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Improving the Microbial Production of Biofuels through Metabolic Engineering

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RÉSUMÉ

Les défis conjoints du changement climatique d'origine anthropique et la diminution des réserves de combustibles fossiles sont le moteur de recherche intense pour des sources d'énergie alternatives. Une avenue attrayante est d'utiliser un processus biologique pour produire un biocarburant. Parmi les différentes options en matière de biocarburants, le bio-hydrogène gazeux est un futur vecteur énergétique attrayant en raison de son efficacité potentiellement plus élevé de conversion de puissance utilisable, il est faible en génération inexistante de polluants et de haute densité d'énergie. Cependant, les faibles rendements et taux de production ont été les principaux obstacles à l'application pratique des technologies de bio-hydrogène. Des recherches intensives sur bio-hydrogène sont en cours, et dans les dernières années, plusieurs nouvelles approches ont été proposées et étudiées pour dépasser ces inconvénients. À cette fin, l'objectif principal de cette thèse était d'améliorer le rendement en hydrogène moléculaire avec un accent particulier sur l'ingénierie métabolique et l'utilisation de bioprocédés à variables indépendantes.

Une de nos hypothèses était que la production d'hydrogène pourrait être améliorée et rendue plus économiquement viable par ingénierie métabolique de souches d'*Escherichia coli* producteurs d'hydrogène en utilisant le glucose ainsi que diverses autres sources de carbone, y compris les pentoses. Les effets du pH, de la température et de sources de carbone ont été étudiés. La production maximale d'hydrogène a été obtenue à partir de glucose, à un pH initial de 6.5 et une température de 35°C. Les études de cinétiques de croissance ont montré que la μmax était 0.0495 h^{-1} avec un Ks de 0.0274 g L^{-1} lorsque le glucose est la seule source de carbone en milieu minimal M9. .Parmi les nombreux sucres et les dérivés de sucres testés, les

rendements les plus élevés d'hydrogène sont avec du fructose, sorbitol et D-glucose; 1.27, 1.46 et 1.51 mol H_2 mol^{-1} de substrat, respectivement.

En outre, pour obtenir les interactions entre les variables importantes et pour atteindre une production maximale d'hydrogène, un design 3^K factoriel complet Box-Behnken et la méthodologie de réponse de surface (RSM) ont été employées pour la conception expérimentale et l'analyse de la souche *d'Escherichia coli* DJT135. Le rendement en hydrogène molaire maximale de 1.69 mol H_2 mol^{-1} de glucose a été obtenu dans les conditions optimales de 75 mM de glucose, à 35°C et un pH de 6.5. Ainsi, la RSM avec un design Box-Behken était un outil statistique utile pour atteindre des rendements plus élevés d'hydrogène molaires par des organismes modifiés génétiquement.

Ensuite, l'expression hétérologue de l'hydrogénases soluble [Ni-Fe] de *Ralstonia eutropha* H16 (l'hydrogénase SH) a tenté de démontrer que la mise en place d'une voie capable de dériver l'hydrogène à partir de NADH pourrait surpasser le rendement stoechiométrique en hydrogène.. L'expression a été démontrée par des tests in vitro de l'activité enzymatique. Par ailleurs, l'expression de SH a restaurée la croissance en anaérobie de souches mutantes pour *adhE*, normalement inhibées en raison de l'incapacité de réoxyder le NADH. La mesure de la production d'hydrogène in vivo a montré que plusieurs souches modifiées métaboliquement sont capables d'utiliser l'hydrogénase SH pour dériver deux moles d'hydrogène par mole de glucose consommé, proche du maximum théorique.

Une autre stratégie a montré que le glycérol brut pourrait être converti en hydrogène par photofermentation utilisant *Rhodopseudomonas palustris* par photofermentation. Les effets de la source d'azote et de différentes concentrations de glycérol brut sur ce processus ont été évalués. À 20 mM de glycérol, 4 mM glutamate, 6.1 mol hydrogène / mole de glycérol

brut ont été obtenus dans des conditions optimales, un rendement de 87% de la théorie, et significativement plus élevés que ce qui a été réalisé auparavant. En prolongement de cette étude, l'optimisation des paramètres a également été utilisée. Dans des conditions optimales, une intensité lumineuse de 175 W/m^2, 30 mM glycérol et 4.5 mM de glutamate, 6.69 mol hydrogène / mole de glycérol brut ont été obtenus, soit un rendement de 96% de la valeur théorique. La détermination de l'activité de la nitrogénase et ses niveaux d'expression ont montré qu'il y avait relativement peu de variation de la quantité de nitrogénase avec le changement des variables alors que l'activité de la nitrogénase variait considérablement, avec une activité maximale (228 nmol de C_2H_4/ml/min) au point central optimal.

Dans la dernière section, la production d'hydrogène à partir du glucose via la photofermentation en une seule étape a été examinée avec la bactérie photosynthétique *Rhodobacter capsulatus* JP91 (*hup*-). La méthodologie de surface de réponse avec Box-Behnken a été utilisée pour optimiser les variables expérimentales de façon indépendante, soit la concentration de glucose, la concentration du glutamate et l'intensité lumineuse, ainsi que d'examiner leurs effets interactifs pour la maximisation du rendement en hydrogène moléculaire. Dans des conditions optimales, avec une intensité lumineuse de 175 W/m^2, 35 mM de glucose, et 4.5 mM de glutamate,, un rendement maximal d'hydrogène de 5.5 (± 0.15) mol hydrogène /mol glucose, et un maximum d'activité de la nitrogénase de 246 (± 3.5) nmol C_2H_4/ml/min ont été obtenus. L'analyse densitométrique de l'expression de la protéine-Fe nitrogenase dans les différentes conditions a montré une variation significative de l'expression protéique avec un maximum au point central optimisé. Même dans des conditions optimales pour la production d'hydrogène, une fraction significative de la protéine Fe a été trouvée dans l'état ADP-ribosylée, suggérant que d'autres améliorations des rendements pourraient être

possibles. À cette fin, un mutant *amtB* dérivé de *Rhodobacter capsulatus* JP91 (*hup*-) a été créé en utilisant le vecteur de suicide pSUP202. Les résultats expérimentaux préliminaires montrent que la souche nouvellement conçue métaboliquement, *R. capsulatus* DG9, produit 8.2 (± 0.06) mol hydrogène / mole de glucose dans des conditions optimales de cultures discontinues (intensité lumineuse, 175 W/m^2, 35 mM de glucose et 4.5 mM glutamate). Le statut d'ADP-ribosylation de la nitrogénase-protéine Fe a été obtenu par Western Blot pour la souche *R. capsulatus* DG9.

En bref, la production d'hydrogène est limitée par une barrière métabolique. La principale barrière métabolique est due au manque d'outils moléculaires possibles pour atteindre ou dépasser le rendement stochiométrique en bio-hydrogène depuis les dernières décennies en utilisant les microbes. À cette fin, une nouvelle approche d'ingénierie métabolique semble très prometteuse pour surmonter cette contrainte vers l'industrialisation et s'assurer de la faisabilité de la technologie de la production d'hydrogène. Dans la présente étude, il a été démontré que l'ingénierie métabolique de bactéries anaérobiques facultatives (*Escherichia coli*) et de bactéries anaérobiques photosynthétiques (*Rhodobacter capsulatus* et *Rhodopseudomonas palustris*) peuvent produire de l'hydrogène en tant que produit majeur à travers le mode de fermentation par redirection métabolique vers la production d'énergie potentielle. D'autre part, la méthodologie de surface de réponse utilisée dans cette étude représente un outil potentiel pour optimiser la production d'hydrogène en générant des informations appropriées concernant la corrélation entre les variables et des producteurs de bio-de hydrogène modifiés par ingénierie métabolique. Ainsi, un outil d'optimisation des paramètres représente une nouvelle avenue pour faire un pont entre le laboratoire et la production d'hydrogène à l'échelle industrielle en fournissant un modèle mathématique

potentiel pour intensifier la production de bio-hydrogène. Par conséquent, il a été clairement mis en évidence dans ce projet que l'effort combiné de l'ingénierie métabolique et la méthodologie de surface de réponse peut rendre la technologie de production de bio-hydrogène potentiellement possible vers sa commercialisation dans un avenir rapproché.

Mots-clés: biocarburants, production d'hydrogène, photofermentation, fermentation, génie métabolique

ABSTRACT

The joint challenges of anthropogenic climate change and dwindling fossil fuel reserves are driving intense research into alternative energy sources. One attractive avenue is to use a biological process to produce a biofuel. Among the various biofuel options, biohydrogen gas is an attractive future energy carrier due to its potentially higher efficiency of conversion to usable power, low to non-existent generation of pollutants and high energy density. However, low yields and production rates have been major barriers to the practical application of biohydrogen technologies. Intensive research on biohydrogen is underway, and in the last few years several novel approaches have been proposed and studied to surpass these drawbacks. To this end the main aim of this thesis was to improve the molar hydrogen yield with special emphasis of metabolic engineering using the interactive effect with bioprocess independent variable.

One investigated hypothesis was that H_2 production could be improved and made more economically viable by metabolic engineering on the facultative hydrogen producer *Escherichia coli* from glucose as well as various other carbon sources, including pentoses. The effects of pH, temperature and carbon source were investigated in batch experiments. Maximal hydrogen production from glucose was obtained at an initial pH of 6.5 and temperature of 35°C. Kinetic growth studies showed that the μmax was 0.0495 h^{-1} with a Ks of 0.0274 g L^{-1} when glucose was the sole carbon source in M9 (1X) minimal medium. Among the many sugar and sugar derivatives tested, hydrogen yields were highest with fructose, sorbitol and d-glucose; 1.27, 1.46 and 1.51 mol H_2 mol^{-1} substrate respectively.

In addition, to obtain the interactions between the variables important for achieving maximum hydrogen production, a 3^K full factorial Box–Behnken design and response surface

methodology (RSM) were employed for experimental design and analysis on a metabolically engineered *Escherichia coli* strain, DJT135. A maximum molar hydrogen yield of 1.69 mol H_2 mol^{-1} glucose was obtained under the optimal conditions of 75 mM glucose, 35°C and pH 6.5. Thus, RSM with Box–Behnken design was a useful statistical tool for achieving higher molar hydrogen yields by metabolically engineered organisms.

Furthermore, the heterologous expression of the soluble [Ni-Fe] hydrogenase from *Ralstonia eutropha* H16 (the SH hydrogenase) was attempted to demonstrate the introduction of a pathway capable of deriving hydrogen from NADH to surpass the stoichiometric molar hydrogen yield. Successful expression was demonstrated by in vitro assay of enzyme activity. Moreover, expression of SH restored anaerobic growth on glucose to *adhE* strains, normally blocked for growth due to the inability to re-oxidize NADH. Measurement of in vivo hydrogen production showed that several metabolically engineered strains were capable of using the SH hydrogenase to derive 2 mol H_2 per mol of glucose consumed, close to the theoretical maximum.

Using another strategy, it was shown that crude glycerol could be converted to hydrogen, a possible future clean energy carrier, by photofermentation using *Rhodopseudomonas palustris* through photofermentation. Here, the effects of nitrogen source and different concentrations of crude glycerol on this process were assessed. At 20 mM glycerol, 4 mM glutamate, 6.1 mol hydrogen/mole of crude glycerol were obtained under optimal conditions, a yield of 87% of the theoretical, and significantly higher than what was achieved previously. As a continuation of this study, multiprocess parameter optimization was also involved. Under optimal conditions, a light intensity of 175 W/m^2, 30 mM glycerol, and 4.5 mM glutamate, 6.69 mol hydrogen/mole of crude glycerol were obtained, a yield 96% of

theoretical. Determination of nitrogenase activity and expression levels showed that there was relatively little variation in levels of nitrogenase protein with changes in process variables whereas nitrogenase activity varied considerably, with maximal nitrogenase activity (228 nmol of C_2H_4/ml/min) at the optimal central point.

In the final section, hydrogen production from glucose via single-stage photofermentation was examined with the photosynthetic bacterium *Rhodobacter capsulatus* JP91 (*hup*-). Response surface methodology with Box–Behnken design was used to optimize the independent experimental variables of glucose concentration, glutamate concentration and light intensity, as well as examining their interactive effects for maximization of molar hydrogen yield. Under optimal condition with a light intensity of 175 W/m^2, 35 mM glucose, and 4.5 mM glutamate, a maximum hydrogen yield of 5.5 (±0.15) mol H_2/mol glucose, and a maximum nitrogenase activity of 246 (±3.5) nmol C_2H_4/ml/min were obtained. Densitometric analysis of nitrogenase Fe-protein expression under different conditions showed significant variation in Fe-protein expression with a maximum at the optimized central point. Even under optimum conditions for hydrogen production, a significant fraction of the Fe-protein was found in the ADP-ribosylated state, suggesting that further improvement in yields might be possible. To this end an AmtB-derivative of *Rhodobacter capsulatus* JP91 (*hup*-) was created by conjugating in *amtB::Km* using the suicide vector pSUP202. Preliminary experimental results showed that the newly metabolically engineered strain, *R. capsulatus* DG9, produced 8.2 (±0.06) mol hydrogen/mole of glucose under optimal conditions in batch cultures (light intensity, 175 W/m^2; 35 mM glucose, and 4.5 mM glutamate). Western blot analyses of the ADP-ribosylation status of the

nitrogenase Fe-protein were investigated on metabolically engineered strain *R. capsulatus* DG9.

In brief, the progress on hydrogen production technology has been limited due to the metabolic barrier. The major metabolic barrier is due to lacking of potential consistent molecular tools to reach or surpass the stochiometric biohydrogen yield since last decades using microbes. To this end a novel approach "metabolic engineering" seems very promising to overcome this constraint towards industrialization to ensure the feasibility of hydrogen production technology. In this present study it has been shown that metabolically engineered facultative (*Escherichia coli*) anaerobe and photosynthetic bacteria (*Rhodobacter capsulatus* and *Rhodopseudomonas palustris*) can produce hydrogen as a major product through fermentative mode by metabolic redirection toward potential energy generation. On the other hand, response surface methodology has depicted in this study as another potential tool to statistically optimize the hydrogen production by generating suitable information concerning interactive correlation between process variables and metabolically engineered biohydrogen producers. Thus, multi process parameter optimization tool has been creating a novel avenue to make a crosslink between lab scale and pilot scale hydrogen production by providing potential mathematical model for scaling up biohydrogen production using metabolically engineered biohydrogen producers. Therefore, it has been clearly revealed in this project that combined effort of metabolic engineering and response surface methodology can make biohydrogen production technology potentially feasible towards its commercialization in near future.

