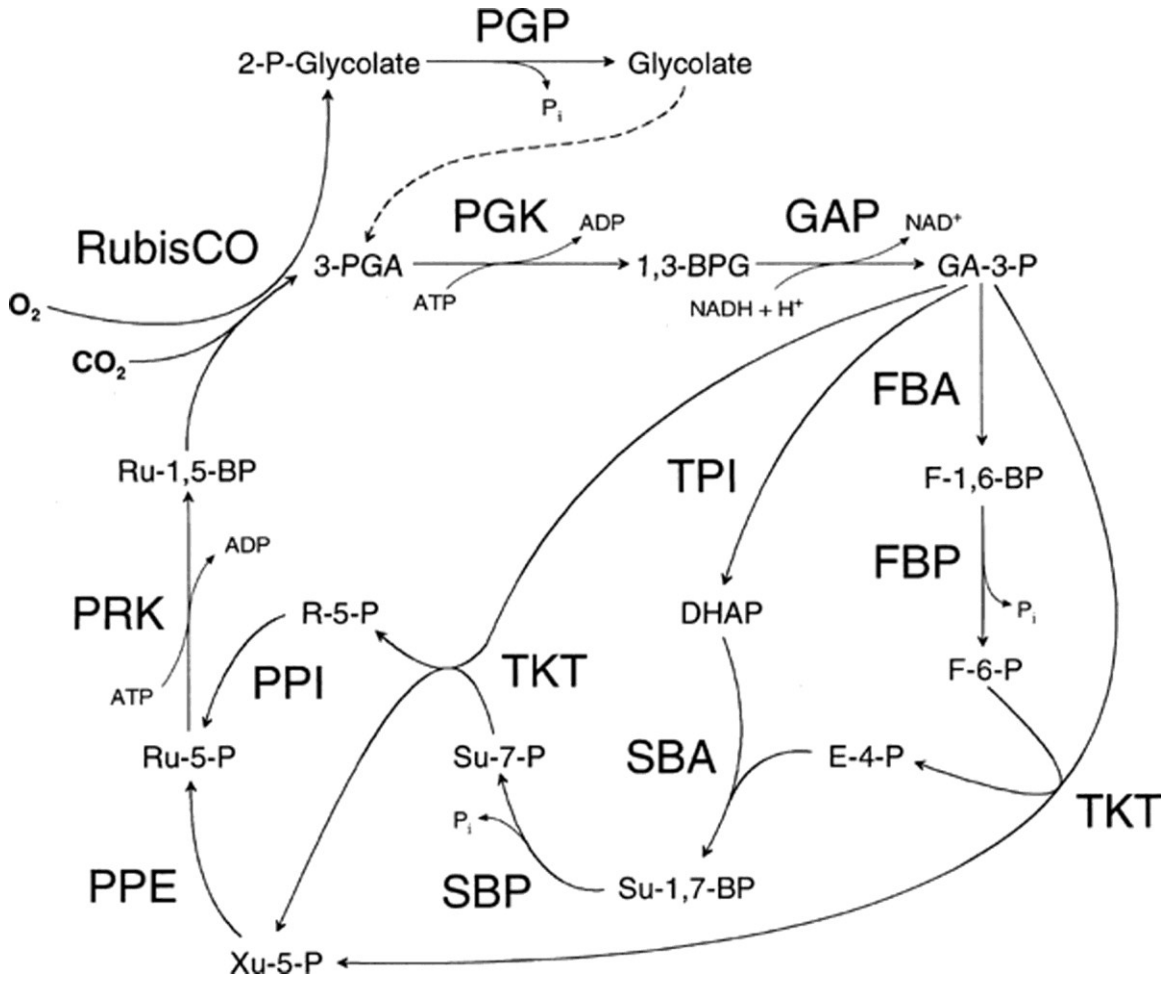


Figure 3. Reactions and enzymes of Calvin-Benson-Bassham cycle in autotrophic bacteria. Abbreviations for metabolites: 3-PGA, 3-phosphoglycerate; 1,3-BPG, 1,3-bisphosphoglycerate; GA-3-P, glyceraldehyde-3-phosphate; DHAP, dihydroxy-acetone-phosphate; F-1,6-BP, fructose-1,6-bisphosphate; F-6-P, fructose-6-phosphate; E-4-P, erythrose-4-phosphate; Su-1,7-BP, sedoheptulose-1,7-bisphosphate; Su-7-P, sedoheptulose-7-phosphate; R-5-P, ribose-5-phosphate; Xu-5-P, xylulose-5-phosphate; Ru-5-P, ribulose-5-phosphate; Ru-1,5-BP, ribulose-1,5-bisphosphate. Abbreviations for enzymes: FBA, frucose-1,6-bisphosphate aldolase, FBP, frutose-1,6-bisphosphate, GAP, glyceraldehyde-3-phosphate dehydrogenase, PGK, 3-phosphoglycerate kinase, PGP, phosphglycolate phosphatase, PPE, pentose-5-phosphate 3-epimerase, PPI, pentose-5-phosphate isomerase, PRK, phosphoribulokinase, RubisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase, SBP, sedoheptulose-1,7-bisphosphatase, TKT, transketolase, TPI, triosephosphate isomerase (adapted from Kusian and Bowien, with permission [60]).

Metabolic engineering approaches as well as other means for improved photofermentative H_2 production will be discussed in more detail in Chapter 2.

1.4.3 Biophotolysis-hydrogen production by algae and cyanobacteria

Biophotolysis is the photosynthetically driven splitting of water to produce hydrogen and oxygen. Hydrogen production by biophotolysis with algae or cyanobacteria is inherently appealing since the major nutrient requirement for these two species are water and solar energy, which exist in abundance. However, earlier studies (before 2000) of this process showed that H_2 can't be produced in a sustained manner and that the small amounts of H_2 produced were soon consumed by the activity of uptake hydrogenase. Thus, H_2 can only be monitored by fast techniques such as that provided by membrane-inlet mass spectrometers. The main reason for the short-lived H_2 production is that the hydrogenase of algae and cyanobacteria which catalyzes proton reduction, is sensitive to O_2, an obligate product of the activity of photosystem II (PSII) [61, 62]. Some O_2 absorbers have been used in attempts to overcome this problem, but obviously such a method won't be economically feasible in a practical large scale H_2 production process [62].

In order to solve this problem, two stage systems have been developed. One such system that has been extensively researched is the use of sulfur deprivation to achieve sustained H_2 production. In this approach, the first stage is a biomass accumulation process, and for the second stage the culture is transferred to a S-depleted medium. Without sulfur, protein can't be synthesized and cells go into a resting stage. Continued photosynthesis without the possibility for repair causes depletion of the D1 protein, which is essential for photosystem II

activity, thus the residual photosynthetic activity becomes very low, dropping below the respiratory oxygen uptake compensation point. Consequently, the intracellular environment becomes anaerobic, allowing for sustained H_2 production. In this way, H_2 production can be sustained for several days. However, the H_2 production rate is very low, about 2 ml/g cell/h, a value about 5-10% of what has been achieved by photosynthetic bacteria [63]. As mentioned above, hydrogenase is very sensitive to O_2, thus protein engineering to make hydrogenase insensitive to O_2 could be a fundamental solution for bio-photolysis based H_2 production. Indeed, some efforts have already been put towards this goal [64-66]. In one study, a mutant strain which can tolerate 8-fold higher amounts of O_2 has been constructed by mutation of amino acids that form a gas channel [64]. Although O_2 tolerance of the mutant is still low, the results are nevertheless encouraging since further modification could in theory lead to even higher levels of oxygen tolerance. The H_2 production pathway of algae and cyanobacteria is shown in Fig. 4 [42].

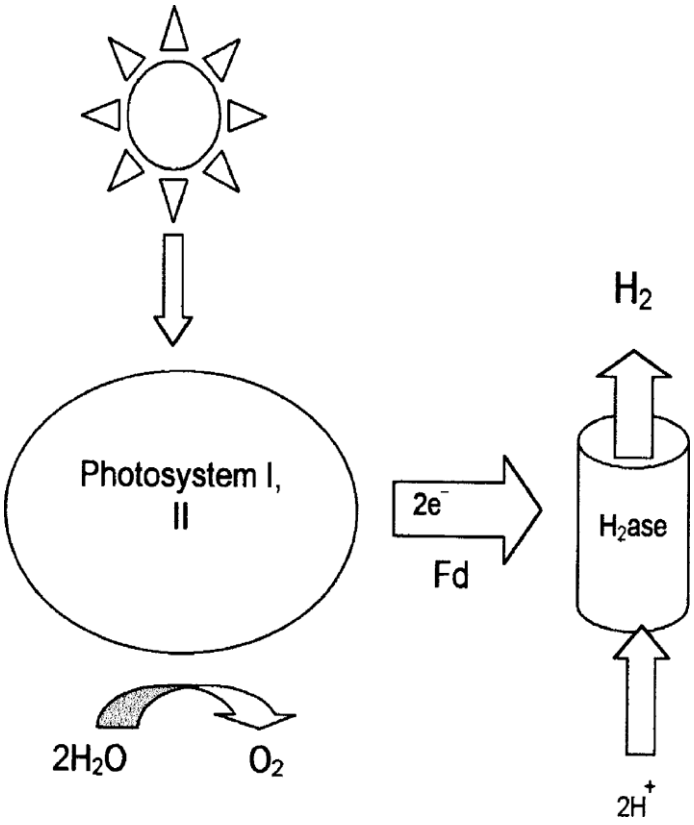


Figure 4. Biophotolysis H_2 production by Algae and Cyanobacteria. The sunlight is captured by the photosynthetic apparatus, then the recovered energy is used to couple water splitting to generate a low potential reductant (ferredoxin), which can be used to reduce a hydrogenase enzyme (adapted from Hallenbeck and Benemann, 2002, with permission [42]).

1.4.4 Improving biofuel production through experimental design

Design of experiments (DOE) methodology includes many subtopics and has been effectively applied to many subjects including life sciences. Examples can be readily found in many reports [67]. In the biological sciences, factorial design coupled with response surface methodology appears to be the most commonly used strategy. The principle of DOE can be found in many textbooks, thus it will not be discussed here.

1.4.4.1 General procedures for performing DOE

Here, a general procedure for applying DOE methodology for H_2 production is given based on my own experience as well as what can been found in the literature (see Fig. 6). The first step of this process is to define the experimental variables and desired responses. Next, a particular design should be selected. Factorial designs are ideal as they are orthogonal and balanced. For a first order design, e.g., 5 variables with each having 2 levels, fractional factorial design is a good choice since it has the advantage of requiring fewer experiments. For a second order design, Box-Behnken design and center composite design are the most commonly used. For 3 variables with 3 levels, Box-Behnken design contains fewer experiments than a center composite design, but for more variables the latter one is preferable. It should be noted that experimental runs should be performed in a randomized manner no matter what type of design is being used. After completion of the experiments, data is fit to a quadratic model with the following formula:

$$y = \sum_{i=1}^{N} a_i X_i + \sum_{i=1}^{N} b_i {X_i}^2 + \sum_{i<j}^{N} c_{ij} X_i X_j$$

(y: response; x_i, x_j: variables; a_i, b_i, c_{ij}: coefficients)

Regression analysis and analysis of variance are the general statistical tools used for validation of the design. In some cases, the validation yields a conclusion that the design variables have no significant effects on the response. In this case, one should consider redesigning the experiment through approaches like changing the step size in the design variables, as shown in Fig. 5.

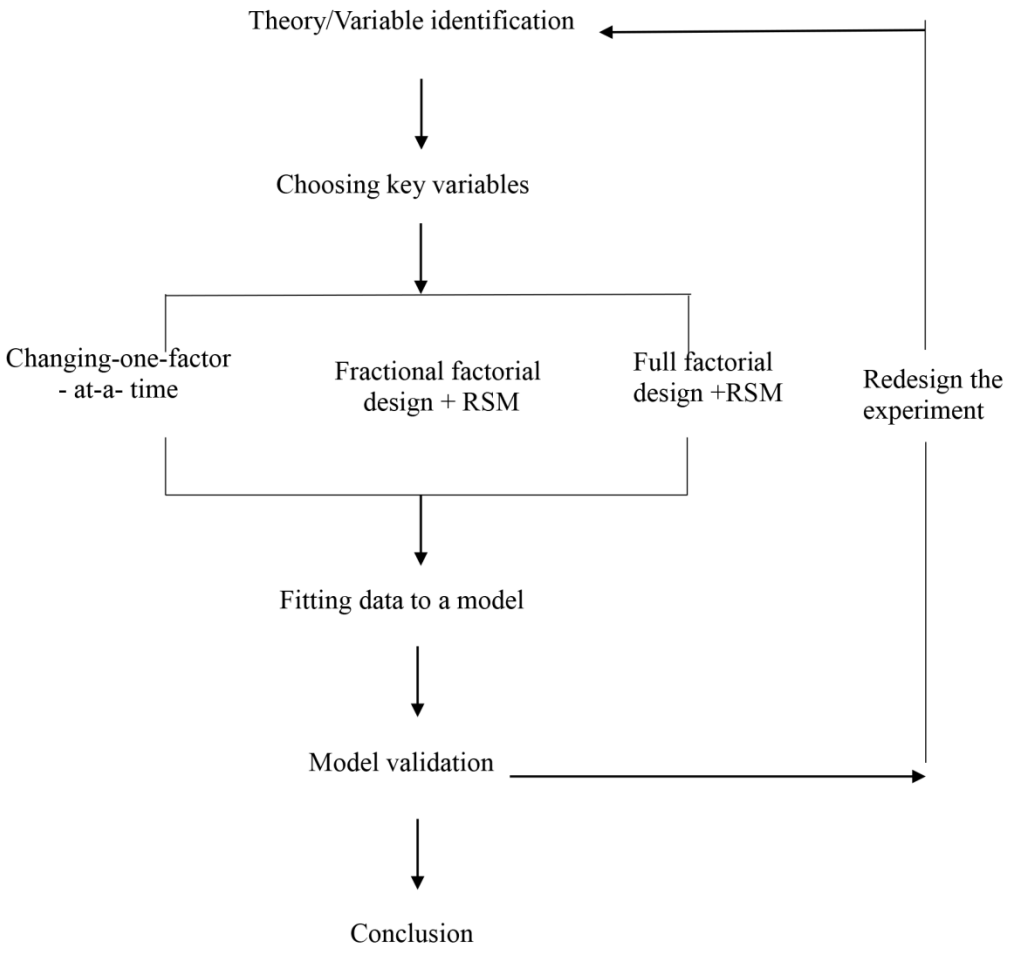


Figure 5. A general procedure for application of DOE approach.

1.4.4.2 Common software for DOE methodology

Typically, general purpose statistical software like Minitab®, or purpose designed and dedicated software like Design Expert®, are suitable. Once the software is installed, only a

few steps are required to choose the design and generate a corresponding experimental run table. Online tutorials are available:

(http://www.minitab.com/uploadedFiles/Documents/gettingstarted/Minitab17_GettingStarted-en.pdf).

When experiments are completed, the data (response values) are input and a regression analysis function or ANOVAR is used to do statistical analysis for a validation of the model.

1.4.4.3 An example of the use of DOE methodology for improved H_2 production

The application of DOE methodology for H_2 production has been recently reviewed [67], thus only two important examples are given here for illustrating purposes. In one study, the Box-Behnken design was used to optimize three variables, glycerol concentration, glutamate concentration and light intensity. The result of this study showed near stoichiometric conversion of glycerol to hydrogen had been achieved - 96% of the theoretical maximum. This corresponds to a 20% percent improvement compared to a previous batch study which only achieved 75% of theoretical maximum [68, 69]. Another study which applied the same strategy achieved a H_2 yield of 1.69 mol/mol glucose with genetically engineered *Escherichia coli* [70]. This suggests that a DOE approach is effective for optimization purposes, and certainly deserves consideration.

Other means for improving photofermentative H_2 production are further discussed in Chapter 2.

2. Regulation of nitrogenase

2.1 Nitrogenase switch-off phenomenon and mechanism induced by ammonium shock

Nitrogenase switch-off is a phenomenon that has been observed when some diazotrophs, grown under nitrogen fixation conditions, are suddenly exposed to environmental stimuli, such as darkness, O_2, or a fixed nitrogen source (ammonia, glutamine, etc.). These stimuli cause nitrogenase activity to cease after a few minutes (or longer), with recovery of nitrogenase activity once the environmental stimulus is removed or metabolized [71]. The study of switch-off effects caused by ammonia is of interest to many research groups.

Rho. rubrum is the best understood phototroph in terms of ammonia switch-off effects and the molecular mechanism behind it has been explored in substantial depth. It was found that the Fe-protein subunit of nitrogenase was covalently modified by an ADP-ribose moiety at arginine-101 when nitrogen starved cells were suddenly exposed to ammonia [72-76]. The enzyme which catalyzes this covalent modification was purified, characterized and named dinitrogenase reductase ADP-ribosyltransferase (DraT) [77]. The modified Fe-protein can no longer transfer electrons to MoFe-protein, thus nitrogenase activity was switched off [78, 79]. However, once the added ammonia was metabolized, the inactivated Fe-protein can be converted back to its active form by the activity of dinitrogenase reductase activating glycohydrolase (DraG) [80]. Sequencing data has shown *draT/G* genes are contiguous on the *Rho. rubrum* chromosome and highly linked to *nifHDK* genes (structural genes of nitrogenase) [81]. Studies have also shown that *draT* and *draG* are cotranscripted in *Rho. rubrum* since a polar mutation in *draT* resulted in undetectable *in vivo* activity of DraG [82].

DraT and DraG are also subject to regulation, a process believed to be linked to PII proteins, which act as sensors of cellular nitrogen, carbon and energy levels and control the activities of a wide range of target proteins by protein-protein interactions. It was found that mutants of *Rho. rubrum* which lack AmtB1 are defective in response to ammonia shock, most probably due to membrane sequestration of DraG in a process dependent upon the involvement of GlnJ and AmtB1 [83, 84]. Another pivotal player in this regulatory process is GlnD, which is a bifunctional uridylyltransferase/uridylyl-removing enzyme. It is thought to be the primary sensor of nitrogen status in the cell and reversibly modifies PII proteins (such as GlnK and GlnB), which in turn regulate a variety of other proteins [85].

R. capsulatus is also one of the most intensively studied diazotrophs for ammonium induced nitrogenase switch-off. AmtB was also found to be necessary for the ammonia induced nitrogenase switch-off and ADP-ribosylation of the Fe-protein [86]. However, there are at least two distinct differences between *Rho. rubrum* and *R. capsulatus* in terms of the molecular mechanism of ammonium induced switch-off effects: (1) a mutant of *R. capsulatus* which has a mutated *glnB* homologue was found to be capable of Fe protein modification, indicating that GlnB is not absolutely required for the ammonia induced covalent modification [87]; (2) a *draT*/*draG* double mutant, W107I, showed ammonia induced switch-off effects, suggesting a second alternative mechanism exists in *R. capsulatus* [88, 89].

Another well studied diazotroph for nitrogenase switch-off is *Rhodopseudomonas palustris*. Here is an example to illustrate a general signal transduction pathway from ammonium shock [90], Fig. 6. Although many pivotal players involved in ammounim induced switch-off effects have been identified and interactions between some of them partially characterized, the thorough understanding of the phenomenon is far from complete.

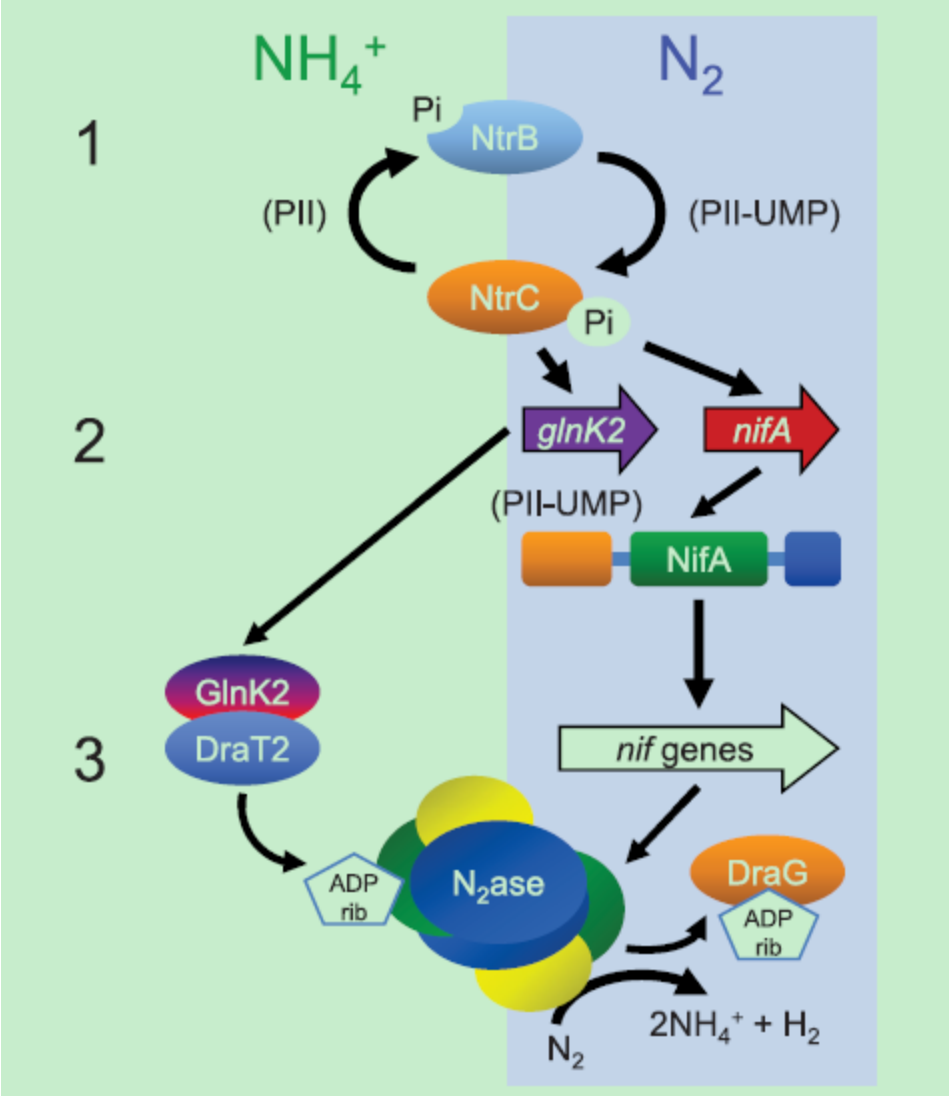


Figure 6. A model for the regulation of nitrogenase activity in *Rhodopseudomonas palustris* strains. When cells are exposed to ammonium, NtrB in combination with a bound nonuridylylated PII protein stimulates dephosphorylation of NtrC, as a result, *glnK2* expresses at low levels and DraT2 restores its ability to ADP-ribosylate nitrogenase reductase. The modified nitrogenase loses its activity. NifA: master transcriptional activator for nitrogenase genes; NtrB: sensor kinase; NtrC: nitrogen regulatory protein C; GlnK2 belongs to PII group; DraT: dinitrogenase reductase ADP-ribosyltransferase; DraG: dinitrogenase-reductase-activating glycohydrolase (adapted from Heinger *et al*., 2011, with permission [90]).

2.2 Transcriptional regulation of nitrogen fixation genes by ammonium

In *Rhodobacter capsulatus*, nitrogen fixation related genes are found dispersed in several groups on the chromosome encoding two nitrogenase systems, which are composed of at least 53 structural and regulatory genes [91, 92]. A number of environmental factors were found to influence nitrogen fixation gene expression, including O_2, availability of fixed nitrogen source and light intensity [93, 94]. Thus, it can be seen that the regulation of nitrogen fixation is extraordinarily complicated. Therefore, in this section, only regulation of nitrogen fixation genes by ammonium will be discussed. The papers reviewed here are those studies in which standard RCV medium was used to ensure that only *nif* nitrogenase will be expressed.

The available data for deducing the nitrogen fixation regulatory cascade include DNA sequence of those genes, their promoters and *in vitro* protein-protein interactions [95-102]. Transcriptional regulation happens at two levels. At the first level, under nitrogen limited conditions, NifR2 phosphorylates NifR1, then NifR1-P works with RpoN to activate NifA. The NifA activity is in turn regulated by GlnB and GlnK. At the second level, NifA works with NifR4 to activate *nif* structural genes-*nifHDK* expression [103-105]. The regulatory cascade is shown in Fig. 7.

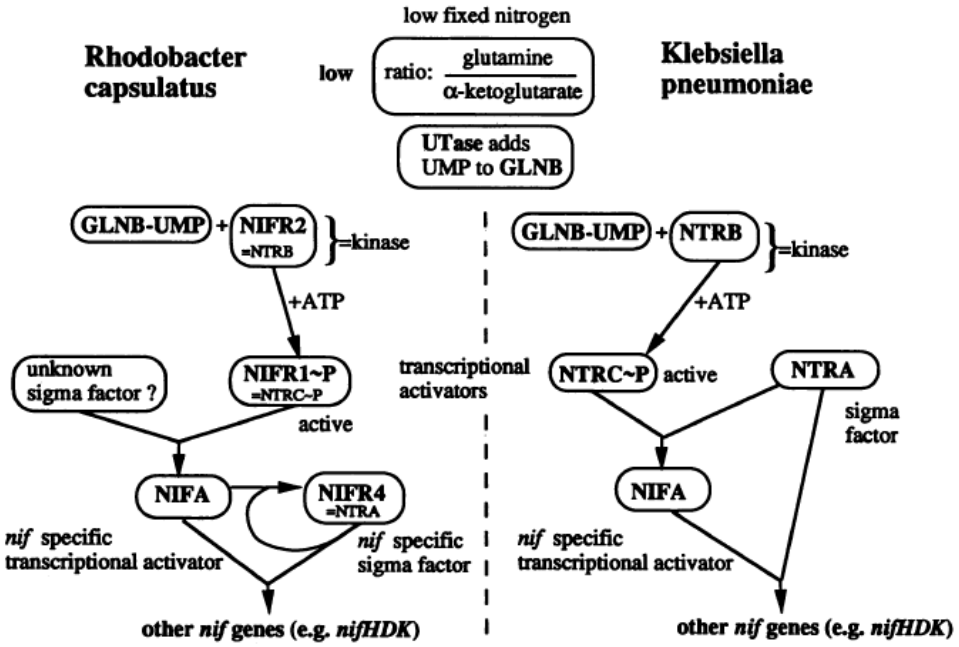


Figure 7. Comparison of nitrogen fixation regulatory models for *Rhodobacter capsulatus* and that for *Klebsiellla pneumonia.* UTase: uridyltransferase (adapted from Hubner *et al*., 1991, with permission [105]).

Chapter 2: Recent Advances in Hydrogen Production by Photosynthetic Bacteria

Patrick C. Hallenbeck[1,2] and Yuan Liu[2]

[1] Life Sciences Research Center, Department of Biology

United States Air Force Academy

2355 Faculty Drive, USAF Academy, Colorado 80840

[2]Department of Microbiology, Infectious Diseases and Immunology

University of Montreal,

CP 6128 Succursale Centre-ville, Montreal, Quebec, Canada H3C 3J7

Author contribution: PCH wrote the outline. YL expanded it. PCH revised and finalized it. PCH and YL made the figures and tables.

Article status: Published, International Journal of Hydrogen Energy, 2016, 41:4446-4454.

Abstract

The photosynthetic bacteria have a very versatile metabolic repertoire and have been known for decades to produce hydrogen during photofermentative growth. Here, recent advances in hydrogen production by these organisms are reviewed and future directions highlighted. Often used as a second stage in two stage hydrogen production processes; first stage fermentative sugar to hydrogen and organic acids; second stage, organic acids to hydrogen, recent studies have highlighted their ability to directly convert sugars to hydrogen. Several studies have attempted to optimize a single stage batch process and these, and a study with continuous cultures have shown that yields approaching 9 mol H_2 / mol glucose can be obtained. One of the drawbacks of this system is the dependency on light, necessitating the use of photobioreactors, thus potentially greatly adding to the cost of such a system. In another approach which avoids the use of light energy, microaerobic fermentation of organic acids to hydrogen, driven by limited oxidative phosphorylation has been demonstrated in principle. Further advances will probably require the use of metabolic engineering and more sophisticated process controls in order to achieve higher stoichiometries, approaches that might be applied to other, light dependent, hydrogen production process by these organisms.

Keywords: Biological hydrogen production, photosynthetic bacteria, photofermentation, energy from wastes, improving rates and yields

1. Introduction

Photosynthetic bacteria have long been studied for their capacity to produce hydrogen from organic acids in a light dependent reaction that is called photofermentation, generating hydrogen from substrates for which this is normally difficult from a thermodynamic point of view [1]. This is possible due to the input of extra energy through bacterial photosynthesis (Figure 1). Although organic acids, available as wastestreams, have traditionally been used as substrate, potentially also allowing for waste treatment credits, a number of novel substrates have recently been shown to be effective, extending the range of these organisms [2]. As discussed below, the nature of this process allows almost stoichiometric conversion of various substrates to hydrogen. Many studies, now numbering in the hundreds, have examined various aspects of hydrogen production by these organisms. However, a number of significant challenges remain, including; low light conversion efficiencies, the high energy demand and low turnover number of nitrogenase, and the potential requirement for expensive hydrogen impermeable photobioreactors. Here the basic mechanisms involved are discussed and some of the recent advances in this field towards higher hydrogen yields are reviewed. These involve integrated approaches, where sugars are degraded using two-stage systems, or attempts to carry out this type of conversion in a single stage. Finally, other approaches for system improvement, including novel physiological approaches or metabolic engineering, are presented.

2. Basic Mechanisms

Hydrogen is evolved by the enzyme nitrogenase which, normally active in N_2 reduction, will reduce protons to hydrogen in its absence [3]. This reaction is ATP dependent and therefore energetically demanding. Normally, this requirement can be met through the action of bacterial photosynthesis, producing ATP through the light driven creation of a proton gradient (Figure 1). This proton gradient is also used to generate, through a process of reverse electron flow, the low potential electrons required for proton reduction. The synthesis of this enzyme is repressed under N-replete conditions [3], so normally substrates must be N-poor, unless special permissive mutants are used [4].

Knowledge of the metabolic pathways involved can be used in attempts, through metabolic engineering, to increase hydrogen yields [5], as discussed further in section 3.4. Basically, it would appear that blocking pathways that divert NADH, CO_2 fixation and PHB (polyhydroxybutyrate) synthesis, should render more reductant available for nitrogenase and its reduction of protons to hydrogen.

3. Different approaches toward improving H_2 production by photosynthetic bacteria

3.1 Photofermentations

As already eluded to, the majority of studies on H_2 production by photosynthetic bacteria have involved the use of organic acids, lactate, acetate, and succinate, as substrates [6-9]. As noted, except with special mutant strains, these reactions take place only in the absence of ammonium. While the metabolic advantage to the cell is not immediately apparent, and in fact might even seem paradoxical given the energetically demanding fixation of carbon

dioxide in the presence of an easily assimilated fixed carbon source, a variety of evidence suggests that proton reduction to hydrogen under these conditions is necessary to maintain the cellular redox poise [10]. Recent results suggest that an additional factor restricting yields is the formation of SMPs (soluble microbial products), and that this can be overcome through the addition of a small amount of ethanol, an effect that is not fully understood at present [11]. Reduction in SMP production would most likely aid in increasing hydrogen production, as this study showed, but metabolic intervention to bring this about is not obvious since SMPs produced by various types of fermentation appear to be a complex mixture of proteins, amino acids, nucleic acids, organic acids, products of energy metabolism, and cellular structural components [12].

3.2 Single and Two-stage Systems

A variety of systems have been investigated where photosynthetic bacteria are used in conjunction with fermentative bacteria in attempts to drive the nearly complete conversion of sugars to hydrogen. In principle, 12 moles of H_2 can be derived from one mole of glucose (equation (1)).

(1) $C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 12H_2$

However, dark fermentation alone can produce at most only 4 moles of H_2, a yield of only 33%, while at the same time producing, as side products, organic acids such as acetate and lactate. Of course, many of these substrates are in fact the products of other microbial fermentations, suggesting that photofermentative hydrogen production could be used to produce additional hydrogen after, or alongside, a dark hydrogen fermentation [1, 2, 13].

Photosynthetic bacteria can be used to convert these organic acids to additional hydrogen, either in a single combined process, or in a two stage process (figure 2). Although elegant in principle, in practice these systems are difficult to operate and optimize. In combined systems, the very different culture requirements of the two different organisms are problematic, and in two stage systems, the effluent from the dark fermentation stage in fact requires substantial treatment and handling, both costly in inefficient processes, to bring it to a state where it is in fact a suitable, non-inhibitory substrate for the photosynthetic bacteria.

Initially, dark fermentation and photofermentation were coupled as a two sequential stages towards the complete extraction of available electrons for H_2 production, which should theoretically give 12 moles of H_2 per mole of glucose (equation (1)). For example, in one study, a hyperthermophile, *Caldicellulosiruptor saccharolyticus*, was used to ferment molasses in a dark stage, followed by the use of several different photosynthetic bacteria for hydrogen production in a light driven process using organic acids generated from the previous stage [14] (Table 1). This approach essentially is of low efficiency considering the high energy input and possible complex design of a bioreactor which is suitable for the two very different processes. Essentially similar results were seen with the use of integrated systems to convert potato starch to hydrogen where combined yields near 5.6 mol H_2 per mol of glucose were noted [15, 16], or with a system consisting of *Clostridium butyricum* and *Rhodopseudomonas palustris* converting sucrose to hydrogen [17]. Finally, a two stage system has been proposed for the conversion of glycerol, available as a biodiesel production waste, to hydrogen, but molar yields were quite low (Table 1) [18]. In fact, it would appear that a simple one-stage process can be quite effective, giving essentially stoichiometric conversion of glycerol to hydrogen (see below and Table 1). Recently, significant

improvement in yields for a two-stage system, to 9.4 moles H_2 per mole of glucose, was demonstrated by the use of the capnophilic fermentation of glucose to lactate and hydrogen by *Thermotoga neapolitana* (Table 1) [19]. Essentially this is possible since increased CO_2 partial pressure shifts the fermentation products towards more lactate at the expense of acetate, thus producing a higher yielding substrate for the photosynthetic bacterium, without affecting the hydrogen yield by *T. neapolitana*. In an alternative approach, which is relatively novel since it has been little studied until now, direct photofermentation of sugars to hydrogen is being developed. Although organic acids have preferentially been used for photosynthetic bacteria, early reports on these organisms noted that some were able to grow on different sugars. However, the conversion of these substrates, potentially quite abundant in different waste streams [2], or as energy crops, to hydrogen has been little studied until now [20-24]. An initial study showed that the direct conversion of glucose to hydrogen using only the photosynthetic bacterium *Rhodobacter capsulatus* JP91 was possible, achieving a H_2 yield of 3 mol H_2 per mole of glucose [20]. Later work which applied response surface methodology or continuous culture mode of operation, further improved hydrogen yields to 5.5 and 9 mol H_2 per mol of glucose, respectively [21-22] (Table 1). In a more practical demonstration of a possible single step process, the conversion of suitably diluted wastes from sugar refining (beet and blackstrap molasses) has been shown [23]. Similar yields (7.07 mol H_2 per mol of glucose) have also been found for a mutant strain of *Rhodovulum sulfidophilum* [24]. Further increases in rates and yields can be sought through the use of metabolic engineering (section 3.4).

Related research has shown that glycerol, an abundant waste product of the biodiesel industry, can be quantitatively converted to hydrogen by photofermentation, achieving a yield

of 6.9 mol of H_2 per mol of crude glycerol, which corresponds to 96% of the theoretical [25] (Table 1), as shown in equation (2).

(2) $C_3H_8O_3 + 3H_2O \leftrightarrow 3CO_2 + 7H_2$

As well, recent results show that *Rhodopseudomonas palustris* CGA009 can be effectively used for the conversion of ethanol to hydrogen in a biological reforming reaction. A yield of 2 mol H_2 per mol of ethanol was achieved, which is comparable to a chemical steam reforming process [26].

3.3 Microaerobic Hydrogen Production

Achieving a yield of above 4 moles of H_2 per mole of glucose requires the input of energy to overcome what is in reality a thermodynamic barrier. During photofermentation, this energy comes from the captured photons. However, photofermentation is inefficient precisely because of the requirement for light and the inherent inefficiency of photosynthesis. Another possibility for adding metabolic energy to the process would be to allow very limited aerobic respiration, which would generate additional ATP and proton gradients, which are needed to drive the reverse electron flow that is required to make the necessary reductant for nitrogenase activity (Figure 3).

However, this is a seemingly paradoxical process since the hydrogen producing process is strictly anaerobic. Nonetheless, there is a precedent for the possible functioning of such a process since these, and some other organisms, are known to be capable of carrying out microaerobic nitrogen fixation.

In order to function, nitrogenase requires energy input in the form of ATP as well as reducing power. Photosynthetic bacteria can generate ATP via different mechanisms,

including photosynthesis in the light, and substrate level phosphorylation or respiration in the dark. However, a low potential reducing agent is also required to act as a direct electron donor to the dinitrogenase reductase subunit of nitrogenase. Early studies showed that there was no nitrogenase activity in the dark under various experimental conditions, leading to the claim that the needed reducing agent could only be generated by a photochemical reaction [27]. In contrast to this result, *Rhodobacter capsulatus*, a well-known hydrogen producer, was found to be able to fix nitrogen in darkness with an *in vivo* nitrogenase activity (acetylene reduction) rate that was significant in darkness, although much lower (<10%) than under light conditions [28]. Cultures grown in the dark on fructose/DMSO had an acetylene reduction rate of 0.2 μmoles/h/mg dry weight when assayed in the dark whereas cultures grown anaerobically in the light on malate had an acetylene reduction rate of 3.2 μmoles/h/mg dry weight. Thus, diazotrophy of *R. capsulatus* is not obligatorily linked to photosynthesis. Microaerobic nitrogenase activity by several purple photosynthetic bacteria was also demonstrated, where low oxygen concentrations were a prerequisite for nitrogenase activity in darkness, with higher levels of dissolved oxygen having a strong inhibitory effect [29, 30].

These studies suggest that hydrogen could possibly be produced under microaerobic conditions in the dark by these organisms; a possible scheme is given in Fig 1. In an initial proof of principle study, the microaerobic production of hydrogen from a variety of substrates has been demonstrated [31]. Remarkably, although molar yields were low, 0.6 mol hydrogen/mol malate followed by 0.41 mol hydrogen/mol lactate, 0.36 mol hydrogen/mol, was obtained from the organic acids, malate, lactate, and succinate, substrates from which it is normally impossible to obtain hydrogen by dark fermentation. Further improvements could be sought through both optimization of bioprocess controls and through metabolic engineering.

Of great importance would be the development of a system for accurate and sensitive dosage of oxygen into the system, enough to give the required energy without being excessive and wastefully oxidizing too much of the substrate. Oxygen levels also need to be carefully controlled as excess oxygen will inhibit nitrogenase activity. Equally important could be the development, through metabolic engineering, of strains where most of the carbon flux is directed towards the hydrogen production pathway.

3.4 Improvements through Process Development and Metabolic Engineering

Improvements in both rates and yields of hydrogen production could be made to occur in a number of ways (Figure 3). For example, effective bioreactor design could lead to a more efficient use of light energy. This aspect has been recently reviewed [32-34], and will not be further discussed here. Metabolic engineering could be used to introduce mutations that would help to redirect electron flux to the metabolic pathways leading to hydrogen production. Metabolic modeling and flux analysis suggests a number of different pathways that might be profitably targeted, including; PHB synthesis, CO_2 fixation, or conversely through the activation of the activation of oxidative pathways such as the oxidative pentose phosphate pathway, or the oxidative TCA cycle. [35, 36].

A number of studies have already shown some partial successes. Compared to the [FeFe] hydrogenase (*Desulfovibrio* spp), the catalytic efficiency of nitrogenase is approximately 1000 times lower, 6.4 s^{-1} versus 9000 s^{-1} [37]. Attempts to directly boost hydrogen production through the expression of a [FeFe] hydrogenase have been unsuccessful so far [38]. However, several photosynthetic bacteria contain the structural gene for [FeFe] hydrogenase, *hydA*. One recent study has shown that this [FeFe] hydrogenase, although inactive in the photosynthetic

bacterium *Rho. rubrum*, could be matured in *Escherichia coli* when overexpressed along with appropriate maturation genes [38]. This strategy is worth pursuing since this could lead to both increased rates and increased yields of hydrogen production due to the elimination of the ATP requirement. In a similar vein, net hydrogen production should be improved through the inactivation of the hydrogen uptake system [39].

One promising strategy to improve the efficiency of light conversion is to use mutants with reduced pigment content, allowing for deeper light penetration into dense cultures and eliminating the wasteful side reactions that occur when photosynthesis is oversaturated by the light intensity. Recent studies have shown that indeed some improvement can be obtained in this way. In one study, a disrupted *pucDE,* the operon for the structural genes for the light-harvesting-II (LH-II) peripheral antenna complex, was created by transposon mutagenesis and was reported to give 50% more hydrogen at high light intensities per unit bacteriochlorophyll. However, since this is on a per bacteriochlorophyll basis and since these mutants had, as originally planned, a 38% reduction in this pigment, the actual volumetric hydrogen production increase was rather small, about 10% [40]. In another study, a mutant of *Rhodobacter capsulatus* having a decreased pigment content was created by inserting PufQ, a bacteriochlorophyll regulatory factor, under the control of the *cbb3* promotor. Although it was claimed that total cumulative hydrogen production was increased 27% (from 2955 ml to 3753 ml), substrate conversion efficiency only increased 5% (from 56.5% to 59.2%) and the maximum rate of hydrogen production actually showed an enormous decrease of 64% (from 28.9 to 18.6 ml $H_2\,L^{-1}\,h^{-1}$) [41]. Moreover, in this study the strain that was created had also lost *cbb3* function and any effect of this secondary mutation was ignored [41]. A similar strategy has also been applied to *Rhodobacter sphaeroides*, where the LH-II complex was

eliminated by mutation of its structural genes, *pucBA*. However, in this study, although the authors claimed hydrogen production was significantly improved, hydrogen production was in fact similar to that of the wild type [42]. Unfortunately, none of these studies examined the effect of mutations in the light harvesting systems on the efficiency of conversion of light energy to hydrogen, something of intrinsic interest, since efficiencies of around 1% are normally found [20] even though the theoretical efficiency is thought to be 8.5% [1].

Of course, it is also of interest to redirect electron flow to the proton reduction process by eliminating other reductant utilizing pathways. Thus, for example, a series of studies examined if improved hydrogen production could be obtained by mutations in which the paradoxical carbon fixation that occurs under photoheterotrophic conditions is eliminated. Although it is counter-intuitive that a microorganism would expend energy (ATP and NADPH) to fix carbon dioxide in the presence of usable fixed carbon, the photosynthetic bacteria use this reaction to maintain redox balance, thus permitting growth on substrates that are more reduced than cell biomass. In theory, this need to expend excess electrons could be met through hydrogen production by nitrogenase, either under conditions where it is normally expressed, or through the accumulation of sponateous mutations that allow its abnormal expression. Growing cultures with electron rich substrates (cyclohexanecarboxylate) under conditions which normally repress nitrogenase (in the presence of ammonium) allows the selection of mutants which have escaped transcriptional control through mutations in the nitrogenase transcriptional regulator NifA [43]. Similarly, elimination of Rubisco alters nitrogenase regulation, allowing for increased hydrogen production [44]. This study was carried out by first introducing NifA*, which permits nitrogenase synthesis in the presence of ammonium and increases its synthesis under the normally permissive conditions, the absence

of ammonium. Introducing NifA* alone under nitrogen deficient conditions led to a 25% increase in molar hydrogen yield (moles H_2 per mole of malate (from 3.79 to 4.73) and an additional mutation in *cbbM* increased this by a small but significant 13% (to 5.34 moles H_2 per mole of substrate). These mutants were originally thought to also be affected in growth due to the toxic accumulation of ribulose-1,5-bisphosphate in the absence of Rubisco activity [45], but this effect was later shown to be due to the inability to adapt to electron imbalance when growing with some substrates [10]. In some cases, the residues that are altered in the spontaneous mutants that cause nitrogenase deregulation have been identified [46] and these are most commonly in the nitrogenase specific transcriptional regulator NifA.

One study examined the effect of interrupting the CBB cycle by mutating *cbbP* (phosphoribulose kinase), but found that in fact, although the hydrogen production rate increased slightly (13%), total hydrogen production and substrate conversion yields were less than wild type levels [47]. A second study gave somewhat similar results with introduction of *cbbP* increasing hydrogen yields by only 1.4% [48]. This study showed that eliminating the CBB (Calvin-Benson-Bassham) cycle can significantly increase hydrogen production, but only in the presence of other mutations such as *ΔccoNOQP*. These results have been rationalized and extended such that a fairly complete picture has been obtained of how electron flux is altered in the absence of carbon dioxide fixation and how the electron distribution is affected by the nature of the substrate [49-51]. Essentially, these authors have shown that two basic metabolic constraints affect H_2 yields, (1) the pathways used for conversion of the substrate to biosynthetic precursors and, (2) the flux through the CO_2-fixing CBB cycle. They conclude that H_2 yields from all substrates can be improved by eliminating flux through the CBB cycle.

One final question, at least for *Rhodopseudomonas palustris* constitutive mutants, of how nitrogenase is active in the presence of the DraT/DraG nitrogenase regulatory system, has also been answered [52]. It appears that nitrogenase escapes post-translational modification because the presence of ammonium is still active in repressing synthesis of the activator, GlnK2, of the nitrogenase modifying enzyme, DraT2. It remains to be seen how this affect is averted in other strains, but some studies have shown that although nitrogenase is active in hydrogen production in the presence of ammonium in constitutive strains, hydrogen production is increased through the addition of a DraT mutation [44].

Depending on species difference, carbon source, cellular physiological state (growing or resting), PHB (polyhydroxybutyrate) synthesis could be a significant electron sink which would potentially decrease H_2 production. Initial studies showed that cultures of *Rhodobacter sphaeroides* actively producing hydrogen from malate produced hydrogen at a rate that was 18% higher when PHB synthesis was blocked through the introduction of a mutation in *phbC* and that this effect was greater, as expected, when combined with a *hup*$^-$ mutation [53, 54]. A large effect can be seen when hydrogen production from acetate is examined since this is the preferred carbon substrate for PHB synthesis [55, 56]. Even though a *Rhodobacter sphaeroides phbC* mutant did not grow as well as wild type (1.19 g/L dcw versus 2.1 g/L) it produced 36% more total hydrogen (657 ml L^{-1} versus 482 ml L^{-1}) with more than double the substrate yield (1.45 versus 0.7) [57]. Similar effects were seen when hydrogen production from acetate was examined with a *phbC* mutant of *Rhodopseudomonas palustris* [58]. A recent study examined the relationship between cell growth, hydrogen production and PHB accumulation using a wild type strain and a PHB synthase deficient one. It was found that the carbon source was a significant factor in PHB accumulation, for example, butyrate gave the

highest amount of PHB, 8.4% in the wild type strain, while with malate as carbon source, PHB accumulation was only 0.1%. As well, the initial amount of substrate was found to have an effect, with low substrate concentrations favoring conversion to hydrogen over synthesis of PHB. Thus, the effect of a *phbC* mutation can be insignificant, depending upon growth conditions [59]. This study also noted the possibility of electron diversion to SMPs (soluble microbial products) and suggested that, depending upon the substrate, this could be more significant than PHB production in reducing hydrogen yields [59]. Indeed, a recent study found that the addition of small amounts of ethanol could effectively shift metabolism away from synthesizing SMPs, which function as another type of electron sink, to H_2 production [11].

In Fig. 3, different approaches toward improving of H_2 production are illustrated, and a comparison of H_2 yields and productivities among different species and different culturing strategies, are compared in Table 1.

Conclusion

Here the recent advances in H_2 production by photosynthetic bacteria have been reviewed and H_2 yields and productivities compared among different species. As can be seen, nearly stoichiometric conversion can be achieved with several substrates, but H_2 productivity is still too low to allow a practical process. However, this problem could be addressed by several approaches, including reactor design, metabolic engineering, and process development. Ultimately of course, the aim would be to create strains in which multiple enhancements through different mutations are combined. We are just beginning to see the development of such superior strains.

Acknowledgements

PCH holds a National Research Council Research Senior Research Associateship Award at the Life Sciences Research Center, Department of Biology, United States Air Force Academy. Research on hydrogen production by photosynthetic bacteria in the author's laboratory is supported by a Discovery Grant from the Natural Sciences and Engineering Council of Canada. YL was supported by a scholarship from CSC, China.

References

[1] Hallenbeck PC. Microbial paths to renewable hydrogen production. Biofuels 2011; 2:285-302.

[2] Keskin T, Abo-Hashesh M, Hallenbeck PC. Photofermentative hydrogen production from wastes. Bioresour Technol 2011; 102:8557-68.

[3] Masepohl B, Hallenbeck PC. Nitrogen and Molybdenum Control of Nitrogen Fixation in the Phototrophic Bacterium *Rhodobacter capsulatus*. Recent Adv in Phototrophic Prokaryotes 2010; 675:49-70.

[4] Adessi A, McKinlay JB, Harwood CS, De Philippis R. A *Rhodopseudomonas palustris nifA** mutant produces H_2 from NH_4^+-containing vegetable wastes. Int J Hydrogen Energy 2012; 37:15893-900.

[5] Hallenbeck PC, Abo-Hashesh M, Ghosh D. Strategies for improving biological hydrogen production. Bioresour Technol 2012; 110:1-9.

[6] Han H, Jia Q, Liu B, Yang H, Shen J. Fermentative hydrogen production from acetate using *Rhodobacter sphaeroides* RV. Int J Hydrogen Energy 2013; 38:10773-78.

[7] Han H, Jia Q, Liu B, Yang H, Shen J. Optimization of photosynthetic hydrogen production from acetate by *Rhodobacter sphaeroides* RV. Int J Hydrogen Energy 2013; 38:12886-90.

[8] Kobayashi J, Yoshimune K, Komoriya T, Kohno H. Efficient hydrogen production from acetate through isolated *Rhodobacter sphaeroides*. J Biosci Bioeng, 2011; 112:602-05.

[9] Han H, Liu B, Yang H, Shen J. Effect of carbonn sources on the photobiological production of hydrogen using *Rhodobacter sphaeroides* RV. Int J Hydrogen Energy 2012; 37:12167-74.

[10] Gordon GC, McKinlay JB. Calvin cycle mutants of photoheterotrophic purple nonsulfur bacteria fail to grow due to an electron imbalance rather than toxic metabolite accumulation. J Bacteriol 2014; 196:1231-37.

[11] Kim DH, Lee JH, Kang S, Hallenbeck PC, Kim EJ, Lee JK, Kim MS. Enhanced photo-fermentative H_2 production using *Rhodobacter sphaeroides* by ethanol addition and analysis of soluble microbial products. Biotechnol Biofuels 2014; 7:79.

[12] Kunacheva C, Stuckey DC. Analytical methods for soluble microbial products (SMP) and extracellular polymers (ECP) in wastewater treatment systems: A review. Water Res. 2014; 61:1-18

[13] Lo YC, Chen CY, Lee CM., Chang JS. Photo fermentative hydrogen production using dominant components (acetate, lactate, and butyrate) in dark fermentation effluents. Int J Hydrogen Energy 2011; 36:14059-68.

[14] Ozgur E, Mars AE, Peksel B, Louwerse A, Yucel M, Gunduz U, Claassen PAM, Eroglu I. Biohydrogen production from beet molasses by sequential dark and photofermentation. Int J Hydrogen Energy 2010; 35:511-17.

[15] Laurinavichene TV, Belokopytov BF, Laurinavichius KS, Khusnutdinova AN, Seibert M, Tsygankov AA. Towards the integration of dark- and photofermentative waste treatment. 4. Repeated batch sequential dark- and photofermentation using starch as substrate. Int J Hydrogen Energy 2012; 37:8800-10.

[16] Laurinavichene TV, Belokopytov BF, Laurinavichius KS, Tekucheva DN, Seibert M, Tsygankou AA. Towards the integration of dark- and photofermentative waste treatment. 3. Potato as substrate for sequential dark fermentation and light driven H_2 production. Int J Hydrogen Energy, 2010; 35:8536-43.

[17] Lo Y, Chen C, Lee C, Chang J. Sequential dark-photo fermentation and autotrophic microbial growth for high yield and CO_2 free biohydrogen production. Int J Hydrogen Energy 2010; 35:10944-53.

[18] Chookaew T, O-Thong S, Prasertsan P. Biohydrogen production from crude glycerol by two stage of dark and photofermentation. Int J Hydrogen Energy 2015; 40 :7433-38.

[19] Dipasquale L, Adessi A, d'Ippolito G, Rossi F, Fontana A, De Philippis R. Introducing capnophilic lactic fermentation in a combined dark-photo fermentation process: a route to unparalleled H_2 yields. Appl Microbiol Biotechnol 2015; 99:1001-10.

[20] Abo-Hashesh M, Ghosh D, Tourigny A, Taous A, Hallenbeck PC. Single stage photofermentative hydrogen production from glucose: An attractive alternative to two stage photofermentation or co-culture approaches. Int J Hydrogen Energy 2011; 36:13889-95.

[21] Ghosh D, Sobro IF, Hallenbeck PC. Optimization of the hydrogen yield from single-stage photofermentation of glucose by *Rhodobacter capsulatus* JP91 using response surface methodology. Bioresour Technol 2012; 123:199-06.

[22] Abo-Hashesh M, Desaunay N, Hallenbeck PC. High yield single stage conversion of glucose to hydrogen by photofermentation with continuous cultures of *Rhodobacter capsulatus* JP91. Bioresour Technol 2013; 128:513-17.

[23] Keskin T, Hallenbeck PC. Hydrogen production from sugar industry wastes using single-stage photofermentation. Bioresour Technol 2012; 112:131-136.

[24] Cai J, Wang G. 2014. Hydrogen production from glucose by a mutant strain of *Rhodovulum sulfidophilum* P5 in single stage photo-fermentation. Int J Hydrogen Energy, 2014; 39:20979-86.

[25] Ghosh D, Sobro IF, Hallenbeck PC. Stoichiometric conversion of biodiesel derived crude glycerol to hydrogen: Response surface methodology study of the effects of light intensity and crude glycerol and glutamate concentration. Bioresour Technol 2012; 106: 154-60.

[26] Liu Y, Dipankar G, Hallenbeck PC. Biological reforming of ethanol to H_2 by *Rhodopseudomonas palustris* CGA009. Bioresour Technol 2015; 176:189-95.

[27] Jacques M, Bruce CK, Vignais PM. Effects of light on nitrogenase function and synthesis in *Rhodopseudomonas capsulata*. J Bacteriol, 1978; 136:201-08.

[28] Michal TM, Judy DM, Howard G. Dark anaerobic dinitrogen fixation by a photosynthetic microorganism. Science 1979; 204:1429-30.

[29] Eike M, Norbert P. Diazotrophic growth of *Rhodopseudomonas acidophila* and *Rhodopseudomonas capsulata* under microaerobic conditions in the dark. Arch Microbiol, 1980; 125:73-77.

[30] Yves J, Eike S, Norbert P. Microaerobic nitrogenase activity in *Thiocapsa* SP. Strain 5811. FEMS Microbiol Lett, 1980; 9:89-93.

[31] Abo-Hashesh M, Hallenbeck PC. Microaerobic dark fermentative hydrogen production by the photosynthetic bacterium, *Rhodobacter capsulatus* JP91. Int J Low Carbon Technol 2012; 7:97-03.

[32] Chen CY, Liu CH, Lo YC, Chang JS. Perspectives on cultivation strategies and photobioreator designs for photofermentative hydrogen production. Bioresour Technol, 2011; 102:8484-92.

[33] Adessi A, De Phillippis R. Photobioreactor design and illumination systems for H_2 production with anoxygenic photosynthetic bacteria: A review. Int J Hydrogen Energy 2014; 39:3127-47.

[34] Jung KW, Kim DH, Kim SH, Shin HS. Bioreactor design for continuous dark fermentative hydrogen production. Bioresour Technol 2011; 102:8612-20.

[35] Haedicke O, Grammel H, Klamt S. Metabolic network modeling of redox balancing and biohydrogen production in purple nonsulfur bacteria. BMC Syst Biol 2011; 5.

[36] Kontur WS, Ziegelhoffer EC, Spero MA, Imam S, Noguera DR, Donohue TJ. Pathways Involved in Reductant Distribution during Photobiological H_2 Production by *Rhodobacter sphaeroides*. Appl Environ Microbiol 2011; 77:7425-29.

[37] Hallenbeck PC, Benemann JR. Biological hydrogen production: fundamentals and limiting processes. Int J Hydrogen Energy 2002; 27:1185-93.

[38]Abo-Hashesh M, Sabourin-Prouost G, Hallenbeck PC. RrHydA is inactive when overexpressed in *Rhodospirillum rubrum* but can be matured in *Escherichia coli*. Int J Hydrogen Energy 2013; 38:11233-40.

[39] Wu X, Bai L, Jiang L, Liu J, Long M. Enhanced photo-fermentative hydrogen production from different organic substrate using *hupL* inactivated *Rhodopseudomonas palustris*. Afr J Microbiol Res 2012; 6:5362-70.

[40] Ma C, Guo L, Yang H. Improved photo-hydrogen production by transposon mutant of *Rhodobacter capsulatus* with reduced pigment. Int J Hydrogen Energy 2012; 37: 12229-33.

[41] Ma C, Wang X, Guo LJ, Wu XM, Yang HH. Enhanced photo-fermentative hydrogen production by *Rhodobacter capsulatus* with pigment content manipulation. Bioresour Technol 2012; 118:490-95.

[42] Ryu MH, Hull NC, Gomelsky M. 2014. Metabolic engineering of *Rhodobacter sphaeroides* for improved hydrogen production. Int J Hydrogen Energy 2014; 39:6384-90.

[43] Rey FE, Heiniger EK, Harwood CS. 2007. Redirection of metabolism for biological hydrogen production. Appl Environ Microbiol 2007; 73:1665-71.

[44]Wang D, Zhang Y, Welch E, Li J, Roberts GP. Elimination of Rubisco alters the regulation of nitrogenase activity and increases hydrogen production in *Rhodospirillum rubrum*. Int J Hydrogen Energy 2010; 35:7377-85.

[45] Wang D, Zhang Y, Pohlmann EL, Li J, Roberts GP. The Poor Growth of *Rhodospirillum rubrum* Mutants Lacking RubisCO Is Due to the Accumulation of Ribulose-1,5-Bisphosphate. J Bacteriol 2011; 193:3293-03.

[46] Farmer RM, Laguna R, Panescu J, McCoy A, Logsdon B, Zianni M, Moskvin OV, Gomelsky M, Tabita FR. Altered residues in key proteins influence the expression and activity of the nitrogenase complex in an adaptive CO_2 fixation-deficient mutant strain of *Rhodobacter sphaeroides*. Microbiol Sgm 2014; 160:198-08.

[47] Öztürk Y, Gökçe A, Peksel B, Gürgan M, Özgür E, Gündüz U. Hydrogen production properties of *Rhodobacter capsulatus* with genetically modified redox balancing pathways. Int J Hydrogen Energy 2012; 37:2014-20.

[48] Wang X, Yang H, Ma C, Guo L. Enhanced photosynthetic hydrogen production performance of *Rhodobacter capsulatus* by deactivating CBB cycle and cytochrome c oxidase. Int J Hydrogen Energy 2014; 39:3176-84.

[49] McKinlay JB, Harwood CS. Carbon dioxide fixation as a central redox cofactor recycling mechanism in bacteria. Proc Natl Acad Sci 2010; 107:11669-75.

[50] McKinlay JB, Harwood CS. Calvin Cycle Flux, Pathway Constraints, and Substrate Oxidation State Together Determine the H_2 Biofuel Yield in Photoheterotrophic Bacteria. MBio 2011; 2.

[51] McKinlay JB, Oda Y, Ruhl M, Posto AL, Sauer U, Harwood CS. Non-growing *Rhodopseudomonas palustris* increases the hydrogen gas yield from acetate by shifting from the glyoxylate shunt to the tricarboxylic acid cycle. J Biol Chem 2014; 289: 1960-0.

[52] Heiniger EK, Oda Y, Samanta SK, Harwood CS. How Posttranslational Modification of Nitrogenase Is Circumvented in *Rhodopseudomonas palustris* Strains That Produce Hydrogen Gas Constitutively. Appl Environ Microbiol 2012; 78:1023-32.

[53] Lee IH, Park JY, Kho DH, Kim MS, Lee JK. Reductive effect of H_2 uptake and poly-beta-hydroxybutyrate formation on nitrogenase-mediated H_2 accumulation of *Rhodobacter sphaeroides* according to light intensity. Appl Microbiol Biotechnol 2002; 60:147-53.

[54] Kim MS, Baek JS, Lee JK. Comparison of H_2 accumulation by *Rhodobacter sphaeroides* KD131 and its uptake hydrogenase and PHB synthase deficient mutant. Int J Hydrogen Energy 2006; 31:121-27.

[55] Kim MS, Kim DH, Cha J, Lee JK. Effect of carbon and nitrogen sources on photo-fermentative H_2 production associated with nitrogenase, uptake hydrogenase activity, and PHB accumulation in *Rhodobacter sphaeroides* KD131. Bioresour Technol 2012; 116:179-83.

[56] Wu SC, Liou SZ, Lee CM. Correlation between bio-hydrogen production and polyhydroxybutyrate (PHB) synthesis by *Rhodopseudomonas palustris* WP3-5. Bioresour Technol 2012; 113:44-50.

[57] Kim MS, Kim DH, Son HN, Ten LN, Lee JK. Enhancing photo-fermentative hydrogen production by *Rhodobacter sphaeroides* KD131 and its PHB synthase deleted-mutant from acetate and butyrate. Int J Hydrogen Energy 2011; 36:13964-71.

[58] Yang CF, Lee CM. Enhancement of photohydrogen production using *phbC* deficient mutant *Rhodopseudomonas palustris* strain M23. Bioresour Technol. 2011; 102:5418-24.

[59] Chen YT, Wu SC, Lee CM. Relationship between cell growth, hydrogen production and poly-beta-hydroxybutyrate (PHB) accumulation by *Rhodopseudomonas palustris* WP3-5. Int J Hydrogen Energy 2012; 37:13887-94.

TABLE

Table 1. Recent studies on single stage and two-stage H_2 production

	Microorganism	Substrate	Yield (mol H_2/mol hexose or glycerol)	Major advantages/ disadvantages	Ref
Single-stage	*Rhodobacter capsulatus* JP91	glucose	3	Simpler operation; suitable for metabolism study; substrates limited to a few sugars and some organic acids	20
	Rhodobacter capsulatus JP91	glucose	5.5		21
	Rhodovulum sulfidophilum P5	glucose	7.07		24
	Rhodobacter capsulatus JP91	glucose	9		22
	Rhodobacter capsulatus JP91	sucrose	7		23
	Rhodopseudomonas palustris CGA009	glycerol	6.69		25
Two-stage	*Thermotoga neapolitana* DSM 4359^T and *Rhodopseudomonas palustris* 42OL	glucose	9.4	Broad substrate range; addition operational cost from treatment of fermentation effluents of first stage	19
	Microbial consortium and mixture of *Rhodobacter capsulatus* B10 and *Rhodobacter sphaeroides* N7	starch	5.3		15
	Caldicellulosiruptor saccharolyticus and *Rhodobacter capsulatus* YO3	beet molasses	6.8		14
	Microbial consortium and *Rhodobacter capsulatus* B10	Potato	5.6		16
	Clostridium butyricum CGS55 and *Rhodopseudomonas palustris* WP3-5	sucrose	5.8		17
	*Klebsiella sp.*TR17 and *Rhodopseudomonas palustris* TN1	glycerol	0.8		18

FIGURES

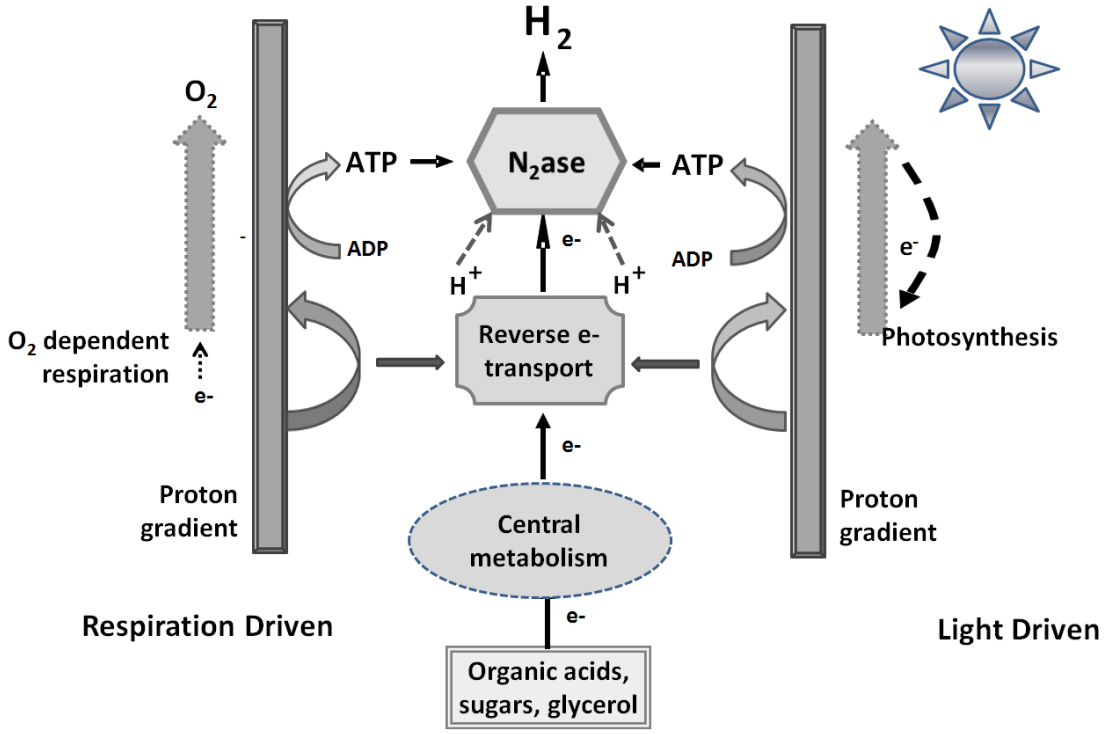


Figure 1. Integration of H_2 production by photosynthetic bacteria with cellular metabolism. A schematic of the interactions with various metabolisms necessary for nitrogenase activity in the photosynthetic bacterium *Rhodobacter capsulatus*. ATP is supplied either by oxidative phosphorylation during microaerobic respirtation (left) or by cyclic photophosphorylation (right). High energy electrons are generated from central metabolism through reverse electron flow driven by proton gradients created during microaerobic respiration (left) or cyclic photosynthesis (right).