Keywords : biofuel, hydrogen production, photofermentation, dark fermentation, metabolic engineering

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ABBREVIATIONS

µg	Microgram
µM	Micromolar
ADP	Adenosine-di-phosphate
AFBR	Anaerobic fluidized bed reactor
ANN	Artificial neural networks
ANOVA	Analysis of variance
ASBR	Anaerobic sequencing batch reactor
ATP	Adenosine-tri-phosphate
BB	Box Behnken
CBB	Calvin–Benson–Bassham
CIGSB	Carrier-induced granular sludge bed
CoA	Coenzyme A
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactors
CW	Chemical wastewater
DALY	Disability adjusted life years
DNA	Deoxyribonucleic acid
DOE	Design of Experiment
DSW	Domestic sewage wastewater
e^-	Electron
EcoCyc	Encyclopedia of *E.coli* metabolism
EGSB	Expanded granular sludge bed

FBBAC	Fixed bed bioreactor with activated carbon
FBR	Fluidized bed reactor
FD	Factorial design
FDH	Formate dehydrogenase
FDHH	Formate dehydrogenase-H
FHL	Formate hydrogen lyase
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase
GC-FID	Gas chromatography-flame ionizing detecter
h	Hour
H_2ase	Hydrogenase
HRT	Hydraulic retention time
Hup	Uptake hydrogenase
Hyd	Hydrogenase
IPTG	Isopropyl-D-thiogalactopyranoside
kb	kilo basepair
kDa	kilo Dalton
LB	Luria Bertani
MATLAB	Matrix Laboratory (software)
MEC	Microbial electrolytic cells
MFC	Microbial fuel cells
mg	milligram
mM	millimolar
mmol	millimole

mol	Mole
NAD	Nicotinamide-adenine dinucleotide (oxidised)
NADH	Nicotinamide-adenine dinucleotide (reduced)
NADP	Nicotinamide-adenine dinucleotide phosphate
NFOR	NADH: ferredoxin oxidoreductase
nmol	Nanomole
^{o}C	Degree Celsius
OD	Optical density
OLR	Organic loading rate
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEP	Phosphoenol pyruvate
PEPC	Phosphoenol pyruvate carboxylase
PFL	Pyruvate formate lyase
PFOR	Pyruvate ferredoxin oxidoreductase
PHB	Polyhydroxybutyrate
PNS	Purple non sulfur
PS	Photo system
PVDF	Polyvinyldifluoride
RNA	Ribonucleic acid
Rnf	RING-type zinc finger protein
rpm	Rotation per minute
RSM	Response surface methodology

Rubisco	Ribulose 1,5-biphosphate carboxylase
SDS–PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH	Soluble hydrogenase
TCA	Tri carboxylic acid
TCD	Thermal conductivity detector
TPP	Thiamine pyrophosphate
UASB	Up flow anaerobic sludge blanket
WHO	World health organization

FOOTNOTES

Note on the text of the Introduction

During my Ph.D study, I co-authored a number of reviews on biological hydrogen production. Some of the sections that I contributed to these reviews have been used in the introduction of this thesis. Where this is the case, the paragraph is followed by one of the footnotes below to indicate the source. It should be noted that these reviews were published by either Elsevier or Springer, both of which allow authors to use published material, in whole or part, without written permission or formal copyright transfer.

[1]Adapted from my contribution in our published review "Hallenbeck P.C., **Ghosh D**., **2012**. Improvements in fermentative biological hydrogen production through metabolic engineering, J Environ. Management, 95: S360-S364."

[2]Adapted from my contribution in our published review "Hallenbeck P.C., Mona Abo-Hashesh, **Ghosh D**., **2012**. Strategies for improving biological hydrogen production. Bioresour. Technol., 110: 1-9. "

[3]Adapted from my contribution in our published chapter "Hallenbeck P.C., **Ghosh D**., Abo-Hashesh M., Wang R., **2011**. Metabolic engineering for enhanced biofuels production with emphasis on the biological production of hydrogen. In: Taylor J.C. (ed.), Advances in chemistry research. Nova Science, Hauppauge, NY, USA, Vol. 6. pp. 125-154."

[4]Adapted from my contribution in our published review "Hallenbeck P.C., **Ghosh D**., **2009.** Advances in Fermentation Biohydrogen Production: the Way Forward?". Trends in Biotechnol., 27(5) : 287-297. "

[5]Adapted from my contribution in our published review " Hallenbeck P.C., **Ghosh D**., Skonieczny T.M., Yargeau V., **2009.** Microbiological and Engineering Aspects of Biohydrogen Production. Ind. J of Microbiol., 49 : 48-59. "

[6]Adapted from my contribution in out published book chapter "**Ghosh D**., Hallenbeck P.C., 2012. Advanced Bioethanol Production. Microbial Technologies in Advanced biofuels production, Springer Science+Business Media, LLC. Vol 3: 165-181. "

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Dipankar Ghosh

INTRODUCTION

1. Climate change and biofuels [1]

The world is faced with serious environmental problems, many due directly or indirectly to fossil fuel utilization. An estimated 40% of annual deaths are thought to be directly linked to environmental degradation (Pimentel et al., 2007) and poor urban air quality, largely due to fossil fuel combustion, plays a significant role in the estimated 3 million people killed worldwide each year by air pollutants (WHO, 2002), including air-borne particulates emanating from vehicle exhaust, which are estimated to be responsible for 20% of the lung cancer deaths in the USA (Pearce, 2002). Fossil fuel driven climate change already has had an effect on human morbidity with conservatively, 150,000 deaths and over 5 million DALYs (disability adjusted life years) attributable to this factor (Campbell-Lendrum and Woodruff, 2007). Of course, impending climate change, driven by fossil fuel derived CO_2 emissions, poses a potentially much greater threat to human health and well-being, the magnitude of which is difficult to assess. The anthropogenic climate change already in place, only a small fraction of what is expected to occur, has already had a significant impact. A recent analysis suggests that there are already, conservatively, 150,000 deaths and over 5 million DALYs attributable to this factor (Campbell-Lendrum and Woodruff, 2007)

There is now a widespread awareness that to avert further environmental degradation and the disastrous consequences of climate change, the present almost total reliance on fossil fuels must be replaced by the development of sustainable alternative energy sources and carriers. This has been given even more impetus recently by the realization that the world is entering a phase of ever decreasing fossil fuel reserves and ever increasing fuel prices. A number of options, for both stationary and mobile (i.e. transportation) power generation, are

under development. Several biofuels, notably bioethanol and biodiesel, are already being produced on a massive scale, and a number of others have been proposed. Biohydrogen appears to have a number of potential advantages as a biofuel. For one thing, there is a significant movement to create a hydrogen economy with a great deal of R&D activity in hydrogen storage and utilization. Every major automobile manufacturer is committed to developing and testing hydrogen-powered prototypes (Anonymous, 2010). To power this proposed future hydrogen economy, a green, sustainable means of producing hydrogen must be developed. Biological production of hydrogen through the dark fermentation of wastes (first) and non-food biomass (second) could potentially provide this. As well, although bioethanol and biohydrogen production could take place in similar facilities, energy costs for biohydrogen might be lower since no distillation step would be required. Another advantage is that hydrogen can be converted to power using fuel cells which are inherently much more efficient than the combustion engines used when the biofuel is bioethanol or biodiesel. Using fuel cells also avoids the generation of various pollutants such as NO_2 and acetaldehyde (bioethanol). Finally, unlike bioethanol, which emits CO_2 when it is combusted, CO_2 is given off during the production of biohydrogen, thus permitting its centralized capture and sequestration. This could potentially even make biohydrogen production carbon negative. Carbon negative is a phrase which describes any activity that removes more carbon (likely CO_2) from the atmosphere. However, a number of problems must be overcome in order to realize the practical application of dark fermentation for biohydrogen production. Engineering issues, such as bioreactor configuration and operation, do not appear to be particularly technically challenging. It should be noted however that significant technical barriers to the implementation of hydrogen as a fuel do exist in terms of achieving practical means for its

purification and storage (Jacoby, 2005;Levin and Chahine, 2010; US DOE Hydrogen Program, 2002, 2006). Likewise, substrate pretreatment problems are either rather easily dealt with, or at worst (cellulosics), are in common with the production of some other biofuels (bioethanol), and are presently the subject of intense investigation. For example, to name only two, $500 million USD has been invested in the formation of energy Biosciences Institute (http://www.energybiosciencesinstitute.org/) and $375 million USD in the formation of federal Bioenergy Research centers (http://www. energy.gov/news/archives/5172.htm) both of which will have as a major focus lignocelluloses conversion. In biohydrogen production through dark fermentation the core issue is the attainable yield, with typical mesophilic fermentations giving at present only about 10-20% of the hydrogen potentially available in the substrate (12 mol H_2/mol hexose) (Hallenbeck, 2005; Hallenbeck and Ghosh, 2009; Hallenbeck et al., 2009; Hawkes et al., 2007; Kraemer and Bagley, 2007). Somewhat higher yields, up to 25% (3 mol H_2/mol hexose) are seen with thermophilic fermentations using either pure cultures or cocultures at the expense of volumetric productivities (Zeidan and van Niel, 2009; Panagiotopoulos et al., 2010). The key obstacle appears to be constraints imposed by the metabolic pathways involved.

2. Microbial processes producing hydrogen [5]

Diversity in microbial physiology and metabolism means that there are a variety of different ways in which microorganisms can produce hydrogen, each one with seeming advantages, as well as problematic issues (Hallenbeck and Benemann, 2002). From an engineering perspective, they all potentially offer the advantages of lower cost catalysts (microbial cells) and less energy intensive reactor operation (mesophilic) than the present industrial process for making hydrogen (steam reformation of methane). Four distinct

approaches for biohydrogen production include: 1) biophotolysis of water using algae/cyanobacteria, 2) photodecomposition (photofermentation) of organic compounds using photosynthetic bacteria, 3) dark fermentative hydrogen production using anaerobic (or facultative anaerobic) bacteria and 4) bioelectrohydrogenesis.

Biophotolysis, the concerted action of the two photo systems of plant-type photosynthesis to split water with absorbed photons and generate reduced ferredoxin to drive the reduction of protons to hydrogen, is carried out by both some green algae and some cyanobacteria **(Figure 1)**. This is an inherently attractive process because it uses water, an abundant and easily obtainable substrate. On the other hand, its simultaneous production of oxygen and hydrogen poses a number of possibly severe problems; the generation of potentially explosive mixtures of these gases, and inhibition of hydrogenase (green algae), highly sensitive to even moderately low concentrations of O_2 Hydrogen production by cyanobacteria, where hydrogen is usually produced by nitrogenase in heterocysts, is much less sensitive to oxygen. However, this comes at a metabolic cost, both due to heterocyst biosynthesis and maintenance, and to the burdensome ATP requirement of nitrogenase. Additional problems arise because of the low solar energy conversion efficiencies obtained, effectively increasing dramatically the surface area requirement for the necessary transparent, hydrogen imperable, enclosed photo bioreactors. Thus these problems have proved daunting, and presently reported rates of solar energy conversion with these systems are not much higher than they were 30 years ago (Hallenbeck, 1978; Miyamoto et al., 1979).

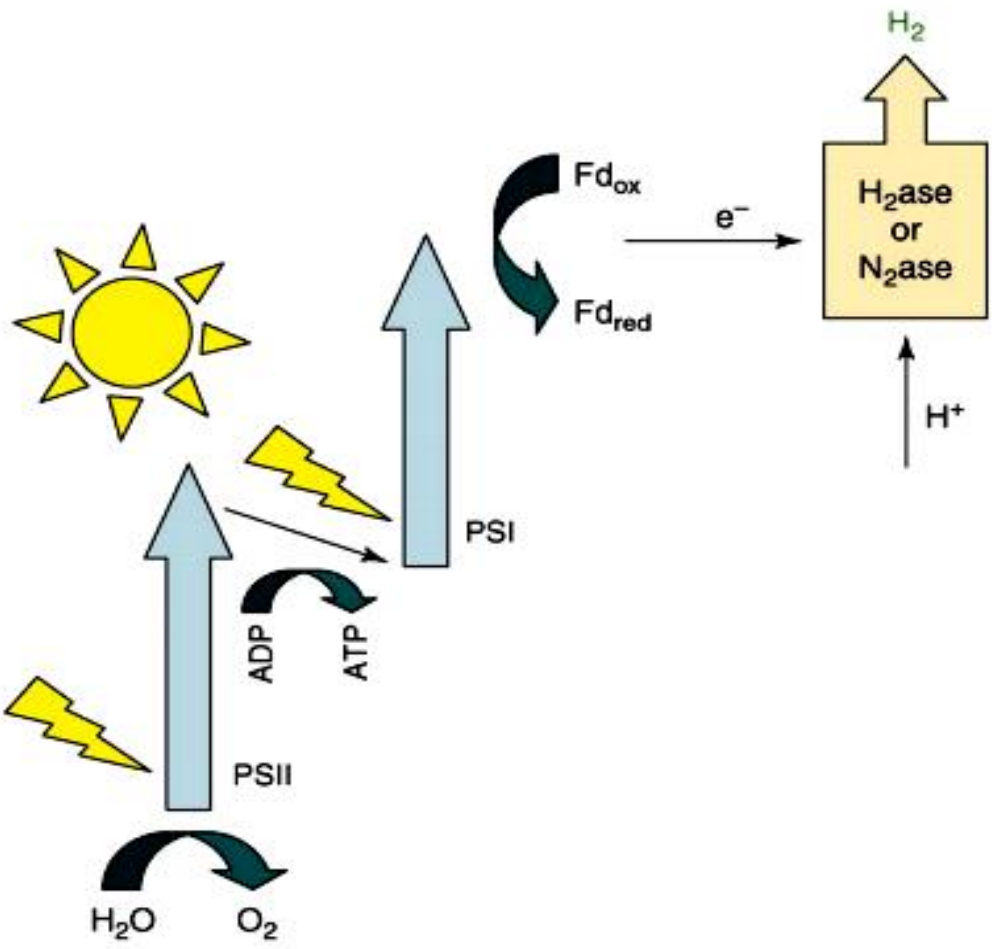

Figure 1. Biophotolysis (green algae – cyanobacteria) [4]

Another process that requires input of light energy is light driven hydrogen production from various substrates, in particular organic acids, by photosynthetic bacteria, a process that has been called photofermentation **(Figure 2)**. Indeed, photosynthetic bacteria have long been studied for their capacity to produce significant amounts of hydrogen due to their high substrate conversion efficiencies and ability to degrade a wide range of substrates. Although pure substrates have usually been used in model studies, some success in using industrial wastewater as substrate has been shown (Yetis et al., 2000; Zhu et al., 2002).

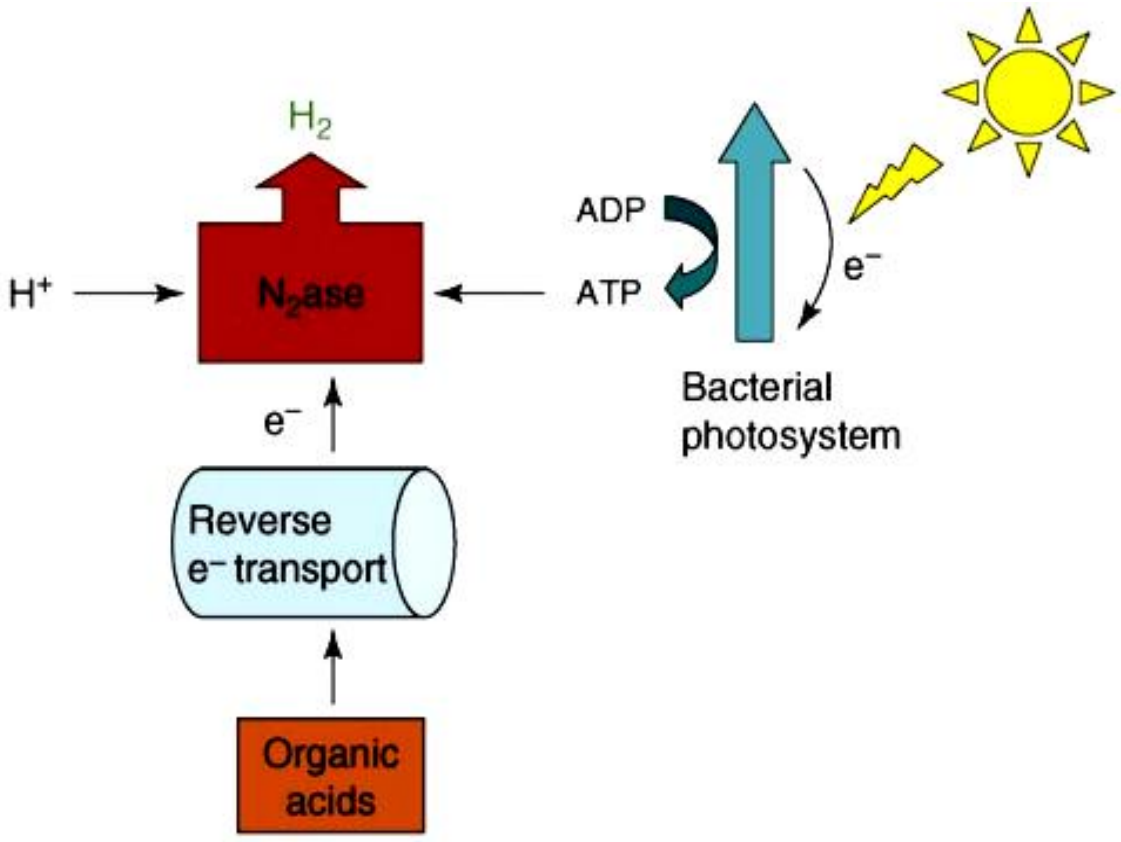

Figure 2. Photofermentation (Photosynthetic bacteria) [4]

However, pre-treatment may be needed prior to photosynthetic biohydrogen gas production due to either the toxic nature of the effluent, or its colour/opaqueness. For example, high biomass concentration is not desirable due to the reduction of light diffusion into the bioreactor. Despite the successes of hydrogen generation via photosynthetic degradation of organic compounds, much work is still needed to create a large-scale, economically attractive process. Though the conversion of substrate is generally high, the production rate of H_2 is slow and hydrogen yields are still far from the theoretical maximum. As with any other light-based production process, light diffusion and intensity play a key role in maximizing product (hydrogen) yield. Increasing light intensity (to a certain threshold) increases the hydrogen yield and production rate, but has a negative effect on light conversion efficiency. Expensive

equipment and the requirement for large reactor surface areas remain serious drawbacks. Though cyclic light process operation (i.e. light–dark cycles) has been shown to increase the amount of hydrogen evolved when compared to continuous illumination (Koku et al., 2003) and a number of other improvements could possibly be made (replace N_2ase with H_2ase, etc.), many questions remain about as to whether overall light conversion efficiencies could ever be high enough to warrant large-scale systems. Photosynthetic hydrogen production might have to be coupled with another process in order to make it economically viable.