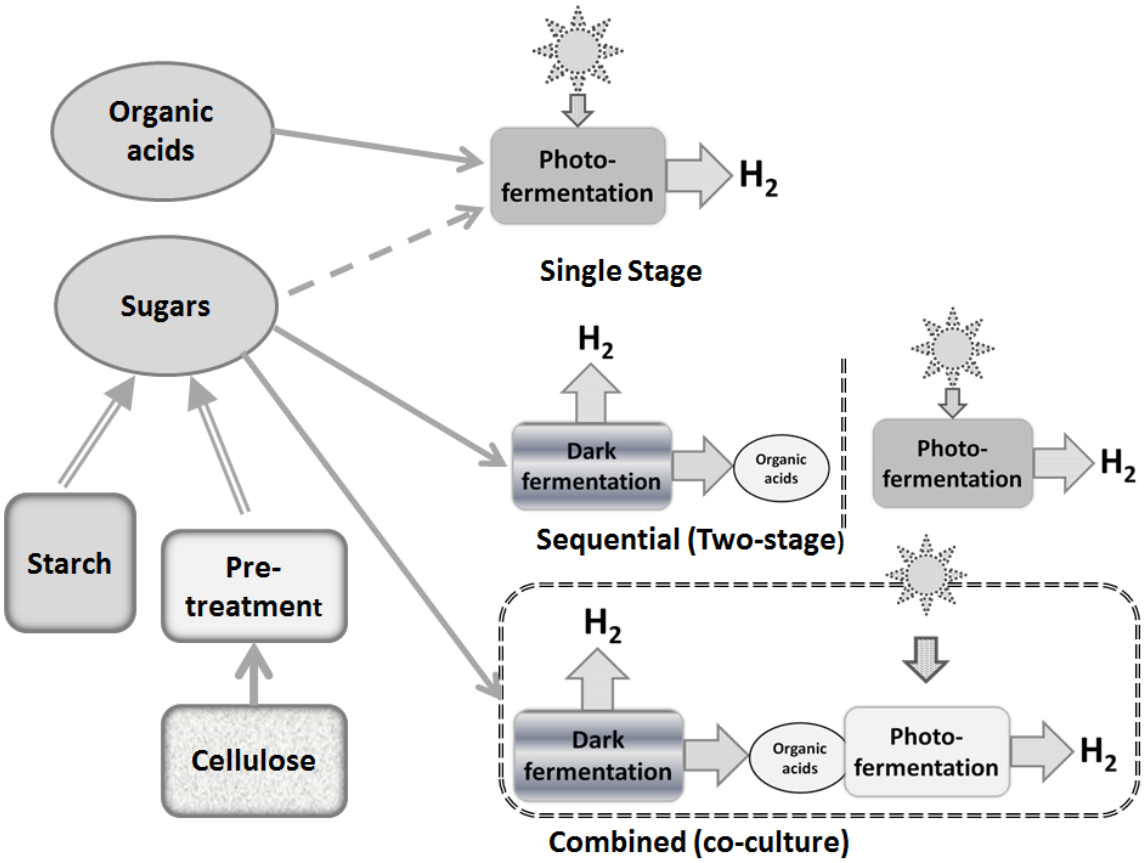


Figure 2. Possible strategies for conversion of various substrates to hydrogen using photofermentation by photosynthetic bacteria. Numerous studies have shown that organic acids can be directly converted to hydrogen through photofermentation (top). Several studies have shown that sugars, derived from various polymeric substances through pretreatment, can first be partially converted to hydrogen through a dark fermentation and the resulting organic acids can be converted to hydrogen through photofermentation either through a sequential, two-stage system (middle) or through a combined co-culture process (bottom). (Figure taken from [2], Figure 2.)

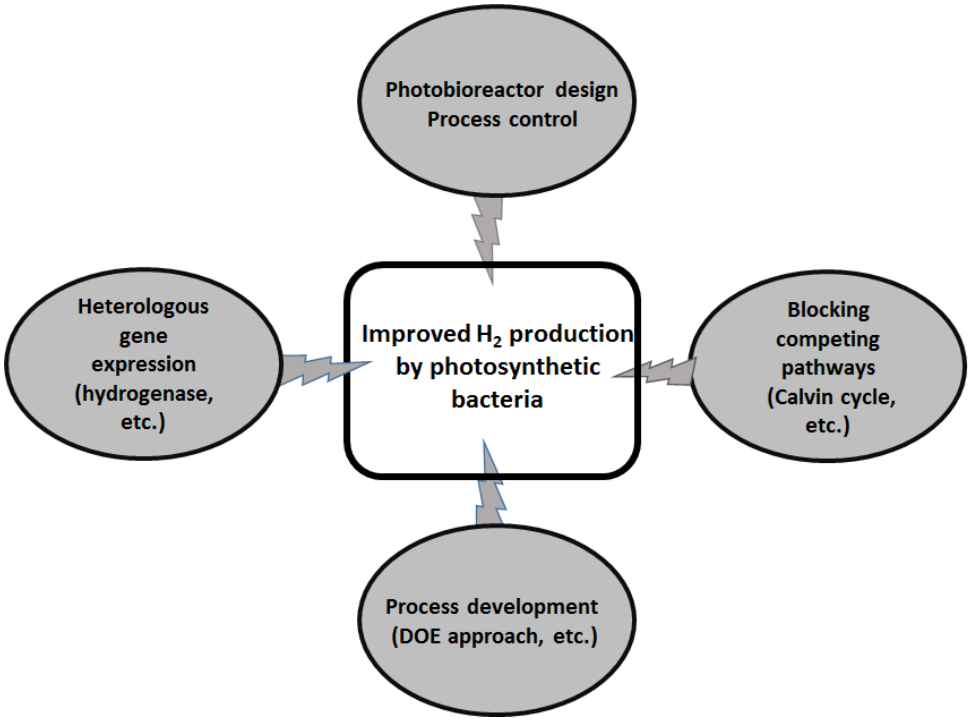


Figure 3. Various approaches towards improving H_2 production by photosynthetic bacteria. Different avenues could be used in attempts to further improve the rates and yields during hydrogen production by photosynthetic bacteria. Photobioreactor design could lead to better light. Blocking competing pathways through metabolic engineering could increase flux from substrate to hydrogen thus improving yields. Process development using optimization approaches such as DOE (design of experiments) can lead to improved substrate utilization and thus higher yields. Finally, heterologous protein expression, in particular substitution of [FeFe] hydrogenase for nitrogenase, could lead to higher rates of hydrogen production.

OBJECTIVES OF THE PRESENT STUDY

The overall objective of this study was to improve photofermentative H_2 production through metabolic engineering and DOE approaches.

One hypothesis for applying a metabolic engineering approach to improve H_2 production with photosynthetic bacterium, *Rhodobacter capsulatus* JP91, was to inactivate the Calvin-Benson-Bassham (CBB) cycle, since under photoheterotrophic condition it serves to maintain redox balance by recycling excess reductant. In theory, this reductant could be used to produce H_2 with nitrogenase. Thus, the metabolic engineering approach applied was to inactivate a key gene in the CBB cycle, namely phosphoribulose kinase, PRK.

The characterization of spontaneous mutants having constitutive nitrogenase expression in the presence of ammonium was focused on identification of key mutations by genome sequencing. This is important because H_2 production with the nitrogenase system requires a thorough understanding of its regulation mechanism at molecular level.

Finally, this thesis examined biological ethanol reformation to H_2 by *Rhodopseudomonas palustris* CGA009. Ethanol transformation to H_2 is thermodynamically difficult. In chemical steam reforming process, the energy requirement is met by using high temperatures. In anaerobic environments in nature, some species are known to live by carrying out ethanol assimilation, producing acetate and H_2. However, this process is only possible in the presence of H_2 consuming microbes which can effectively change the free energy of the reaction to make it favorable. Some photosynthetic species can grow on ethanol and generate chemical energy in the form of ATP with photosynthesis to drive this reaction. Thus, our hypothesis was that ethanol could be reformed to H_2 in a sustained manner.by a single microorganism, *Rhodopseudomonas palustris* CGA009

RESULTS

Chapter 3: A Kinetic study of H_2 production by a Calvin-Benson-Bassham cycle mutant, PRK (phosphoribulose kinase), of the photosynthetic bacterium *Rhodobacter capsulatus*

Yuan Liu and Patrick C. Hallenbeck*

Département de Microbiologie, infectiologie, et immunologie, Université de Montréal CP 6128 Succursale Centre-ville, Montréal, Québec H3C 3J7 Canada

Author contribution: The experimental design and wet laboratory experiments were carried out by YL. YL performed all dataset analysis. All the work was under supervision of Professor Patrick Hallenbeck.

Article status: Published, International Journal of Hydrogen Energy, 2016, 41:11081-11089.

Abstract

In purple non-sulfur photosynthetic bacteria, the Calvin-Benson-Bassham (CBB) cycle plays a role in maintaining intracellular redox balance under photoheterotrophic growth conditions, potentially consuming reductant and chemical input (ATP) that could be used for H_2 production by nitrogenase. Previous studies have shown *R. capsulatus* JP91 to be a remarkable H_2 producer and in the present study a PRK, phosphoribulose kinase, knock out mutant of this strain was created in an attempt to further improve H_2 yield. Analysis of growth with different carbon sources showed that this mutant could only grow in acetate medium without, however, producing any H_2. A spontaneous mutant was recovered which had gained the ability to grow on glucose and produce H_2 with a yield that was 20-40% greater than the wild type, JP91 strain. Analysis shows that growth and hydrogen production are strongly linked with electrons from glucose mostly directed to H_2 production and biomass formation.

.

Key words: *Rhodobacter capsulatus* JP91; phosphoribulose kinase mutant; spontaneous mutant, YL1; single stage photofermentation; Calvin cycle; kinetics study; H_2 yield and productivity; organic acids analysis; electron allocation analysis; bacteriochlorophyll.

1. Introduction

As early as 1949, a photosynthetic bacterium, *Rhodospirillum rubrum*, was found to produce H_2 photosynthetically in media containing one of several different organic acids as carbon source and glutamate or aspartate as nitrogen source [1, 2]. Many studies since then have shown that the capacity of the photosynthetic bacteria to produce H_2 is a general phenomenon, as many strains are capable of producing H_2 under the appropriate conditions through a reaction catalyzed by an inducible nitrogenase system [3]. In addition to organic acids, which were initially used as carbon source, many other potentially utilizable, abundant carbon sources, such as glucose, glycerol, and wastewater, can be readily metabolized to H_2 by a number of photosynthetic bacteria [4-7]. Glutamate, which is permissive for nitrogenase synthesis and can induce high nitrogenase activity, is the most widely used nitrogen source [8-12].

Along with theory development, new techniques, including improved photo-bioreactor design, response surface methodology, and metabolic engineering, have also been developed, as detailed in several recent reviews [13, 14], leading to improvements in hydrogen yields. Although, the composition of the complete electron transport chain to nitrogenase is still uncertain; for example, it is still not clear how ferredoxin is reduced, it is generally accepted that the necessary reducing power is ultimately derived from the carbon source used, such as organic acids and sugars. Thus, improved H_2 production can be expected through the inactivation of competing pathways, for example the Calvin-Benson-Bassham (CBB) cycle, or the polyhydroxybutyrate (PHB) biosynthetic pathway [15, 16].

Two recent studies examined how CBB cycle mutations can achieve higher H_2 production in practice. Indeed, the mutants generated showed improved H_2 production, either H_2 yield or productivity. However, these studies were somewhat preliminary since they lacked detailed kinetic analyses and important information about the relationship of H_2 production, nitrogenase activity and biommass accumulation [17, 18]. In the present study a *cbbP* (PRK, phosphoribulose kinase) knockout mutant of *Rhodobacter capsulatus* JP91 was created and a spontaneous derivative of the PRK mutant showing improved hydrogen production was isolated. A detailed optimization and kinetic analysis of both H_2 yields and H_2 productivities was carried out as well as an analysis of electron allocation under hydrogen producing conditions.

2. Materials and Methods

2.1 *Strains and growth conditions*

All strains and plasmids used in this study are given in Table 1. *Escherchia coli* strains were routinely grown in LB medium with the necessary antibiotics. *R. capsulatus* strains were grown in RCV minimal salts [22] supplemented with glutamate (3.5 mM) and different carbon sources; 30 mM acetate (RCVG-A, abbreviated as AG), 20 mM glucose (RCVG-G, abbreviated as GG), 30 mM lactate (RCVG-L, abbreviated as LG) and 40 mM malate (RCVG-M, abbreviated as MG). YPS [22] plates were used for conjugation and strain purification. Antibiotics were used at the following final concentrations: for *E. coli* strains, kanamycin, 25 µg/ml, tetracycline, 15 µg/ml, and ampicillin, 50 µg/ml; for *R. capsulatus* strains, kanamycin, 10 µg/ml, and tetracycline, 1 µg/ml.

2.2 PRK mutant construction.

General cloning techniques, including plasmid construction and PCR were carried out according to reference [23]. The *cbbP* gene was amplified using genomic DNA of *Rhodobacter capsulatus* SB1003 as template and the primers given in Table 1. For the construction of the knockout generating suicide plasmid, a 0.6 kb *Pvu*I genomic DNA fragment encoding part of the *cbbP* gene was cloned into pSUP202, resulting in pYN1. The kanamycin resistance gene from pBSL15 was inserted into the *Bcl*I site of the *cbbP* fragment contained in pYN1, yielding pYN2. The *cbbP::Km* of pYN2 was then subcloned into pAY10 to yield the final plasmid pYN3. This plasmid was transformed into *E. coli* S17-1 for subsequent conjugation.

2.3 Conjugation

One ml of late log phase *E. coli* S17-1 harboring pYN3 and *R. capsulatus* JP91 were pelleted by centrifugation and resuspended with 50 µl of YPS medium for each, then mixed thoroughly by repeatedly pipetting. The mixture was spread on 2 cm^2 center of YPS plates and incubated in darkness aerobically, at 30 °C for 12 hours. A cell suspension was then collected with 1 ml of RCV minimal salts solution and diluted 10 times before being plated on YPS plates supplemented with 5% of sucrose and kanamycin (10 µg/ml). Four small, red colonies which appeared after about 40 hours of incubation at 30 °C, aerobically in darkness, were subsequently verified to be the result of double crossover events by PCR using the primer set used for the original amplification of *cbbP*. One mutant was purified by repeated streaking on YPS-agar plates supplemented with kanamycin and used in the following

experiments. The correct insertional mutation of *cbbP* gene was also confirmed by genome sequencing (Results not shown, to be published elsewhere).

2.4 Isolation of spontaneous mutants

The PRK mutant was first grown on YPS medium and then transferred into RCVG-G, RCVG-L, RCVG-A and RCVG-M media. Unexpectedly, normal growth was only observed in RCVG-A medium. However, after extended incubation (4 to 7 days depending upon carbon source) at 30 °C, 80 W/m^2, vigorous growth started in the other media (RCVG-G, RCVG-L, and RCVG-M), suggesting outgrowth of spontaneous mutants. The mutant which arose in RCVG-G medium was purified, named YL1, and characterized in terms of H_2 production.

2.5 H_2 production

RCVG-G pre-grown cells were inoculated (2.5%, v/v) into 120 ml serum bottles filled with the same medium and sparged with oxygen-free argon before transfer to the H_2 production setup which was equipped with four 50 W halogen bulbs, a water tank to maintain temperature, and four inverted graduated cylinders pre-filled with water for gas collection [7]. The bottles were connected to the graduated cylinders with hydrogen impermeable FEP 890 tubing with their ends extended into the bottom of the cylinder.

2.6 Analytical techniques

Optical density was determined with a double-beam spectrophotometer (Shimadzu UV 2101 PC). Briefly, anaerobic sampling of 1 ml culture was done using a sterile syringe pre-flushed with H_2O and this culture was first used for *in vivo* nitrogenase assay before undergoing proper dilution (OD_{660} to 0.2 ~ 0.4) for measuring optical density at 660 nm. H_2 was measured with a Shimadzu GC-8A gas chromatograph as previously described [7]. The light intensity at front surface, vertical center of the serum bottle was determined with a Delta OHM photo/radiometer (HD 2102.1) and a voltage regulator was used to adjust it to the set value. Organic acids were measured using a Beckman System Gold HPLC, equipped with a 168 UV-VIS detector and a Prevail organic acid column at room temperature. 25 mM KH_2PO_4 (pH adjusted to 2.5 with concentrated H_3PO_4) with a flow rate of 1 ml/min was used as mobile phase. Dry cell weight and bacteriochlorophyll content were measured as described by Madigan et al [24]. Nitrogenase activity assay was carried out as described by Hallenbeck et al [25]. Glucose concentration was quantified using the DNS assay [26].

2.7 Calculation of electron recovery

Electrons in H_2, biomass and organic acids were calculated as described by Battley [27], and summed, then divided by the electrons in the glucose consumed. Here we assume that the strains used in the present study have the same biomass composition as that of *Rhodopseudomonas palustris*, 22.416 g/mol, as determined by McKinlay and Harwood [28].

3. Results and discussion

3.1 Growth characteristics of the R. capsulatus PRK mutant

It is generally accepted in purple non-sulfur photosynthetic bacteria that one of the most important physiological roles of CBB cycle is to maintain intracellular redox balance when cells are cultured photoheterotrophically [29-32]. However, alternative mechanisms exist for redox balancing in these microorganisms under these conditions that are operative under specific conditions; the nitrogenase system and the DMSOR (dimethyl sulfoxide reductase) system [30].

R. capsulatus has two operons encoding CBB cycle enzymes, namely cbb_{I} and cbb_{II}. However, there is only a single *cbbP* gene (encoding phosphoribulokinase, PRK), located in the cbb_{II} operon. Inactivation of Rubisco of *Rho. rubrum* has been shown to result in poor growth due to the accumulation of ribulose-1, 5-biphosphate [33]. Previously, a PRK mutant of *R. capsulatus* was made and found to have negligible amounts of Rubisco activity, suggesting that inactivation of this gene should eliminate CBB cycle flux [30]. In addition, this mutant was reported to be unable to grow photoheterotrophically on malate.

In the present study, a *cbbP::kan* mutant (PRK mutant) was created as described in Materials and Methods and its physiological growth properties were studied with RCVG-A (AG), RCVG-G (GG), RCVG-L (LG) and RCVG-M (MG) medium, respectively. The wild type strain *R. capsulatus* JP91 grew well in all four media and produced large amounts of H_2. However, the PRK mutant grew only in AG medium without producing any H_2 (Fig. 1A, B). It has been reported that a Rubisco mutant of *Rhodopseudomonas palustris* (*Rps. palustris*) is unable to grow with acetate as carbon source, whereas a Rubisco mutant of *Rhodobacter*

sphaeroides (*R. sphaeroides*) can, possibly due to the fact that *R. sphaeroides* metabolizes acetate through the ethylmalonyl-CoA pathway, a pathway with integral steps of CO_2 reduction [34]. The report that *R. capsulatus* cultured with acetate as carbon source had no isocitrate lyase activity seems to favor the expectation that *R. capsulatus* assimilates acetate using this pathway [35]. However, results from other groups seemingly contradict these results [36-39]. Thus, at present, it is unclear how acetate is metabolized by *R. capsulatus*.

We speculated growth failure in the other three media was due to redox imbalance, since, at least when malate was used as carbon source, neither of the two Rubisco mutants discussed above can grow [34]. We tested this speculation by adding 30 mM DMSO (dimethylsulfoxide) to the media. If the growth was indeed due to redox imbalance, the addition of DMSO would correct this by providing an electron sink. Indeed, growth was observed with the three carbon sources, glucose, lactate and malate, after only a short period of adaption from YPS to RCV medium containing DMSO (Fig. 1C). Therefore, it appears inactivation of CBB cycle results in growth failure of the PRK mutant with glucose, malate or lactate as carbon source due to redox imbalance, and that this mutation has an effect on nitrogenase expression.

3.2 Effect of light intensity on H_2 production by strains JP91 and YL1

Interestingly, we were able to isolate three putative spontaneous mutants in all of the three media after extended incubation time which didn't support growth of the PRK mutant initially. One spontaneous mutant YL1, isolated from RCVG-G medium, was purified and verified by PCR to retain the mutation in *cbbP*, i.e. it was a pseudorevertant. Since it was a gain of

function mutant, able to grow under conditions where the parental strain could not, it was also of interest to analyze its ability to produce hydrogen.

Even though it has previously been demonstrated that nitrogenase synthesis is not strictly light dependent, light does have a strong stimulatory effect and light intensity has been shown to be a significant factor affecting nitrogenase activity [40, 7]. Here we examined three light intensities: 120 W/m^2, 175 W/m^2 and 240 W/m^2 (Fig. 2). As can be seen from Figure 2A, the overall H_2 productivity of strain JP91 is higher than that of strain YL1 at a light intensity of 120 W/m^2. Average volumetric productivities were 15.6 and 12.0 ml H_2/L culture/h, respectively. At higher light intensities, 175 W/m^2 and 240 W/m^2, the hydrogen productivity of both strains were comparable, as shown in Fig. 2B and Fig. 2C. On the other hand, the H_2 yield of strain YL1 was 20 – 40% higher than strain JP91 under all light intensities tested (Table 3).

In order to better understand metabolic flux distribution during hydrogen production by both strains, the organic acids in the culture medium were determined by HPLC (Table 2). There were some interesting differences between strain JP91 and strain YL1 under the some of the conditions, and interesting differences for each strain with respect to light intensity. For example, strain JP91 showed relatively high levels of lactate production at low (120 W/m^2) and moderate (175 W/m^2) light intensities whereas strain YL1 did not. Both strains showed some pyruvate production under all light intensities examined, and both strains gave very little organic acid production under the high light intensity tested (240 W/m^2).

In addition, an electron allocation analysis was carried out for the experiments shown in Figure 3. Although there was some variation, electron recovery was relatively close to complete (Table 3). It is difficult to get 100% closure with this type of analysis since the

variation in determination of each of main electron allocations is additive. Nevertheless, several generalizations can be made. For one, especially at high light intensity, 240 W/m^2, the majority of the electrons went into H_2 production and biomass accumulation, The H_2 yield of strain YL1 is higher than in all cases than strain JP91. Especially at moderate (175 W/m^2) and low (120 W/m^2) light intensity this was due to a much smaller diversion of substrate into organic acid byproducts. Thus, the percent electrons found in hydrogen is higher.

3.3 Kinetics of H_2 production under low light intensity

As shown in Fig. 2A, the H_2 productivity of strain JP91 was superior under low light intensity, 120 W/m^2, thus it was of interest to determine the kinetics of biomass accumulation, glucose consumption and daily *in vivo* nitrogenase activity in order to gain insights into this phenomenon (Fig 3A-C).

From the results shown in Fig. 3A it can be seen that H_2 production is closely linked to growth, in agreement with a previously described model which predicted that highest H_2 production would be obtained with growing cultures [41]. For both strains, initial productivities were high, dropping remarkably after 2-3 days. Glucose analysis showed that some glucose remained in final culture broth, hence this drop in hydrogen productivity was not due to glucose exhaustion. As well, glutamate, as readily detected by HPLC, also remained in the final fermentation broth. The overall H_2 productivity of strain JP91 was better than strain YL1, although initially they were comparable (day 1-day 2), in good agreement with the previous experiment (compare Fig. 2A and Fig. 3A). From day 3 to day 5, H_2 production by strain YL1 nearly stopped, while strain JP91 kept producing H_2, although at a

slower rate. During this period the total nitrogenase activity, i.e. nitrogenase specific activity (determined at saturating light intensity) multiplied by the total biomass, of strain JP91 was about 1.5-2 times higher than strain YL1; however, it produced 3.5 times greater amount of H_2, 0.84 versus 0.24 mmoles.

We also observed that the culture color of strain YL1 was much stronger than that of strain JP91, although it had a lower amount of biomass. Thus, we quantified the bacteriochlorophyll content of both strains. It was found that strain YL1 had about a 3-fold higher amount of bacteriochlorophyll content, 0.5 versus 0.18 (unit: OD772/mg dry cell weight). As well, under the other light intensities examined, the culture color of strain YL1 was also stronger than that of strain JP91. This could explain why the biomass yield of strain JP91 in the experiment shown in Figure 3 was significantly higher than that of YL1, 25 mg versus 22.4 mg/40 ml culture. In addition, when the light intensity was increased from 120 W/m^2 to 240 W/m^2, there was a corresponding 26.4% increase in the biomass of strain JP91, whereas the biomass of strain YL1 only increased by 7.1%, indicating that light shading effects for strain YL1 was stronger than for strain JP91. Although it is as yet unclear why strain YL1 synthesizes more bacteriochlorophyll (Bchl), it is apparent that this phenomenon directly affects hydrogen production (Figure 3A). The major avenue through which the Bchl levels affect hydrogen production is through an effect on biomass production since specific hydrogen production (H_2 mmol/OD660) was essentially the same for both strains (Fig. 3A).

3.4 Comparison with previous studies

An examination of results from previous studies (Table 4) shows that strain YL1, described in the present article, performs very well in comparison with other studies that have examined the single-stage photofermentation of glucose. At a light intensity of 120 W/m^2, strain YL1 had a yield of 7.63 mol H_2/ mol glucose (Table 3), representing 63% of the maximum possible (12 mol H_2/ mol glucose). This is higher than all other previously reported batch studies, including one that used DOE (Design of Experiments) to achieve maximal hydrogen production, and is 11% higher than the yield achieved with strain JP91 in the present study. In addition, the yield by strain YL1 determined here is higher than in a previous study of single stage photofermentation of glucose by *Rhodobacter sphaeroides* where metabolic flux to hydrogen was increased by mutating *phbC*, encoding an enzyme essential for polyhydroxybutyrate synthesis [45]. In the past, higher yields were reported for single stage photofermentation of glucose carried out under continuous culture conditions [4]. In the future it would be interesting to test hydrogen productivities and yields with strain YL1 under these conditions.

Previous studies have also examined the effects of knocking out the CBB cycle of various photosynthetic bacteria on hydrogen production from different organic acids (Table 4) [15, 17, 28]. Here too, eliminating this electron drain appears to increase, at least to some extent, hydrogen yields from either acetate or malate. Although we have not directly examined hydrogen production from organic acids by the newly described *R. capsulatus* YL1, given what was found for photofermentation of glucose, it is highly likely that yields from other substrates, in particular organic acids, would be increased. Again, this could be studied in some detail in the future.

Conclusion

In an attempt to further improve H_2 yields through elimination of a potential metabolic drain, a PRK (phosphoribulose kinase) knock-out mutant of *R. capsulatus* JP91 was created. However, growth studies with four different carbon sources, glucose, lactate, malate and acetate, showed that this mutant could only grow in RCVG-A (acetate) medium, and, unlike the parental strain, didn't produce any H_2. A pseudorevertant of the PRK mutant, strain YL1 was recovered and studied. This spontaneous mutant had gained the ability to grow on RCVG-G (glucose) and produce hydrogen, with an increased H_2 yield (20-40%) under some conditions. Growth studies showed that growth and H_2 production are closely linked. Electron allocation analysis suggests that electrons from glucose are mostly directed into H_2 production and biomass formation, thus approaching the theoretical maximum H_2 yield for a growing culture. Interestingly, it was found that the bacteriochlorophyll content of strain YL1 was about 3-fold higher than the parental strain, JP91, which could explain why total hydrogen production by strain YL1 was lower than that of strain JP91 at low light intensities.

Acknowledgements

This research was supported by a Discovery Grant from the Natural Sciences and Engineering Council of Canada (RGPIN/04605-2015) to PCH. YL was supported by a scholarship from CSC, China.

References

[1] Gest H, Kamen MD. Photoproduction of molecular hydrogen by *Rhodospirillum rubrum*. Science. 1949; 109:558-59.

[2] Gest H, Kamen MD. Study on the metabolism of photosynthetic bacteria IV. Photochemical production of molecular hydrogen by growing cultures of photosynthetic bacteria. J. bacteriol. 1949; 58:239-44.

[3] Sigel JM, and Kamen MD. Studies on the metabolism of photosynthetic bacteria VII. Comparative studies on the photoproduction of H_2 by *Rhodopsedomonas gelatinosa* and *Rhodospirillum rubrum*. J. Bacteriol. 1951; 61: 215-28.

[4] Abo-Hashesh M, Desaunay N, Hallenbeck PC. High yield single state conversion of glucose to hydrogen by photofermentation with continuous culture of *Rhodobacter capsulatus* JP91. Bioresour. Technol. 2013; 128:513-17.

[5] Ghosh D, Sobro IF, Hallenbeck PC. Stoichiometric conversion of biodiesel derived crude glycerol to hydrogen: response surface methodology study of the effects of light intensity and crude glycerol and glutamate concentration. Bioresour. Technol. 2012; 106: 154-60.

[6] Keskin T, Abo-Hashesh M, Hallenbeck PC. Photofermentative hydrogen production from wastes. Bioresour. Technol. 2011; 102: 8557-68.

[7] Liu Y, Ghosh D, Hallenbeck PC. Biological reformation of ethanol to hydrogen by *Rhodopesudomonas palustris* CGA009. Bioresour. Technol. 2015; 176:189-95.

[8] Hillmer P, Gest H. H_2 metabolism in the photosynthetic bacterium *Rhodopseudomonas capsulata*: H_2 production by growing culture. J. Bacteriol. 1977; 129:724-31.

[9] Hillmer P, Gest H. H_2 metabolism in the photosynthetic bacterium *Rhodopseudomonas capsulata*: production and utilization of H_2 by resting cells. J. Bacteriol. 1977; 129:732-39.

[10] Liu T, Li XF, Zhou Z. Improvement of hydrogen yield by *hupR* gene knock-out and *nifA* gene overexpression in *Rhodobacter sphaeroides* 6016. Int. J. Hydrogen Energy. 2010; 35:9603-10.

[11] Kim DH, Lee JH, Kong S, Hallenbeck PC, Kim EJ, Lee JK et al. Enhanced photofermentative H_2 production using *Rhodobacter sphaeroides* by ethanol addition and analysis of soluble microbial products. Biotechnol. Biofuels. 2014; 7:79-88.

[12] Ma C, Wang XQ, Guo LJ, Wu XM, Yang HH. Enhanced photofermentative hydrogen production by *Rhodobacter capsulatus* with pigment content manipulation. Bioresour. Technol. 2012; 118:490-95.

[13] Hallenbeck PC, Liu Y. Recent advances in hydrogen production by photosynthetic bacteria. Int. J. Hyd. Energy. 2016; 41:44446-4454.

[14] Kontur WS, Noguera DR, Donohue TJ. Maximazing reductant flow into microbial H_2 production. Curr. Opin. Biotechnol. 2012; 23:382-89.

[15] McKinlay JB, Harwood CS. Calvin cycle flux, pathway constrains, and substrate oxidation state together determine the H_2 biofuel yield in photosynthetic bacteria. mBio. 2011; 2:e00323-0.

[16] McKinlay JB, Oda Y, Ruhl M, Posto AL, Sauer U, Harwood CS. Non-growing *Rhodopseudomonas palustris* increases the hydrogen gas yield from acetate by shifting from glyoxylate shunt to the tricarboxylic acid cycle. J. Biol. Chem. 2014; 289:1960-70.

[17] Wang D, Zhang YP, Welch E, Li JL, Roberts GP. Elimination of Rubisco alters the regulation of nitrogenase activity and increase hydrogen production in *Rhodospirillum rubrum*. Int. J. Hydrogen Energy. 2010; 35:7377-85.

[18] Ozturk Y, Gokce A, Peksel B, Gurgan M, Ozgur E, Gundze U. Hydrogen production properties of *Rhodobacter capsulatus* with genetically modified redox balancing pathways. Int. J. Hydrogen Energy. 2012; 37:2014-20.

[19] Simon R, Priefer U, Puhler, A. A broad-host-range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. Nat. Biotechnol 1983; 1:784-91.

[20] Colbeau A, Magnin J-P, Cauvin B, Champion T, Vignais PM. Genetic and physical mapping of an hydrogenase gene cluster from *Rhodobacter capsulatus*. Mol. Gen. Genetics. 1990; 220, 393-99

[21] Alexeyev MF. Three kanamycin resistance gene cassettes with different polylinkers. BioTechniques. 1995; 18:52, 54, 56.

[22] Weaver PF, Wall JD, Gest H. Characterization of *Rhodopseudomonas capsulata*. Arch. Microbiol. 1975; 105:207-16.

[23] Sambrook J and Russell DW. Molecular cloning: a laboratory manual. 3rd Ed. Cold Spring Harbor Lab. NY: Plainview; 2001.

[24] Madigan MT, Gest H. Growth of the photosynthetic bacterium *Rhodopseudomonas capsulata* chemoautotrophically in darkness with H_2 as the energy source. J. Bacteriol. 1979; 137:524-30.

[25] Hallenbeck PC, Meyer CM, Vignais PM. Nitrogenase from the photosynthetic bacterium *Rhodopseudomonas capsulata*: purification and molecular properties. J. Bacteriol. 1982; 149:708-17.

[26] Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 1959; 31:426-28.

[27] Battley EH. General procedures for writing growth-process equations. In: Battley, EH, editor. Energetics of microbial growth. Wiley; 1987, pp 363.

[28] McKinlay JB, Harwood CS. Carbon dioxide fixation as a central redox cofactor recycling mechanism in bacteria. Proc. Natl. Acad. Sci. USA. 2009; 107:11669-75.

[29] Tichi MA, Tabita FR. Interactive control of *Rhodobacter capsulatus* redox-balancing systems during phototrophic metabolism. J. Bacteriol. 2001; 183:6344-83.

[30] Paoli GC, Vichivanives P, Tabita FR. Physiological control and regulation of the *Rhodobacter capsulatus cbb* operons. J. Bacteriol. 1998; 180:4258-69.

[31] Wang X, Falcone DL, Tabita FR. Reductive pentose phosphate-independent CO_2 fixation in *Rhodobacter sphaeroides* and evidence that Ribulose Bisphosphate carboxylase/oxygenase activity serves to maintain the redox balance of the cell. J. Bacteriol. 1993; 175:3372-79.

[32] Hallenbeck PL, Lerchen R, Hessler P, Kaplan S. Phosphoribulokinase activity and regulation of CO_2 fixation critical for photosynthetic growth of *Rhodobacter sphaeroides.* J. Bacteriol. 1990; 172:1749-61.

[33] Wang D, Zhang Y, Pohlmann EL, Li J, Roberts GP. The poor growth of *Rhodospirillum rubrum* mutants lacking Rubisco is due to accumulation of ribulose-1,5-bisphosphate. J. Bacteriol. 2011; 193:3293-03.

[34] Laguna R, Tabita FR, Alber BE. Acetate-dependent photoheterotrophic growth and the differential requirement for the Calvin-Benson-Bassham reductive pentose phosphate cycle in *Rhodobacter sphaeroides* and *Rhopseudomas palustris*. Arch. Microbiol. 2011; 193:151-54.

[35] Meister M, Saum S, Alber BE, Fuchs G. L-malyl-coenzyme A/beta methylmalyl-coenzyme A lyase is involved in acetate assimilation of the isocitrate lyase-negative bacterium *Rhodobacter capsulatus*. J. Bacteriol. 2005; 187:1415-25.

[36] Willison JC. Pyruvate and acetate metabolism in the photosynthetic bacterium *Rhodobacter capuslatus*. Microbiology. 1988; 134:2429-39.

[37] Kornberg HL, Lascelles J. The formation of isocitratase by the Athiorhodaceae. Microbiology. 1960; 23:511-17.

[38] Albers H, Gottschalk G. Acetate metabolism in *Rhodopseudomonas gelatinosa* and several other Rhodospirillaceae. Arch. Microbiol. 1976; 111:45-49.

[39] Kondratieva EN. Interrelation between modes of carbon assimilation and energy production in phototrophic purple and green bacteria. In International review of biochemistry, Microbial Biochemistry, vol. 21, pp117-75, Edited by Quayle JR. Baltimore: University Park Press.

[40] Michal, T.M., Judy, D.M., Howard, G. Dark anaerobic dinitrogen fixation by a photosynthetic microorganism. Science. 1979; 204:1429-30.

[41] Hadicke O, Grammel H, Klamt S. Metabolic network modeling of redox balancing and biohydrogen production in purple nonsulfur bacteria. BMC Syst. Biol. 2011; 5:150-68.

[42] Abo-Hashesh M, Ghosh D, Tourigny A, Taous A, Hallenbeck PC. Single-stage photofermentative hydrogen production from glucose: an attractive alternative to two stage photofermentation or co-culture approaches. Int. J. Hydrogen Energy. 2011; 36:13889-95.

[43] Ghosh D, Sobro IF, Hallenbeck PC. Optimization of hydrogen yield from single-stage photofermentation of glucose by *Rhodobacter capsulatus* JP91 using response surface methodology. Bioresour. Technol. 2012; 123:199-06.

[44] Ozturk Y, Gokce A. Characterization of hydrogen production by an ammonium insensitive pseudo-revertant of a CBB deficient *Rhodobacter capsulatus* strain. Int. J. Hydrogen Energy. 2012; 37:8811-19.

[45] Tao YZ, Liu D, Yan X, Zhou ZH, Lee JK, Yang C. Network identification and flux quantification of glucose metabolism in *Rhodobacter sphaeroides* under photoheterotrophic H_2-producing conditions. J. Bacteriol. 2012; 194:274-83.

TABLES

Table 1. Strains and plasmids used in this experiment.

Strains and Plasmids	Relevant characteristics	Source or reference
E. coli		
DH5α	Host for cloning vectors	This study
S17-1	RP4-2 Tc::Mu Km::Tn*7*	[19]
GM2163	*dam-13:*Tn*9*	G Szatmari
R. capsulatus		
JP91	*hup*$^-$	[20]
JP91 *cbbP*$^-$ (PRK mutant)	*hup*$^-$ *cbbP*$^-$	This study
YL1	Spontaneous nif$^-$ mutant (with respect to glutamate as nitrogen source) of JP91 *cbbP*$^-$	This study
Plasmids		
pSUP202	Ap^r Tc^r Chl^r, mobilizable *R.capsulatus* suicide vector	[19]
pBSL15	contains kanamycin resistance cassette	[21]
pYN1	pSUP202 derivative carrying most of *cbbP*	This study
pYN2	Kanamycin resistance gene cloned into the BclI site of *cbbP* on pYN1	This study
pAY10	*sacB* cloned into pSUP202	A Yakunin
pYN3	*cbbP*::Km subcloned into pAY10	This study

Primers used in this study (The first pair was used for amplication of *cbbP* gene, and both pairs were used for validation of the mutant)

For: CAC CTC GAC CGT CAA GGC GAC CTT
Rev: CT TCG CGC ACC AGA CGC TCG ATCA

For: ACA TGA AGG CGG AAC TGG AA
Rev: TCA TCG AGG TCA GAT AGG GGA A

Table 2. Organic acids analysis by HPLC[a].

	120 W/m^{2}[b]		175 W/m^{2}		240 W/m^{2}	
	JP91	YL1	JP91	YL1	JP91	YL1
Pyruvate	0.56 ± 0.08	0.74 ± 0.12	1.77 ± 0.26	2.08 ± 0.2	0.51 ± 0.16	0.46 ± 0.007
Lactate	8.49 ± 0.91	ND[c]	8.65 ± 0.6	1.46 ± 0.33	ND	ND
Succinate	0.2 ± 0.28	0.95 ± 0.06	8.49 ± 0.6	ND	ND	ND

[a] Concentrations are given as mM. Acetate, malate, and formate were not detected by HPLC in the culture medium of either strain under any of the culture conditions tested.

[b] This is the power, in joules per second, received per square meter.

[c] ND, not detectable.

Table 3. H_2 yield and electrons allocation analysis at different light intensities with glucose as carbon source.

	120 W/m^2		175 W/m^2		240 W/m^2	
	JP91	YL1	JP91	YL1	JP91	YL1
H_2 yield[a]	6.19 ± 0.42	7.63 ± 0.13	3.87 ± 0.49	5.42 ± 0.33	5.71 ± 0.06	6.09 ± 0.88
Electrons in H_2 [b](%)	54.16 ± 3.16	65.25 ± 1.14	29.44 ± 3.28	44.02 ± 5.43	43.12 ± 0.47	50.76 ± 7.3
Electrons in Biomass[c]	29.22 ± 2.56	42.53 ± 1.28	34.96 ± 0.38	35.34 ± 3.42	44.81 ± 1.8	42.15 ± 5.24
Electrons in organic acids	24.88 ± 2.31	8.02 ± 0.37	59.31 ± 2.57	11.83 ± 0.03	1.4 ± 0.42	1.64 ± 0.1
Electron recovery[d]	108.26 ± 2.71	115.79 ± 2.79	123.71 ± 3.08	91.19 ± 2.87	89.34 ± 0.91	94.55 ± 12.68

[a] H_2 yield: mol H_2/mol glucose consumed

[b] expressed as %. The same calculated method was used for organic acids and biomass.

[c] Here it is assumed that the biomass composition of *R. capsulatus* JP91 and *R. capsulatus* YL1 are the same as that of *Rhodopseudomonas plaustris* [28], which is $CH_{1.8}N_{0.18}O_{0.38}$.

[d] Here it is assumed that the molecular weight of biomass of *R. capsulatus* JP91 and *R. capsulatus* YL1 are the same as that of *Rhodopseudomonas plaustris* [28], which is 22.416 g/mol. H_2, organic acids and biomass were considered as main electron reservoirs.

Table 4. H_2 yield comparison with closely related studies

Microorganisms	Genotype	Strategy (ies) used	Light source and intensity	H_2 yield[a]	Ref
R. capsulatus JP91	*hup*$^-$	Single-stage photofermentation of glucose	I[b]; 200 W/m^2	25%	42
R. capsulatus JP91	*hup*$^-$	Single-stage photofermentation of glucose; Response surface methodology	I; 100, 175, 250 W/m^2	46%	43
R. capsulatus JP91	*hup*$^-$	Single-stage photofermentation of glucose; Continuous culture	I; NA[c]	75%	4
R. capsulatus JP91	*hup*$^-$	Single-stage photofermentation of glucose	I; 120, 175, 240 W/m^2	52%	This study
R. capsulatus YL1	*hup*$^-$, *cbb*$^-$	Single-stage photofermentation of glucose; Inactivation of CBB cycle	I; 120, 175, 240 W/m^2	63%	This study
R. capsulatus YO7	*hup*$^-$, *cbb*$^-$	Inactivation of CBB cycle	I; 2500-3000 lux	66%[d]	18
R. capsulatus YO7-R3	*hup*$^-$, *cbb*$^-$, *nifA**	Inactivation of CBB cycle; introduces constitutionally expressed transcriptional activator of nitrogenase genes	I; 2500-3000 lux	56%[d]	44
Rhodospirillum rubrum UR5251	*cbb*$^-$	Inactivation of CBB cycle	NA	ND[d,e]	17
Rhodospirillum rubrum UR2562	*cbb*$^-$, *nifA**	Inactivation of CBB cycle; introduces constitutionally expressed transcriptional activator of nitrogenase genes	NA	68%[d, f]	17
Rhodopseudomonas palustris CGA679	*cbb*$^-$, *nifA**	Inactivation of CBB cycle; introduces constitutionally expressed transcriptional activator of nitrogenase genes	I; NA	20%[g]	28
Rhodopseudomonas palustris CGA679 complemented	*cbb*$^-$, *nifA** (pBBPcbbLSX)	Inactivation of CBB cycle; introduces constitutionally expressed transcriptional activator of nitrogenase genes; complemented with Rubisco genes	I; NA	14.5%[g]	15
Rhodobacter sphaeroides KD131 derived	*hup*$^-$, *phbC*$^-$	Inactivation of uptake hydrogenase and PHB synthesis pathway	I; 4000 lux	56%	45

[a] Percentage of the theoretical maximum

[b] I, incandescent

[c] NA, not available

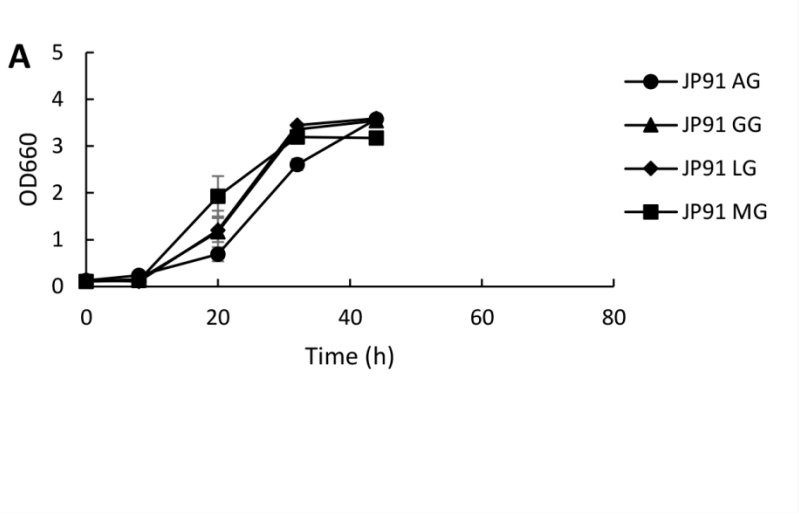
[d] Malate was used as carbon source.

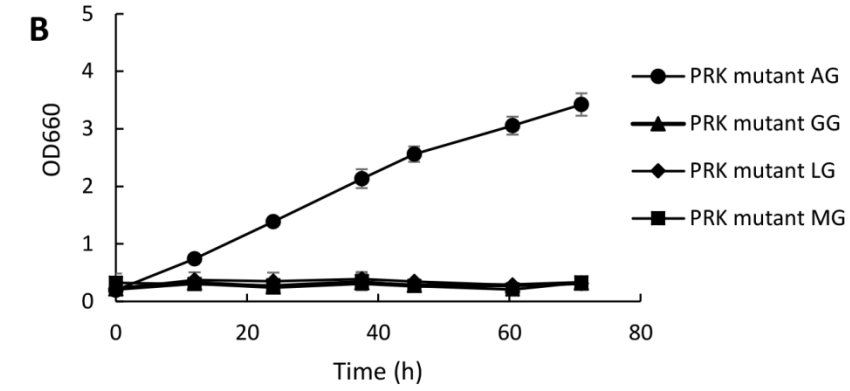
[e] ND: not determined door to very slow growth of the mutant.

[f] The value was corrected since the one originally reported is in error.

[g] Acetate was used as carbon source.

Figures


A
OD660
Time (h)
JP91 AG
JP91 GG
JP91 LG
JP91 MG


B
OD660
Time (h)
PRK mutant AG
PRK mutant GG
PRK mutant LG
PRK mutant MG

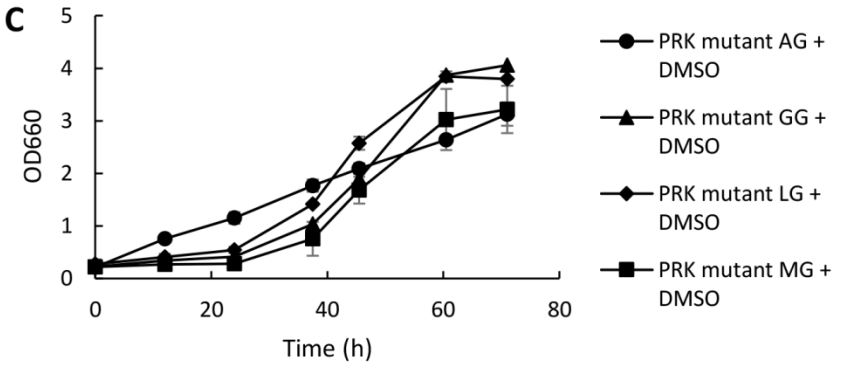
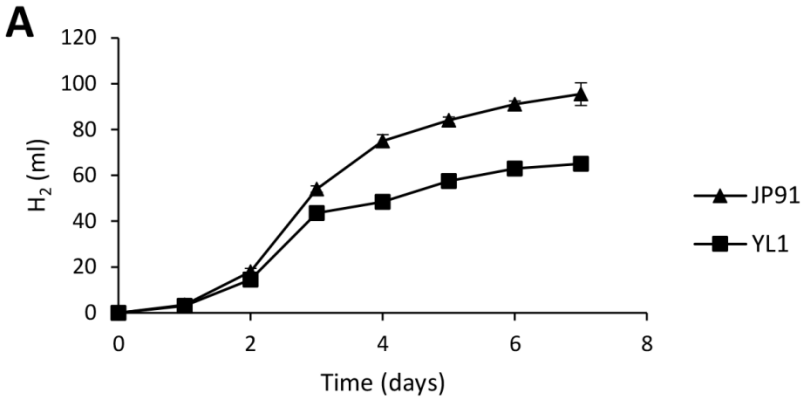
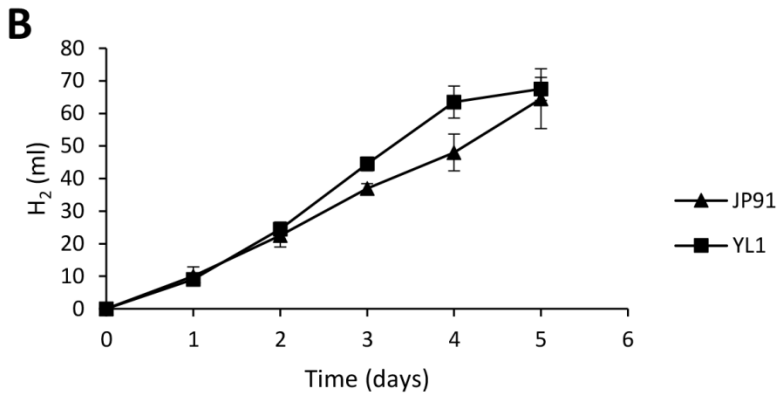

C
OD660
Time (h)
PRK mutant AG + DMSO
PRK mutant GG + DMSO
PRK mutant LG + DMSO
PRK mutant MG + DMSO

Figure 1. Growth characteristics of strains JP91and its PRK mutant in different media. Strains JP91(**A**) and its PRK mutant (**B**) were grown in RCVG-A, RCVG-G, RCVG-L and RCVG-M media at 30 °C with a light intensity of 80 W/m^2. The inocula were pre-grown in YPS medium. (**C**): The PRK mutant was grown in RCVG-A, RCVG-G, RCVG-L and RCVG-M media supplemented with 30 mM DMSO. Each data point is an average of two biological repeats with error bars indicating standard deviation.


A
120
100
80
60
40
20
0
H2 (ml)
0
2
4
6
8
Time (days)
JP91
YL1


B
80
70
60
50
40
30
20
10
0
H2 (ml)
0
1
2
3
4
5
6
Time (days)
JP91
YL1

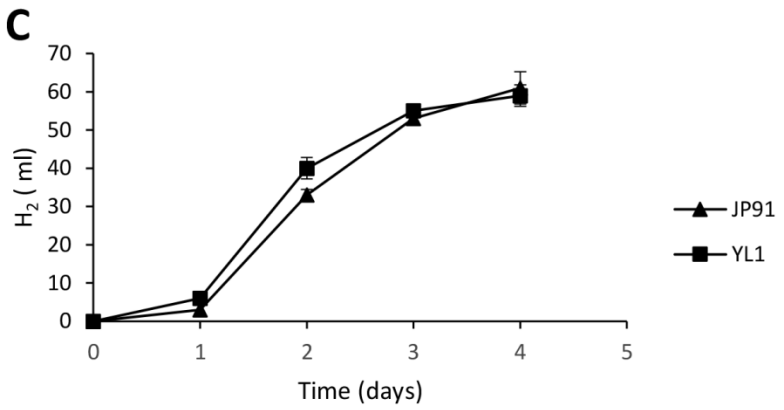
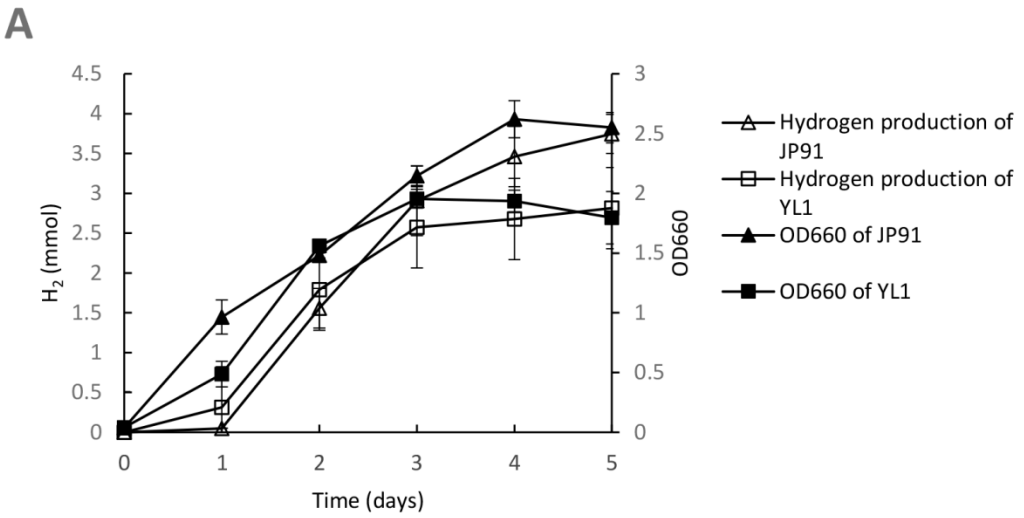
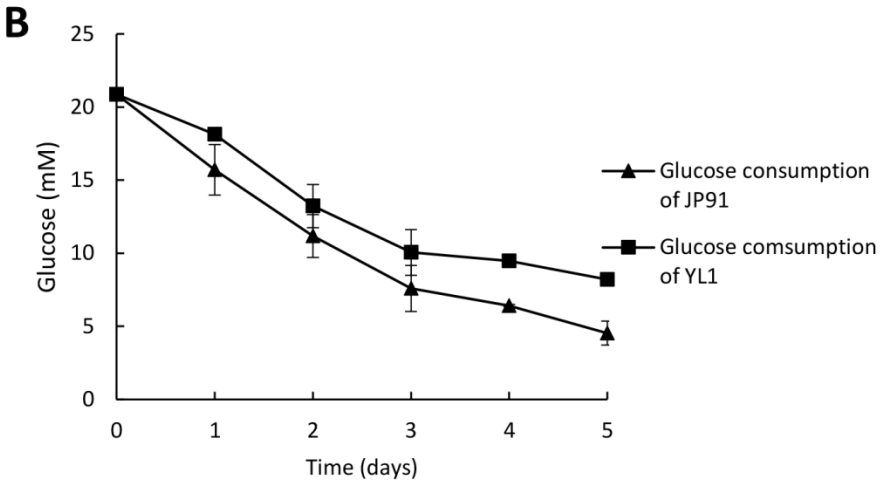

C
70
60
50
40
30
20
10
0
H2 (ml)
0
1
2
3
4
5
Time (days)
JP91
YL1

Figure 2. Effects of different light intensities on H_2 production by strains YL1 and JP91 grown in RCVG-G. (**A**) 120 W/m^2; (**B**) 175 W/m^2; (**C**) 240 W/m^2. The cultures were maintained at 30 °C. Each data point is an average of two biological repeats with error bars indicating standard deviation.


A
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
H2 (mmol)
3
2.5
2
1.5
1
0.5
0
OD660
0
1
2
3
4
5
Time (days)
Hydrogen production of JP91
Hydrogen production of YL1
OD660 of JP91
OD660 of YL1


B
25
20
15
10
5
0
Glucose (mM)
0
1
2
3
4
5
Time (days)
Glucose consumption of JP91
Glucose comsumption of YL1

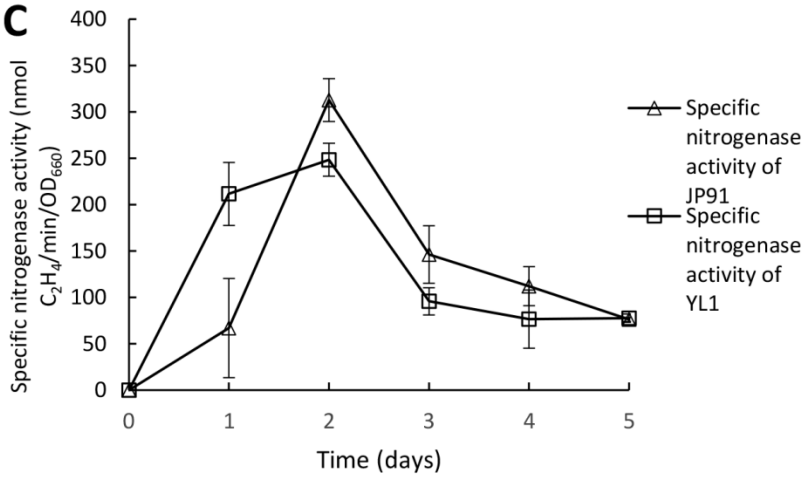

C
400
350
300
250
200
150
100
50
0
Specific nitrogenase activity (nmol C2H4/min/OD660)
0
1
2
3
4
5
Time (days)
Specific nitrogenase activity of JP91
Specific nitrogenase activity of YL1

Figure 3. Comparison of H_2 production, glucose consumption, biomass formation and *in vivo* nitrogenase activity of strains JP91 and YL1. The experiment bottles were incubated at 30 °C with illumination of 120 W/m^2. Each day H_2 quantity was determined with GC at conditions detailed in the text. The liquid culture was sampled each day with a 1-ml syringe which was made anaerobic before sampling by rinsing it with H_2O and used for *in vivo* N_2ase assay, OD determination, and glucose assay, respectively. The values shown are average of two biological repeats, and error bars indicate standard deviation. (**A**) relationship between H_2 production and biomass accumulation; (**B**) Glucose consumption kinetics; (**C**) each day *in vivo* nitrogenase activity. Each data point is an average of two biological repeats with error bars indicating standard deviation.

Chapter 4: Characterization of mutants of *Rhodobacter capsulatus* having constitutive nitrogenase expression in the presence of ammonium

Yuan Liu and Patrick C. Hallenbeck*

Département de Microbiologie, infectiologie, et immunologie, Université de Montréal CP 6128 Succursale Centre-ville, Montréal, Québec H3C 3J7 Canada

Author contribution: The experimental design and wet laboratory experiments were carried out by YL. YL and PCH performed all dataset analysis. All the work was under supervision of PCH.

Article status: This article is currently in preparation to be submitted.

Abstract

The nitrogenase system, which catalyzes nitrogen fixation, is a highly energy-demanding process, thus, its expression and activity are normally under tight control by ammonium availability. In the present study, we characterized two secondary spontaneous mutants, YL3 and YL4, derived from a pseudo-revertant YL2 of the PRK (phosphoribulose kinase) mutant described in chapter 3. An initial assessment of H_2 production showed that these two mutants were excellent H_2 producers in ammonium-containing media, suggesting their possible use in the future to produce H_2 using some ammonium containing organic wastes. Indeed, Western blot results showed that nitrogenase is constitutively expressed in YL3 and YL4 in the presence of various amounts of ammonium. In order to understand how nitrogenase was constitutively expressed in the presence of ammonium, the genomes of YL2, YL3 and YL4 were sequenced. We found mutations in both *nifA1* and *nifA2*, the master regulators for nitrogenase expression, for both YL3 and YL4. It is likely that a conformational change of NifA alters the protein-protein interaction between NifA and PII proteins, such as GlnB or GlnK, permitting its activity to be maintained and therefore capable of activating nitrogenase expression in the presence of ammonium.

Keywords: ammonium; constitutive expression of nitrogenase; switch-off; *Rhodobacter capsulatus* YL3 and YL4; posttranslational regulation; genome sequencing; NifA; SNAP.

1. Introduction

In many diazotrophs, nitrogen fixation is tightly controlled by ammonium availability in the medium [1-5]. It has been shown that this regulation occurs on at least three levels through the involvement of some pivotal proteins, such as the PII proteins GlnK and GlnB: (1) the activation of transcription of the *nif*-specific activator, *nifA*; (2) the post-translational control of NifA activity; (3) the post-translational control of nitrogenase activity through ADP-ribosylation of NifH or by an unknown mechanism [6-9]. NifA is one of the key components of this regulatory circuit [10, 11]. It has been proposed that NifA has four to five functional domains: N-terminal domain, Q-linker, central domain, IDL region and C-terminal domain [10]. The N-terminal domain is likely to be involved in posttranslational regulation of NifA activity through interaction with GlnK/GlnB. The central domain catalyzes open transcription complex formation. The C-terminal domain is involved in DNA binding [12, 13]. In *Rhodobacter capsulatus*, there are two copies of NifA, namely NifA1 and NifA2. NifA1 and NifA2 differ in their N-19 and N-22 amino residues and have a total of 579 and 582 residues, respectively [14]. The transcription of NifA is controlled by NtrB/NtrC or a NtrC-independent mechanism [15, 16]

As mentioned in chapter 3, we isolated a pseudo-revertant, YL2 from RCVG-M medium after prolonged incubation. In this study, the growth characteristics of YL2 in ammonium media with four different carbon sources were tested and the results showed that YL2 could only grow with acetate as carbon source. However, two secondary spontaneous mutants, YL3 and YL4 were isolated after prolonged incubation. Such mutants could be potentially be used for H_2 production with organic wastes containing ammonium. Thus it was of interest to study

these mutants in some detail and to attempt to understand how their nitrogenase system has escaped regulation by ammonium.

2. Materials and Methods

2.1 Bacterial strains, media and growth conditions

The strains used in this study are listed in Table 1. For the growth study of YL2, RCV media [17] supplemented with 30 mM ammonium and four different carbon sources, 20 mM glucose, 30 mM malate, 40 mM lactate or 60 mM acetate was used. For examination of nitrogenase expression, YL3, YL4 and SB1003 were grown with RCV supplemented with 30 mM malate and 10-30 mM ammonium as carbon and nitrogen sources. For the examination of ammonium switch-off, strains were cultured as described by Yakunin [18]. For the H_2 production study, JP91, YL3 and YL4, were inoculated into RCV supplemented with 30 mM malate and 10 (and 30) mM ammonium as carbon and nitrogen sources. All cultures were incubated at 30 °C in a Biotronette Mark III (Lab-line instruments) environmental chamber equipped with three 150 W incandescent bulbs.

2.2 Analytical methods

Optical density was determined with a double-beam spectrophotometer (Shimadzu UV 2101 PC). Briefly, 100 µl of culture were sampled anaerobically using a sterile syringe pre-flushed with H_2O and diluted (OD_{660} to 0.2 ~ 0.4) before reading the optical density at 660 nm. H_2 was measured with a Shimadzu GC-8A gas chromatograph as previously described [19].

Nitrogenase activity was determined by the acetylene reduction method as described by Hallenbeck [20]. Nitrogenase expression levels were determined by Western blot as described by Yakunin and Hallenbeck [21]. The genomic DNA of *R. capsulatus* strains was extracted using Geneaid bacterial genome extraction kit following the manufacturer's user guide (Presto^{TM} Mini gDNA Bacteria Kit). The Genomes were sequenced at Mr DNA (Shallowater, TX) by using a 600 cycles v3 reagent kit (Illumina) in MiSeq (Illumina). The SNP report was analyzed with Microsoft Excel (Microsoft).

3. Results and Discussion

3.1 Isolation of secondary spontaneous mutants of Rhodobacter capsulatus

In a previous study we reported that a PRK (phosphoribulose kinase) mutant failed to grow in RCV-glutamate medium with glucose, malate and lactate as carbon sources presumably due to intracellular electron imbalance. However, after prolonged incubation growth was observed, indicating the outgrowth of spontaneous mutants. A pseudo-revertant, YL2, was isolated from RCVG-M (abbreviated as MG) medium. The relationships between different strains were shown in Fig.1. In this study, YL2 was tested for growth in ammonium media with malate, glucose, lactate and acetate as carbon sources. Among these potential carbon sources, only acetate supported its growth (Fig.2). The fact that YL2 can only grow with acetate as carbon source might be due to the fact that acetate is metabolized through the ethylmalonyl-CoA pathway which includes a built-in CO_2 reduction step, enabling re-establishment of redox balance without a functional Calvin-Benson-Bassham cycle [22, 23]. However, after prolonged incubation (7-20 days depending on carbon source), growth was observed (Fig. 2B),

indicating outgrowth of secondary spontaneous mutants. Such mutants are important because they can potentially be used for H_2 production with various kinds of organic wastes which normally contain ammonium and they also can be used to study nitrogenase regulation mechanisms. The spontaneous mutant isolated from malate (MN) medium was named YL3, and the one from lactate (LN) medium was named YL4.

3.2 Study of growth rates and H_2 production properties

A practical H_2 industry based on biotechnology would require the lowest medium cost, thus various kinds of organic wastes which normally contain different amounts of ammonium are ideal for such a purpose. A recent study has demonstrated the competency of an ammonium-tolerant strain of *Rhodopseudmonas palustris* in this regard by using it to produce H_2 with vegetable waste derived medium [24]. Although JP91 has been shown to be a remarkable H_2 producer with glutamate as nitrogen source in previous studies [25, 26, 27], its H_2 production ability in the presence of ammonium has never been tested.

Here, we first determined the growth rates of JP91, YL3 and YL4 in MN medium and found that YL3 and YL4 grew slightly slower than JP91, with doubling times of 7.87 and 7.65 versus 6.47 h (Table 2). As an initial experiment, the H_2 production properties of these strains were examined using synthetic medium with 10 or 30 mM ammonium as nitrogen source and 30 mM malate as carbon source. When 10 mM ammonium was used, all three strains produced H_2, however the amounts of H_2 produced by JP91 were significantly lower than those of strain YL3 or YL4, 7.25 versus 13.5 or 12.5 ml (Table 2). When the ammonium concentration was increased to 30 mM, no H_2 production was observed for JP91, and an *in*

vivo nitrogenase assay showed there was no nitrogenase activity. In contrast, H_2 was still produced by YL3 and YL4, reaching 60% of that under ammonium limiting conditions (10 mM), indicating active nitrogenase at high ammonium concentrations (Table 2).

3.3 Influence of different ammonium concentrations on nitrogenase expression

Since substantial levels of H_2 were produced by YL3 and YL4 even under a high level of ammonium (30 mM), it was of interest to determine nitrogenase expression levels at different ammonium concentrations. Strain SB1003 was chosen as a control since this strain has been highly studied in the past for nitrogen switch-off, but it probably would have been better to have done this experiment using JP91. In fact, since this strain is more closely related to the strains under study it would have been a better control [18, 28]. Here, 10 mM, 20 mM, 23 mM, 26 mM, and 30mM of ammonium were added into RCV medium using 30 mM malate as carbon source. As shown in Fig. 3, nitrogenase expression of strain YL3 (YL4 had the same pattern as YL3, data not shown) remained the same, regardless of the ammonium concentrations. Quantification of band intensity using Image J software showed there is very little variation between samples. For example, band intensities at 10 and 30 mM ammonium are 11663 and 11597, respectively. In contrast, SB1003 showed decreased nitrogenase expression with increasing ammonium concentrations, a trend that is in agreement with previous results [28]. The band intensity at 20 mM ammonium decreased by 67.2% compared to that at 10 mM ammonium. There was no appreciable change when ammonium concentration was between 20 to 26 mM. However, the band intensity at 30 mM ammonium was only 3.04% of that at 10 mM ammonium. Thus, for YL3 and YL4, the expression of nitrogenase appears to escape transcriptional regulation by ammonium.