A third method of the biological production of hydrogen is dark fermentation, where hydrogen production is inherently more stable since it takes place in the absence of oxygen. Indeed, anaerobic systems have an advantage over their photosynthetic counterparts in that they are simpler, less expensive, and produce hydrogen at much higher rates. The major drawback of course is that these bacteria are unable to overcome the inherent thermodynamic energy barrier to full substrate decomposition. Thus, in general fermentative systems suffer from low hydrogen yields (Hallenbeck, 2005). The reason for this is that anaerobic metabolism is evolutionarily optimized for maximizing biomass and not hydrogen (Hallenbeck and Benemann, 2002). Typically, anaerobic species (ex. from the genus *Clostridium*) generate gas in the exponential growth phase, and then the metabolism shifts from H_2/acid production to solventogenesis when the culture reaches stationary growth phase (Hawkes et al., 2002; Kapdan and Kargi, 2006). Poor hydrogen yields have also been linked to high hydrogen partial pressure, high substrate concentration, low iron concentration, and/or low pH (Van Ginkel et al., 2001; Yu et al., 2002; Yang and Shen, 2006). Current maximum hydrogen yields obtained do not make the fermentative process an attractive one from an economic point of view when compared to conventional reforming techniques. Ongoing

research is attempting to address this issue and identify a set of parameters under which both yield and production rate can be maximized.

It has been argued that in order for hydrogen production by dark fermentation to be economically feasible and sustainable, a two-step/hybrid biological hydrogen production process would be necessary (Benemann, 1996). By combining the anaerobic and photosynthetic steps, as shown in **Figure 3**, higher overall substrate conversion efficiency is possible as the photosynthetic microbes can degrade the soluble metabolites from the fermentative step using sunlight to overcome the energy barrier. Volatile fatty acids are the main soluble breakdown products from the first step, and these are preferred substrates of photo-heterotrophic bacteria (Fang et al., 2006; Kim et al., 2006a; Kim et al., 2006b).

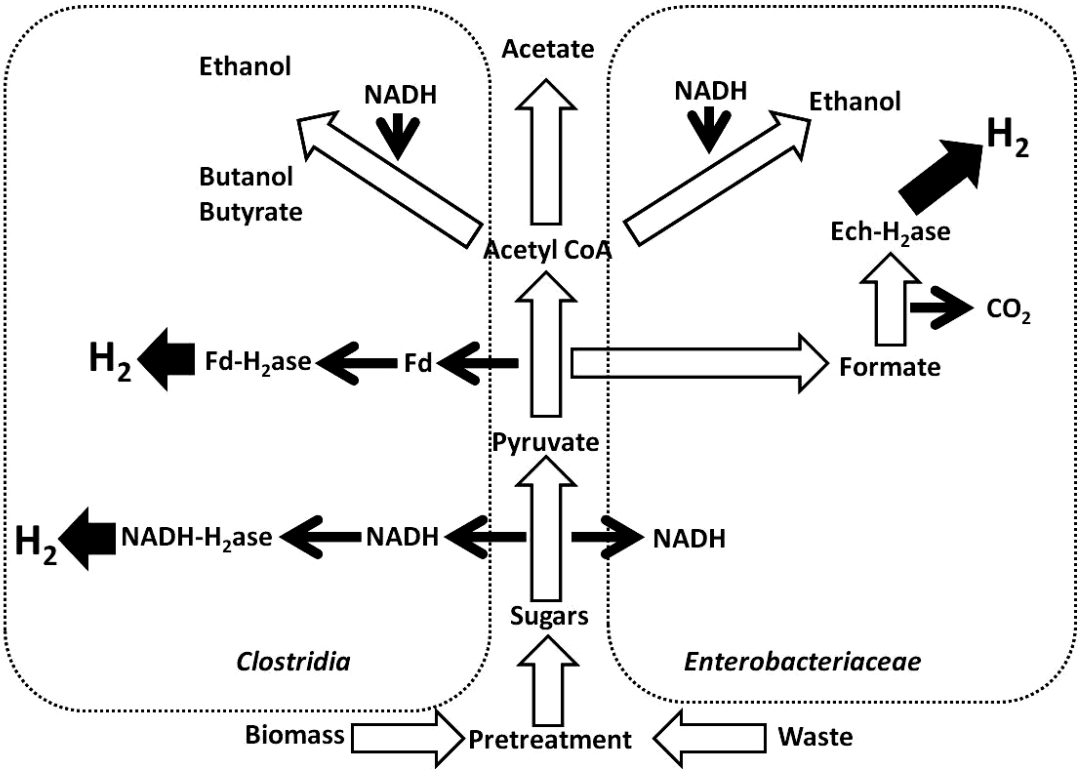

Figure 3. Dark fermentation (*Clostridia*, *Enterobacteriacae*)

Theoretically, 12 moles of hydrogen can be produced from 1 mole of glucose in the two step process. It should be pointed out that some photosynthetic bacteria are theoretically capable of doing this in a single step since species are known which can use some sugars as substrate. Thus, the only real advantage of such a two-step process might be to decrease the time and volumes required for initial substrate conversion. Indeed, complex substrates, i.e. most carbohydrate-containing wastes, would not be readily degraded by photosynthetic bacteria, and probably require the use of mixed consortia. There have been a number of recent reports on two-stage systems as shown in **Figure 4** (Fang et al., 2006; Kim et al., 2006a; Kim et al., 2006b; Eroðlu et al., 2006; Tao et al., 2007; Nath et al., 2008; Chen et al., 2008; Asada et al., 2006). One study successfully produced hydrogen using olive mill wastewater for the production of biohydrogen in a two-stage process, with a threefold increase in hydrogen production when compared to photo-fermentation alone, and a chemical oxygen demand (COD) conversion efficiency of ~55% (Eroðlu et al., 2006). High COD concentrations may have had an inhibitory effect since COD removal could be increased by diluting the wastewater. In similar work, an almost 70% conversion efficiency of *Chlamydomonas reinhardtii* biomass (mainly glucose-starch) was achieved in a two-step process that utilized *Clostridium butyricum* and *Rhodobacter sphaeroides.*

There have been several studies in which co-cultures of fermentative and photosynthetic organisms were examined (Fang et al., 2006; Asada et al., 2006). Work with co-cultures of both *C. butyricum* and *R. sphaeroides* showed only a slight increase in the hydrogen yield when compared with production obtained from pure cultures separately (Fang et al., 2006); even at high *Rhodobacter* ratios (~6:1) it appeared that *R. sphaeroides* was not able to compete with *Clostridium* for substrate (glucose). However, it is difficult to draw

conclusions as to the efficacy of having both types of organisms present in the same reaction vessel since molar yields, either alone or in co-culture, were very low, < 1 mol H_2/mole glucose, and large quantities of fermentation products, acetate and butyrate accumulated (i.e. were not used as a substrate for photofermentation).

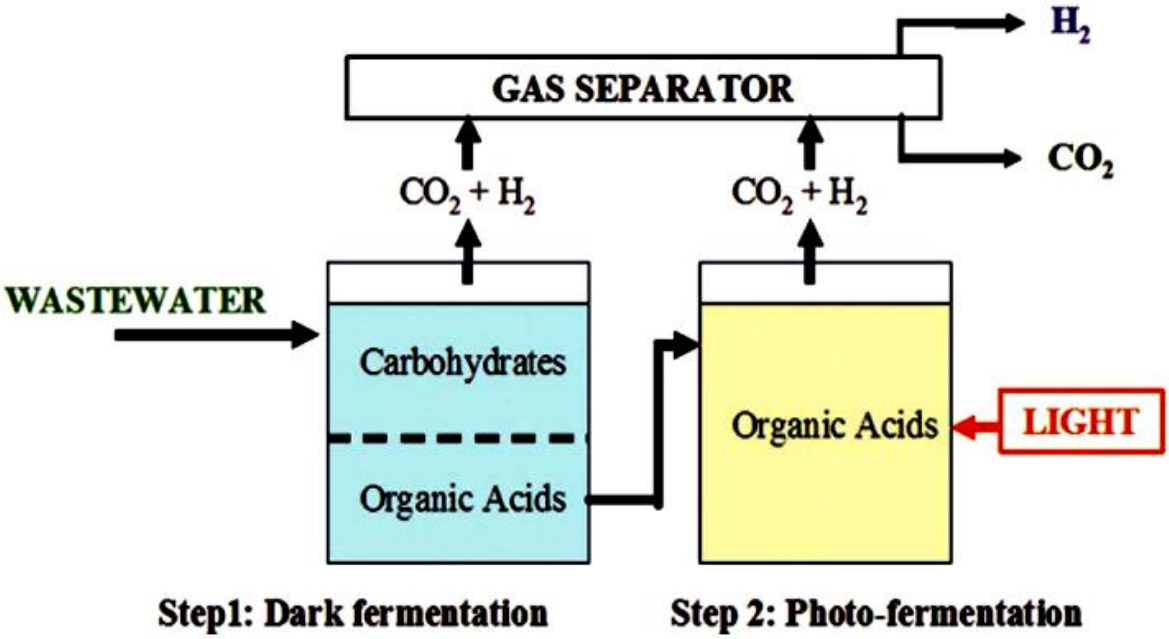

Figure 4. A two-step approach for biohydrogen production [5]

A co-immobilized two stage system using *Lactobacillus* and *R. sphaeroides* was much more successful with a maximum yield of 7 mol H_2/mole glucose (Asada et al., 2006). However, it remains to be seen if the extra manipulation and costs involved, especially if the system is run in batch mode, can be justified for any practical application. Indeed it may be difficult to rationalize the use of any type of co-culture system. Thus, a two-step approach to two-stage fermentations, where the two species are separated, may show more promise in achieving economical hydrogen production yields in large scale applications.

Finally, a new hybrid biological hydrogen production process has very recently been described and is under active study (Liu et al., 2005; Rozendal et al., 2006; Ditzig et al., 2007; Cheng and Logan , 2007; Rozendal et al., 2007; Call and Logan, 2008; Rozendal et al., 2008; Tartakovsky et al., 2008). It is based on the concept and practice of a microbial fuel cell (MFC). In fact, the idea is to add a little electrical potential to that generated by a microbial fuel cell, thus reaching a sufficient force to reduce protons to hydrogen, in a process that can be called bioelectrohydrogenesis as shown in **Fig. 5.** Thus the cell could be called a microbial electrohydrogenesis cell (MEC). (Microbial electrolysis cell is an unacceptable term since it implies that the protons are derived from water splitting.) The advantage of MFCs and MECs is that the energy available in waste streams can be directly recovered as electricity (MFC) or hydrogen (MEC). The metabolic pathways involved are not clear, and in fact thus far MEC studies have been carried out only with mixed cultures, often using those already enriched and active in microbial fuel cells (MFC).

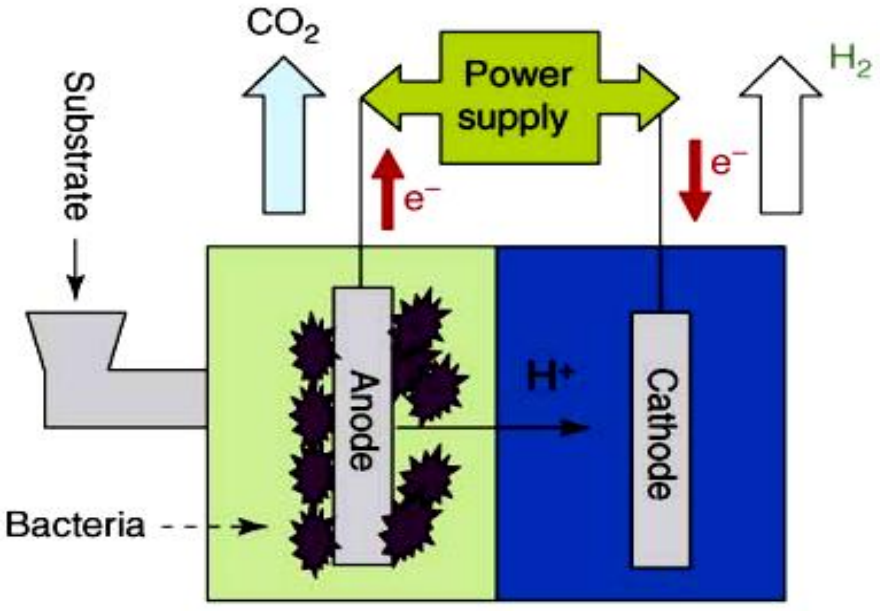

Figure 5. Hybrid biological hydrogen productions [5]

However, MFCs usually contain bacteria such as *Geobacter* and *Shewanella*, which are known to effectively couple their metabolism to electrode surfaces. These reactions are essentially anaerobic respirations where the external electron acceptor is an electrode instead of the more usual oxidized compound (nitrate, TMAO, fumurate, etc.). Thus bioelectrohydrogenesis utilizes electrochemically active micro-organisms which, with a small to moderate voltage input, convert dissolved organic matter into hydrogen inside an electrochemical cell/microbial fuel cell via coupled anode-cathode reactions. Thus, in principal, and in practice, sufficient energy can be added to allow the conversion of compounds such as acetate, products of dark fermentation, to hydrogen. Ordinarily of course, as discussed elsewhere, microbes such as these are unable to do this conversion on their own except in syntrophic association with a hydrogen-consuming organism capable of maintaining very low hydrogen partial pressures (Cord-Ruwisch et al., 1998; Methe et al., 2002). Although an appealing concept, and obviously one with the potential to permit the complete conversion of simple substrates, sugars or acetate, or even wastewaters (Ditzig et al., 2007) to hydrogen, there are a number of serious challenges in several problem areas to overcome. Not surprisingly, many of these are also faced in the further development of microbial fuel cells (Cord-Ruwisch et al., 1998). Power densities at the electrode surface are low, which translates into low volumetric hydrogen production. However, a variety of manipulations involving electrode materials and cell construction (Rozendal et al., 2007; Call and Logan, 2008; Rozendal et al., 2008; Tartakovsky et al., 2008) have increased volumetric hydrogen production by several orders of magnitude over the original reports, so that values near 1 m^3H_2/m^3 reactor liquid volume/day can now be obtained (Cheng and Logan , 2007; Tartakovsky et al., 2008). However, this remains well below that obtainable in a standard dark

fermentation. While yields of 50% or greater can be demonstrated, higher yields require increased voltage, adversely affecting energy efficiency. Other issues include the need for a noble metal catalyst in cathode fabrication, and decreased hydrogen production due to potential methanogenic reactions in both the anodic and cathodic chambers. In fact, it has been proposed that syntrophic reactions are the basis for the normal functioning of a MFC (Freguia et al., 2008), which very well could apply to MECs. Much remains to be learned about the microbiology involved in these processes, and it is likely that novel organisms can be isolated from functioning MFCs and MECs (Kodama and Watanabe, 2008; Zuo et al., 2008). Obviously, much more research is needed to address key limitations. Nonetheless, bioelectrohydrogenesis appears to be a promising future approach to hydrogen generation from wastewater, especially for effluents with low organic content.