3.4 Nitrogenase switch-off phenomenon induced by ammonium

It has been shown that when highly nitrogen-limited cultures of strain B10S, a close relative of SB1003, was exposed to 200 μM ammonia, nitrogenase activity was switched off within 10 min [6], while there was no change of nitrogenase activity upon an ammonium shock for a *glnK-glnB* double mutant. Since there was H_2 production when using ammonium as nitrogen source for YL3, an *in vivo* nitrogenase assay was carried out as described in the Methods section where 5 mM ammonium was added into the assayed cultures at 44 min. SB1003 was also used as a control in this experiment. Although, the nitrogenase activity of SB1003 decreased substantially after 20 min of ammonium shock, it wasn't completely switched off, as shown in Fig 4A. On the other hand, the nitrogenase activity of YL3 remained completely unchanged after exposure to ammonium (Fig 4B), suggesting that in this mutant nitrogenase activity has also escaped the post-translational control by ammonium.

3.5 Identification of mutations resulting in constitutive nitrogenase expression by genome sequencing

In order to identify the mutations permitting constitutive nitrogenase expression of YL3 and YL4 grown with high concentrations of ammonia, the genomes of strains YL2, YL3 and YL4 were sequenced (YL3 and YL4 are directly derived from YL2). By polymorphism comparison of YL2 to YL3 and YL2 to YL4, several putative mutations were found, shown in Table 3. In YL3, the first mutation was mapped to *nifA1* coding region in which there is a 6 bp insertion between position 598 and 599. The second mutation was mapped to the *nifA2* coding region in which the same 6 bp insertion was found between position 598 and 599 and a 15 bp

insertion was found between position 105 and 106. In addition to mutations in *nifA*, we also found that the malate synthase G gene was disrupted by a 1 bp insertion between position 950 and 951, however, no evidence has shown that it belongs to the *nif* regulon. More mutations were found on non-coding sequences which are located far from CDS (more than 10 kb) with known function (data not shown). In YL4, a G-A substitution was found at position 590 in the coding regions of both *nifA1* and *nifA2*, which is a non-synonymous mutation since arginine is replaced by histidine (R197H). In many diazotrophs, such as *Klebsiella pneumoniae, Azotobacter vinelandii*, NifA is required for nitrogenase expression. However, In *Herbaspirillum seropedicae*, a NifA variant which lost its GAF domain (in N-terminal) could still activate nitrogenase transcription, but was insensitive to ammonium regulation [13, 29, 30]. In *Rps. palustris*, it has been shown that a single mutation in NifA at nearby positions (202, 213, 209 and 212) was sufficient to circumvent ammonium regulation [9]. In *R. capsulatus*, a L66Q substitution at N-terminal of NifA1 also has the same effect. [12]. Thus, it seems that the mutations found here can potentially explain the Western blot results. A likely mechanism for regulation of NifA activity is through protein-protein interaction between NifA and GlnB (or GlnK), since a *glnB/glnK* double mutant showed no ammonium control of *nifA* activity and a yeast two-hybrid study directly demonstrated the interactions of GlnB-NifA1, GlnB-NifA2, GlnK-NifA1 and GlnK-NifA2 [6, 31]. We noticed that the polymorphism percentage of *nifA* in YL3 and YL4 are less than 100% (Table 3A, B). One likely reason is due to reversion, as it was also found by other authors that *nifA* mutation wasn't stable [9]. Thus, in the future it would be good to confirm these mutations through resequencing and to introduce them back into proper strains to verify their physiological role in nitrogen-fixation conditions. In addition, another important question that remains to be answered is how is *nifA*

transcribed in these strains in the presence of ammonium? In *Rps. palustris* it has been reported that there is a basal level of *nifA* expression in ammonium-containing media, and that this amount of NifA is sufficient to activate nitrogenase expression [9]. Thus, it also would be of value to examine the NifA levels in future work.

Conclusion

In this study, we characterized two secondary spontaneous mutants, YL3 and YL4, isolated from ammonium-containing media. An initial H_2 production study showed that YL3 and YL4 produced more hydrogen than JP91 when cultured in synthetic medium with different levels of ammonium as nitrogen source. Thus, both strains could be applied in a practical H_2 industry using organic wastes containing ammonium as feedstock. Western blot results showed that, regardless of ammonium concentrations (10 to 30 mM), the nitrogenase of YL3 and YL4 was constitutively expressed. Through analyzing SNP reports, we found mutations in *nifA1* and *nifA2* for both YL3 and YL4. It is likely that a conformation change of NifA altered protein-protein interaction, thus allowing NifA activity to be maintained, thereby can activating nitrogenase expression in the presence of ammonium.

Acknowledgements

This research was supported by a NSERC Discovery grant to PCH. YL was supported by a scholarship from CSC, China.

Reference

[1] Dixon R, Kahn D. Genetic regulation of biological nitrogen fixation. Nat. Rev. Microbiol. 2004; 2:621-631.

[2] Zhang Y, Pohlmann EL, Ludden PW, Roberts GP, Mutagenesis and functional characterization of the *glnB*, *glnA*, and *nifA* genes from the photosynthetic bacterium *Rhodospirillum rubrum*. J. Bacteriol. 2000; 182:983-92.

[3] Yelton MM, Yoch DC. Nitrogen metabolism in *Rhodospirillum rubrum*: characterization of glutamate synthase. Microbiology. 1981; 123:335-42.

[4] Neilson AH, Nordlund S. Regulation of nitrogenase synthesis in intact cells of *Rhodospirillum rubrum*: inactivation of nitrogen fixation by ammonia, glutamine and L-asparagine. Microbiology. 1975; 91:53-62.

[5] Hubner P, Masepohl B, Klipp W, Bickle TA. *nif* gene expression studies in *Rhodobacter capsulatus*: *ntrC*-independent repression by high ammonium concentrations. Mol. Microbiol. 1993; 10:123-32.

[6] Drepper T, Groβ S, Yakunin AF, Hallenbeck PC, Masepohl B, Klipp W. Role of *glnB* and *glnK* in ammonium control of both nitrogenase systems in the phototrophic bacterium *Rhodobacter capsulatus*. Microbiology. 2003; 149:2203-12.

[7] Hubner P, Willison JC, Vignais PM, Bickle TA. Expression of regulatory *nif* genes in *Rhodobacter capsulatus*. J. Bacteriol. 1991; 173:2993-99.

[8] Kranz RG, Foster-Hartnett D. Transcriptional regulatory cascade of nitrogen-fixation genes in anoxygenic photosynthetic bacteria: oxygen and nitrogen responsive factors. Mol. Microbiol. 1990; 4:1793-00.

[9] Heiniger EK, Oda Y, Samanta SK, Harwood, CS. How posttranslational modification of nitrogenase is circumvented in *Rhodopseudomonas palustris* strains that produce hydrogen gas constitutively. Appl. Eniviron. Microbiol. 2012; 78:1023-32.

[10] Hans-Martin F. Genetical regulation of nitrogen fixation in Rhizobia. Microbiol. Rev. 1994; 58:352-86.

[11] Reyes-Ramirez F, Little R, Dixon R. Mutant forms of the *Azotobacter vinelandii* transcriptional activator NifA resistant to inhibition by the NifL regulatory protein. J. Bacteriol. 2002; 184:6777-85.

[12] Paschen A, Drepper T, Masepohl B, Klipp W. *Rhodobacter capsulatus nifA* mutants mediating *nif* gene expression in the presence of ammonium. FEMS Microbiol. Lett. 2001; 200:207-13.

[13] Aquino B, Stefanello AA, Oliveira MAS, Pedrosa FO, Souza EM, Monteiro RA, Chubatsu LS. Effect of point mutations on *Herbaspirillum seropedicae* NifA activity. Braz. J. Med. Biol. Res. 2015; 48:683-90.

[14] Masepohl B, Klipp W, Puhler A. Genetic characterization and sequence analysis of the duplicated *nifA/nifB* gene region of *Rhodobacter capsulatus*. Mol. Gen. Genet. 1988; 212:27-37.

[15] Bowman WC, Kranz RG. A bacterial ATP-dependent, enhancer binding protein that activates the housekeeping RNA polymerase. Genes. Dev. 1998; 12:1884-93.

[16] Masepohl B, Klipp W. Organization and regulation of genes encoding the molybdenum nitrogenase and the alternative nitrogenase in *Rhodobacter capsulatus*. Arch. Microbiol. 1996; 165:80-90.

[17] Weaver PF, Wall JD, Gest H. Characterization of *Rhodopseudomonas capsulata*. Arch Microbiol. 1975; 105:207-6.

[18] Yakunin AF, Hallenbeck PC. Short-term regulation of nitrogenase activity by NH_4^+ in *Rhodobacter capsulatus*: multiple *in vivo* nitrogenase response to NH_4^+ addition. J. Bacteriol. 1998; 180:6392–95.

[19] Liu Y, Ghosh D, Hallenbeck PC. Biological reformation of ethanol to hydrogen by *Rhodopesudomonas palustris* CGA009. Bioresour. Technol. 2015; 176:189-95.

[20] Hallenbeck PC, Meyer CM, Vignais PM. Nitrogenase from the photosynthetic bacterium *Rhodopseudomonas capsulata*: purification and molecular properties. J. Bacteriol. 1982; 149:708-17.

[21] Yakunin AF, Hallenbeck PC. A lumino/iodophenol chemiluminescent detection system for western immunoblots. Anal. Biochem. 1998; 258:146-49.

[22] Laguna R, Tabita FR, Alber BE. Acetate-dependent photoheterotrophic growth and the differential requirement for the Calvin-Benson-Bassham reductive pentose phosphate cycle in *Rhodobacter sphaeroides* and *Rhopseudomas palustris*. Arch. Microbiol. 2011; 193:151-54.

[23] Meister M, Saum S, Alber BE, Fuchs G. L-malyl-coenzyme A/beta methylmalyl-coenzyme A lyase is involved in acetate assimilation of the isocitrate lyase-negative bacterium *Rhodobacter capsulatus*. J. Bacteriol. 2005; 187:1415-25.

[24] Adessi A, McKinlay JB, Harwood CS, Philippis RD. A *Rhodopseudomonas palustris nifA** mutant produces H_2 from NH_4^+-containing vegetable wastes. Int. J. Hydrogen Energy. 2012; 37:15893-00.

[25] Abo-Hashesh M, Desaunay N, Hallenbeck PC. High yield single state conversion of glucose to hydrogen by photofermentation with continuous culture of *Rhodobacter capsulatus* JP91. Bioresour. Technol. 2013; 128:513-17.

[26] Abo-Hashesh M, Ghosh D, Tourigny A, Taous A, Hallenbeck PC. Single-stage photofermentative hydrogen production from glucose: an attractive alternative to two stage photofermentation or co-culture approaches. Int. J. Hydrogen Energy. 2011; 36:13889-95.

[27] Ghosh D, Sobro IF, Hallenbeck PC. Optimization of hydrogen yield from single-stage photofermentation of glucose by *Rhodobacter capsulatus* JP91 using response surface methodology. Bioresour. Technol. 2012; 123:199-06.

[28] Yakunin AF, Laurinavichene TV, Tsygankov AA, Hallenbeck PC. The presence of ADP-Ribosylated Fe Protein of nitrogenase in *Rhodobacter capsulatus* is correlated with cellular nitrogen status. J. Bacteriol. 1999; 181:1994-00.

[29] Monteiro RA, Souza EM, Funayama S, Yates MG, Pedrosa, FO, Chubatsu LS. Expression and functional analysis of an N-truncated NifA protein of *Herbaspirillum seropedicae*. FEMS Microbiol. Lett. 1999; 447:283-86.

[30] Souza EM, Pedrosa FO, Drummond M, Rigo LU, Yates MG. Control of *Herbaspirillum seropedicae* NifA activity by ammonium ions and oxygen. J. Bacteriol. 1999; 181:681-84.

[31] Pawlowski A, Riedel K, Klipp W, Dreiskemper P, Groβ S, Bierhoff H, Drepper T, Masepohl B. Yeast two-hybrid studies on interaction of proteins involved in regulation of nitrogen fixation in the phototrophic bacterium *Rhodobacter capsulatus*. J. Bacteriol. 2003; 185:5240-47.

[32] Colbeau A, Magnin J-P, Cauvin B, Champion T, Vignais PM. Genetic and physical mapping of an hydrogenase gene cluster from *Rhodobacter capsulatus*. Mol. Gen. Genetics. 1990; 220, 393-99.

[33] Strnad H, Lapidus A, Paces J, Ulbrich P, VIced C, Paces V, Haselkorn R. Complete geonome sequence of the purple nonsulfur bacterium *Rhodobacter capsulatus* SB1003. J. Bacteriol. 2010; 192:3545-46.

TABLES

Table 1. Strains used in this study

R. capsulatus Strains	Relevant characteristics	Reference
JP91	*hup*$^-$, control strain	[32]
SB1003	Control strain	[33]
YL2	Pseudo-revertant of the PRK (*cbbP*) mutant	This study
YL3	*nif*^{c}, *cbbP*$^-$	This study
YL4	*nif*^{c}, *cbbP*$^-$	This study

Note: *nif*^{c}, constitutive nitrogenase expression in the presence of ammonia.

Table 2. Growth rates and H_2 production properties with ammonium medium

	Doubling time (h)[a]	H_2 production (ml)[b]	
		10 mM NH_4^+	30 mM NH_4^+
JP91	6.47 ± 1.54	7.25 ± 0.07	0
YL2	NG[c]	NG	NG
YL3	7.87 ± 1.67	13.5 ± 0.07	8.75 ± 1.06
YL4	7.65 ± 1.46	12.5 ± 2.12	7 ± 0.71

[a] The strains were grown with 30 mM malate and 30 mM ammonium as carbon and nitrogen sources, respectively.

[b] H_2 was quantified when the cultures reached stationary phase.

[c] NG: no growth.

Table 3A Identity of mutations in YL3 by comparison of SNP reports of YL3 to YL2

Gene	Mutation	Poly[a] %	P not reference %	Q call (60)	Depth Ave: 116.83	Protein domain[b]	Gene product	Impact
RCAP-RS02860	598_599, 6 bp insertion: AACGCG	32.3	99.9	50.9	137	N terminal or Q-linker	Nitrogenase transcriptional activator, NifA1	Inframe insertion, conservative
RCAP_RS04205	950_951 1bp insertion: T	38.1	100	60	152	Unknown	Malate synthase G	Frame shift, disruptive
RCAP_RS16135	598_599, 6 bp insertion: AACGCG	33.3	99.9	58.36	125	N terminal or Q-linker	Nitrogenase transcriptional activator, NifA2	Inframe insertion, conservative
	105_106, 15 bp insertion: ATCGCGAAGACCTTT	29.4	99.3	26.37	158			

Table 3B Identity of mutations in YL4 by comparison of SNP reports of YL4 to YL2

Gene	Mutation	Substitution	Poly, %	P not ref, %	Q call	Depth Ave: 137.07	Putative protein domain	Gene product	Impact
RCAP-RS02860	SNP, 590 G-A	R197H	55.8	100	60	136	N terminal or Q-linker	Nitrogenase transcriptional activator, NifA1	Non-synonymous
RCAP_RS16135	SNP, 590 G-A	R197H	53.8	100	60	167	N terminal or Q-linker	Nitrogenase transcriptional activator, NifA2	Non-synonymous

[a] Poly-polymorphism

[b] Protein domain designation is taken from Fischer [8]

FIGURES

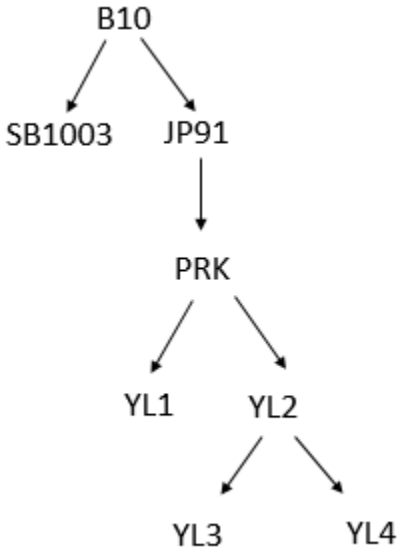


Figure 1. Strains used in this chapter and chapter 3 and their relationships. Both SB1003 and JP91 were derived from wild type B10. PRK mutant was derived from JP91. YL1 and YL2 were derived from PRK, and YL3 and YL4 were derived from YL2.

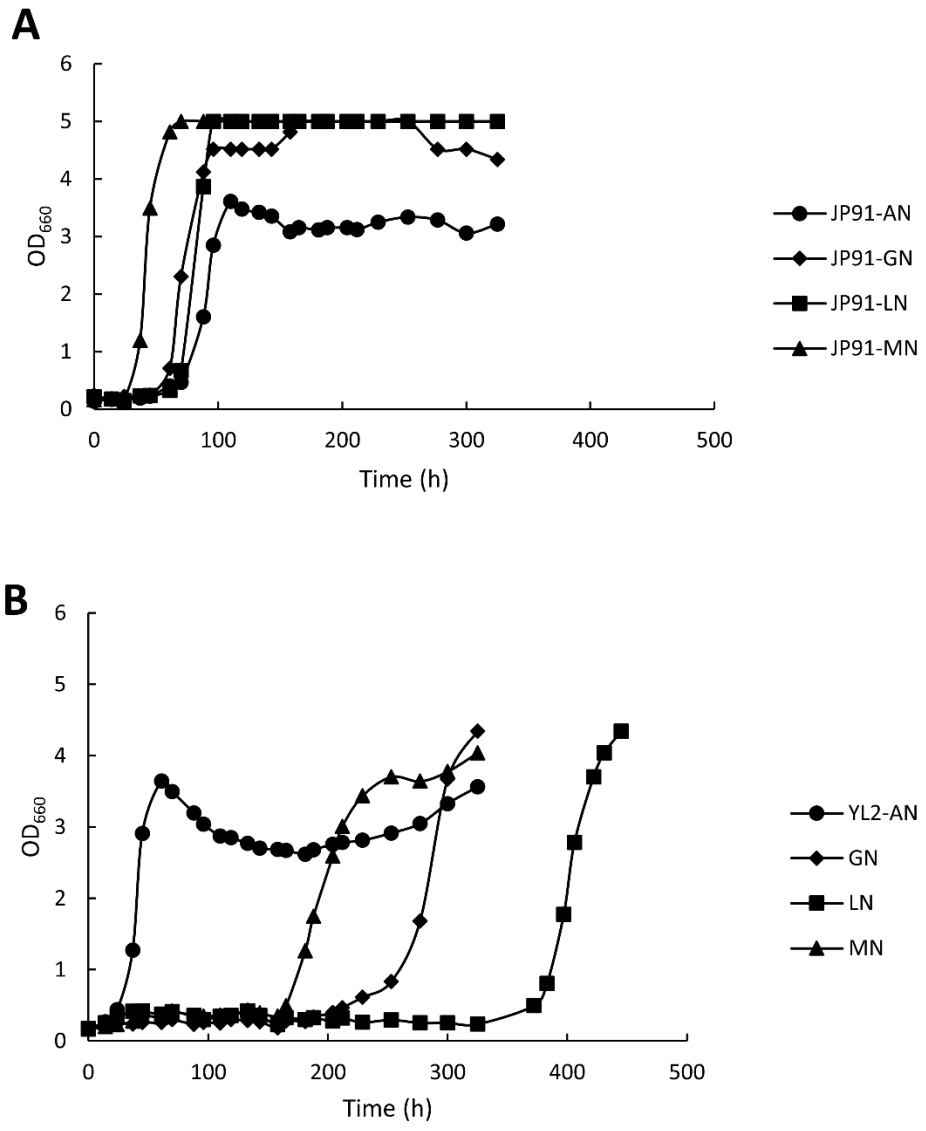


Figure 2. Growth study of JP91 and YL2 in different media. The JP91 **(A)** and YL2 **(B)** were grown in AN (acetate and ammonium), GN (glutamate and ammonium), MN (malalte and ammonium) and LN (lactate and ammonium) at 30 °C and The inoculum of both strains were pre-grown in YPS medium at the same condition for 48 h. The OD660 was monitored every 12 h or 24 h. Note here in Fig. 2B, the growth curves in GN, MN and LN media are representing spontaneous mutants instead of YL2, see text for details.

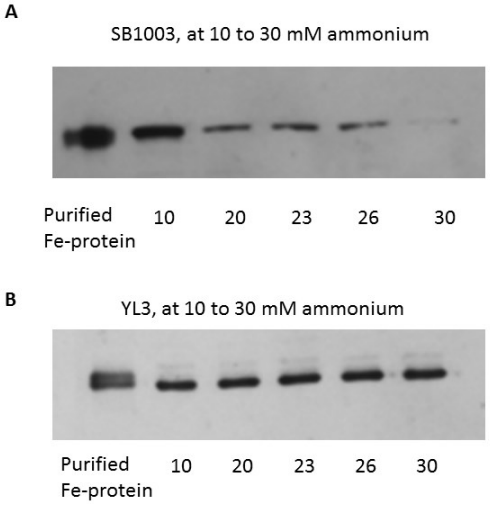


Figure 3. Immunoblotting analysis of Fe-protein expression of nitrogenase. Purified Fe protein was used as control, shown in lane1. Upper panel: cell extracts of *R. capsulatus* SB1003 grown in RCV minimum salts supplemented with 30 mM malate as carbon source, and 10, 20, 23, 26, 30 mM of NH_4^+ as nitrogen source. Lower panel: cell extracts of YL3 grown in above medium. 1 μg of total protein was loaded in each lane.

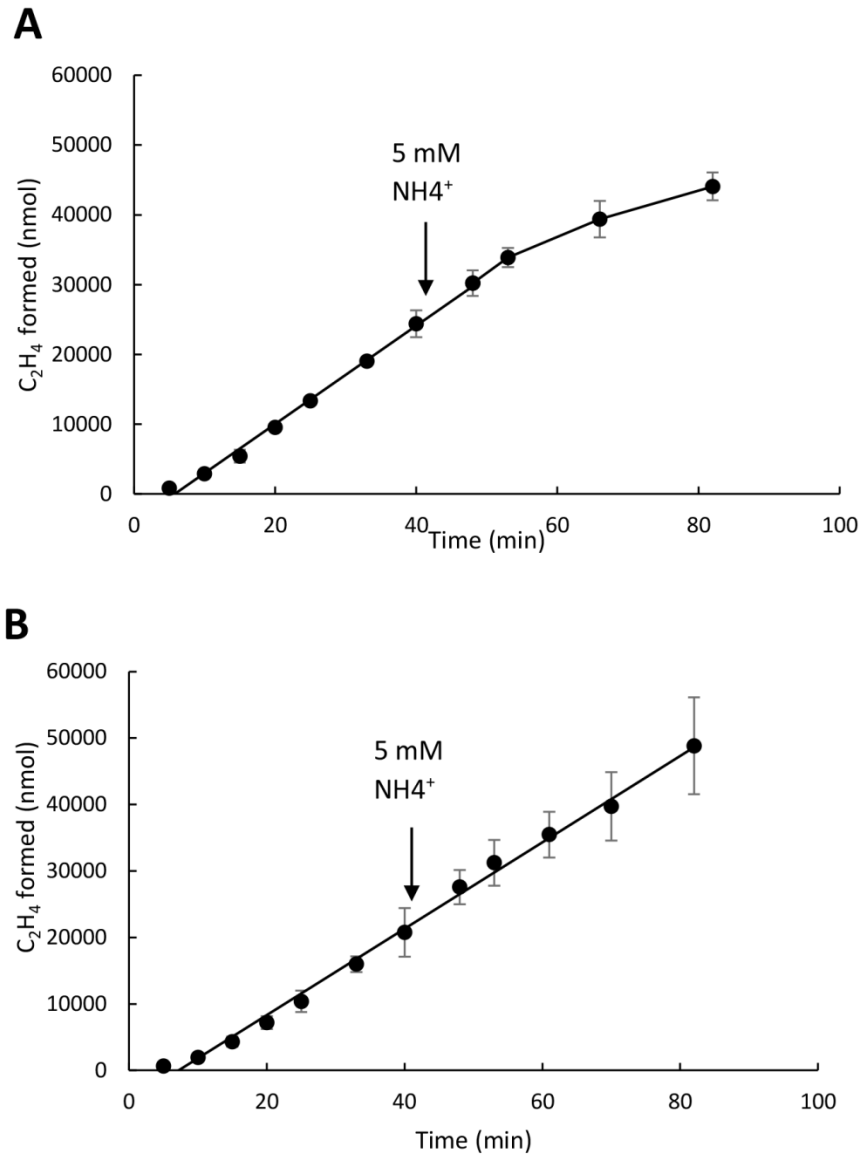


Figure 4. Effect of ammonium shock on nitrogenase activity of SB1003 and YL3. (A) SB1003, (B) YL3. The highly nitrogen limited (HNL) culture were obtained as described in the text. After 44 min of the assay, ammonium was added to each bottle at a final concentration of 5 mM. Each data point shown is the average of two independent biological repeats, with error bars indicating standard deviation.

Chapter 5: Biological reformation of ethanol to hydrogen by *Rhodopseudomonas palustris* CGA009

Yuan Liu, Dipankar Ghosh, Patrick C. Hallenbeck*

Département de Microbiologie, infectiologie, et immunologie, Université de Montréal CP 6128 Succursale Centre-ville, Montréal, Québec H3C 3J7 Canada

Author contribution: The majority of experimental design and wet laboratory experiments were carried out by YL. DG provided technical support on *in vivo* nitrogenase assay and western blot. YL and PCH performed all dataset analysis. All the work was under supervision of PCH.

Article status: This article was published in Bioresource Technology, 2015; 176:189-195.

Abstract

A future hydrogen economy requires the sustainable production of renewable hydrogen. One method to produce hydrogen from stored renewable energy could be through reformation of bioethanol. However, chemically catalyzed reformation processes, although well studied, still present a number of significant technical challenges. Here, bioreformation of ethanol to hydrogen by photofermentation with the photosynthetic bacterium *Rhodopseudomonas palustris*, is described. Cultures were shown to tolerate up to 2% ethanol. A RSM (response surface methodology) analysis was carried out in which three key factors, light intensity, ethanol and glutamate concentrations were varied. The results showed that nearly 2 moles of H_2 could be obtained from one mole of ethanol, 33% of what is theoretically possible.

Keywords: bioreformation of ethanol; renewable hydrogen; photosynthetic bacteria; Response Surface Methodology.

1. Introduction

Hydrogen has been undergoing extensive research and development as a potential future alternative fuel which could help alleviate global energy shortages as well as alleviate environmental problems caused by combustion of fossil fuels (Hallenbeck 2011). Hydrogen is especially attractive since fuel cells using hydrogen are much more energy efficient than combusting conventional or alternative fuels. Currently, most of the world's hydrogen supply, 50 million tons annually, is met by steam reforming of natural gas (Report of the hydrogen production expert panel, 2014).

Thus, if a future hydrogen economy is to be sustainable, a renewable method of producing the required hydrogen needs to be found. A variety of means are available for generating renewable electricity which can be used to make hydrogen through electrolysis. However, most such power sources are intermittent, and hydrogen generated in this way is most suitably used for centralized stationary power. There has been a great deal of interest in reforming bioethanol to hydrogen, since in principle, very little energy need be lost (Deluga *et al*., 2004). Ethanol, presently produced at high yields from corn and cane sugars, and possibly in the future from lignocellulosic materials, would make an excellent energy (hydrogen) carrier, facilitating storage and refueling. However, although a great deal of research has gone into chemical means of reforming, and much progress has been made, there remain significant challenges for chemically catalyzed ethanol reformation (Chen *et al*., 2004; Dominguez *et al*., 2012; Haryanto *et al*., 2005; Mattos *et al*., 2012; Sun and Wang., 2014). These include the possible requirements for noble metal catalysts, catalyst inactivation, high temperature requirements, and the production of problematic side products, such as CO and acetaldehyde.

Therefore, an alternative reformation process operating at ambient temperatures, robust and without special metal requirements, and producing hydrogen without the co-production of toxic compounds would be desirable. Biological systems fulfill many of these requirements since enzymes have exquisite specificity, and living organisms grow at moderate temperatures and are self-replicating. However, the main problem of course is that reformation of ethanol is thermodynamically unfavorable, Eq. (1):

$$C_2H_5OH + 3H_2O \rightarrow 2CO_2 + 6H_2 \ (\Delta G^0 = 97 \text{ kJ/mol}) \qquad (1)$$

In chemical catalysis, the required energy is provided by the relatively high temperatures used, 400-700 °C, but generating the required energy biologically would be very challenging. Some organisms have been found to convert ethanol to acetate and hydrogen, a reaction that is less energy demanding but even so impossible under standard conditions, Eq. (2):

$$C_2H_5OH + H_2O \rightarrow CH_3COOH + 2H_2 \ (\Delta G^0 = 9.5 \text{ kJ/mol}) \qquad (2)$$

The organisms that live by performing this reaction can only do so in the presence of a hydrogen consuming organism which effectively changes the free energy of the reaction to make it favorable. This metabolic relationship between the two organisms is called syntrophy, widely found in anaerobic environments in nature (Hallenbeck, 2012; McInerney *et al*., 2008; Morris *et al*., 2013; Sieber *et al*., 2012). A few organisms are capable of the co-fermentation of ethanol and acetate to hydrogen, driven by the co-production of butyric acid (Hallenbeck, 2012). However, until now, no organism has been shown to be capable of the sustained production of hydrogen from ethanol alone.

Photosynthetic bacteria are well known for their relatively broad substrate utilization in the light, being particularly adept at growing on organic acids (van Neil, 1944). In order to

maintain redox balance when using substrates more reduced than cellular biomass, these organisms carry out CO_2 fixation, or, in the absence of easily metabolized fixed nitrogen, the reduction of protons to hydrogen through the action of the enzyme nitrogenase, a process called photofermentation (Gest and Kamen, 1949, Hallenbeck, 2013). In addition to organic acids, some species are capable of the conversion of molecules such as glycerol (Ghosh *et al*., 2012) and glucose (Abo-Hashesh *et al*., 2013) to hydrogen, achieving high substrate conversion yields of 95% and 75% respectively.

It seemed likely that this process could be applied to the bioreformation of ethanol by photosynthetic bacteria for a number of reasons: (1) many species can grow on ethanol in the light (van Niel, 1944), showing that they can use light energy to drive the assimilation of ethanol, (2) the key enzyme for hydrogen production, nitrogenase, catalyzes an irreversible process which functions even in the presence of 100% hydrogen, and (3) a preliminary study demonstrated hydrogen production from ethanol, although the yield or the duration of this reaction were not examined (Fujii *et al*., 1983). Here the ability to use this process for bioreformation of ethanol was examined and RSM (response surface methodology) was applied in an attempt to optimize hydrogen yields by varying three key factors; light intensity, ethanol concentration and glutamate.

2. Materials and Methods

2.1 Bacterial strain and pre-culture conditions

The purple non-sulfur bacterium *Rps. palustris* CGA009 (*hup*$^-$) was routinely maintained in screw-cap sealed tubes completely filled with RCV glycerol (Sabourin-Provost and Hallenbeck, 2009; Weaver *et al*., 1975), with biotin (15 μg/L) and para-amino benzoic acid (200 μg/L), and incubated at 30 °C in a Biotronette Mark III (Lab-line instruments) environmental chamber equipped with three 150 W incandescent bulbs. Pre-cultures were made by transferring 1 ml of glycerol-grown cells into 17 ml Hungate tubes completely filled with RCV-ethanol, and culturing for about 10 days. The pre-culture was used to inoculate the cultures for hydrogen production.

2.2 Experimental set-up for ethanol bioreformation

Light was provided by two panels of six 50 W halogen bulbs and light intensity was measured with a Delta OHM photo/radiometer (HD 2102.1) and adjusted by a voltage regulator. Ethanol pre-grown cells were inoculated (5% (v/v)) into 120 ml serum bottles filled with 80 ml RCV medium and sparged with oxygen-free argon. Ethanol was injected into bottles with sterile syringes after sparging to prevent losses and the bottles were then incubated at 30 °C in a water bath. Produced gases were introduced into inverted cylinders pre-filled with water using hydrogen impermeable FEP 890 tubing.

2.3 Analytical methods

Hydrogen was measured using a gas chromatograph (Shimadzu GC-8A) equipped with a 1 M column packed with a 5A molecular sieve and a thermal conductivity detector with argon as carrier gas. Ethanol concentrations were determined by an assay using ADH (alcohol dehydrogenase) (Poklis and Mackell, 1982). Nitrogenase activity was determined by the acetylene reduction method (Hallenbeck *et al*., 1982). Protein determination was done using Pierce BCA protein assay kit (Thermo Scientific). Nitrogenase expression levels were determined by Western blotting followed by densitometric analysis using Image J software. Phosphoenolpyruvate carboxylase (PEPC) was assayed using a coupled enzyme system where the oxaloacetate formed was reduced to malate by NADH in the presence of malate dehydrogenase (Bio Basic Canada Inc). The oxidation rate of NADH was quantified spectrophotometrically. The reaction mixture contained crude enzyme preparation, 10 µmol of sodium phosphoenopyruvate, 10 µmol $KHCO_3$, 20 µmol $MgCl_2$, 80 µmol Tris-HCl (pH 9.0) buffer, 14 units of malate dehydrogenase and 0.15 µmol NADH in 1 ml total volume (Sadaie *et al*., 1997).

2.4 Optimization with Response Surface Methodology

A 3^K Box–Behnken model was used to examine the key parameters for improved ethanol conversion and to analyze possible interactive effects of key factors (Box and Behnken, 1960). The combined effects of ethanol and glutamate concentration, ethanol and light intensity, and glutamate and light intensity, on hydrogen yield and nitrogenase activity were investigated. Design Expert 8.0® was used for experimental design, making RSM plots, and statistical

analysis. A total of 17 experimental runs were made, with the central point repeated 5 times (Table 1).

3 Results and Discussion

3.1 Effects of different ethanol concentrations on growth.

Ethanol is an especially electron rich carbon source, making it more reduced than biomass. Thus an appreciable number of electrons might be available for hydrogen production. First, the effects of various concentrations of ethanol, from 25 mM to 1.09 M, as sole carbon source were assessed using RCV medium with 4 mM glutamate as nitrogen source. Bacterial growth curves were similar with ethanol concentrations less than 440 mM (2%, v/v), with an OD_{660} at day 4 of close to 0.25 (Fig. 1). However, increasing the ethanol concentration to 1.09 M (5% v/v) caused significant growth inhibition. This established an initial range that could be used in a DOE (design of experiments approach) to determine the effects of varying different parameters at the same time.

3.2 Optimization of ethanol biological reforming with response surface methodology.

Response surface methodology has been shown to work well to maximize the ability to make hydrogen by optimizing key factors (Hallenbeck and Ghosh, 2009). Here a 3^K Box-Behnken model was chosen to optimize the hydrogen production process with three independent variables; light intensity, ethanol concentration and glutamate concentration. Light is necessary for nitrogenase activity and hence for hydrogen production (Meyer *et al*.,

1978). Glutamate concentration is another key factor since it can support high levels of nitrogenase synthesis without a significant negative effect on activity (Masepohl and Hallenbeck, 2010; Yakunin *et al*., 1999). Ethanol, being the sole carbon substrate and the compound to be converted to hydrogen, is also an obvious key factor. The DOE software was used to set up a series of 17 experimental runs where these three factors were varied at three different levels and hydrogen yield (moles of hydrogen produced per mole of ethanol consumed) was examined as the response (Table 1). ANOVA analysis showed that the response surface for hydrogen yield was well fit by a quadratic model with an F-value of 8.15 (Table 2). The model of F-value of 8.15 implies the model is significant. There is only a 0.57% chance that an F-value this large could occur due to noise. Based on P-value ($P < 0.05$), the software predicted the following terms as significant: [glutamate], light intensity x [ethanol], $(\text{light intensity})^2$, $[\text{ethanol}]^2$ and $[\text{glutamate}]^2$ (Table 2). The ANOVA values show the extent to which the experimental data is statistically significant (Table 2). For a number of reasons, there were appreciable deviations between runs. Replication of the central point gave a rather large standard deviation of ± 38% (see below). However, the strength of the DOE approach used here is that, even with large variability in individual data points, it allows a statistically significant model to be built which can the used in more precise future studies.

In the experimental setup used here, hydrogen production occurred during growth on ethanol as the sole carbon source with final OD600s ranging, depending upon the run, from 0.27 to 0.83. Hydrogen yields were calculated with respect to substrate (ethanol) utilized during the incubation period and residual ethanol, depending upon the run, varied between 10% and 78%.

3.3 Perturbation plot analysis of hydrogen yields

A perturbation plot can be used to compare the effect of all the factors at one point in the chosen design space. Perturbation plots trace out the variations in a response due to changes in each individual factor as it is varied away from the reference point while the

ratios of all the other components are held constant. This way all the possible variables are viewed on a single plot, allowing the visualization of the sensitivity of the response to deviations from the central point. Essentially this allows the direct visualization of how sensitive the system is to deviations from the central point. Thus, trace plots (also called perturbation plots in response surface and factorial designs) help one to compare the effects of all the components in the design space. The steepness in curvature of a given factor is an indication of how sensitive the response is to changes in that factor. Therefore, relatively flat lines can be interpreted as a general lack of significant effect on the response by variation in the factor under examination. Here this analysis was used to examine changes in hydrogen yields as the three factors under study, light intensity (A), ethanol concentration (B) and glutamate concentration (C) were varied from the design central point (Figure 2).

Seen from this perspective, yield was most sensitive to increases in glutamate (i.e. curve C is the steepest), with the response dropping off to 20% of its central point value at the highest glutamate concentration (6.5 mM). On the other hand, yield was least sensitive to a decrease away from the central point in this factor, with a drop off to only 74% of the central value at the lowest value for this factor (0.5 mM). Variations in light intensity (A) and ethanol (B) had less drastic effects with drop offs to 71% and 54%, respectively, of the central point value when these were increased to their highest values (250 W/m^2 and 30 mM, respectively). Likewise, at the lowest values for these factors, 100 W/m^2 and 10 mM, the yield drops to 56%

and 49% of the central point value respectively. Finally, this analysis suggests that the actual yield optimum lies at slightly lower values of glutamate, with a predicted yield of 1.96 mol H_2 / mol ethanol at 190 W/m^2, 20 mM ethanol and 2.45 mM glutamate.

3.4 Response surface for hydrogen yields and factor interactions

Response surfaces were generated to allow pairwise comparison of the effects of variations in the chosen parameters on hydrogen yields. When light intensity and the concentration of ethanol are varied at a medium glutamate concentration (3.5 mM), it can be seen that hydrogen yields are particularly low at low light intensities and high ethanol concentrations (Fig 3A). Hydrogen yield is the highest, nearly 2 mol H_2 / mol ethanol, at intermediate levels of these two factors which appear to have an interactive effect, as seen from the concentric ellipses of the two-dimensional projection.

Varying the concentrations of ethanol and glutamate at an intermediate level of light (175 W/m^2) had a number of interesting effects on hydrogen yields (Fig 3B). Hydrogen yields are greatly suppressed at high glutamate concentrations, more or less independently of the ethanol concentration. Hydrogen yields approaching 2 mol H_2 / mol ethanol were obtained at low to moderate glutamate concentrations and medium ethanol concentrations. Thus at the central point, replicated 5 times, 1.85 ± 0.71 mol of H_2 per mole of ethanol, was obtained. Again, the concentric ellipses of the two-dimensional projection demonstrate interaction between these two factors.

Finally, somewhat similar effects to those noted above for ethanol and glutamate (Fig 3B) are seen when variations in light intensity and glutamate at constant ethanol (20 mM) are

examined. Hydrogen yields at high glutamate concentrations are low irrespective of the light intensity. A clear optimum in hydrogen yield, again approaching 2 mol H_2 / mol ethanol are found at intermediate levels of glutamate and light, with concentric ellipses of the two-dimensional projection showing that these factors as well interact in their effects on the hydrogen yield response.

Of course, in addition to high yields, practical ethanol reformation to hydrogen would also require significant volumetric production rates. As noted previously in many studies of the photofermentative conversion of different substrates to hydrogen by the photosynthetic bacteria, volumetric production rates are very low (Hallenbeck *et al*., 2012; Hallenbeck, 2013). The conversion of ethanol to hydrogen, examined here, is no exception, with average volumetric production of 47 ml H_2/L/day at the central point. Therefore, in order for this to be in the range required for a practical system, volumetric production rates would need to be increased by several orders of magnitude. This is the major challenge for future studies on this process.

3.5 Nitrogenase expression and its relationship to hydrogen yields

Photofermentation by photosynthetic bacteria has been studied for over half a century and the overwhelming consensus is that, regardless of substrate, hydrogen evolution is catalyzed by nitrogenase (Hallenbeck, 2013). Therefore, especially since hydrogen evolution is not observed in the presence of ammonium which represses nitrogenase, it is safe to assume that the enzyme responsible for hydrogen production under the conditions employed here is

nitrogenase, which in the absence of N_2 reduces protons to hydrogen. Thus it was useful to examine the levels of nitrogenase present under the different conditions. The expression levels of the Mo-Fe and Fe components of nitrogenase were analyzed by Western blotting (see Fig. 4) The amounts of nitrogenase proteins, Mo-Fe protein + Fe protein, in general show relatively little variations between the different conditions (data not shown for run 16 and 17), shown here as run number (for levels of the three factors for each run number see Table1). Thus, it would appear that parameters other than the total amount of enzyme present exert major control over nitrogenase activity and consequent hydrogen yields.

3.6 PEPC activity of ethanol grown cells and acetate grown cells

Previously it was reported that *Rhodopseudomonas* sp. No.7 had three fold higher levels of PEPC (phosphoenolpyruvate carboxylase) activity when grown on ethanol as compared to acetate (Sadaie *et al*., 1997). Hence it was of interest to examine the PEPC activity levels of the strain used here while producing hydrogen in comparison to acetate grown cells. Indeed, PEPC activity was found to be two fold higher in ethanol grown cells, 75.4 ± 15.9 nmol/min/mg protein, than in acetate grown cells, 37.4 ± 6.2 nmol/min/mg protein. It is known that acetate is assimilated through the glyoxylate pathway (Laguna *et al*., 2011), but the finding that PEPC activity is higher when the cells are grown on ethanol suggests that ethanol is converted to acetyl-CoA through a two-step oxidation and then enters the citric acid cycle (Fig. 5). Although the need for increased PEPC activity might be related to a requirement to maintain redox balance, a need that seems to drive much of the metabolism in these organisms (Larimer *et al*., 2004; McKinlay and Harwood, 2010), it is more likely related to biosynthetic requirements. Growth on ethanol as sole carbon source requires metabolites

found in the gluconeogenesis pathway, and these are most easily furnished through the formation of phosphoenolpyruvate from oxaloacetate by PEPC since direct synthesis of pyruvate from acetyl-CoA requires reduced ferredoxin and is therefore difficult (Fig. 5). Future work could be aimed at increasing hydrogen yields through metabolic engineering since the present yield, 2 mol H_2/mol of ethanol only represents 33% of the theoretical maximum. This would require detailed knowledge about the metabolic pathways involved.

Conclusion

Here the sustained bioreformation of ethanol by *Rps. palustris* was demonstrated for the first time, and characterized in some detail. Response surface methodology was used to examine the role and possible interaction of three key factors, light intensity, and ethanol and glutamate concentrations, on hydrogen yields. Under optimal conditions, 190 W/m^2, 20 mM ethanol and 2.45 mM glutamate, nearly 2 mol H_2 /mol ethanol were obtained. Little variation in the amount of the key enzyme, nitrogenase, were seen when cells grown under the different conditions were examined by Western blot. This suggests that future work to increase yields should focus on metabolic engineering of the pathways involved.

Acknowledgements

This research was supported by a NSERC Discovery grant to PCH. YL was supported by a scholarship from CSC, China, and DG was supported by a scholarship from PBEEE/FQRNT (Le Fonds Québécois de la recherché sur la nature et les technologies).

References

Abo-Hashesh, M., Desaunay, N., Hallenbeck, P.C., 2013. High yield single stage conversion of glucose to hydrogen by photofermentation with continuous cultures of *Rhodobacter capsulatus* JP91. Bioresour. Technol. 128, 513–517.

Box, G.E.P., Behnken, D.W., 1960. Three level design for the study of quantitative variables. Technometrics. 2, 455–475.

Chen, Z.X., Yan, Y.B., Elnashaie, S.S.E.H., 2004. Catalyst deactivation and engineering control for steam reforming of higher hydrocarbons in a novel membrane reformer. Chem. Eng. Sci. 59, 1965–1978.

Deluga, G.A., Salge, J.R., Schmidt, L.D., Verykios, X.E., 2004. Renewable hydrogen from ethanol by autothermal reforming. Science. 303, 993–997.

Dominguez, M., Taboada, E., Molins, E., Llorca, J., 2012. Ethanol steam reforming at very low temperature over cobalt talc in a membrane reactor. Catal. Today. 193, 101–106.

Fujii, T., Nakazawa, A., Sumi, N., Tani, H., Ando, A., Yabuki, M., 1983. Utilization of alcohols by *Rhodopseudomonas sp*. No. 7 isolated from n-propanol-enrichment cultures. Agric. Biol. Chem. 47, 2747–2753.

Gest, H., Kamen, M.D., 1949. Studies on the metabolism of photosynthetic bacteria; photochemical production of molecular hydrogen by growing cultures of photosynthetic bacteria. J. Bacteriol. 58, 239–245.

Ghosh, D., Sobro, I.F., Hallenbeck, P.C., 2012. Stoichiometric conversion of biodiesel derived crude glycerol to hydrogen: response surface methodology study of the effects of light intensity and crude glycerol and glutamate concentration. Bioresour. Technol. 106, 154–160.

Hallenbeck, P.C., 2011. Microbial paths to renewable hydrogen production. Biofuels. 2, 285–302.

Hallenbeck, P.C., 2012. Fundamentals of dark hydrogen fermentations; multiple pathways and enzymes. In:Azbar,N., Levin, D.B. (Eds.), State of the Artand Progress in the Production of Biohydrogen. Bentham Science Publishers, pp. 94–111.

Hallenbeck, P.C., 2013. Photofermentative biohydrogen production. In: Pandey, A., Chang, J.S., Hallenbeck, P.C., Larroche, C. (Eds.), Biohydrogen. Elsevier, pp. 145–160.

Hallenbeck, P.C., Ghosh, D., 2009. Advances in fermentative biohydrogen production: the way forward? Trends Biotechnol. 27, 287–297.

Hallenbeck, P.C., Meyer, C.M., Vignais, P.M., 1982. Nitrogenase from the photosynthetic bacterium Rhodopseudomonas capsulata: purification and molecular properties. J. Bacteriol. 149, 708–717.

Hallenbeck, P.C., Abo-Hashesh, M., Ghosh, D., 2012. Strategies for improving biological hydrogen production. Bioresour. Technol. 110, 1–9.

Haryanto, A., Fernando, S., Murali, N., Adhikari, S., 2005. Current status of hydrogen production techniques by steam reforming of ethanol: a review. Energy Fuels. 19, 2098–2106.

Laguna, R., Tabita, F.R., Alber, B., 2011. Acetate-dependent photoheterotrophic growth and the differential requirement for the Calvin–Benson–Bassham reductive pentose phosphate cycle in Rhodobacter sphaeroides and *Rhodopseudomonas palustris*. Arch. Microbiol. 193, 151–154.

Larimer, F.W., Chain, P., Hauser, L., Lamerdin, J., Malfatti, S., Do, L., Land, M.L., Pelletier, D.A., Beatty, J.T., Lang, A.S., Tabita, F.R., Gibson, J.L., Hanson, T.E., Bobst, E., Torres,

J.L.T., Peres, C., Harrison, F.H., Gibson, J., Harwood, C.S., 2004. Complete genome sequence of the metabolically versatile photosynthetic bacterium *Rhodopseudomonas palustris*. Nat. Biotechnol. 22, 55–61.

Masepohl, B., Hallenbeck, P.C., 2010. Nitrogen and molybdenum control of nitrogen fixation in the phototrophic bacterium *Rhodobacter capsulatus*. In: Hallenbeck, P.C. (Ed.), Recent Advances in Phototrophic Prokaryotes. Springer, pp. 49–70.

Mattos, L.V., Jacobs, G., Davis, B.H., Noronha, F.B., 2012. Production of hydrogen from ethanol: review of reaction mechanism and catalyst deactivation. Chem. Rev. 112, 4094–4123.

McInerney, M.J., Struchtemeyer, C.G., Sieber, J., Mouttaki, H., Stams, A.J., Schink, B., Rohlin, L., Gunsalus, R.P., 2008. Physiology, ecology, phylogeny, and genomics of microorganisms capable of syntrophic metabolism. Ann. N. Y. Acad. Sci. 1125, 58–72.

McKinlay, J.B., Harwood, C.S., 2010. Carbon dioxide fixation as a central redox cofactor recycling mechanism in bacteria. Proc. Natl. Acad. Sci. U. S. A. 107, 11669–11675.

Meyer, J., Kelley, B.C., Vignais, P.M., 1978. Effect of light nitrogenase function and synthesis in *Rhodopseudomonas capsulata*. J. Bacteriol. 136, 201–208.

Morris, B.E.L., Henneberger, R., Huber, H., Moissl-Eichinger, C., 2013. Microbial syntrophy: interaction for the common good. FEMS Microbiol. Rev. 37, 384–406.

Poklis, A., Mackell, M.A., 1982. Evaluation of a modified alcohol dehydrogenase assay for the determination of ethanol in blood. Clin. Chem. 28, 2125–2127.

Report of the Hydrogen Production Expert Panel, 2014. (<http://www. hydrogen.energy.gov/pdfs/hpep_report_2013.pdf> (accessed 01.10.14).

Sabourin-Provost, G., Hallenbeck, P.C., 2009. High yield conversion of a crude glycerol

fraction from biodiesel production to hydrogen by photofermentation. Bioresour. Technol. 100, 3513–3517.

Sadaie, M., Nagnao, T., Suzuki, T., Shinoyama, H., Fujii, T., 1997. Some properties and physiological roles of phosphoenopyruvate carboxylase in *Rhodopseudomonas sp*. No.7 grown on ethanol under anaerobic-light conditions. Biosci. Biotechnol. Biochem. 61, 625–630.

Sieber, J.R., McInerney, M.J., Gunsalus, R.P., 2012. Genomic insights into syntrophy: the paradigm for anaerobic metabolic cooperation. Annu. Rev. Microbiol. 66, 429–452.

Sun, J., Wang, Y., 2014. Recent advances in catalytic conversion of ethanol to chemicals. Catalysis. 4, 1078–1090.

van Niel, C.B., 1944. The culture, general physiology, morphology, and classification of non-sulfur purple and brown bacteria. Bacteriol. Rev. 8, 1–118.

Weaver, P.F., Wall, J.D., Gest, H., 1975. Characterization of *Rhodopseudomonas capsulata*. Arch. Microbiol. 105, 207–216.

Yakunin, A.F., Laurinavichene, T.V., Tsygankov, A.A., Hallenbeck, P.C., 1999. The presence of ADP-ribosylated Fe protein of nitrogenase in *Rhodobacter capsulatus* is correlated with cellular nitrogen status. J. Bacteriol. 181, 1994–2000.

TABLES

Table 1. Box–Behnken experimental design with three independent variables and one response.

Run No.	Factor 1 A: light intensity (W/m2)	Factor 2 B: ethanol (mM)	Factor 3 C: glutamate (mM)	Response H_2 yield (mol/mol)
1	100	10	3.5	0.58
2	175	20	3.5	1.07
3	250	10	3.5	0.14
4	175	20	3.5	1.5
5	175	20	3.5	1.85
6	175	30	6.5	0.06
7	175	10	6.5	0.02
8	250	20	6.5	0.03
9	250	20	0.5	1.28
10	100	30	3.5	0.2
11	100	20	6.5	0.15
12	175	10	0.5	0.85
13	100	20	0.5	0.41
14	175	20	3.5	1.85
15	250	30	3.5	1.23
16	175	20	3.5	2.99
17	175	30	0.5	0.32

Table 2. ANOVA for Response Surface Quadratic model Response 1, H_2 Yield

ANOVA for Response Surface Quadratic model
Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	3.32	9	0.37	8.15	**0.0057**
A-Light Intensity (W/m2)	0.038	1	0.038	0.84	0.3902
B-Ethanol (mM)	3.519E-003	1	3.519E-003	0.078	0.7885
C-Glutamate (mM)	0.67	1	0.67	14.76	0.0064
AB	0.28	1	0.28	6.08	0.0432
AC	0.12	1	0.12	2.74	0.1416
BC	0.053	1	0.053	1.17	0.3159
A^2	0.32	1	0.32	7.14	0.0319
B^2	0.65	1	0.65	14.26	0.0069
C^2	0.98	1	0.98	21.56	0.0024
Residual	0.32	7	0.045		
Lack of Fit	0.058	3	0.019	0.30	0.8252
Pure Error	0.26	4	0.065		
Cor Total	3.64	16			

The Model F-value of 8.15 implies the model is significant. There is only a 0.57% chance that an F-value this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case C, AB, A^2, B^2, C^2 are significant model terms. The "Lack of Fit F-value" of 0.30 implies the Lack of Fit is not significant relative to the pure error. There is a 82.52% chance that a "Lack of Fit F-value" this large could occur due to noise.

Final Equation in Terms of Actual Factors:

Sqrt(H2 Yield) =
-1.24198
+0.013907 * Light Intensity (W/m^2)
+0.084133 * Ethanol (mM)
+0.33870 * Glutamate (mM)
+3.49751E-004 * Light Intensity (W/m^2) * Ethanol (mM)
-7.83502E-004 * Light Intensity (W/m^2) * Glutamate (mM)
+3.83164E-003 * Ethanol (mM) * Glutamate (mM)
-4.92600E-005 * Light Intensity $(W/m^2)^2$
-3.91632E-003 * Ethanol $(mM)^2$
-0.053510 * Glutamate $(mM)^2$

FIGURES

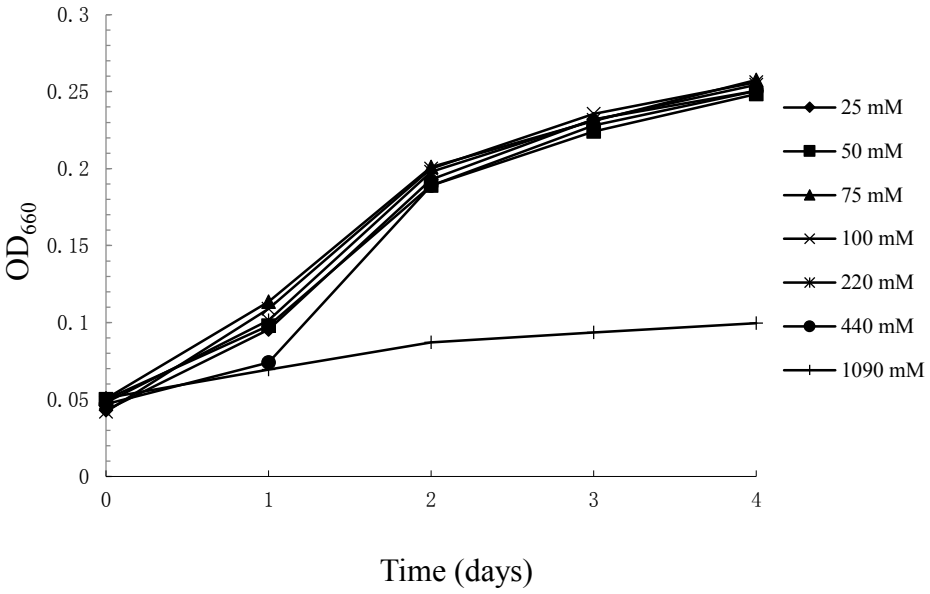


Figure 1. Growth of *Rhodopesudomonas plaustris* CGA009 at different ethanol concentrations. Cultures were incubated in the light in RCV-glutamate with different ethanol concentrations as shown.

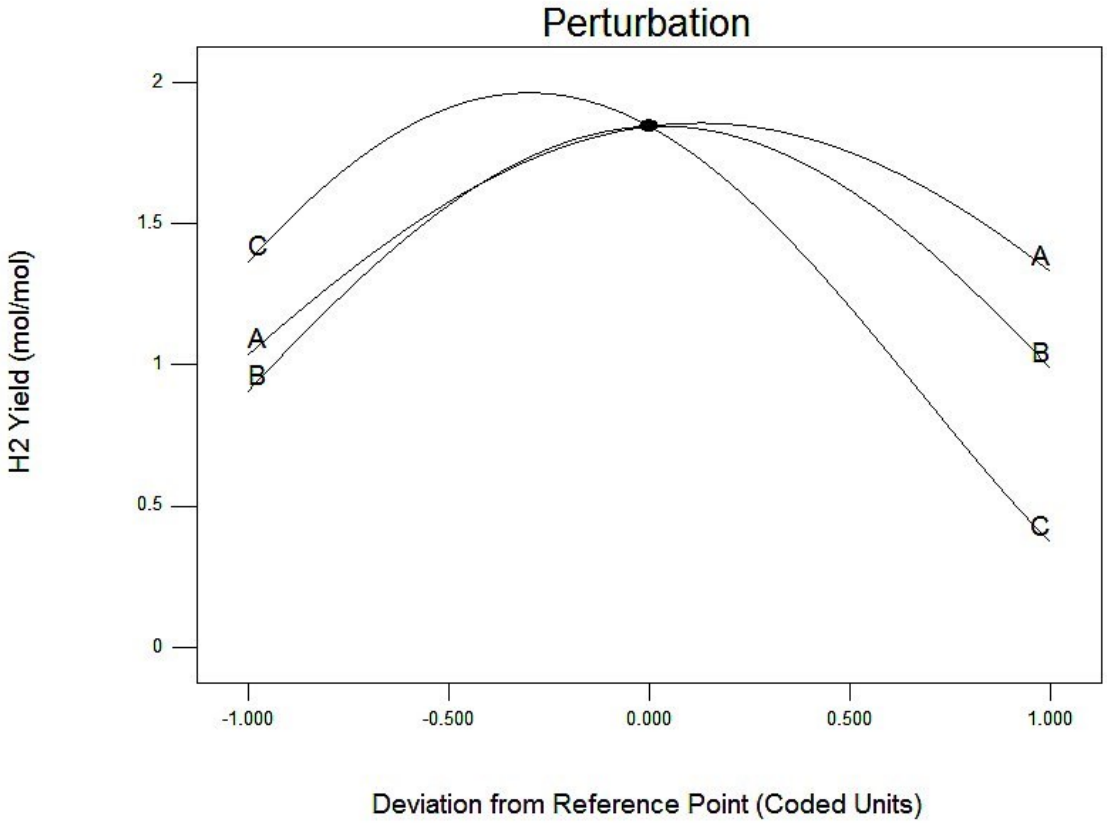


Figure 2. Perturbation plot analysis of the influence of light intensity, ethanol concentration and glutamate concentration on hydrogen yields. DesignExpert 8® was used to generate a perturbation plot centered around the central design point. Traces shown are the predicted response (hydrogen yields) to variation in a single factor when the other factors are held constant. Factors are varied throughout the design range, from lowest to highest value. (A), light intensity; (B), ethanol concentration; (C), glutamate concentration.

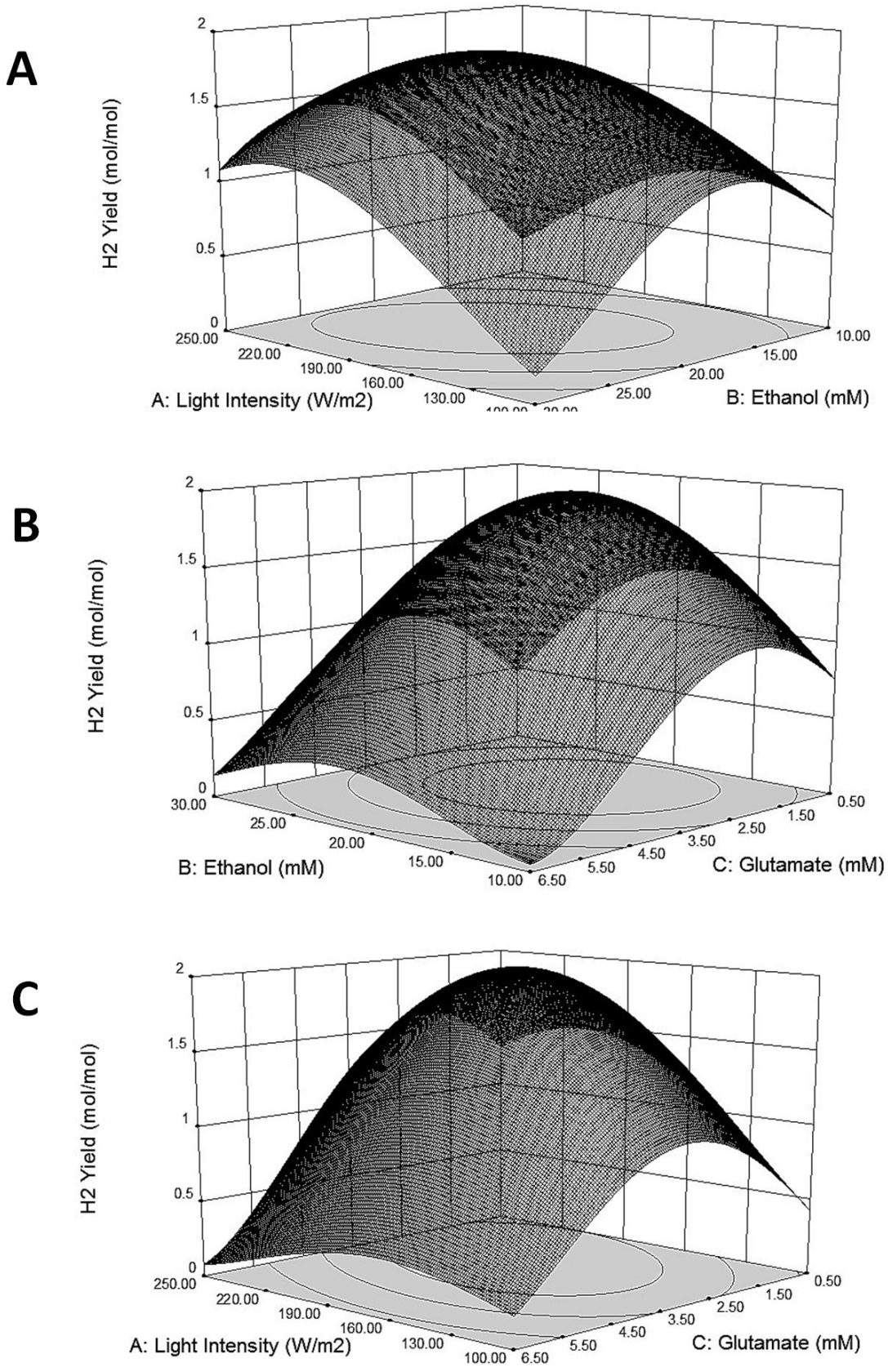

A
H2 Yield (mol/mol)
2
1.5
1
0.5
0
250.00
220.00
190.00
160.00
130.00
A: Light Intensity (W/m2)
10.00
15.00
20.00
25.00
B: Ethanol (mM)
B
H2 Yield (mol/mol)
2
1.5
1
0.5
0
30.00
25.00
20.00
15.00
10.00
B: Ethanol (mM)
0.50
1.50
2.50
3.50
4.50
5.50
6.50
C: Glutamate (mM)
C
H2 Yield (mol/mol)
2
1.5
1
0.5
0
250.00
220.00
190.00
160.00
130.00
100.00
A: Light Intensity (W/m2)
0.50
1.50
2.50
3.50
4.50
5.50
6.50
C: Glutamate (mM)

Figure 3. Response surfaces for hydrogen yields as a function of variations in the different factors. A, The response of the hydrogen yield to different light intensities and [ethanol] at a constant glutamate concentration of 3.5 mM. B. The response of the hydrogen yield to different ethanol and glutamate concentrations at a light intensity of 175 W/m^2. C. The response of the hydrogen yield to different light intensities and [glutamate] at a constant ethanol concentration of 20 mM.

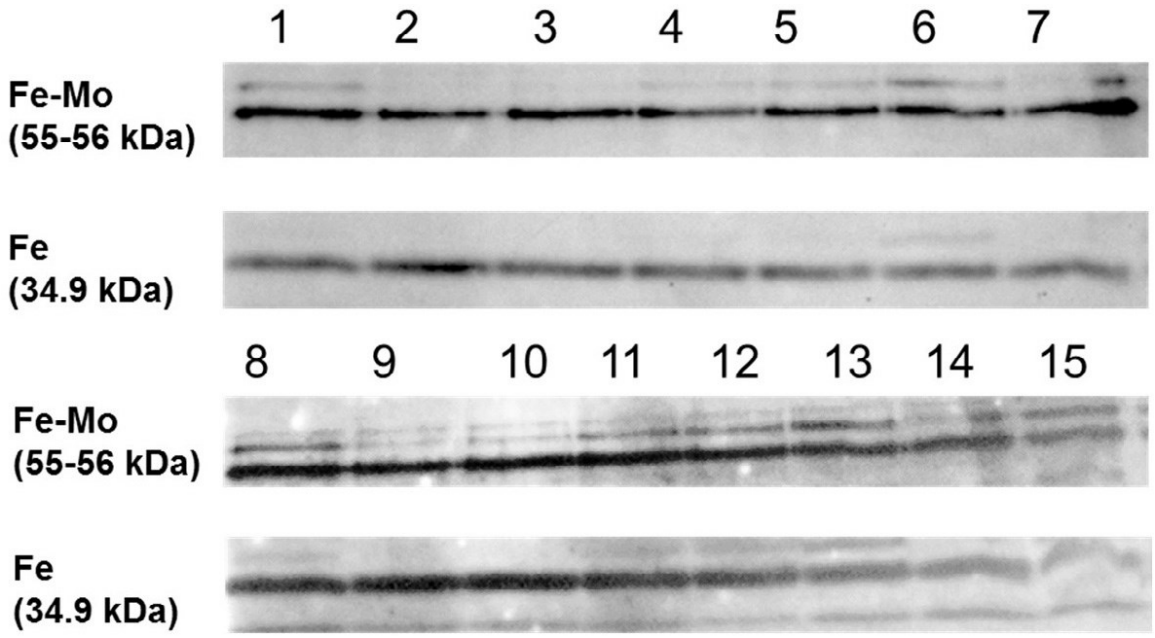


Figure 4. Levels of expression of the nitrogenase proteins under different experimental conditions. The Mo-Fe protein and Fe protein components of nitrogenase were assayed by extraction and Western blot analysis.