3. Techniques for improved biohydrogen

3.1. Reactor configurations [4]

Possible improvements to biohydrogen production have been sought through specialized bioreactor configurations (**Table 1**). This has led to systems with more robust, reliable performance that are stable over long periods of time (months) and resistant to short-term fluctuations in operational parameters. In addition, optimized volumetric production rates could be obtained. Biohydrogen fermentations, as most other fermentations, can be carried out in either batch or continuous mode. Batch mode fermentations have been shown to be more suitable for initial optimization studies (Kapdan and Kargi, 2006; Davila-Vazquez et al., 2008), but any industrially feasible process would most likely have to be performed on a continuous or at least semi-continuous (fed or sequencing batch) basis. Many studies have employed continuous stirred tank reactors (CSTR) with either purified strains or microbial

mixtures (Kapdan and Kargi, 2006; Bartacek, 2007; Davila-Vazquez et al., 2008). CSTRs, the most commonly used continuous reactor systems, offer simple construction, ease of operation and effective homogenous mixing. However, in these reactors, hydraulic retention time (HRT) controls the microbial growth rate and therefore HRTs must be greater than the maximum growth rate of the organism(s), because faster dilution rates cause washout. A second conceptual category of continuous flow reactors, characterized by the means of the physical retention of the microbial biomass, overcomes this problem and offers several advantages for a practical bioprocess. Because microbial growth and the concentration of microbial biomass are rendered independent of HRT, high cell concentrations can be achieved, fostering high volumetric production rates, and high throughput is possible, allowing the use (and treatment) of dilute waste streams with relatively small volume reactors.

Indeed, many recent studies (Hawkes et al., 2007; Bartacek, 2007; Davila-Vazquez et al., 2008; Ren et al., 2007; Cheong et al., 2007; Chang et al., 2002; Zhang et al., 2007; Chang et al., 2004; Wu and Chang, 2007; Lee et al., 2006; Wu et al., 2007; Zhang et al., 2007; Oh et al., 2004; Kim et al., 2005) have shown that high volumetric production rates of hydrogen can be achieved in these reactors, and some examples are given in Table 1. Physical retention of microbial biomass has been accomplished by several different means, including the use of naturally forming flocs or granules of self immobilized microbes, microbial immobilization on inert materials, microbial-based biofilms or retentive membranes. In fact, there are so many variations that there are almost as many different reactor types, with associated acronyms (UASB, FBBAC, AFBR, ASBR, CIGSB, FBR), as there are laboratories carrying out research in this area (**Table 1**). Unfortunately, this also creates a situation in which it is very difficult to ascertain whether the differences in the various studies are due to different reactor

configurations or to differences in operational parameters. Future studies could help to resolve this ambiguity by directly comparing different reactor configurations or, at the very least, by operating a novel configuration under conditions used in previous studies. One study that specifically compared two different reactor types showed that granules gave superior performance over a biofilm, with an approximately threefold higher hydrogen production rate (Oh et al., 2004). In general, the major advance of these types of reactors over a CSTR is their greatly increased volumetric production rate, which in some cases can be up to an astounding>50-fold larger (**Table 1**). In addition, these reactor types present a favorable environment for the development and maintenance of mixed microbial consortia, which have advantages that are discussed below. A potential problem with these types of reactors is the loss of hydrogen through the formation of methane. Because cell growth is no longer directly controlled by HRT in systems using retained microbial biomass, slow growing methanogens can flourish even at high rates of liquid throughput. It should be noted that the hydrogen yields obtained with these systems are no greater than those achieved with CSTRs, and indeed there is no reason to think that the reactor type would influence the yield.

Table 1. Available dark fermentation reactors [4]

Microorganisms	Substrate	Type of reactor	H_2 rate (l H_2/l/h)
Sludge (wastewater treatment plant)	Molasses	Continuous stirred-tank reactor (CSTR)	0.20
Sludge (wastewater treatment plant)	Glucose	Anaerobic sequencing batch reactor (ASBR)	0.23
Sludge (wastewater treatment plant)	Sucrose	Fixed bed bioreactor with activated carbon (FBBAC)	1.2
Activated sludge and digested sludge	Glucose	Anaerobic fluidized bed reactor (AFBR)	2.4
Sludge (wastewater treatment plant)	Sucrose	Upflow anaerobic sludge blanket reactor (UASB)	0.27
Anaerobic sludge	Sucrose	Polymethymethacrylate (PMMA) immobilized cells	1.8
Sludge (wastewater treatment plant)	Sucrose	Carrier-induced granular sludge bed (CIGSB)	9.3
Sludge (wastewater treatment plant)	Sucrose	Fluidized bed reactor (FBR)	1.4
Sludge (wastewater treatment plant)	Glucose	Anaerobic fluidized bed reactor (AFBR)	7.6 biofilm; 6.6 granules
Sludge (wastewater treatment plant)	Sucrose	Continuously stirred anaerobic bioreactor (CSABR)	15.0
Heat-treated soil	Glucose	Membrane bioreactor (MBR)	0.38

3.2. The use of mixed microbial consortia [4]

Most of the reactors described above depend upon the formation of flocs or granules, which are macroscopic aggregates of microbial cells. The ability to form these particles is a rare trait in a pure culture, but it can be easily selected for when a mixed culture is used as an inoculum. There are also a number of other potential advantages of using microbial consortia instead of pure cultures. Industrial hydrogen fermentations will have to be carried out under non-sterile conditions using readily available complex feedstocks with only minimal pretreatment. Microbial consortia address these issues as they have been selected for growth and dominance under non-sterile conditions. As a complex community they are also likely to contain a suite of the necessary hydrolytic activities, and they are potentially more robust to changes in environmental conditions (Kleerebezem and Van Loosdrecht, 2007).

A large number of recent studies have examined hydrogen production using microbial consortia, and some relevant examples are given in **Table 1** (Li and Fang, 2007). The use of microbial consortia has indeed been proven useful in reactor systems that yield high volumetric rates of hydrogen production, as discussed above. However, there are also several issues associated with their use. As complex communities, their composition can vary over time, with changes in process parameters and from reactor to reactor, as was shown by molecular (16sRNA) studies (Koskinen et al., 2007; Maintinguer et al.,2008; Lin et al., 2008; Lin et al., 2006). A possible way to overcome this issue might be to construct 'designer' consortia (Brenner et al., 2008) with the goal of creating a community of diverse members, each contributing a unique and essential metabolic capacity. The total community metabolic range would be greater than any individual member, while at the same time mutual interdependence would assure stable maintenance of individual members. However, little is

known about the complex interactions that occur in natural consortia or how stable synthetic microbial communities could be built (Weibel, 2008). Thus, much additional fundamental work might be required before practically useful synthetic hydrogen-producing consortia could become a reality. It seems that spatial organization within a consortium might be important (Kim et al., 2008).

3.3. Isolation and properties of novel hydrogen producers [5]

Modern molecular techniques have revolutionized and expanded the scope of microbial systematics and physiology. We are now aware that only a very small fraction of what is out there has been isolated and studied in the laboratory. In the present perspective of hydrogen production technology, there is a no evidence that any naturally isolated microbe can produce more that 4 moles of H_2 per mole of glucose. Moreover, there is no clear contender for a robust, industrially capable microorganism that can be used as a platform for research to genetically alter its metabolic pathway to produce more than 4 moles of H_2 per mole of glucose equivalent. Therefore, data mining of genomic and metagenomic sequences might provide insight into potentially useful hydrogenases. As well, it is possible that continued isolation and cultivation efforts might yield novel isolates with some unique properties for waste decomposition and higher hydrogen yields and production rates.

An initial data mining study targeted genomes containing both a Ni-Fe hydrogenase and formate dehydrogenase, and found a number of interesting possibilities (Kalia et al., 2003). This screen would presumably find systems similar to the well known *Escherchia coli* Fhl system which consists of a formate dehydrogenase and hydrogenase 3, the paradigm of the Ni-Fe hydrogenase subclass known as *E. coli* hydrogenases (Hedderich and Forzi, 2005; Vignais and Billoud, 2007; Vignais, 2008) , although other possible

combinations might be found as well. Although the *E. coli* hydrogenases are now known to be wide spread, occurring in at least 56 different bacterial and 28 different archeal genomes (Vignais, 2008), sequence homology alone cannot assign physiological function and some of *E. coli* are known to preferentially carry out hydrogen oxidation. The recently described isolates that belong to the genera *Bacillus* and *Proteus* (Porwal et al., 2008) may contain this enzyme complex. Reported hydrogen yields were quite low, but conditions were not optimized. More commonly, hydrogen fermenting bacteria are thought to contain a [Fe-Fe] hydrogenase. However, their occurrence in the environment, at least in an initial metagenomic survey, appears to be quite limited (Meyer, 2004), and this is borne out by a recent blast search of over one billion base pairs of non-redundant sequence where only 10 hits to a [Fe-Fe] hydrogenase bait sequence were found (Meyer, 2007). A search of 371 fully sequenced microbial genomes only found 25, and not all these appeared to be bonafide [Fe-Fe] hydrogenase sequences (Meyer, 2007). A large number of the sequences were in microbes belonging to the Class *Clostridia*. There were a number found in the δ-proteobacteria, but many of these, as well as the lone γ-proteobacterium, *Shewanella oneidensis*, are thought to function in hydrogen oxidation, not proton reduction. Thus, in this case too, caution is required in interpreting data mining results.

A number of somewhat novel fermentative hydrogen producing bacteria have been isolated recently. *Enterobacter asburiae* SNU-1 was isolated from a domestic landfill and gave a yield of 0.43 mol hydrogen/ mol formate. It showed rather high maximum and overall hydrogen production productivities (398 and 174 ml/l/h) with glucose. Unlike many hydrogen producers, this strain produced hydrogen in both the exponential and stationary phase (Shin et al., 2007). A new thermophilic hydrogen producer *Thermoanaerobacterium*

thermosaccharolyticum PSU-2 was found to carry out an ethanolacetate type fermentation with inorganic nitrogen medium medium, whereas a butyrate-acetate type fermentation was found with a medium containing organic nitrogen. Maximum hydrogen yields were 2.53 mol $H_2.mol^{-1}$ hexose and rates were 270 ml $H_2.l^{-1}.h^{-1}$. As is typical of most fermentations, hydrogen production slowed dramatically with time due to acidification (O-Thong et al., 2008). *Citrobacter* sp. Y19, originally isolated for CO-dependent H_2 production can also ferment glucose over a wide range of temperatures (25- 40^oC) and pH (5–9) with a maximum H_2 yield of 2.49 mol H_2. mol^{-1}glucose and an H_2 production rate of 32.3 mmol $H_2.g^{-1}$cells. h^{-1} (Oh et al., 2003). Other major metabolic end products are acetate and ethanol. Another bacterium, *Rhodopseudomonas palustris* P4, isolated by the same group for its CO-dependent H_2 production abilities was also studied for its capacity for fermentative H_2 production in batch mode. Maximum H_2 yields were 2.76 mol H_2. mol^{-1}glucose and H_2 production rates were 29.9 mmol H_2. g^{-1}cell. $h^{-1}$with ethanol, acetate and CO_2 as the other major metabolites. As to be expected from the well-known effects of pH and high hydrogen partial pressures on fermentative hydrogen production (Hallenbeck, 2005), a high concentration of phosphate buffer or intermittent sparging with argon improved overall performance (Oh et al., 2002). A novel extreme thermophile, *Caldicellulosiruptor saccharolyticus* (Class *Clostridia*), was found to produce hydrogen from a variety of substrates including: glucose, xylose, and an industrial waste stream, paper sludge, as a renewable cheap feedstock, with major metabolic end products acetate and lactate. As is typically found for extreme thermophiles (Hallenbeck, 2005), maximal volumetric H_2 production rates were quite low, 9–10 mmol $H_2.l^{-1}.h^{-1}$ with simple sugars, and even lower rates, 5 to 6 mmol $H_2.l^{-1}.h^{-1}$, were found with the complex substrate indicating the possible presence of inhibiting components in paper sludge

hydrolysate (Kadar et al., 2004). Two strains of the mesophilic anaerobic bacterium *Sporacetigenium mesophilum* (Class *Clostridia*) were isolated from ananaerobic sludge digester treating municipal waste. Unusually, optimal hydrogen production was detected at pH 8.8, with a moderate yield of 1.4 mol H_2.mol^{-1}glucose and the major metabolic end products were acetate, ethanol, and CO_2, typical for a *clostridial*-type fermentation [60]. As noted previously in various other studies (Hallenbeck, 2005), yields under thermophilic conditions tend to be higher. Thus, a thermophilic bacterium, *Thermotoga neapolitana*, gave a hydrogen yield of 2.4 ± 0.3 mol H_2. mol^{-1}glucose with acetic acid and lactic acid as additional metabolic end products (Eriksen et al., 2008). For reasons that aren't clear, malonic acid addition increased H_2 yields to 3.5–3.8 mol H_2. mol^{-1} glucose. These authors were unable to reproduce the earlier surprising and even somewhat fantastic claims of microaerobic hydrogen production by this bacterium with yields greater than 4 mol H_2. mol^{-1} mol glucose (Van Ooteghem et al., 2002; Van Ooteghem et al., 2004).

3.4. Modeling and optimization [4]

Modeling and optimization have been carried out in attempts to improve biohydrogen production. Rates and yields of hydrogen production, as for many other bioprocesses, are a function of several variables, including pH, temperature, substrate concentration and nutrient availability, among others. Although many studies have reported the effects of varying individual parameters one-at-a-time (Davila-Vazquez et al., 2008), modeling and analysis could be used to determine the optimal values of the important relevant parameters. A variety of modeling methods have been developed that are broadly applicable to a spectrum of diverse fields, including engineering, biology, environmental science, food processing and industrial processing, and their application in biohydrogen production is the subject of a very recent

review (Wang and Wan, 2008). Although the metabolic pathways and fluxes are relatively well understood for a pure culture fermenting a defined substrate, this type of analysis can nevertheless have some value for modeling fermentations of complex substrates by microbial consortia that involve multiple metabolic types with unknown interactions.

One type of treatment, principal component analysis (Bernard and Bastin, 2005), was recently used to assess the effects of pH, HRT and mixing on hydrogen production (Aceves-Lara et al., 2008). Two main types of widely used modeling approaches that are pertinent to BioH_2 production are artificial neural networks (ANNs) and design of experiments (DOE). ANNs were successfully used to model the results of hydrogen production and chemical oxygen demand (COD) removal with an upflow anaerobic sludge blanket reactor (UASB) (Mu and Yu, 2007) and an expanded granular sludge bed (EGSB) reactor (Guo et al., 2008). DOE uses statistical modeling to analyze the relationship between a set of controllable independent experimental factors and to propose an appropriate design for their experimental verification (Ilzarbe et al., 2008). A common and powerful DOE approach is afforded by factorial design (FD) combined with response surface methodology (RSM) (Hanrahan and Lu, 2006). FD investigates how responses of multiple factors depend on each other and permits the identification of the most important parameters that control the process and the degree of interaction among them. FD approaches can be coupled to experimental systems by RSM, which uses a response function to fit the obtained experimental data to the theoretical design.

Several recent studies investigating dark fermentative hydrogen production have applied FD and RSM with the aim of optimizing hydrogen production (**Table 2**). Their results demonstrate that this type of analysis can indeed be used to optimize various aspects of a biohydrogen process, including pretreatment strategies (Espinoza-Escalante et al., 2008; Wang

et al., 2007), conditions for spore germination (Fan et al., 2004), micronutrient formulations (Lin and Lay, 2005; Guo et al., 2009; O-Thong et al., 2008), substrate composition (Turcot et al., 2008), carbon/ nitrogen (C/N) and carbon/phosphate (C/P) (O-Thong et al., 2008). The most useful application has been to determine the optimal operating conditions with regard to HRT (Cheong et al., 2007; Karlsson et al., 2008; Zhao et al.,2008), pH and substrate concentration, that is, the organic loading rate (OLR) (Zhao et al.,2008; Davila-Vazqueza et al., 2008; Jo, et al., 2008a; Jo et al., 2008b; Mu et al., 2008; Wang and Wan, 2008). Although all mesophilic fermentations will require similar conditions, there are sufficient differences in some of the parameters to justify the need for optimization of a particular system. In addition, these methods could prove helpful in establishing process parameters for novel consortia or complex substrates (waste streams). Finally, it should be noted that although these methods can quickly optimize a particular fermentation, they cannot further improve the yield over what could be obtained by more classical methods of optimization, as can been seen by the results reported in **Table 3**.

Table 2. Application of FD and RSM for biohydrogen production [4]

Bioprocess aspect	Parameters studied	Results		
		Optimized value	H_2 yield (mol H_2/mol hexose)	H_2 rate (l/l/day)[a]
Pretreatment	Temp, cavitation, alkalinization	Alkalinization most effective	1.55	0.63
	Temp, pH, enzyme/substrate	35 °C, 7.0, 2.5%	0.98	2.1
Seed spore germination	pH, [substrate]	5.5, 5 g/l	0.66	2.2
Micronutrient formulation	[Fe^{2+}]	3 mg/l	1.72	3.2
		180 mg/l	2.2	n/a
		260 mg/l	2.6	0.58
Substrate composition	Food waste/sewage	87:13	0.9	3.0
	C/N, C/P	74, 450	n/a	n/a
Fermenter operation	HRT	48 h	n/a	n/a
		16 h	1.6	2.9
		8 h	0.77	5
	pH	7.5[b], 7.5[c], 6.0[d]	1.8[b], 1.5[c], 1.6[d]	3.3[b], 5.2[c], 4.9[d]
		6.1	n/a	n/a
		6.5	n/a	n/a
		5.5	1.74	0.5
		7.2	2.05	6.8
	Substrate concentration (g/l)	14.5[e]	1.6	2.95
		5[b], 5[c], 15[d]	1.8[b], 1.5[c], 1.6[d]	3.3[b], 5.2[c], 4.9[d]
		21[c]	n/a	n/a
		18[c]	n/a	n/a
		25[c]	1.74	0.5
		28[c]	2.05	6.8

n/a – could not be calculated either due to the use of a complex substrate, or to lack of sufficient information.
[a]Note that in Table 1 the volumetric rates are per hour, whereas here they are per day.
[b]Lactose.
[c]Glucose.
[d]Cheese whey powder.

3.5. Moving towards complete substrate conversion [4]

However, even with the improvements noted above, hydrogen yields are restricted by the existing metabolic pathways to either 2 H_2 mol/ mol glucose (*Enterobacteriaceae*) or, at most 4 H_2 mol/ mol glucose (*Clostridia*) at very low hydrogen partial pressures. The techniques already discussed have not, and cannot, increase yields beyond these limits. These yields are, however, practically unacceptable for several reasons. For one, they are not competitive with the production of other biofuels, such as biomethane or bioethanol, from the same starting materials, because the efficiency of substrate conversion to these biofuels is 80% or greater. Furthermore, incomplete substrate conversions during hydrogen fermentations (two-thirds of the substrate is used to make other products; **see Figures 6 and 7**) lead to the production of a large amount of side products (i.e. COD), which subsequently need to be disposed of. Therefore, development of a practical biohydrogen fermentation process requires either the introduction of additional pathways that would allow near stoichiometric conversion (12 H_2 mol/ mol glucose) within the microbial cell or the development of a two-stage system that would allow nearly complete energy recovery by introducing a second process to generate energy, preferably hydrogen, from the fermentation side products that are produced in the first hydrogen-producing dark fermentation stage.

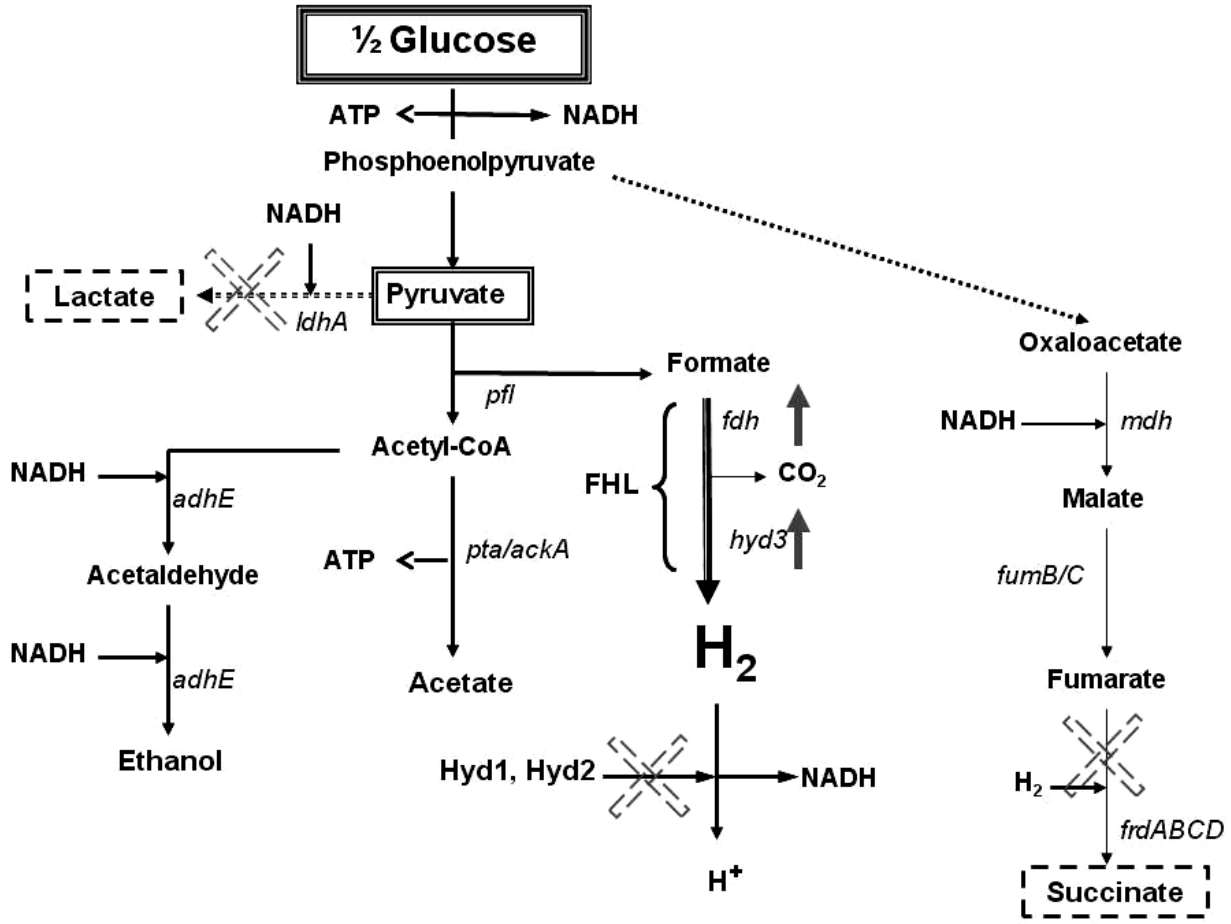

Figure 6. Metabolic engineering possibilities for increasing hydrogen production during mixed-acid fermentation by *Escherichia coli* [The mixed acid fermentation carried out by *E. coli* is shown in schematic form. Different products are possible with their relative proportion changing depending upon the redox state of the substrate and the pH. Lactate formation and formate degradation to CO_2 and H_2 are induced at acid pHs which serves to relieve the acid stress. Proportions of reduced products, principally ethanol, and acetate vary in order to maximize ATP production (via acetate production from acetyl-CoA by the pta/ackA pathway) while at the same time reoxidizing NADH to provide the NAD needed by the glycolytic pathway for further substrate utilization. Acetyl-CoA and formate are formed from pyruvate by pyruvate:formate lyase. Formate is the key metabolite in hydrogen production. Increases in hydrogen production can come from either gene inactivation, shown by a hashed X, or by increased synthesis of a particular pathway, shown by a grey filled arrow. The standard designations for the genes encoding the enzymes for the various steps are given in italics: *pfl*, pyruvte:formate lyase; *ldhA*, lactate dehydrogenase; *fdh*, formate dehydrogenase; *hyd1*, (for convenience this stands for the operons necessary to express hydrogenase 1 activity); *hyd2*, (for convenience this stands for the operons necessary to express hydrogenase 2 activity); *hyd3*, (for convenience this stands for the operons necessary to express hydrogenase 3 activity); *adhE*, alcohol dehydrogenase; *mdh*, malade dehydrogenae; *fumBC*, fumarate reductase; *frdABCD*, fumarate reductase; *pta*/*ackA*, phosphotransacetylase/acetate kinase.]

The mixed-acid fermentation carried out by *E. coli* schematically shown. Different fermentation products can arise and their relative amounts can change depending upon the redox state of the substrate and the pH of the culture. Lactate formation and formate degradation to CO_2 and H_2 are induced at acidic pH, which serves to relieve the acid stress. Succinate production from phosphoenol pyruvate is also possible but usually represents only a minor fraction of total fermentation products. The relative proportions of products are balanced to maximize ATP production (via acetate production from acetyl-CoA by the *pta–ackA* pathway) while at the same time reoxidizing NADH through the formation of reduced products, such as ethanol, to provide the NAD needed by the glycolytic pathway for further substrate utilization. Acetyl coenzyme A (acetyl-CoA) and formate are formed from pyruvate by pyruvate–formate lyase. Pathways that have been eliminated with the aim of increasing hydrogen production are shown by dashed red crosses. Enzymes in the FHL (formate hydrogen lyase) pathway that have been unregulated to increase hydrogen production are shown by green arrows. The standard designations for the genes encoding the enzymes for the various steps shown are given in italics: *adhE*, alcohol dehydrogenase E; *fdh*, formate dehydrogenase; *frdABCD*, fumarate reductase A, B, C and D; *fumBC*, fumarate hydratase B and C; *hyd1*, (for convenience this stands for the operons necessary to express hydrogenase 1 activity); *hyd2*, (for convenience this stands for the operons necessary to express hydrogenase 2 activity); *hyd3*, (for convenience this stands for the operons necessary to express hydrogenase 3 activity); *ldhA*, lactate dehydrogenase A; *mdh*, malate dehydrogenase; *pfl*, pyruvate–formate lyase; *pta–ackA*, phosphotransacetylase–acetate kinase A [4]

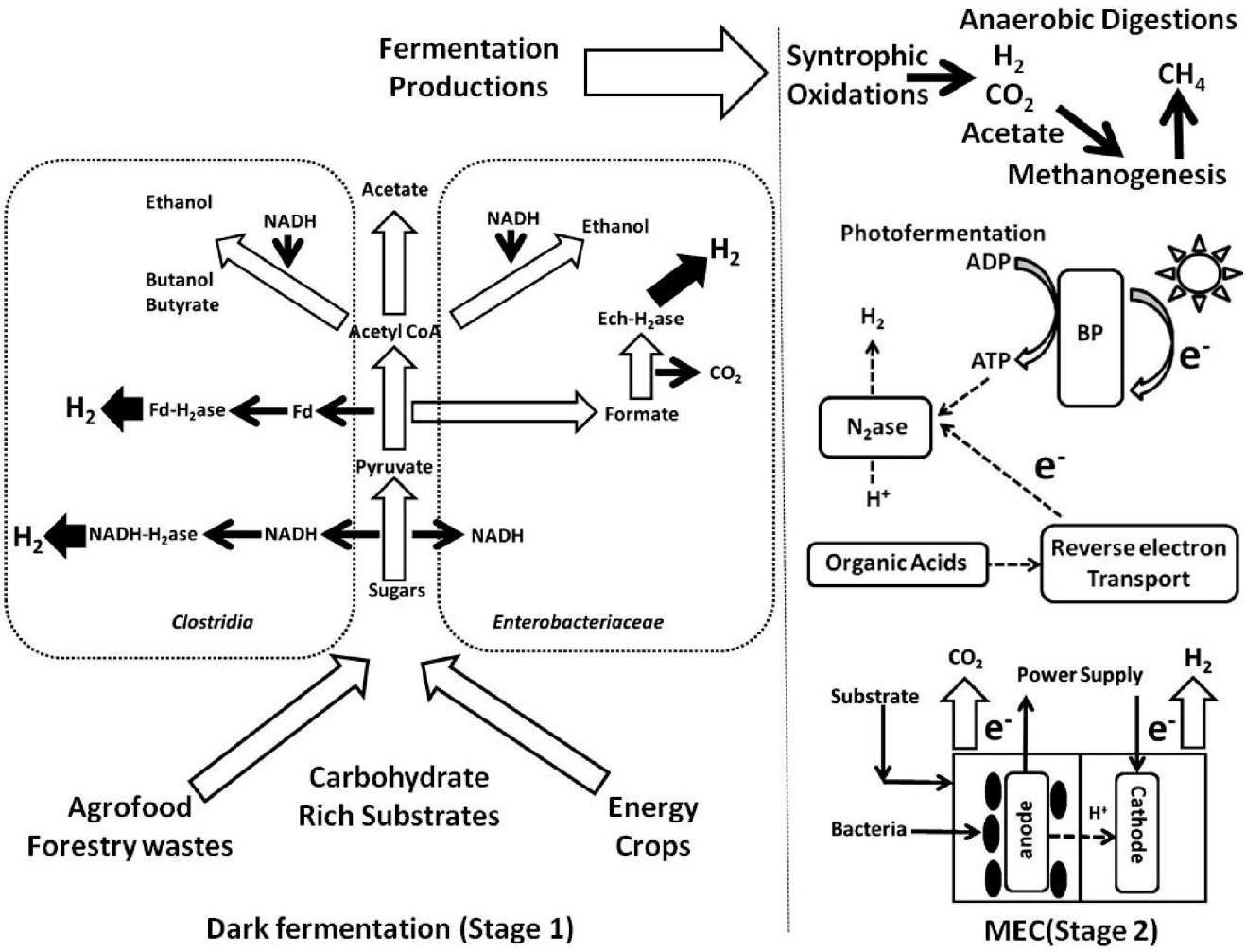

Figure 7. Different possible two-stage systems for the complete conversion of substrate. In the first stage (shown on the left) substrate is fermented to hydrogen and various fermentation products, which vary with organism and conditions. The two types of fermentation pathways are shown within the box on the left. Glycolysis of sugars produces pyruvate, from which hydrogen and other fermentation products are derived. Hydrogen is either produced from formate via an *E.coli* (Ni-Fe) hydrogenase (H_2ase) in enterobacterial-type fermentation (right-hand side box) or from reduced ferredoxin via an Fe-Fe hydrogenase in *Clostridia*-type fermentations (left-hand side box). Additionally, hydrogen can be derived from NADH in Clostridia-type fermentations, although the molecular details are unclear and might involve an NADH dependent Fe-Fe hydrogenase, at least in a few cases (as shown by the dashed pink box). In both cases, the formation of acetate provides ATP synthesis. As discussed in the main text, acetate and the other fermentation products can then be fed into a second stage reactor (right panels), in which additional energy is extracted, either in the form of methane in the case of anaerobic digestion or hydrogen for photofermentations or MECs. In the latter case, additional energy is required for the second stage of the process: either light for photofermentation or electricity for MECs. Anaerobic digestion as a second stage is shown on the upper right side. Anaerobic digestors comprise a community of different organisms whose concerted action is to convert the various fermentation products of the first stage into methane.