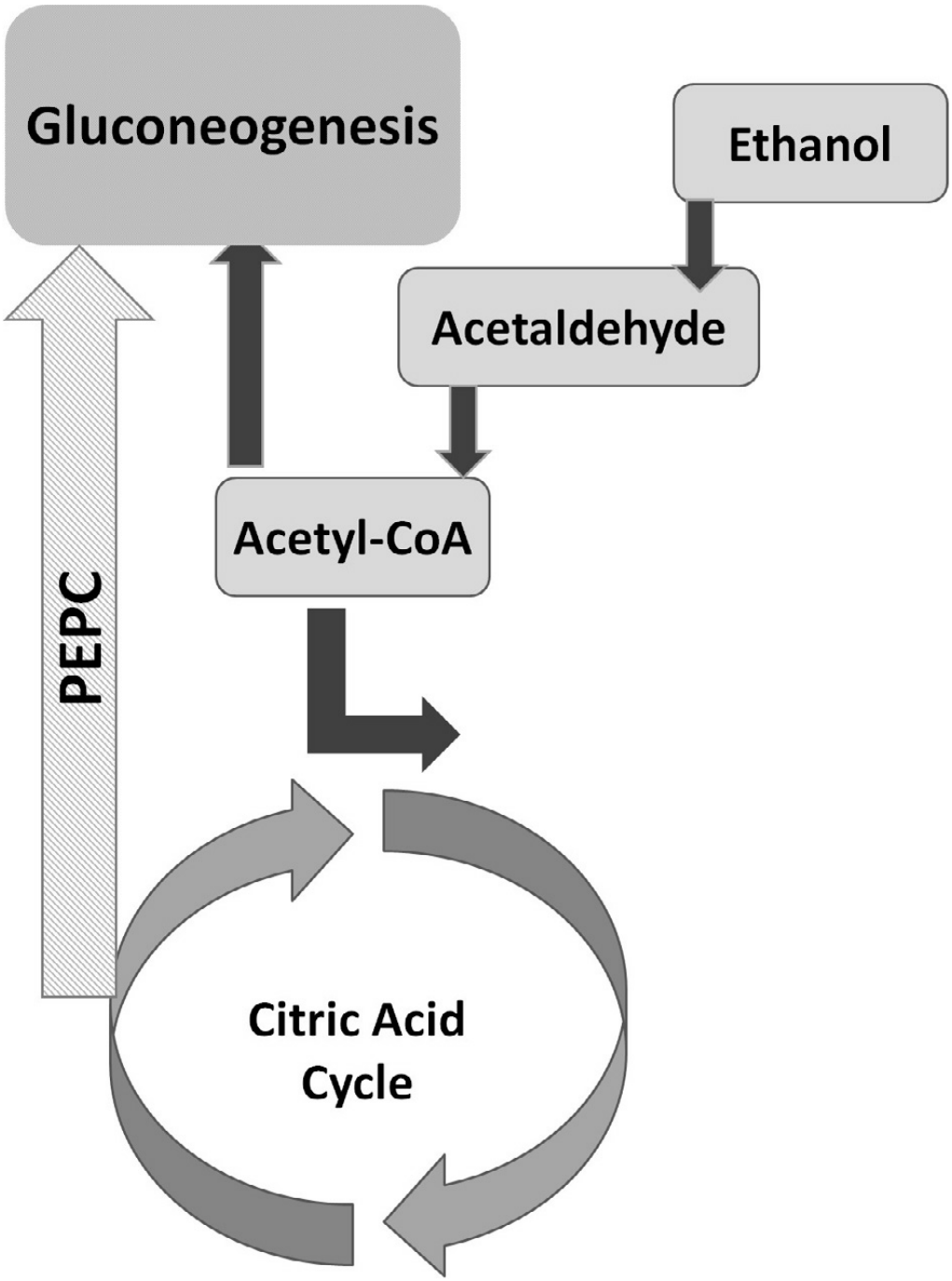


Figure 5. Probable pathways involved in ethanol assimilation by *Rps. palustris*. How ethanol is probably fed into central metabolic pathways during growth and photofermentation of ethanol as sole carbon substrate. In a first step, ethanol would be converted to acetyl-CoA through the action of alcohol dehydrogenase and acetaldehyde dehydrogenase. Acetyl-CoA then is fed primarily into the citric acid cycle with conversion of some oxaloacetate to phosphoenolpyruvate by PEPC (phosphoenolpyruvate carboxylase) to feed gluconeogenique pathways.

DISCUSSION

In this thesis, improvement of H_2 production by purple non-sulfur photosynthetic bacteria through metabolic engineering and DOE (design of experiments) was examined. In addition, two mutants having constitutive nitrogenase expression in the presence of ammonium were characterized in some detail.

1. A Kinetic study of H_2 production by a Calvin-Benson-Bassham cycle mutant, PRK (phosphoribulose kinase), of the photosynthetic bacterium *Rhodobacter capsulatus*

It was first observed by Gest and Kamen that H_2 was produced vigorously by illuminated cultures of the purple non-sulfur photosynthetic bacterium *Rhodospirillum rubrum* when grown with organic acids as carbon source and glutamate or aspartate as nitrogen source [33]. Numerous photofermentative H_2 production studies were carried out since then [68-70, 106, 107], and *Rhodobacter capsulatus* is one of the most intensively studied species for this purpose. This species is particularly well adapted to grow on organic acids [108]. High substrate conversion efficiencies were achieved with several organic acids, such as pyruvate, lactate, succinate and malate. Taking lactate as an example, a H_2 yield as high as 72% can be achieved, however, with glucose as carbon source, the H_2 yield was only 32% [106]. In *R. capsulatus*, the ED pathway is responsible for glucose degradation [109, 110]. Glucose would be metabolized to pyruvic acid which has been demonstrated as an ideal carbon source for H_2 production [106]. Thus, glucose should in theory give a high H_2 yield.

R. capsulatus JP91 (*hup*$^-$, (encoding uptake hydrogenase)), was recently tested for H_2 production using glucose as carbon source in a single stage [111]. An alternative is two-stage conversion where glucose is first metabolized into organic acids in dark fermentation by *E. coli* or *Clostridium*, then the fermentation broth is used to feed photosynthetic bacteria for H_2 production. Their results showed that 3 moles of H_2 could be obtained from 1 mole of

glucose, 25% of theoretical maximum, equivalent to the previous study [106, 111]. A follow up study using response surface methodology further improved the yield to 46% [112]. However, even with optimization, the substrate conversion efficiency is still less than half of the theoretical maximum, suggesting process development is not adequate and that further yield improvement could be achieved through metabolic engineering.

It has been demonstrated that CO_2 fixation with the Calvin cycle serves as the preferred pathway to maintain redox balance during photoheterotrophic growth of the purple non-sulfur photosynthetic bacteria, such as *R. capsulatus*, *Rps. palustris* and *R. sphaeroides* [58]. Even under H_2 production conditions, it was found that a significant portion of reductants was recycled by the Calvin cycle [113]. However, in theory the energy input and reducing power input into the Calvin cycle could otherwise be used for H_2 production through the activity of nitrogenase. Several recent studies examined this hypothesis [114, 115], as shown in chapter 3, Table 4. However, these results are somewhat incomplete and contradictory. For example, in one study, it was shown that the wild type strain achieved substrate conversion of 62.6 ± 2.61, whereas its PRK mutant yielded comparable results (65.7 ± 1.17). In addition, the H_2 production rate of the two strains was almost the same [115]. These results suggest that the Calvin cycle plays a negligible role with the strains and the conditions used in their study. In the present study, a PRK knock-out mutant was created in order to verify this hypothesis. Since in the previous studies less than half the total electrons from the substrate consumed were recovered in the form of H_2 even after RSM optimization [112], it was of interest to determine the electron distribution by quantifying the electron allocation to biomass, H_2, and organic acids. Of course, other metabolites, such as PHB, SMP could also exist in fermentation broth. However, the required techniques to quantify them have so far not been

established. Under low to intermediate light intensities, it was found that the proportion of organic acids was high. This might be due to insufficient energy input, especially for cells deep in culture where little light can penetrate. In fact, at high biomass levels, this portion of the culture might essentially be in the dark. Darkness can induce switch-off of nitrogenase due to modification of nitrogenase reductase [116]. Thus, the nitrogenase synthesized at an early stage could be subsequently modified or degraded, as a result, organic acids were accumulated without being further oxidized. At high illumination levels, the proportion of electrons going into organic acids dropped dramatically, and, biomass levels increased. In addition, analysis showed that only 89% or 94% of electrons was recovered in the form of H_2, biomass or organic acids, suggesting that a minor fraction of other metabolites were co-produced. Thus, although the highest yield reported here is significantly higher than the two previous studies, 7.73 versus 3 or 5.53 H_2 mol/mol glucose [111, 112], additional improvement is still possible.

As mentioned earlier, the PRK mutant failed to grow in glutamate medium which is permissive for nitrogenase expression and growth was only observed in medium containing DMSO, suggesting that growth failure was due to electron imbalance. Similarly, a Calvin cycle mutant of *Rho. rubrum* also grew poorly in glutamate medium. It was once thought that poor growth was due to electron imbalance [114]. However, it was later found that the poor growth was actually due to accumulation of ribulose-1,5-bisphosphate, the product of phosphoribulose kinase (PRK). In addition, a *Rho. rubrum* PRK mutant grew normally with a similar growth rate as the wild type grown under the same conditions [117]. For these reasons, in the present study, a PCK knockout mutant was created instead of a Rubisco knockout mutant. In *R. capsulatus*, there are two Calvin cycle operons, however, there is only one copy of the *cbbP* gene [118]. Thus, elimination of PRK activity should be sufficient to disrupt

Calvin cycle flux. In addition, a PRK mutant of *R. capsulatus* was found to have negligible amounts of Rubisco activity [118]. Thus, it is safe to rule out the possibility that accumulation of ribulose-1,5-bisphosphate resulted in growth failure.

So far, it is not clear why the PRK knockout mutant has the phenotypic growth properties that it shows. Possibly, a deleterious chromosomal rearrangement or other secondary mutation occurred during the construction of the PRK mutant affecting its ability to express the nitrogenase complex. This could be verified by complementation of the PRK mutant with a plasmid containing *cbbP* and a promoter which can be expressed in *R. capsulatus*. It has been reported that PRK mediates nitrogenase induced repression of Calvin cycle genes in *R. sphaeroides* [119], however, it is not known whether a disrupted Calvin cycle in turn can affect nitrogenase expression or not. Thus, it seems that the interaction between the Calvin cycle and the nitrogenase system is very complex. Nevertheless, it might be possible to find mutations that could explain the growth failure by genome sequencing of YL1 and the PRK mutant.

During an examination of the H_2 production rate by different strains, it was found that JP91 was superior under low light intensity. This is probably due to the fact that the bacteriochlorophyll content of YL1 was significantly higher, which could cause stronger self-shading effects. However, both strains had comparable performances at intermediate to high light intensities. A detailed kinetic study gave a number of interesting observations: (1) H_2 production and biomass accumulation are closely linked; (2) The majority of H_2 was produced during the stage of rapid growth, in agreement with a previously described model which predicted that the highest H_2 production rate would be obtained with growing cultures [120]; (3) At day 3 to day 5, the *in vivo* nitrogenase activity of JP91, as determined at saturating

light intensities, was about 1.5-2 fold higher than that of YL1, as well, it produced 3.5 fold higher amounts of H_2. This strongly suggests that the lower H_2 production rate of YL1 was due to a shortage of light energy, caused by higher amounts of bacteriochlorophyll. Under all conditions used in this study, YL1 achieved higher H_2 yields than JP91, suggesting that elimination of Calvin cycle flux can improve H_2 production when nitrogenase system is functional.

2. Characterization of mutants of *Rhodobacter capsulatus* having constitutive nitrogenase expression in the presence of ammonium.

Purple non-sulfur bacteria, such as *R. capsulatus* and *R. sphaeroides*, are well known for their metabolic versatility with at least five growth modes described so far [121], thus the study of redox balance mechanisms under a variety of physiological conditions has been an interesting subject. It is generally accepted that the major role of the Calvin cycle under photoheterotrophic conditions is to maintain redox balance by disposing of excess reductant, since a Rubisco mutant can't grow photoheterotrophically unless an electron acceptor (such as DMSO) is provided, or H_2 production is permitted [58]. However, a recent study found that the poor growth of the Rubisco mutant of *Rho. Rubrum* was due to an accumulation of ribulose-1,5-bisphosphate (RuBP, substrate of Rubisco) rather than a redox imbalance [117]. Indeed, disruption of PRK or enzymes upstream of PRK in Calvin cycle restored normal growth of this Rubisco mutant. Therefore, it was concluded that the Calvin cycle is not essential for *Rho. Rubrum* under photoheterotrophic conditions and its role in controlling redox balance needs to be further elucidated. However, another study noted that the medium previously used was permissive for H_2 production, and validation experiments confirmed that

substantial levels of H_2 were produced during growth of the wild type and several Calvin cycle mutants of *Rho. rubrum* [122]. Thus, it appears that although accumulation of RuBP indeed impeded growth of the Rubsico mutant, the role of Calvin cycle in maintaining redox balance can't be completely ruled out. Interestingly, Gordon *et al*., also found that *Rho. rubrum* and *Rps. palustris* Calvin cycle PRK mutants that couldn't produce RuBP couldn't grow photoheterotrophically on succinate unless an electron acceptor was provided or H_2 production was permitted. Thus, the Calvin cycle is still needed to oxidize electron carriers even in the absence of toxic RuBP [122]. This result reestablished the role of the Calvin cycle as a mean of recycling excess reductant under photoheterotrophic conditions. More interestingly, a Calvin cycle mutant of *Rho. rubrum* was isolated which could grow photoheterotrophically on malate without a need for electron acceptors or H_2 production. The mechanism which permit growth of this mutant under these conditions remains to be elucidated.

In the present study it was initially planned to grow the *R. capsulatus* Calvin cycle mutant YL2 on four different ammonium media containing carbon sources with different oxidation states and to study the individual growth mechanisms. However, the results showed that YL2 can only grow with acetate as carbon source without producing any H_2. It can be suggested that since acetate is metabolized through the ethylmalonyl-CoA pathway, which includes a build-in step of CO_2 reduction, the need for the Calvin cycle is eliminated, as discussed in chapter 3. Two secondary spontaneous mutants, YL3 and YL4 were isolated after prolonged incubation. Isolation of such mutants with suppressor mutation (s) can help to understand how these mutants managed to grow without a functioning Calvin cycle to maintain redox balance.

The growth of both strains with simultaneous H_2 production, confirms that the Calvin cycle is essential for photoheterotrophic growth unless H_2 production is permitted.

The discovery that YL3 and YL4 produced substantial amounts of H_2 reshaped the research plan, since understanding nitrogenase regulation is critical for improving H_2 production with the photosynthetic bacteria. First, nitrogenase expression under different levels of ammonium was examined and it was found that, unlike SB1003 whose nitrogenase expression is under tight control of ammonium, YL3 and YL4 showed constitutive nitrogenase expression. *In vivo* nitrogenase assays also showed that addition of ammonium to 5 mM didn't have any effect on the nitrogenase activity of YL3 and YL4. Of course, this could be speculated from the initial growth study in which 30 mM of ammonium was used to grow these strains. The genomes of YL2, YL3 and YL4 were then sequenced in order to discover the mutations responsible for the observed phenomena. Interestingly, mutations in *nifA1* and *nifA2* were found for both YL3 and YL4, as described in chapter 4. NifA serves as the transcriptional activator of nitrogenase, and its activity is under the control of PII proteins, presumably through protein-protein interaction. Two pieces of evidence back this hypothesis: (1) a *glnK-glnB* double mutant of *R. capsulatus* has constitutive nitrogenase expression [123]; (2) A yeast hybrid study confirmed interactions between these protein pairs: NifA1 and GlnK, NifA1 and GlnB, NifA2 and GlnK, NifA2 and GlnK [101]. When *R. capsulatus* was grown in ammonium containing medium, it was found that a moderate level of NifA was expressed [105].

Thus, a model to explain the constitutive nitrogenase expression observed here could be the following: moderate levels of NifA were expressed in the presence of ammonium, and these NifA variants were locked into active forms due to conformational changes which allow them

to escape regulation by PII proteins. In addition, these amounts of NifA are sufficient to activate nitrogenase expression. However, based on the results obtained so far, it is not yet possible to explain how the synthesized nitrogenase wasn't subsequently modified by the activity of DraT or a DraT-independent mechanism. Many studies have shown that synthesized nitrogenase is instantly switched off by as little as 200 μM of ammonium [86, 88, 89]. In *R. capsulatus*, nitrogenase switch-off is due to DraT activity which covalently modifies dinitrogenase reductase or to a DraT-independent mechanism [88]. The SNP analysis carried out here did not find mutations in DraT or the PII proteins which regulate DraT activity. In a study of how posttranslational modification of nitrogenase is circumvented in *Rps. palustris* strains that produce hydrogen gas constitutively, Heiniger *et al*., found that GlnK2 wasn't well expressed. As a result, insufficient levels of GlnK2 couldn't activate DraT2 activity, which is responsible for the inactivation of nitrogenase [90]. Thus, it would be of interest to examine expression levels of PII in a future study.

The initial H_2 production results with YL3 and YL4 using ammonium media is encouraging. At 10 mM and 30 mM ammonium, both strains produced significantly higher amounts of H_2 than JP91, suggesting that they might be good candidates for a process to recycle various kinds of ammonium-containing wastes, such as kitchen wastes, etc. At present, using organic wastes for H_2 production is mostly studied with dark fermentation [124]. However, dark fermentation is inefficient since electrons from organic wastes can't be effectively extracted to produce H_2. In fact, large quantities of electron-rich compounds are left unused. Two-stage fermentation, which combines dark and photofermentative H_2 production, has also been tested in some studies [125-127]. One major disadvantage of two-stage fermentation is that it would require extensive bioprocess development before reaching a

practical stage. For example, the bioreactor that is used needs to be appropriate for two types of fermentation, namely dark fermentation and photofermentation. In contrast, single-stage photofermentation with ammonium-tolerant strains seems to be more practical and was actually demonstrated in a recent study [128]. Thus, it would be of interest to study H_2 production with YL3 and YL4 using organic wastes as substrate in the future.

3. Biological reformation of ethanol to hydrogen by *Rhodopseudomonas palustris* CGA009

Currently, as mentioned before, most of the world's hydrogen supply is met by steam reforming of natural gas (Report of the hydrogen production expert panel, 2014). This process, although well established, still has a number of drawbacks, including high energy input and emission of toxic gases. Thus, it is necessary to find an environmentally friendly and sustained method for H_2 production. Biological H_2 production with purple non-sulfur photosynthetic bacteria is a promising alternative since they are adapted to a vast range of substrates and can capture sunlight to drive their nitrogenase system to produce H_2. Of the many potential substrates that could be used for biological H_2 production, ethanol is one of the most attractive ones since it has been produced in large quantities with microbial fermentation using renewable resources as substrates. Many species can grow on ethanol with illumination [108], showing that they can use light energy to drive the assimilation of ethanol. A preliminary study has demonstrated hydrogen production from ethanol with *Rhodopseudomonas* sp No.7, however, in this work, neither the yield nor the duration of this reaction were examined [129]. Although a few strains of *R. capsulatus* have been shown to be remarkable H_2 producers in many studies using a range of substrates [106, 107, 111, 112], this

species had been previously characterized as unable to catabolize ethanol in the light [130-132]. Thus, *Rps. palustris* CGA009 was used in this project. However, it should be noted that it was recently found that *R. capsulatus* can actually grow on ethanol, but with a minimum doubling time of 33 h [133]. Such slow growth is probably why earlier researchers concluded that they couldn't grow on ethanol. In addition, this would make it a poor candidate for reformation of ethanol.

Our initial growth study showed that the strain of *Rps. palustris* used here could tolerate ethanol up to 414 mM and that growth was coupled with H_2 production. The doubling time determined at 30 °C, 80 W/m^2 was 24 h when ethanol concentrations were between 25 to 212 mM. Since response surface methodology (RSM), which uses a response function to fit the obtained experimental data to the theoretical design, has been successfully used for photofermentative H_2 production in a few studies [69, 70, 112], it was of interest to examine its robustness in this study. Three parameters, light intensity, glutamate concentration, ethanol concentration, were used as variables and H_2 yield was used as the response. Light is essential for this process, since for one thing, reformation of ethanol to H_2 is thermodynamically difficult [134], thus requiring energy input which needs to be met by photosynthesis; for another, it was found that the synthesis of the nitrogenase system was strongly stimulated by light [116]. Of many nitrogen sources tested, glutamate appears to support maximal nitrogenase expression and activity [90, 114]. However, high levels of glutamate tend to give lower H_2 production, presumably because excess glutamate can be converted to glutamine, which, when at a certain level, triggers regulation of nitrogenase by PII proteins [135].

Thus, there is a need to optimize this process for the best H_2 production performance. Ethanol, being used as carbon source, is a key factor as it determines the N/C ratio which in

turn regulates metabolic flux distribution between H_2 production and biomass accumulation [106]. The values chosen for these three parameters, shown in Table 1 of chapter 5, were based on results of a previous central composite design and a publication from our laboratory [112]. In total, 17 experimental runs were conducted with the central point replicated 5 times. The H_2 yield was quantified under each environmental condition. A mathematical model which considered the effects of single variables as well as interactive effects of pairwise variables was constructed, as shown in chapter 5. ANOVA analysis suggests that this model is adequate and that the lack of fit is non-significant. By keeping one parameter at a constant value, and varying the other two parameters, a number of response surface plots were generated. The optimal H_2 yield was found when glutamate, light and ethanol were at intermediate levels. The highest H_2 yield predicted by this model is close to 2 mol H_2/ mol ethanol, which deviates slightly from our validation data, which is 1.85 mol H_2/ mol ethanol.

The nitrogenase expression on day 10 (final day of the experiment) was also examined with Western blotting in attempts to correlate H_2 production and nitrogenase amount (Mo-Fe protein + Fe-protein). The results show that although H_2 yield varied several fold under different conditions, the amount of nitrogenase showed relatively little variation (less than 1.5-fold). This result suggests these two parameters can't be simply correlated. This is probably due to the fact that H_2 amount is an accumulative parameter, whereas nitrogenase amount and its existing form (modified or unmodified) is dynamic and changes with environmental factors such as cell age and cell density which affect light supply for cells in dense cultures. A better solution would be to monitor daily nitrogenase activity and nitrogenase expression.

The H_2 yield achieved here was 2 mol/mol ethanol, 33% of the theoretical maximum, which is equivalent to chemical reforming process [136]. However, like many studies of photofermentative H_2 production [69, 111, 112], the volumetric production rates achieved here, 2 ml/L culture/h was too low to be practical. This may require the application of a combination of modern techniques, such as metabolic engineering, protein engineering and bioreactor development in the future to achieve sufficient levels of H_2 production rate.

CONCLUSIONS AND PERSPECTIVES

1. A Kinetic study of H_2 production by a Calvin-Benson-Bassham cycle mutant, PRK (phosphoribulose kinase), of the photosynthetic bacterium *Rhodobacter capsulatus*.

In this work, a Calvin cycle mutant of the photosynthetic bacterium *R. capsulatus*, the PRK mutant, was created in order to redirect Calvin cycle flux to the H_2 production pathway. However, my results show that this mutant fails to grow in a typical H_2 production medium in which glutamate is used as nitrogen source, presumably due to intracellular electron imbalance. Nevertheless, I was able to isolate several pseudo-revertants after prolonged incubation of the PRK mutant in those media which didn't support its growth. These pseudo-revertants are gain of function mutants (*nif*$^+$). In addition, the *cbbP* mutation was retained, as confirmed by PCR, thus they are also Calvin cycle mutants. One of them, YL1 was used for a kinetics study of H_2 production. Under low light intensity-120 W/m^2, the H_2 production rate of JP91 was better than YL1. Under intermediate and high light intensities, 175 and 240 W/m^2, both strains had comparable performance. However, in all cases, the H_2 yield of YL1 was improved compared to JP91, by 20-40% depending on different illumination levels. At low to intermediate light intensities, organic acids took a significant portion of total consumed electrons from the substrate used (glucose), and this was especially the case for JP91. At a high intensity level, the proportion of organic acids decreased dramatically, instead, the levels of biomass increased. Therefore, a proper balance between H_2 production, biomass formation and organic acids production needs to be found. Response surface methodology could be used to optimize the whole process in which levels of biomass, organic acids and H_2 are used as responses. This would allow us to get a better idea about how metabolites are distributed under different environmental conditions. Based on an electron allocation analysis, I found in some cases, for example, JP91 at 240 W/m^2, only 89% electrons were recovered, indicating

that other metabolites were produced in relatively significant amounts. These may include PHB and SMP, as reported in similar studies [137, 138]. Thus, it would be of value to quantify these metabolites in future work. In addition, a new bioreactor allowing deeper light penetration needs to be developed. Of course, biomass levels can be maintained at a constant value using a continuous culture method, or perhaps a turbidostat arrangement.

I observed that YL1 had a darker color compared to JP91 under all culture conditions, and analysis showed that the Bchl content of YL1 was 3 times higher than JP91. Since YL1 is a gain of function mutant (*nif*$^+$), it would appear that there is a regulatory link between nitrogenase expression and photosynthesis. Indeed, it was previously reported that HvrA, a protein involved in low light activation of the photosynthetic apparatus, might serve as a regulatory link between nitrogen fixation and photosynthesis [139]. Under nitrogen-fixation conditions, the *hvrA* mutant had significant increase of bacteriochlorophyll a content [140]. However, at this stage it is difficult to compare this report with the results of this thesis. In this regard it would be helpful to sequence the genomes of YL1 and the PRK mutant. Relevant work to this end is currently ongoing in our laboratory.

It is also of interest to figure out why the PRK mutant reported here failed to grow in a medium permitting H_2 production. Although a Rubisco mutant of *Rho. rubrum* had poor growth in the same medium, nevertheless, it did grow [114]. This work could be done by complementation of the PRK mutant with a plasmid which can express the *cbbP* gene in *Rhodobacter capsulatus*.

2. Characterization of mutants of *Rhodobacter capsulatus* having constitutive nitrogenase expression in the presence of ammonium

As mentioned earlier, I isolated another pseudo-revertant, YL2, by prolonged incubation of the PRK mutant in MG medium. I first examined the growth of YL2 in four different media with ammonium, which represses nitrogenase expression, as nitrogen source, and the results showed that only acetate could support the growth of YL2. However, two secondary spontaneous mutants grew out after prolonged incubation. Such mutants are of importance due to two reasons: (1) they can be used for photofermentative H_2 production with various ammonium-containing organic wastes as feedstock; (2) They can be used to study the regulatory mechanism of nitrogen fixation.

Initial H_2 production using synthetic medium with two levels of ammonium as nitrogen source demonstrated that YL3 and YL4 were superior to JP91, previously shown to be an excellent H_2 producer, thus, ammonium containing organic wastes, such as waste water or vegetable wastes could be used for H_2 production in future studies. By SNP analysis, we found a disruptive mutation in malate synthase G of YL3. Since this enzyme is involved in the glyoxylate shunt which competes with TCA cycle, it could have a significant impact on H_2 yield, even on H_2 production rate. When acetate was used as carbon source, it has been reported that non-growing *Rps. palustris* had a higher H_2 yield due to a shift from glyoxlate shunt to tricarboxylic acid cycle [141]. I had compared the *in vivo* nitrogenase activity of YL3 and YL4 grown with malate as carbon source, and initial results showed that YL3 had about 5-fold higher nitrogenase activity. However, I was unable to reproduce this result in following experiments. It is not clear that this was due to mutation lost or other reasons. It would be

helpful to examine H_2 production kinetics and yield of YL3 and YL4 grown with acetate as carbon source, since this may provide some idea of possible differences in central metabolism.

Western blot results showed that the nitrogenase was constitutively expressed under different levels of ammonium in both YL3 and YL4. *In vivo* nitrogenase assay results suggested that the regulation of nitrogenase activity was also altered. In agreement with derepression of nitrogenase, I found mutations in both strains in *nifA*, the master transcriptional activator. Future work should be directed at introducing these *nifA* variants into JP91 and examining nitrogenase expression and activity in the presence of ammonium. At present, it is not clear how the regulation of nitrogenase activity is circumvented at high ammonium concentration, since *draT*, an enzyme which covalently modifies nitrogenase reductase did not seem to be mutated according to the SNP analysis.

3. Biological reformation of ethanol to H_2 with *Rhodopseudomonas palustris* CGA009.

In this work, the sustained bioreformation of ethanol by *Rps. palustris* was demonstrated for the first time, and characterized in some detail. Response surface methodology was used to examine the role and possible interaction of three key factors, light intensity, and ethanol and glutamate concentrations, on hydrogen yields. Under optimal conditions, 190 W/m^2, 20 mM ethanol and 2.45 mM glutamate, it was predicted that up to 2 mol H_2/mol ethanol could be obtained, in good agreement with validation data. Future work could be directed at quantifying other gaseous and liquid metabolites. This is important because, even under optimal condition, only 2 mol H_2/ mol ethanol was achieved, representing 33% of theoretical maximum. This would help us identify the potential competing pathways, thus making further

improvement through metabolic engineering. So far, one can speculate that PHB could be a major sink for electrons, since it is expected that ethanol would be converted to acetate first, and acetate stimulates PHB synthesis, as has been reported in a few photosynthetic species [142-144]. Another issue is that the H_2 production rate reported here was only 2 ml/L culture/h, nearly 10-fold lower than what we have achieved with *R. capsulatus* using glucose as carbon source. Many studies have shown that H_2 production is growth linked and a model also predicted maximum H_2 production would be achieved with growing cultures [120]. The doubling time of *Rps. palustris* grown with ethanol as sole carbon source at 30 °C and 80 W/m^2 of illumination was about 24 h. Compared to ethanol, acetate is a readily usable substrate for H_2 production and it supports a nearly 10-fold higher H_2 production rate under similar conditions [145]. I discussed earlier that ethanol might be fed into central metabolism through the activity of alcohol dehydrogenase and acetaldehyde dehydrogenase, which catalyze ethanol to acetyl-CoA, which in turn can then be fed into the central metabolic pathway. Thus, one can speculate that this initial conversion of ethanol to acetyl-CoA might be the rate-limiting step. Of course, it might be better to compare transcriptomes of *Rps. palustris* grown with ethanol and acetate and find out the differences. In doing so one may get some insights into how each is metabolized and then apply metabolic engineering theory to accelerate ethanol assimilation. In addition, one could select for fast growing photosynthetic species capable of H_2 production using ethanol as carbon source. Compared to *Rps. palustris* CGA009 used here, *Rsp.* sp. No.7 had about 7-fold higher H_2 production rate [129], therefore, it should be possible to get a strain which grows even faster under these conditions.

BIBLIOGRAPHY

[1] Nichols, N. N., Monceaux, D. A., Dien, B. S., Bothast, R. J., 2008. Production of ethanol from corn and sugarcane, in: Wall, J. D., Harwood, C. S., Demain, A. (Eds), Bioenergy, ASM Press, pp 3-13.

[2] Kim, T. H., Kim, T. H., 2014. Overview of technical barriers and implementation of cellulosic ethanol in the U.S. Energy, 66:13-19.

[3] Ingledew, W. M., 1999. Alcohol production by *Saccharomyces cerevisiae*: a yeast primer, in: Jacques, K. A., Lyons, T. P., Kelsall, D. R. (Eds), The Alcohol Text Book, 3rd ed. Nottingham University Press, Nottingham, United Kindom, pp27-50.

[4] Lee, H., Biely, P., Latta, R. K., Barbosa, M. F. S., Schneider, H., 1986. Utilization of xylan by yeasts and its conversion to ethanol by *Pichia stipites* strains. Appl. Environ. Microbiol, 52:320-324.

[5] Jeffries, T. W., Shi, N. Q., 1999. Genetic engineering for improved xylose fermentation by yeasts. Adv. Biochem. Eng. Biot, 65:117-161.

[6] Jeffries, T. W., Jin, Y. S., 2004. Metabolic engineering for improved fermentation of pentoses by yeasts. Appl. Micriobiol. Biotechnol, 63:495-509.

[7] Lutke-Eversloh, T., Bahl, H., 2011. Metabolic engineering of *Clostridium acetobutylicum*: recent advances to improve butanol production. Curr. Opin. Biotechnol, 22:634-647.

[8] Ponnusamy, S., Reddy, H. K., Muppaneni, T., Downes, C. M., Deng, S., 2014. Life cycle assessment of biodiesel production from algal bio-crude oils extracted under subcritical water conditions. Bioresour. Technol, 170:454-461.

[9] Moody, J. W., McGinty, C. M., Quinn, J. C., 2014. Global evaluation of biofuel potential from microalgae. Proc. Natl. Acad. of Sci. U.S.A, 111:8691-8696.

[10] Resurreccion, E. P., Colosi, L. M., White, M. A., Clarens, A. F., 2012. Comparison of algae cultivation methods for bioenergy production using a combined life cycle assessment and life cycle costing approach. Bioresour. Technol, 126: 298-306.

[11] Quinn, J. C., Yates, T., Douglas, N., Weyer, K., Butler, J., Bradley, T. H., Lammers, P. J. 2012. Nannochloropsis production metrics in a scalable outdoor photobioreactor for commercial applications. Bioresour. Technol, 117:164-171.

[12] Schenk, P. M., Thomas-Hall, S. R., Stephens, E., Marx, U. C., Stephens, E., Marx, U. C., Mussgnug, J. H., Posten, C., Kruse, O., Hankamer, B., 2008. Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production. Bioenerg. Res, 1: 20-43.

[13] Hallenbeck, P. C., Ghosh, D., 2012. Improvements in fermentative biological hydrogen production through metabolic engineering. J. Environ. Manage, 95: S360-S364.

[14] Bisaillon. A., Turcot, J., Hallenbeck, P. C., 2006. The effect of nutrient limitation on hydrogen production by batch cultures of *Escherichia coli.* Int. J. Hydrogen Energy, 31: 1504-1508.

[15] Yoshida, A., Nishimura, T., Kawaguchi, H., Inui, M., Yukawa, H., 2006. Enhanced hydrogen production from glucose using *ldh*- and *frd*-inactivated *Escherichia coli* strains. Appl. Microbiol. Biotechnol, 73:67-72.

[16] Turcot, J., Bisaillon, A., Hallenbeck, P. C., 2008. Hydrogen production by continuous cultures of *Escherichia coli* under different nutrient regimes. Int. J. Hydrogen Energy, 33:1465-1470.