Archaea carry out methanogenesis and can directly use the acetate produced in the first stage. Associated syntrophic bacteria oxidize other fermentation products from the first stage to substrates that can be used in methanogenesis, such as acetate, H_2 and CO_2. Photofermentation as a second-stage process is shown in the middle right scheme. Usually, a pure culture of a purple non-sulfur photosynthetic bacterium is used to convert the organic acids produced in the first stage dark fermentation to hydrogen. These organisms use the captured light energy to drive reverse electron flow, producing the necessary low potential electrons as well as the ATP required for the nitrogenase (N_2ase), which in this case is the hydrogen-producing enzyme system. A variation on this theme would be inclusion of both metabolic types of organisms in a single photo bioreactor. Indeed, it has been shown that glucose could be converted to hydrogen at a respectable yield of 7 H_2/glucose (60%) by an immobilized co-culture consisting of *Lactobacillus*, which converted glucose to lactate, and the photosynthetic bacterium *Rhodobacter sphaeroides*, which converted the lactate to hydrogen (Asada et al., 2006). A microbial electrohydrogenesis cell as a second stage is shown in the lower right scheme. Here, bacteria in the anodic chamber degrade the fermentation products from the first stage and donate electrons to the anode. Additional voltage is added via a power supply and hydrogen is evolved at the cathode in the cathodic chamber

3.6. Choice of substrates

3.6.1. Wastewater substrates [5]

Biohydrogen production from simple sugars has been well researched; however, relatively few studies have dealt with using industrial/domestic wastewater as a potential feedstock. In a recent study, the ability of a mixed culture to produce hydrogen from composite chemical wastewater (CW) in conjunction with co-substrates was studied (Venkata Mohan et al., 2007), The CW was an heterogenous mixture of pharmaceuticals, pesticides, wastes from numerous chemical processing units and synthetic wastewater (SW) containing glucose (2 g/L) and nutrients, domestic sewage wastewater (DSW) and glucose served as co-substrates. It was found that a 40%/60% mixture of CW/DSW gave the highest yield and highest relative H_2 production rate, followed by a 40%/60% mixture of CW/SW+1 g/L glucose. Interestingly, synthetic wastewater alone showed poor hydrogen evolution, as did increasing the glucose co-substrate concentration. Various studies have shown that addition of trace nutrients does not consistently increase hydrogen gas production (Oh and Logan, 2005; Van Ginkel et al., 2005).

Of course with waste streams of complex composition, COD is a convenient measure of substrate potentially available for conversion to hydrogen while at the same

3.6.2. Biomass substrates [5]

Plant biomass, agricultural wastes and industrial effluents from such sectors as the pulp/paper and food industries represent an abundant potential source of substrate for the production of biohydrogen. Production of hydrogen by dark fermentation of cellulosic and lignocellulosic material usually requires substrate pretreatment procedures (**Table 3**), which significantly increase the hydrogen production cost. (Note that the same would be true for the production of bioethanol or most other biofuels). Different microorganisms have been studied in order to obtain higher efficiencies in microbial hydrolysis of a variety of cellulosic materials (Lo et al., 2008). Significant amounts of hydrogen can be produced from cellulosic feedstocks (straw, wood chips, grass residues, paper waste, saw dust, etc) using natural consortia and conventional fermentors operated under conditions that favor H_2-producing bacteria able to degrade cellulose for example, *Clostridium thermocellum* (Levin et al., 2006). The hydrogen production obtained is however variable and depends greatly on the bacterial consortium and culture medium. As an alternative, a two-stage hydrolysis-fermentation approach can be used with a comparable biohydrogen production efficiency to that reported in other studies using cellulose or hydrolyzed cellulose as the substrate (Lo et al., 2008). Other types of waste such as municipal food waste and sewage sludge have also been studied and a combination of feedstocks in optimal ratios can significantly enhance the production of hydrogen as compared with the individual wastes (Zhu et al., 2008).

Table 3. Comparison of pretreatment strategies for lignocellulosic degradation [6]

Process	Possible advantages	Possible disadvantages
Separate hydrolysis and fermentation	– Reactor size and operating conditions easily optimized – Enzyme specificity and efficiency can be adjusted to substrate – Newly discovered or engineered enzymes easily incorporated	– Enzyme production costly – Enzymes may need to be cloned from different sources – Two stage system required
Native consolidated bioprocessing	– Direct conversion of cellulose to biofuel possible – Single stage process; simpler facility, ease of operation – Avoidance of glucose inhibition of cellulose degradation – Uses existing metabolic machinery	– Optimal temperatures for cellulose degradation and fermentation may be different – Low rates and yields of useful products by native organism – Low titres of active enzymes due to inefficient anaerobic growth
Engineered consolidated bioprocessing	– Optimal cellulose degradation capacity in efficient fermenter – Single stage process – Cost-effective production of cellulases – Designer cellulosomes can be constructed	– Need for complex metabolic engineering, expression of multiple components – May lack synergy factors found in native organism

3.7. Microbial Platforms and Metabolic engineering for biofuels [3]

A variety of biofuels are possible through metabolic engineering (**Figure 8**). Alternative fuels that are currently being investigated include; bioethanol, biobutanol, longer-chain alcohols, biohydrogen, and fatty acid derivatives such as biodiesels and alkanes. It should be noted in addition that some of these compounds are useful chemical feedstocks. The advanced liquid biofuels under development could have a number of advantages over bioethanol as alternative fuels; an energy content closer to gasoline (giving higher fuel economy), less corrosive (permitting use of existing pipelines) and less volatile, and the ability to be used undiluted in existing internal combustion engines. However, since microorganisms do not naturally produce these compounds in high quantities, application of metabolic

engineering is necessary to obtain efficient and economical bioconversion of feedstocks to biofuels at high rates and near stoichiometric yields.

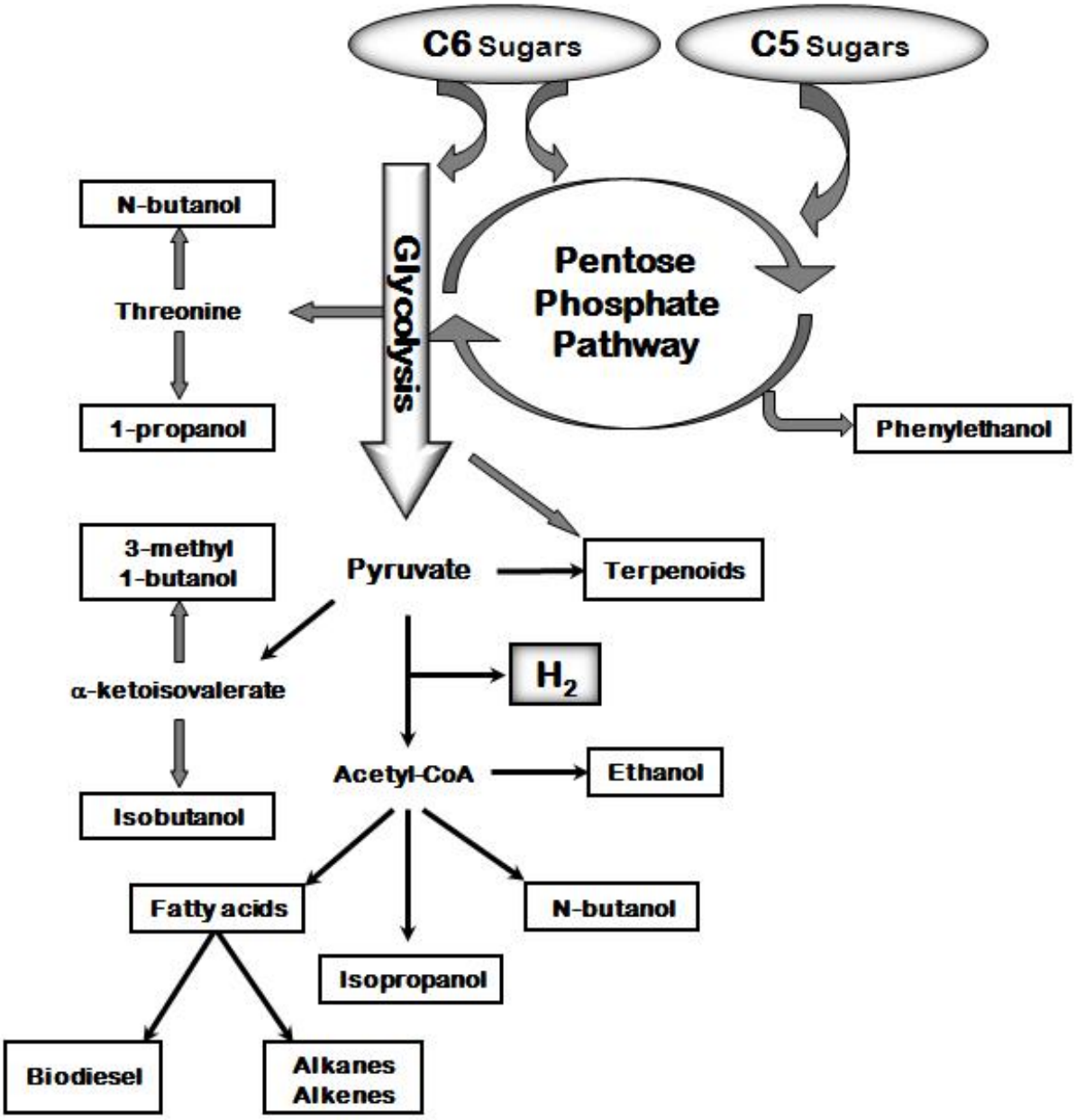

Figure 8. Possible Biofuels. [A variety of different biofuels are accessible using microbial metabolic capabilities. Some products can be made through traditional fermentation, with suitable modifications to increase yields and rates (solid outlines). In addition, existing biosynthetic pathways can be subverted to make a variety of biofuels (dashed outlines).]

Two strategies can be used to produce advanced biofuels from the metabolic intermediates generated from the breakdown of hexoses and pentoses (**Figure 9**). Metabolic engineering can be used to increase flux through an existing pathway thus increasing rates and yields of a naturally occurring metabolic production that can usefully serve as a biofuel. Examples here include ethanol, butanol, and, as described in greater detail below, hydrogen. Strategies that can be used include blocking side pathways that divert the metabolite pool to undesirable side products, or boosting the levels of key enzymes in the desired pathway. N-butanol (also often called biobutanol), has some favorable characteristics in comparison with ethanol and is currently under active commercial development by a number of companies, including Gevo Technologies and a joint BP-Dupont venture. Butanol is a natural fermentation product of solventogenic Clostridial species, and there are a number of ways to enhance its production, including metabolic engineering (Lee et al., 2008a; Lee et al., 2008b; Nakayama et al., 2008; Gheshlaghi et al., 2009).

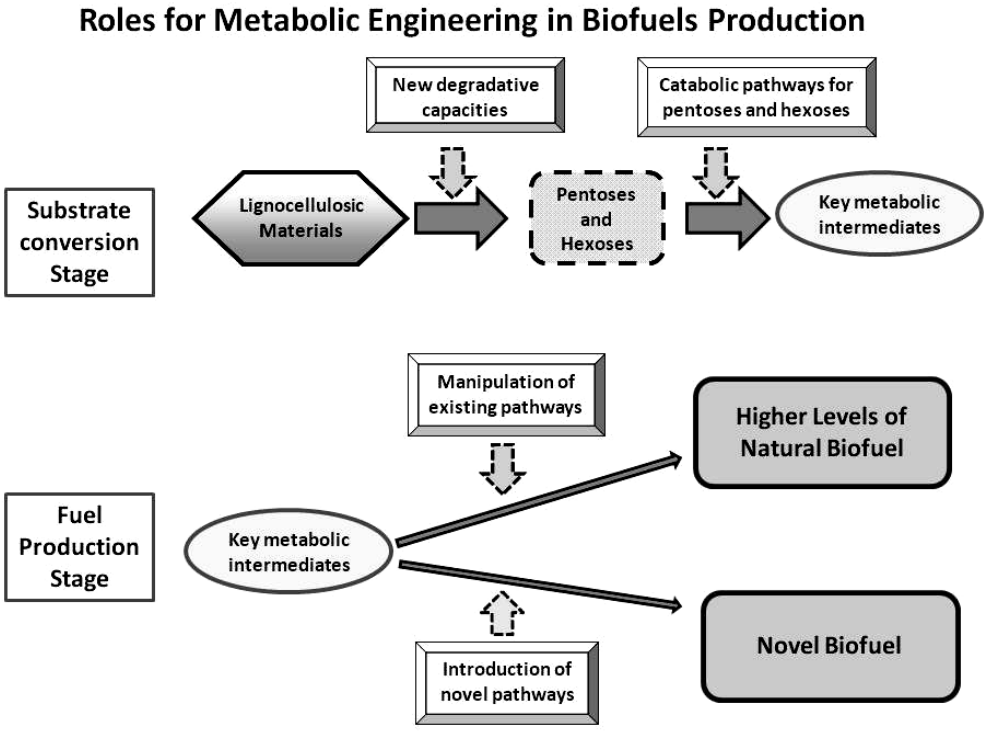

Figure 9. Roles for Metabolic Engineering in Biofuels Production. [Metabolic Engineering can be used to expand biofuels production in a number of ways. First, in substrate conversion, metabolic engineering makes it possible to create organisms with the newly acquired ability to degrade complex lignocellulosic substrates, by far the largest substrate pool available from non-food biomass. As well, organisms with strong downstream capabilities can have their substrate range extended to include the capacity to use the pentoses and hexoses derived from lignocellulosic substrates. Secondly, the conversion of key metabolic intermediates to biofuels can be augmented in two ways. Changes in existing pathways can increase the production of a biofuel that is normally made by that organism. New enzymes and pathways can be added to enable the organism to produce novel biofuels.]

Numerous studies have now shown that it is possible to make novel biofuels by the judicious addition of enzymes and pathways to an organism, most commonly of course *Escherchia coli* (Shen et al., 2008; Yan et al., 2009). Thus, *E. coli* can be made to make isopropanol in a number of ways (Hanai et al. 2007; Atsumi et al., 2008a), n-butanol [Atsumi et al., 2008b; Inui et al., 2008) or even isobutanol (Atsumi et al., 2010). Similarly, a butanol producing strain of *S. cerevisiae* has been created (Steen et al., 2008). The keto-acid pathways associated with synthesis of the amino acids threonine and norvaline have successfully been manipulated to produce a variety of butanol derivatives (Shen and Liao, 2008; Atsumi and Liao, 2008; Cann and Liao, 2008; Connor and Liao, 2008). Similarly, terpenoid biosynthetic pathways (Dewick, 1999) can be successfully adated to produce a variety of molecules of potential interest as biofuels (Rude and Schirmer, 2009; Fortman et al., 2008; Withers et al., 2007). A variety of fatty acid ethyl esters (biodiesel) and alkanes and alkenes are available through fatty acid biosynthesis pathways which use acetyl-CoA as precursor (Kalscheuer et al., 2006; Steen et al., 2010).

3.8. Metabolic engineering for hydrogen production [3]

Biological hydrogen production has been extensively studied over the past several decades as a means of producing a sustainable, renewable biofuel (Hallenbeck and Benemann,

2002; Hallenbeck, 2005; Hallenbeck, 2009; Hallenbeck and Ghosh, 2009). While the biofuels discussed above are liquid and thus most could be either blended with gasoline or diesel for use in existing engines or even used neat, hydrogen, a gas, would require novel power conversion technologies and infrastructure. Thus, while other biofuels can be short term solutions, hydrogen could only be adopted on a large scale over a longer term. Nevertheless, much effort is being devoted towards developing a future hydrogen economy, particularly in regards to its use as a mobile fuel source, and worldwide there are a already a number of demonstration projects, and prototype hydrogen powered vehicles have been created by most of the major automotive manufacturers. The impetus for this is that, at least in theory, hydrogen appears to have a number of advantages over other biofuels. First, it can be converted to electricity to power the drive train by fuel cells, a process considerably more efficient than the combustion currently required for the conversion of other potential fuels to mechanical energy. Secondly, its breakdown generates no pollutants whereas combustion of other biofuels gives a number problematic compounds potentially leading to appreciable levels of carcinogens, and smog. Thirdly, although all biofuels are largely carbon neutral since the carbon released by their combustion is derived, directly or indirectly, from recently fixed atmospheric CO_2, carbon emissions associated with biohydrogen occur during its production rather than during its utilization, thus potentially permitting easy capture of CO_2 and sequestration. Thus, in this scenario, biological hydrogen production could even be carbon negative.

Due to these factors, much research effort is going into developing biological process for hydrogen production, necessary if there is to be a renewable source of hydrogen since >90% is now made by steam reformation of methane. Although hydrogen can be made by

electrolysis using renewably generated electricity, e.g. wind turbines, photovoltaic cells, electrolysis with current technologies is only about 65% efficient and, in addition, would represent the conversion of a high quality energy carrier which could be used to supply stationary power demands currently supplied mainly by burning fossil fuels to one of lower quality. There are several possible routes to biological hydrogen production, including the use of photosynthetic systems to capture solar energy and convert the captured energy to hydrogen, and dark fermentation systems that could potentially use various waste streams or energy crops containing energy stored in lignocellulose (Hallenbeck and Benemann, 2002; Hallenbeck and Ghosh, 2009). Many different aspects of fermentative hydrogen production are currently under investigation, including optimization of reactor configuration, use of mixed microbial consortia and co-cultures, development of novel isolates, reverse micelles and the introduction of hybrid two-stage systems (Hallenbeck and Ghosh, 2009). However, the conversion efficiencies and hydrogen yields achieved using these approaches are still suboptimal (Hallenbeck, 2009; Hallenbeck and Ghosh 2009; Hallenbeck et al., 2009). Here we examine efforts underway to use metabolic engineering to increase the rates and yield of hydrogen production. As suggested in **Figure 9**, improvement strategies could include either mutation of existing pathways to increase metabolic flux towards product formation, or the introduction of novel pathways.

3.8.1. Mutation of existing pathways with special emphasis on dark fermentation [3]

Metabolic engineering has only relatively recently been applied for the improvement of dark biohydrogen production, with several different technologies and approaches being used in the last few years. Increasing dark biohydrogen production by existing metabolic networks can be attempted by increasing the metabolic flux (Stephanopoulos and Simpson,

1997; Stephanopoulos, 1999) through gene knockouts of competing pathways or enhanced homologous expression of specific enzymes involved in the biohydrogen production. Most of studies have used the laboratory workhorse for metabolic engineering, *E. coli* for the usual reasons; its genome can be easily manipulated, its metabolism is the best understood amongst all available bacteria, and it readily utilizes a wide range of carbon sources. In addition, a highly curated metabolic pathway database, *EcoCyc*, is available for metabolic pathway prediction (Keseler, 2005). As well, *E. coli* has potential advantages over at least some other microorganisms which produce hydrogen through dark fermentation in that it exhibits rapid growth and has simple nutritional requirements.

E. coli's natural metabolism constrains biohydrogen molar yields to 2 H_2/glucose, **figure 11**. On the other hand, strict anaerobic bacteria, in particular *Clostridia*, are in theory capable of generating up to 4 H_2/glucose, **figure 10** (Hallenbeck, 2005; Hallenbeck, 2009). However, although theoretical hydrogen yields are greater with strict anaerobes such as *Clostridium* sp., facultative anaerobes have some possible advantages for hydrogen production; as they are less sensitive to oxygen, they are able to readily recover if accidentally exposed to oxygen, and have faster growth and hydrogen production rates (Shin et al., 2007).

Most work on metabolic engineering for hydrogen production has been with *E. coli*, and recent studies have provided evidence that in principle metabolic engineering can be used to achieve the theoretically predicted maximal biohydrogen yield. As can be seen from the metabolic pathway for glucose fermentation by *E. coli* shown in **figure 11**, the possible products of fermentation are hydrogen, acetate, ethanol, lactate, formate and succinate **(**Clark, 1989**)**. *E. coli* is well adapted for anaerobic metabolism and possesses four different hydrogenase operons (*hya, hyb, hyc, hyf*); all encoding Ni-Fe hydrogenases (**Table 4**).

Table 4. Genes of four hydrogenase operons (*hya, hyb, hyc, hyf*) of *E.coli*

H_2ase type		Gene	Size, bp	Description
Hydrogen oxidizing	H_2ase1	*hyaA*	372	small subunit
		hyaB	597	large subunit
		hyaC	235	*b*-type cytochrome subunit
		hyaD	195	maturation protease
		hyaE	132	processing of HyaA and HyaB
		hyaF	285	nickel incorporation H_2ase1
	H_2ase2	*hybO*	372	small subunit
		hybA	328	4Fe-4S ferredoxin-type
		hybB	392	component
		hybC	567	*b*-type cytochrome subunit
		hybD	164	large subunit
		hybE	162	maturation protease
		hybF	113	specific chaperone
		hybG	82	nickel chaperone H_2ase 1 and 2 accessory protein
Energy conserving proton reducing	H_2ase3	*hycA*	153	Regulator of the transcriptional
		hycB	203	regulator FhlA
		hycC	608	Fe-S subunit
		hycD	307	membrane subunit
		hycE	569	membrane subunit
		hycF	180	Hydrogenase large subunit
		hycG	255	Fe-S protein
		hycH	136	Hydrogenase small subunit
		hycI	156	maturation protease

	H_2ase4	*hyfA*	205	4Fe-4S subunit
		hyfB	672	membrane subunit
		hyfC	315	membrane subunit
		hyfD	479	membrane subunit
		hyfE	216	membrane subunit
		hyfF	526	membrane subunit
		hyfG	555	subunit
		hyfH	181	Fe-S subunit
		hyfI	252	Fe-S subunit
		hyfJ	137	processing
		hyfR	670	formate sensing transcriptional activator,

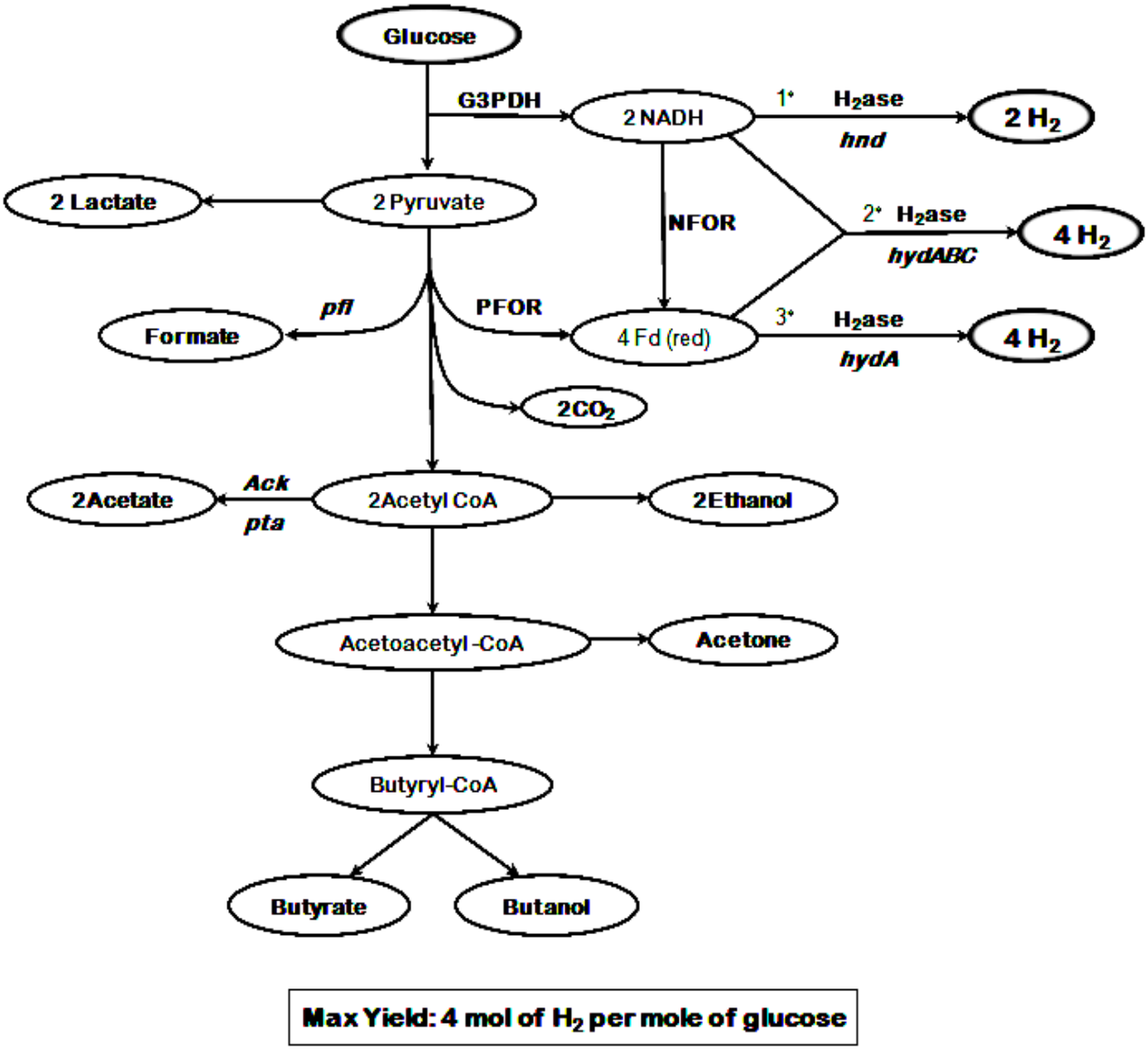

Figure 10. Metabolic pathways relevant for hydrogen production in *Clostridia.* [Fermentative hydrogen production from glucose by *C. acetobutylicum*, a strict anaerobic bacterium. Hydrogen can be produced through the action of PFOR and NFOR. The maximum theoretical hydrogen yield is 4 mol of H_2 per mole of glucose, with acetate or acetone as the fermentation end-product. The glucose metabolic pathway results in lactate, acetate, ethanol, acetone, butanol and butyrate as fermentation end-products. The proteins shown in bold with an asterisk have been studied in *Clostridium* species through metabolic engineering in order to enhance biohydrogen production. G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PFOR, pyruvate ferredoxin oxidoreductase; NFOR, NADH:ferredoxin oxidoreductase; NADH, nicotinamide-adenine dinucleotide; red, reduced. Three different types of [Fe-Fe] hydrogenases have been described in Clostridia. HydA, the classical clostridial hydrogenase that reacts with reduced ferredoxin, Hnd, a NAD(P)H reactive hydrogenase, and HydABC a novel bifurcating hydrogenase that reacts simultaneously with NADH and reduced ferredoxin.]

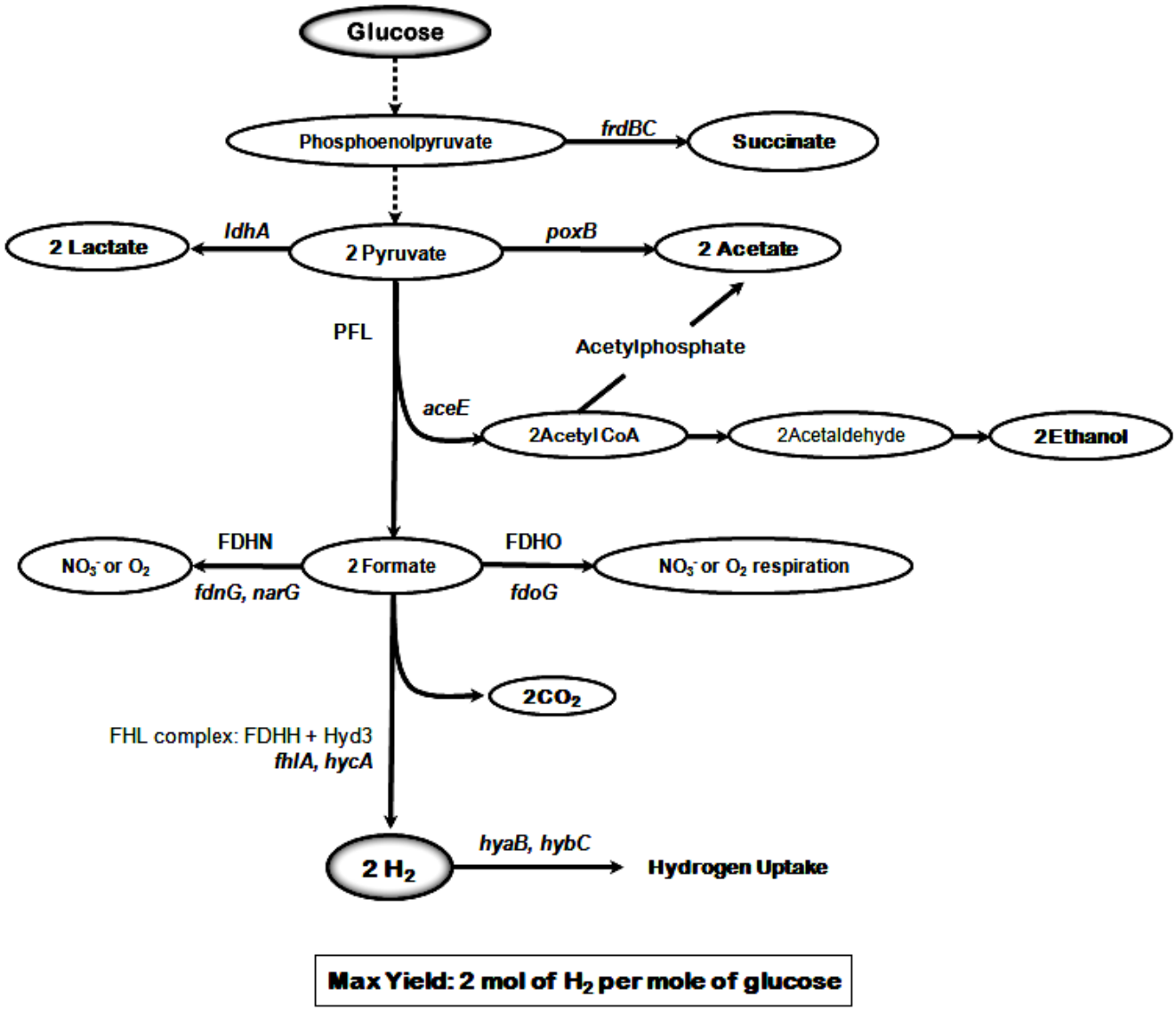

Figure 11. **Metabolic pathways relevant for hydrogen production in *Escherchia coli.*** [Fermentative hydrogen production from glucose by *E. coli*, a well-studied facultative anaerobic bacterium. Hydrogen is produced through the action of the FHL complex. The maximum theoretical hydrogen yield is 2 mol of H_2 per mole of glucose. The glucose metabolic pathway yields succinate, lactate, acetate, ethanol and formate as fermentation end-products. The proteins shown in bold with an asterisk have been studied through metabolic engineering in order to enhance the biohydrogen production. PFL, pyruvate formate lyase; FDH, formate dehydrogenase; FHL, formate hydrogen lyase; Hyd, hydrogenase; CoA, coenzyme A.]