[17] Maeda, T., Sanchez-Torres, V., Wood, T. K., 2007. Enhanced hydrogen production from glucose by metabolically engineered *Escherichia coli.* Appl. Microbiol. Biotech, 77:879-890.

[18] Belaich, A., Belaich, J. P., 1976. Microcalorimetric study of the anaerobic growth of *Escherichia coli*: growth thermograms in a synthetic medium. J. Bacteriol, 125:14-18.

[19] Clark, D. P., 1989. The fermentation pathways of *Escherichia coli.* FEMS. Microbiol. Rev, 63:223-234.

[20] Alam, K.Y., Clark, D. P., 1989. Anaerobic fermentation balance of *Escherichia coli* as observed by *in vivo* nuclear magnetic resonance spectroscopy. J. Bacteriol, 63:6213-6217.

[21] Akhtar,M. K., Jones, P. R., 2008. Engineering of synthetic *hydE-hydF-hydG-hydA* operon for biohydrogen production. Analyt. Biochem, 373:170-172.

[22] Veit, A., Kalim Akhtar, M., Mizutani, T., Jones, P. R., 2008. Constructing and testing the thermodynamic limits of synthetic NAD[P]H: H_2 pathways. Microb. Biotechnol, 1:382-394.

[23] Ghosh, D., Bisaillon, A., Hallenbeck, P. C., 2013. Increasing the metabolic capacity of *Escherichia coli* for hydrogen production through heterologous expression of the *Ralstonia eutropha* SH operon. Biotechnol. Biofuels, 6:122.

[24] Akhtar, M. K., Jones, P. R., 2009. Construction of a synthetic YdbK-dependent pyruvate: H_2 pathway in *Escherichia col*i BL21 (DE3). Metab. Eng, 11:139-147.

[25] Tracy, B. P., Jones, S. W., Fast, A. G., Indurthi, D. C., Papoutsakis, E. T., 2012. Clostridia: the importance of their exceptional substrate and metabolite diversity for biofuel and biorefinery applications. Curr. Opin. Biotech, 23:364-381.

[26] Hallenbeck, P. C., Ghosh, D., 2009. Advances in fermentative biohydrogen production: the way forward? Trends. Biotechnol, 27: 287-297.

[27] Hallenbeck, P. C., 2009. Fermentative hydrogen production: principles, progress, and prognosis. Int. J. Hydrogen Energy, 34:7379-7389.

[28] Klein, M., Ansorge-Schumacher, M. B., Fritsch, M., Hartmeier, W., 2010. Influence of hydrogenase overexpression on hydrogen production of *Clostridium acetobutylicum* DSM 792. Enzyme. Microb. Tech, 46:384-390.

[29] Jo, J. H., Jeon, C. O., Lee, S. Y., Lee, D. S., Park, J. M., 2010. Molecular characterization and homologous overexpression of FeFe-hydrogenase in *Clostridium tyrobutyricum* JM1. Int. J. Hydrogen Energy, 35:1065-1073.

[30] Cai, G., Jin, B., Monis, P., Saint, C., 2013. A genetic and metabolic approach to redirection of biochemical pathways of *Clostridium butyricum* for enhancing hydrogen production. Biotechnol. Bioeng, 110:338-342.

[31] Wang, R. F., Zong, W. M., Qian, C. L., Wei, Y. J. Yu, R., Zhou, Z., 2011. Isolation of *Clostridium perfringens* strain W11 and optimization of its hydrogen production by genetic modification. Int. J. Hydrogen Energy, 36:12159-12167.

[32] Yu, R. S., Wang, R. F., Bi, T., Sun, W., Zhou, Z., 2013. Blocking the butyrate formation pathway impairs hydrogen production by *Clostridium perfringens*. Acta. Biochim. Biophys. Sin, 45:408-415.

[33] Gest, H., Kamen, M. D., 1949. Photoproduction of molecular hydrogen by *Rhodospirillum rubrum.* Science, 109:558-559.

[34] Gest, H., Kamen, M. D., Bregoff, H. M., 1950. Studies on the metabolism of photosynthetic bacteria. 5. photoproduction of hydrogen and nitrogen fixation by *Rhodospirillum rubrum*. J. Biol. Chem, 182:153-170.

[35] Kamen, M. D., Gest, H., 1949. Evidence for a nitrogenase system in the photosynthetic bacterium *Rhodospirillum rubrum*. Science, 109:560.

[36] Bulen, W. A., LeComte, J. R., 1966. The nitrogenase system from Azotobacter: two enzyme requirement for N_2 reduction, ATP dependent H_2 evolution, and ATP hydrolysis. Proc. Natl. Acad. Sci. U. S. A, 56:979-986.

[37] Nordlund. S., Eriksson. U., Baltscheffsky. H., 1978. Properties of the nitrogenase system from a photosynthetic bacterium, *Rhodospirillum rubrum*. Biochim. Biophys. Acta, 504:248-254.

[38] Ludden, P. W., Burris, R. H., 1978. Purification and properties of nitrogenase from *Rhodospirillum rubrum*, and evidence for phosphate, ribose and an adenine-like unit covalently bound to the iron protein. Biochem J, 175:251-259.

[39] Burris, R. H., 1991. Nitrogenases. J. Biol. Chem, 266:9339-9342.

[40] Dilworth, M. J., 1966. Acetylene reduction by nitrogen-fixing preparations from *Clostridium pasleurianum*. Biochim. Biophys. Acta, 127:285-294.

[41] Simpson, F. B., Burris, R. H., 1984. A nitrogen pressure of 50 atmospheres does not prevent evolution of hydrogen by nitrogenase. Science, 224:1095-1097.

[42] Hallenbeck, P. C., Benemann, J. R., 2002. Biological hydrogen production; fundamentals and limiting processes. Int. J. Hydrogen Energy, 27:1185-1193.

[43] Voet, D., Voet J. G., 2004. Rates of enzymatic reactions. In: Donald Voet and Judith G. Voet, (Ed.) Wiley Jhon Wiley & Sons, Inc. pp. 490-491.

[44] Burns, R. C., Hardy, R. W. F., 1975. Nitrogen fixation in bacteria and higher plants, Springer-Verlag, Berlin, pp 1-189.

[45] Paul, F., Colbeau, A., Vignais, P. M., 1979. Phosphorylation coupled to the oxidation by chromatophores from *Rhodopseudomonas capsulate*. FEMS. Microbiol. Lett, 106:29-33.

[46] Ludden, P. W., Burris, R. H., Siefert, E., in: Proceedings of the Second International Symsposium on Photosynthetic Prokaryotes, Codd, G. A., and Stewart, W. D. P (Ed.) (Federation of European Microbiological Societies, Dundee, Scotland, 1976), pp. 149-151.

[47] Madigan, M. T., Wall, J. D., Gest, H., 1979. Dark anaerobic dinitrogen fixation by a photosynthetic microorganism. Science, 204:1429-1430.

[48] Keister, D. L., Yike, N. J., 1966. Studies on an energy-linked pyridine nucleotide transhydrogenase in photosynthetic bacteria. Biochem. Biophys. Res. Commun, 24:519-525.

[49] Fukuyama, K., Okada, T., Kakuta, Y., Takahashi, Y., 2002. Atomic resolution structures of oxidized [4Fe-4S] ferredoxin from *Bacillus thermoproteolyticus* in two crystal forms: systematic distortion of [4Fe-4S] cluster in the protein. J. Mol. Biol, 315:1155-1166.

[50] Hallenbeck, P. C., Jouanneau, Y., Vignais, P. M., 1982. Purification and molecular properties of a soluble ferredoxin from *Rhodopseudomonas capsulate*. Biochim. Biophys. Acta, 681:168-176.

[51] Hallenbeck, P. C., 1991. *Rhodobacter capsulatus* nitrogenase reduction by natural *in vivo* electron carriers: reactivity with FdI reduced by chloroplasts. Biochim. Biophys. Acta, 1057:97-101.

[52] Hallenbeck P. C., Gennaro, G, 1998. Stopped-flow kinetic studies of low potential electron carriers of the photosynthetic bacterium: ferredoxin I and NifF. Biochim. Biophys. Acta, 1365:435-442.

[53] Yoch, D. C. and Arnon, D. I., 1975, Comparison of two ferredoxins from *Rhodospirillum rubrum* as electron carriers for the native nitrogenase. J. Bacteriol, 121:743-745.

[54] Tomas, E., Stefan, N., 2005. Electron transport to nitrogenase in *Rhodospirillum rubrum*: Identification of a new *fdxN* gene encoding the primary electron donor to nitrogenase. FEMS. Microbiol. Lett, 245:345-351.

[55] Schmehl, M., Jahn, A., Meyer zu, V. A., Hennecke, S., Masepohl, B., Schuppler, M., Marxer, M., Oelze, J., Klipp, W., 1993. Identification of a new class of nitrogen fixation genes in *Rhodobacter capsulatus*: a putative membrane complex involved in electron transport to nitrogenase. Mol. Gen. Genet, 241:602-615.

[56] Ho-Sang, J., Yves, J., 2000. Enhanced nitrogenase activity in strains of *Rhodobacter capsulatus* that overexpress the *rnf* genes. J. Bacteriol, 182:1208-1214.

[57] Tichi, M. A., Tabita, F. R., 2001. Interactive control of *Rhodobacter capsulatus* redox-balancing systems during phototrophic metabolism. J. Bacteriol, 183:6344-6354.

[58] Tichi, M. A., Tabita, F. R., 2000. Maintenance and control of redox poise in *Rhodobacter capsulatus* strains deficient in the Calvin-Benson-Bassham pathway. Arch. Microbiol, 174:322-333.

[59] Vichivanives, P., Bird, T. H., Bauer, C. E., Tabita, F. R., 2000. Mutiple regulators and their interactions *in vivo* and *in vitro* with the *cbb* regulons of *Rhodobacter capsulatus*. J. Mol. Biol, 300:1079-1099.

[60] Kusian, B., Bowien, B., 1997. Organization and regulation of *cbb* CO_2 assimilation genes in autotrophic bacteria. FEMS. Microbiol. Lett, 21:135-155.

[61] Benemann, J., 1996. Hydrogen biotechnology: Progress and prospects. Nat. Biotechnol. 14:1101-1103.

[62] Hallenbeck, P. C., Abo-Hashesh, M., Ghosh, D., 2012. Strategies for improving biological hydrogen production. Bioresour. Technol, 110:1-9.

[63] Melis, A., Zhang, L. P., Forestier, M., Ghirardi, M. L., Seibert, M., 2000. Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*. Plant. Physiol, 122:127-135.

[64] Seibert, M., King, P., Zhang, L. P., Mets, L. Ghirardi, M., Molecular engineering of algal H_2 production. Proceedings of the 2002 U.S. DOE Hydrogen Program Review, NREL/CP-610-32405.

[65] Buhrke, T., Lenz, O., Krauss, N., Friedrich, B., 2005. Oxygen tolerance of the H_2 sensing NiFe hydrogenase from *Ralstonia eutropha* H16 is based on limited access of oxygen to the active site. J. Biol. Chem. 280:23791-23796.

[66] Abbas, A. H., Benedicte, B., Oscar, G., Pierre-Pol, L., 2013. O_2-independent formation of the inactive states of NiFe hydrogenase. Nat. Chem. Biol, 9:15-17.

[67] Wang, J., Wan, W., 2009. Experimental design methods for fermentative hydrogen production: A review. Int. J. Hydrogen Energy, 34:235-244.

[68] Sabourin-Provost, G., Hallenbeck, P. C., 2009. High yield conversion of a crude glycerol fraction from biodiesel production to hydrogen by photofermentation. Bioresour. Technol, 100:3513-3517.

[69] Ghosh, D., Sobro, I. F., Hallenbeck, P. C., 2012. Stoichiometric conversion of biodiesel derived crude glycerol to hydrogen: response surface methodology study of the effects of light intensity and crude glycerol and glutamate concentration. Bioresour. Technol, 106:154-160.

[70] Ghosh, D., Sobro, I. F., Hallenbeck, P. C., 2010. Response surface methodology for process parameter optimization of hydrogen yield by the metabolically engineered strain *Escherichia coli* DJT135. Bioresour. Technol, 101:1820-1825.

[71] Nordlund, S., Metabolic regulation of nitrogen fixation in annoxygenic phototrophic bacteria. In: Nitrogen Fixation (Proc, 5th Int Symp on Nitrogen Fixation with Non-legumes), Polsinelli, M., Materassi, R., Vincenzini, M., (Ed.) 1991, Kluwez Acad Public, Dordrecht/Boston/London, pp. 491-502.

[72] Ludden, P. W., Burris, R. H., 1979. Removal of an adenine-like molecule during activation of dinitrogenase reductase from *Rhodospirillum rubrum*. Proc. Natl. Acad. Sci. U. S. A, 76:6201-6205.

[73] Ludden, P. W., Burris, R. H., 1978. Purification and properties of nitrogenase from *Rhodospirillum rubrum*, and evidence for phosphate, ribose, and adenine-like unit covalently bound to the iron protein. Biochem. J, 175:251-259.

[74] Dowling, T. E., Preston, G. G., Ludden, P. W., 1982. Heat activation of the Fe-protein of nitrogenase from *Rhodospirillum rubrum*. J. Biol. Chem, 257:13987-13992.

[75] Sweet, W. J., Burris, R. H., 1982. Effects of *in vivo* treatment of the activity of nitrogenase isolated from *Rhodospirillum rubrum*. Biochim. Biophys. Acta, 680:17-21.

[76] Saari, L. L., Triplett, E. W.,and Ludden, P. W., 1984. Purification and properties of the activating enzyme for iron protein of nitrogenase from photosynthetic bacterium *Rhodospirillum rubrum*. J. Biol. Chem, 259:15502-15508.

[77] Lowery, R. G., and Ludden, P. W., 1988. Purification and properties of dinitrogenase reductase ADP-ribosyltransferase from photosynthetic bacterium *Rhodospirillum rubrum*. J. Biol. Chem, 263:16714-16719

[78] Guth, J. H., Burris, R. H., 1983. Comparative study of the active and inactive forms of dinitrogenase reductase from *Rhodospirillum rubrum*. Biochim. Biophys. Acta, 749:91-100.

[79] Lowery, R. G., Chang, C. L., Davis, L. C., Mckenna, M., Stephens, P. J., Ludden, P. W., 1989. Substitution of histidine for arginine-101 of dinitrogenase reductase disrupts electron transfer to dinitrogenase. Biochemistry, 28:1206-1212

[80] Pope, M. R., Saari, L. L., Ludden, P. W., 1986. N-Glycohydrolysis of adenosine diphosphoribosyl arginine linkages by dinitrogenase reductase activating glycohydrolase from *Rhodospirillum rubrum*. J. Biol. Chem, 261:10104-10111

[81] Fitzmaurice, W. P., Saari, L. L., Lowery, R. G., Ludden, P. W., Roberts, G. P., 1989. Genes coding for the reversible ADP-ribosylation system of dinitrogenase reductase from *Rhodospirillum rubrum*. Mol. Gen. Genet, 218:340-347.

[82] Liang, J., Nielsen, G. M., Lies, D. P., Burris, R. H., Roberts, G. P., Ludden, P. W., 1991. Mutations in the *draT* and *draG* genes of *Rhodospirillum rubrum* results in loss of regulation of nitrogenase by reversible ADP-ribosylation. J. Bacteriol, 173;6903-6909.

[83] Zhang, Y., Wolfe, D. M., Pohlmann, E. L., Conrad, M. C., Roberts, G. P., 2006. Effect of AmtB homologues on the posttranslational regulation of nitrogenase activity in response to ammonia and energy signals in *Rhodospirillum rubrum*. Microbiology, 152:2075-2089.

[84] Wang, H., Franke, C. C., Nordlund, S., Noren, A., 2005. Reversible membrane association of dinitrogenase reductase activating glycohydrolase in the regulation of nitrogenase activity in *Rhodospirillum rubrum*; dependence on the GlnJ and AmtB1. FEMS. Microbiol. Lett, 253:273-279

[85] Zhang, Y., Pohlmann, E. L., Roberts, G. P., 2005. GlnD is essential for NifA activation, NtrB/NtrC-regulated gene expression, and posttranslational regulation of nitrogenase activity in the photosynthetic, nitrogen fixation bacterium *Rhodospirillum rubrum*. J. Bacteriol, 187:1254-1265.

[86] Yakunin, A. Y., Hallenbeck, P. C., 2002, AmtB is necessary for NH_4^+-induced nitrogenase switch-off and ADP-ribosylation in *Rhodobacter capsulatus*. J. Bacteriol, 184:4081-4088.

[87] Hallenbeck, P. C., 1992. Mutations affecting nitrogenases switch-off in *Rhodobacter capsulatus*. Biochim. Biophys. Acta, 1118:161-168

[88] Fedorov, A., Troshina, O. Y., Laurinavichene, T. V., Glazer, V. M., Babykin, M., Zinchenko, V., Yakunin, A. F., Tsygankov, A. A., 1998. Regulatory effect of ammonium on the nitrogenase activity of *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* is not mediated by ADP-ribosylation of the Fe-protein of nitrogenase. Microbiology, 67:736-742.

[89] Yakunin, A. Y., Hallenbeck, P. C., Short-term regulation of nitrogenase activity by NH_4^+ in *Rhodobacter capsulatus*: multiple *in vivo* nitrogenase responses to NH_4^+ addition. J. Bacteriol, 180:6392-6395.

[90] Heiniger, E. K., Oda, Y., Samanta, S. K., Harwood, C. S., 2012. How posttranslational regulation of nitrogenase is circumvented in *Rhodopseudomonas palustris* strains that produce hydrogen gas constitutively. Appl. Environ. Microbiol, 78:1023-1032.

[91] Willison, J. C., Ahombo, G., Chabert, J., Magnin, J., Vignais, P. M., 1985. Genetic mapping of the *Rhodopseudomona capsulata* chromosome shows non-clustering of genes involved in nitrogen fixation. Microbiology, 131:3001-3015.

[92] Masepohl. B., Klipp, W., 1996. Organization and regulation of genes encoding the molybdenum nitrogenase and the alternative in *Rhodobacter capsulatus*. Arch. Microbiol, 165:80-90.

[93] Kranz, R. G., Haselkorn, R., 1986. Anaerobic regulation of nitrogen fixation genes in *Rhodopseudomonas capsulata*. Proc. Natl. Acad. Sci. U. S. A, 83:6805-6809.

[94] Kranz, R. G., Foster-Hartnett, D., 1990. Transcriptional regulatory cascade of nitrogen fixation genes in anoxygenic photosynthetic bacteria: oxygen and nitrogen response factors. Mol. Microbiol, 4:1793-1800.

[95] Foster-Hartnett, D., Kranz, R. G., 1994. The *Rhodobacter capsulatus glnB* gene is regulated by NtrC at tandem *rpoN*-independent promoters. J. Bacteriol, 176:5171-5176,

[96] Cullen, P. J., Foster-Hartnett, D., Gabbert, K. K., Kranz, R. G., 1994. Structure and expression of the alternative sigma factor, RpoN, in *Rhodobacter capsulatus*; physiological relevance of an autoactivated *nifU2-rpoN* superoperon. Mol. Microbiol, 11:51-65.

[97] Foster-Hartnett, D., Cullen, P, G., Gabbert, K. K., Kranz, R. G., 1993. Sequence, genetic, and *lacZ* fusion analyses of a *nifR3-ntrB-ntrC* operon in *Rhodobacter capsulatus*. Mol. Microbiol, 8:903-914.

[98] Foster-Hartnett. D., Kranz, R. G., 1992. Analysis of the promoters and upstream sequences of *nifA1* and *nifA2* in *Rhodobacter capsulatus*; activation requires *ntrC* but not *rpoN*. Mol. Microbiol, 6:1049-1060.

[99] Kranz, R. G., Pace, V. M., Caldicott, I. M., 1990. Inactivation, sequence, and *lacZ* fusion analysis of a regulatory locus required for repression of nitrogen fixation genes in *Rhodobacter capsulatus*. J. Bacteriol, 173:53-62.

[100] Jones, R., Haselkorn, R., 1989. The DNA sequence of the *Rhodobacter capsulatus ntrA, ntrB* and *ntrC* genes analogues required for nitrogen fixation. Mol. Gen. Genet, 215:507-516.

[101] Pawlowski, A., Riedel, K., Klipp, W., Dreiskemper, P., Grob, S., Bierhoff, H., Drepper, T., Masepohl, B., 2003. Yeast two-hybrid studies on interaction of proteins involved in regulation of nitrogen fixation in the phototrophic bacterium *Rhodobacter capsulatus*. J. Bacteriol, 185:5240-5247.

[102] Borghese, R., Wall, J. D., 1995. Regulation of the *glnBA* operon of *Rhodobacter capsulatus*. J. Bacteriol, 177:4549-4552.

[103] Paschen, A., Drepper, T., Masepohl, B., Klipp, W., 2001. *Rhodobacter capsulatus nifA* mutants mediating *nif* gene expression in the presence of ammonium. FEMS. Microbiol. Lett, 200:207-213.

[104] Richard, C. L., Tandon, A., Kranz, R. G., 2004. *Rhodobacter capsulatus nifA1* promoter: high GC-10 regions in high-GC bacteria and the basis for their transcription. J. Bacteriol, 186:740-749.

[105] Hubner, P., Willison, J. C., Vignais, P. M., Bickle, T. A., 1991. Expression of regulatory *nif* genes in *Rhodobacter capsulatus*. J. Bacteriol, 173:2993-2999.

[106] Hillmer, P., Gest, H., 1977. H_2 metabolism in the photosynthetic bacterium *Rhodopseudomonas capsulata*: H_2 production by growing cultures. J. Bacteriol, 129:724-731.

[107] Hillmer, P., Gest, H., 1977. H_2 metabolism in the photosynthetic bacterium *Rhodopseudomonas capsulata*: production and utilization of H_2 by resting cells. J. Bacteriol, 129:732-739.

[108] van Niel, C. B., 1944. The culture, general physiology, morphology, and classification of non-sulfur purple and brown bacteria. Bacteriol. Rev, 8:1–118.

[109] Conrad, B., Schlegel, H. G., 1978. An alternative pathway for the degradation of endogenous fructose during the catabolism of sucrose in *Rhodopseudomonas capsulata*. Microbiology, 105:305-313.

[110] Eidels, I., Preiss, J., 1970. Carbohydrate metabolism in *Rhodopseudomonas capsulata*: enzyme titers, glucose metabolism and polyglucose polymer synthesis. Arch. Biochem. Biophys, 140:75-89.

[111] Abo-Hashesh, M., Ghosh, D., Tourigny, A., Taous, A., Hallenbeck, P. C., 2011. Single stage photofermentative hydrogen production from glucose: An attractive alternative to two stage photofermentation or co-culture approaches. Int. J. Hydrogen Energy, 36:13889-13895.

[112] Ghosh, D., Sobro, I. F., Hallenbeck, P. C., 2012. Optimization of the hydrogen yield from single-stage photofermentation of glucose by *Rhodobacter capsulatus* JP91 using response surface methodology. Bioresour. Technol, 123:199-206.

[113] McKinlay, J. B., Harwood, C. S., 2009. Carbon dioxide fixation as a central redox cofactor recycling mechanism in bacteria. Proc. Natl. Acad. Sci. U. S. A, 107:11669-11675.

[114] Wang, D., Zhang, Y. P., Welch, E., Li, J. L., Roberts, G. P., 2010. Elimination of Rubisco alters the regulation of nitrogenase activity and increase hydrogen production in *Rhodospirillum rubrum*. Int. J. Hydrogen Energy, 35:7377-7385.

[115] Ozturk, Y., Gokce, A., Peksel, B., Gurgan, M., Ozgur, E., Gundze, U., 2012. Hydrogen production properties of *Rhodobacter capsulatus* with genetically modified redox balancing pathways. Int. J. Hydrogen Energy, 37:2014-2020.

[116] Meyer, J., Kelley, B. C., Vignais, P. M., 1978. Effect of light on nitrogenase function and synthesis in *Rhodopseudomonas capsulata*. J. Bacteriol, 136:201-208.

[117] Wang, D., Zhang, Y., Pohlmann, E. L., Li, J., Roberts, G. P., 2011. The poor growth of *Rhodospirillum rubrum* mutants lacking Rubisco is due to accumulation of ribulose-1,5-bisphosphate. J. Bacteriol, 193:3293-3303.

[118] Paoli, G. C., Vichivanives, P., Tabita, F. R., 1998. Physiological control and regulation of the *Rhodobacter capsulatus cbb* operons. J. Bacteriol, 180:4258-4269.

[119] Farmer, R. M., Tabita, F, R., 2015. Phosphoribulokinase mediates nitrogenase induced carbon dioxide fixation gene repression in *Rhodobacter sphaeroides*. Microbiology, 161:2184-2191.

[120] Hadicke, O., Grammel, H., Klamt, S., 2011. Metabolic network modeling of redox balancing and biohydrogen production in purple nonsulfur bacteria. BMC Syst. Biol. 5:150-68.

[121] Madigan, M. T., Gest, H., 1979. Growth of the photosynthetic bacterium *Rhodopseudomonas capsulata* chemoautotrophically in darkness with H_2 as the energy source. J. Bacteriol, 137:524-530.

[122] Gordon, G. C., McKinlay, J, B., 2014. Calvin cycle mutants of photoheterotrophic purple nonsulfur bacteria fail to grow due to an electron imbalance rather than toxic metabolite accumulation. J. Bacteriol, 196:1231-1237.

[123] Drepper, T., Groβ, S., Yakunin, A. F., Hallenbeck, P. C., Masepohl, B., Klipp, W., 2003. Role of *glnB* and *glnK* in ammonium control of both nitrogenase systems in the phototrophic bacterium *Rhodobacter capsulatus*. Microbiology, 149:2203-2212.

[124] Show, K. Y., Lee, D. J., Tay, J. H., Lin, C. Y., Chang, J. S., 2012. Biohydrogen production: current perspectives and the way forward. Int. J. Hydrogen Energy, 37:15616-15631.

[125] Chookaew, T., O-Thong, S., Prasertsan, P., 2015. Biohydrogen production from crude glycerol by two stage of dark and photofermentation. Int. J. Hydrogen Energy, 40:7433-7438

[126] Nath, K., Muthukumar, M., Kumar, A., Das, D., 2008. Kinetics of two stage fermentation process for the production of hydrogen. Int. J. Hydrogen Energy, 33:1195-1203.

[127] Su, H., Cheng, J., Zhou, J., Song, W., Cen, K., 2009. Combination of dark and photo-fermentation to enhance hydrogen production and energy conversion efficiency. Int. J. Hydrogen Energy, 34:8846-8853.

[128] Adessi, A., McKinlay, J. B., Harwood, C. S., Philippis, R. D., 2012. A *Rhodopseudomonas palustris nifA** mutant produces H_2 from NH_4^+-containing vegetable wastes. Int. J. Hydrogen Energy, 37:15893-15900.

[129] Fujii, T., Nakazawa, A., Sumi, N., Tani, H., Ando, A., Yabuki, M., 1983. Utilization of alcohols by *Rhodopseudomonas* sp. No. 7 isolated from n-propanol-enrichment cultures. Agric. Biol. Chem, 47:2747–2753.

[130] Imhoff, J. F., Trüper, H. G., 1989. The purple nonsulfur bacteria. In: Bergey's Manual of Systematic Bacteriology, Vol. 3. Staley, J. T., Bryant, M. P., Pfennig, N., and J.G. Holt, J. G., (Eds). Williams and Wilkins, Baltimore. pp. 1658–1682.

[131] van Niel, C. B., 1944. The culture, general physiology, morphology and classification of the non-sulfur purple and brown bacteria. Bacteriol. Rev, 8: 1–118.

[132] Weaver, P. F., Wall, J. D., Gest, H., 1975. Characterization of *Rhodopseudomonas capsulata*. Arch. Microbiol, 105: 207–216.

[133] Pantazopoulous, P. E., Madigan, M. T., 2000. Primary alcohols and di-alcohols as growth substrates for the purple nonsulfur bacterium *Rhodobacter capsulatus*. Can. J. Microbiol, 46: 1166–1170.

[134] Liu, Y., Ghosh, D., Hallenbeck, P. C., 2015. Biological reformation of ethanol to hydrogen by *Rhodopesudomonas palustris* CGA009. Bioresour. Technol, 176:189-195.

[135] Yakunin, A. Y., Laurinavichene, T. V., Tsygankov, A. A., Hallenbeck, P. C., 1999. The presence of ADP-ribosylated Fe protein of nitrogenase in *Rhodobacter capsulatus* is correlated with cellular nitrogen status. J. Bacteriol, 181:1994-2000.

[136] Dominguez, M., Taboada, E., Molins, E., Llorca, J., 2012. Ethanol steam reforming at very low temperature over cobalt talc in a membrane reactor. Catal. Today, 193: 101–106.

[137] Kontur, W. S., Noguera, D. R., Donohue, T. J., 2012. Maximizing reductant flow into microbial H_2 production. Curr. Opin. Biotechnol. 23:382-389.

[138] Kim, D., Lee, J., Kang, S., Hallenbeck, P. C., Kim, E., Lee, J., Kim, M., 2014. Enhanced photofermentative H_2 production using *Rhodobacter sphaeroides* by ethanol addition and analysis of soluble microbial products. Biotechnol. Biofuel, 7:79.

[139] Karsten. R., 2002. The H-NS-like protein HvrA modulates expression of nitrogen fixation genes in the phototrophic purple bacterium *Rhodobacter capsulatus* by binding to selected *nif* promoters. FEMS. Microbiol. Lett, 216:151-158.

[140] Kern, M., Kamp, P., Paschen, A., Masepohl, B., Klipp, W., 1998. Evidence for a regulatory link of nitrogen fixation and photosynthesis in *Rhodobacter capsulatus* via HvrA. J. Bacteriol, 180:1965-1969.

[141] McKinlay, J. B., Oda, Y., Rühl, M., Posto, A. L., Sauer, U., Harwood, C. S., 2014. Non-growing *Rhodopseudomonas palustris* increases the hydrogen gas yield from acetate by shifting from glyoxylate shunt to the tricarboxylic acid cycle. J. Biol. Chem, 289; 1960-1970.

[142] Chen, Y., Wu, S., Lee, C., 2012. Relationship between cell growth, hydrogen production and poly-β-hydroxybutyrate accumulation by *Rhodopseudomonas palustris* WP3-5. Int. J. Hydrogen Engergy, 37:13887-13894.

[143] Kim, M. S., Kim, D. H., Son, H. N., Ten, L. N., Lee, J. K., 2011. Enhancing photo-fermentative hydrogen production by *Rhodobacter sphaeroides* KD131 and its PHB synthase deleted-mutant from acetate and butyrate. Int. J. Hydrogen Energy, 36:13964-13971.

[144] Hustede, E., Steinbüchel, A., Schlegel, H. G., 1993. Relationship between the photoproduction of hydrogen and the accumulation of PHB in non-sulfur purple bacteria. Appl. Microbiol. Biotechnol, 39:87-93.

[145] Barbosa, M. J., Rocha, J. M. S., Tramper, J., Wijffels, R. H., 2011. Acetate as a carbon source for hydrogen production by photosynthetic bacteria. J. Biotechnol. 85:25-33.

ADDITIONAL INFORMATION

Medium

RCV basic salts

Super-salts	50ml/L
K-phosphate buffer (0.64M)	30ml/L

Super-salts (stock solution):

1% EDTA	40ml/L
20% $MgSO_4 \cdot 7H_2O$	20ml/L
7.5% $CaCl_2 \cdot 2H_2O$	20ml/L
1% $FeSO_4 \cdot 7H_2O$	20ml/L
0.1% Thiamine·HCl	20ml/L
Trace elements	20ml/L

Trace elements (mg/250ml):

$MnSO_4 \cdot H_2O$	398
H_3BO_3	700
$Cu(NO_3)_2 \cdot H_2O$	10
$ZnSO_4 \cdot 7H_2O$	60
$Na_2MoO_4 \cdot 2H_2O$	188

K-phosphate buffer (0.64M):

KH_2PO_4	20g/500ml
K_2HPO_4	30g/500ml

YPS medium:

$CaCl_2$	2 mM

$MgSO_4$	2 mM
Yeast extract	3g/L
Peptone	3g/L
Agar	15g/L

SDS-PAGE and Western Blot

Separating gel 12.5%

Tris-HCl (1.9 M, pH 8.8)	3.69 ml
Acrylamide-Bis (30:0.2)	7.81 ml
SDS 10%	0.19 ml
H_2O	6.25 ml

Degas for 15 min; then add 10 µl TEMED, 50 µl freshly made 10% AP just before pouring. Overlay immediately with water saturated isobutanol.

Stacking gel

Tris HCl (0.63 M pH 6.8)	2 ml
Acrylamide-Bis (30:0.8)	1.7 ml
SDS 10%	0.1 ml
H_2O	6.2 ml

Degas for 15 min; then add 10 µl TEMED, 50 µl freshly made 10% AP. Place the comb about half-way down.

Electrode buffer (per one liter)

Tris-Glycine 10X buffer	100 ml
SDS 10%	10 ml
H_2O	890 ml

Sample buffer (2X)

Tris HCl (0.63 M, pH 6.8)	2 ml

SDS 20%	2 ml
Mercapteothanol	1 ml
Glycerol 50%	4 ml
BPB 1%	20 µl
H_2O	1 ml

Mix sample (cell culture) with 2X buffer of equal volume; then put it in boiling water bath for 5 min.

PVDF Transfer buffer

Tris-Glycine 10X buffer	100 ml
Methanol	100 ml
H_2O	800 ml

TBS

Tris	3.03 g
NaCl	8.78 g

Ajust pH to 7.4 with 3 N HCl, fill to 1 liter with H_2O.

TBST

Add 0.05% tween 20 into TBS solution.

Blocking soulution

1% skim milk powder in TBS buffer.