In addition, it has an operon, *hyp*, encoding additional factors necessary for the maturation of these hydrogenases. The formate hydrogen lyase (FHL) complex enzyme system is the key player in hydrogen production by this organism and catalyzes the reversible generation of equimolar amounts of H_2 and CO_2 by oxidizing formate in a homeostatic reaction that reduces the acidification caused by mixed acid fermentation (Rossman et al., 1991). Thus, metabolic flux into formate, formed from pyruvate under anaerobic conditions by pyruate:formate lyase (Pfl), is a key factor. Therefore there is an obvious role for metabolic engineering in attempting to manipulate the flux into and out of the formate pool. On the one hand, knock out mutations can direct greater metabolite partitioning into formate, and up-regulation of downstream enzymes, the FHL pathway, can ensure efficient conversion of formate into hydrogen (**Table 5 and figure 6**). Another factor to be considered is the possible decrease in net product (hydrogen) formation which might be caused by so-called uptake hydrogenases.

Table 5. Targets for Modification of Existing Pathways in *E.coli*

Gene	***Function***	***Mode of action***	***H_2 Production***
ldhA	Lactate dehydrogenase	Inactivation eliminates a drain on the pyruvate pool	Small increase (~20-30%)
frdBC	Fumarate reductase	Inactivation eliminates side reaction in EMP, increases pyruvate pool	Small increase (~20-30%)
hycA	Inhibitor of *fhlA* expression	Inactivation increases synthesis of FhlA, increase in FHL complex	No to small increase
fhlA	Activator of Fhl expression	Introduction of constitutive allele (FhlA*), increase in FHL complex	Small (5-10%)
hyd1, hyd2	Uptake hydrogenases	Inactivation prevents H_2 oxidation	Modest increase (~35%)
hyd3	Ech hydrogenase	H_2 evolving, FHL H_2ase	Large increase over low basal level

A variety of recombinant strains of *E. coli* have been developed with mutations in several different genes, for example, in the large subunit of uptake Hyd-1 and -2 (*hyaB* and *hybC* respectively) (Maeda et al., 2007a; Maeda et al., 2007b), in lactate dehydrogenase (*ldhA*) (Bisaillon et al., 2006; Yoshida et al., 2006; Maeda et al., 2007b) in the FHL repressor (*hycA*) (Penfold et al., 2003; Yoshida et al., 2005; Yoshida et al., 2006; Penfold et al., 2006; Maeda et al., 2007a; Maeda et al., 2007b) in the FHL activator (*fhlA*) (Yoshida et al., 2005; Maeda et al., 2007a; Maeda et al., 2007b; Bisaillon et al., 2006; Yoshida et al., 2006) in fumarate reductase (*frdBC*) (Maeda et al., 2007b; Yoshida et al., 2006), in the Tat system (*tatA–E*) (Penfold et al., 2003; Penfold et al., 2006), in the alpha subunit of the formate dehydrogenase-N and -O (*fdnG* and *fdoG* respectively) (Maeda et al., 2007a; Maeda et al., 2007b), in the alpha subunit of nitrate reductase A (*narG*) (Maeda et al., 2007a; Maeda et al., 2007b), in pyruvate dehydrogenase (*aceE*) (Maeda et al., 2008), in pyruvate oxidase (*poxB*) (Maeda et al., 2007b) and in proteins that transport formate (*focA* and *focB*) (Maeda et al., 2007a; Maeda et al., 2007b) (**Figures 11 and 6**). Some of the salient points are discussed below. There are several pathways which can drain the pyruvate pool and thus affect proton reduction (Gottschalk, 1986; Mathews and Wang, 2009). Two competing pathways that have been specifically targeted are lactate production via lactate dehydrogenase (*ldhA*) and succinate production, involving malate dehydrogenase (*mdh*) and succinate dehydrogenase (*frdABCD*) and these have been mutated in attempts to increase hydrogen production. Lactate dehydrogenase deficient mutants show small but significant increases in net hydrogen production (Sode et al., 1999; Bisaillon et al., 2006; Yoshida et al., 2006; Maeda et al., 2008). The action of fumarate reductase (*frdBC*) could in principal also drain the pyruvate pool since it catalyses the synthesis of succinate from phosphoenolpyruvate, a glycolytic intermediate on

the pathway to pyruvate formation. However, little gain is to be expected from inactivation of fumarate reductase since normally there is only a relatively small (10-15%) carbon flux through this pathway (Belaich and Belaich, 1976; Clark, 1989; Alam and Clark, 1989). Indeed, inactivation of the *frd* genes leads to only a modest increase in H_2 yields (Maeda et al., 2007; Yoshida et al., 2006). Net hydrogen production may be compromised by hydrogen oxidation in some of these strains which show higher than expected ethanol production (Sode et al., 1999). Although it may seem paradoxical for the cell to use reductant to produce hydrogen and then to capture the hydrogen to remake reductant (NADH), since the two hydrogenase activities involved have different cellular localizations this process of hydrogen recycling could potentially allow the formation of a proton gradient across the membrane which could then drive ATP synthesis (**Figure 12**).

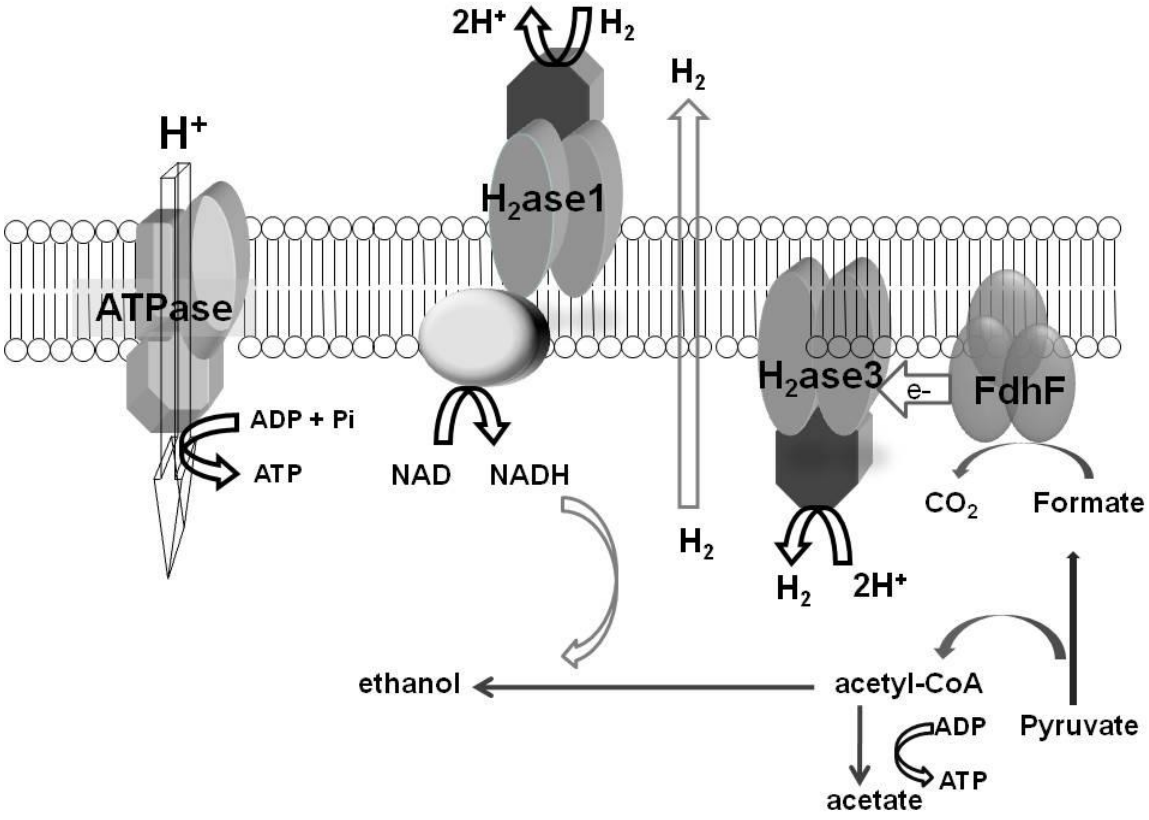

Figure 12. Potential hydrogen recycling in *E. coli.* [The differential localization of different hydrogenases in the same organism creates a potential for hydrogen recycling and the development of a proton gradient across the cytoplasmic membrane. In *E. coli*, Hyd3, the hydrogenase responsible for hydrogen production is oriented towards the cytoplasm, where it reduces protons with electrons derived from formate to hydrogen. Hyd3 and FdhF, the membrane associated formate dehydrogenase that acts under fermentative conditions, along with two membrane bound electron carriers, constitute the FHL complex. The hydrogen that is produced freely diffuses across the cytoplasmic membrane to the periplasm where it encounters Hyd1. Hyd1 oxidizes the hydrogen, leaving protons in the periplasm and the extracted electrons are used to reduce NAD to NADH on the cytoplasmic side. Note that the overall reaction represents the transfer of protons from the cytoplasm to the periplasm. The resultant proton gradient can then be used to drive ATP synthesis or other energized membrane dependent processes, for example chemotaxis. NADH is used to reduce acetyl-CoA to ethanol to maintain redox balance.]

Several different approaches can, and have, been used to attempt to increase flux out of the formate pool and into hydrogen. The key target here is the FHL complex, composed of a specific formate dehydrogenase (FdhF), several membrane bound electron carriers, and a hydrogenase poised for hydrogen evolution (Hyd3), which is normally only fully expressed under acidic conditions and is regulated by several control elements. In fact, there is no reason for the cell to dispose of formate other than to reduce acid accumulation and at pHs near neutrality appreciable levels of formate can accumulate, which obviously negatively impacts net hydrogen yields. One obvious remedy is to drive the overexpression of the FHL complex. Thus, for example, *E. coli* strain HD701, which cannot synthesize the FHL complex repressor (*hyc*A), shows twice the volumetric hydrogen production rate of the parent strain *E. coli* MC4100 (Penfold et al., 2003) at a glucose concentration of 100 mM. The difference in rates between wild-type and the recombinant strain were even greater at lower glucose concentrations. Similar effects were seen when industrial waste water with high sugar content was used as substrate instead of glucose (Penfold et al., 2003) and introduction of an invertase gene, which converts sucrose to glucose and fructose, enabled strain HD701 to utilize sucrose

and produce hydrogen with a rate of 3.2 mmol (mg protein)$^{-1}$ h^{-1} (Penfold and Macaskie, 2004).

Likewise, *E. coli* WDHL (Δ*hyc*A and Δ*lac*I) which was constructed to improve the molar hydrogen yield from lactose through overexpression of FHL activator (*fhl*A) and constitutive expression of *lac* operon gave 2.74 mol H_2.mol^{-1} lactose consumed, which is comparable to the yields achieved with various Clostridial strains or mixed microbial consortia (Rosales-Colunga et al., 2009). In another study, several different mutations were introduced in the FHL control system. A strain in which *fhlA* was overexpressed had 1.7-fold higher hydrogen production, whereas a *hycA*-inactivated strain had 1.2-fold higher hydrogen production. *E. coli* SR13 was constructed by combining FHL repressor (*hycA*) inactivation with FHL activator (*fhlA*) overexpression caused a 6.5-fold increase in formate dehydrogenase (*fdhF*) expression and a sevenfold increase in Hyd-3 large subunit (*hycE*) expression. This increased hydrogen production from formate 2.8 fold in comparison to the wild type *E. coli* W3110, and use of high cell densities of this strain and formic acid allowed high volumetric rates of hydrogen production (Yoshida et al., 2005).

As discussed above, net biohydrogen evolution in *E. coli* is potentially lowered by the action of uptake hydrogenases (Hyd1 and Hyd2), which would recycle a portion of the hydrogen produced, and respiratory formate dehydrogenases, which could oxidize a portion of the formate generated from pyruvate. The respiratory (uptake) hydrogenases, Hyd-1 and -2, and two of the formate dehydrogenases, FdH-N and FdH-O of *E. coli* are located on the periplasmic side of the cytoplasmic membrane and are transported across this membrane by the twin-arginine (Tat) protein translocation system, composed of TatABCE (Berks et al., 2005). Thus, inactivation of the Tat protein translocation system might be expected to improve

hydrogen production. Indeed deletion mutagenesis of the Tat protein translocation system (Δ*tat*C and Δ*tat*A-E) gave an increase in hydrogen evolution rates for several different derivative strains (Penfold et al., 2006). Although this approach demonstrates that improvement in net hydrogen yields can be achieved by decreasing or removing Hyd1 and Hyd2 activity, it might be problematic to use it on a more practical level due to pleiotrophic phenotypes associated with Tat mutants including slow growth and defective outer membranes (Stanley et al 2001). Indeed, direct elimination of *E. coli* Hyd1 and Hyd2 leads to modest increases in hydrogen yields (Penfold et al., 2003; Maeda et al., 2007; Bisaillon et al., 2007; Turcot et al., 2008), with, in one case, elimination of *hyd*1 and *hyd*2 giving a 37% increase in H_2 production rate compared to the wild type strain (Bisaillon et al., 2007).

Since different pathways are involved, multiple mutations can have an additive effect. In *E. coli* suitable strains can be easily constructed by successive rounds of P1 transduction from the Keio *E. coli* K-12 library allowing the introduction of multiple stable mutations into a single bacterium. One example of the use of this approach to direct the metabolic flux toward hydrogen evolution is the metabolically engineered *E. coli* BW25113 strain, which carries mutations in *hyaB*, *hybC*, *hycA*, *fdoG*, *frdC*, *ldhA*, *aceE*. In this strain hydrogen production from glucose was 4.6 fold greater than wild-type with a molar hydrogen yield of 1.3 mol $H_2.mol^{-1}$ glucose (Maeda et al., 2007b).

Another example of the effect of introducing multiple mutations is given by a series of studies aimed at maximizing hydrogen production from formate. Multiple mutations were introduced to block hydrogen consumption and to increase the synthesis of the FHL system (Maeda et al., 2007a, b). To enhance hydrogen production from formate, the *hyaB* and *hybC* genes were deleted to remove the hydrogen uptake activity of Hyd1 and 2; in order to increase

synthesis of the FHL complex the *hycA* gene (FHL repressor) was deleted and the *fhlA* gene (FHL activator) was over expressed. Several modifications were introduced to eliminate formate consumption by pathways other than FHL. The alpha subunit of formate dehydrogenase-N (encoded by *fdnG*), the alpha subunit of formate dehydrogenase-O (encoded by *fdoG*), and the alpha subunit of nitrate reductase A (encoded by *narG*) were deleted. *focA* and *focB* deletions were also made to prevent export of formate [Maeda et al., 2007a]. The multiple mutant derivative of BW25113 with *hyaB*, *hybC*, *hycA* and *fdoG* deleted and *fhlA* overexpressed had a much higher rate of hydrogen production rate from formate than the wild-type strain BW25113 and produced the theoretical maximum hydrogen yield of 1 mol of H_2 per mole of formate (Maeda et al., 2007a).

Of course, similarly mutated strains are also useful for obtaining higher molar yields from glucose. Thus introduction of additional mutations to the strain just discussed causes an increase in hydrogen production from glucose (Maeda et al., 2007a). The pyruvate pool was maximized by deleting fumarate reductase (encoded by *frdC*) to prevent diversion of phosphoenolpyruvate to succinate, lactate dehydrogenase (*ldhA*), pyruvate dehydrogenase (*aceE*) and pyruvate oxidase (*poxB*). The resultant strain (also carrying *hyaB*, *hybC*, *hycA*, *fdoG*, *frdC*, *ldhA* and *aceE* deletions) had a 4.6-fold higher hydrogen production rate from glucose than the wild-type strain and a hydrogen yield of 1.3 mol of H_2 per mole of glucose, two-fold higher than wild-type BW25113 cells. Likewise, further mutation of *E. coli* SR13, described earlier, to inactivate the lactate dehydrogenase (*ldh*A) and succinate dehydrogenase (*frd*BC) pathways, thus directing a greater proportion of glucose metabolism through the pyruvate formate lyase (PFL) system and into formate, gave a molar hydrogen yield of 1.82 mol H_2. mol^{-1} glucose, close to the theoretical 2 mol H_2. mol^{-1} glucose (Yoshida et al., 2006).

Similar results were seen in other studies where a constitutively active FhlA was expressed in a strain containing mutations in *hyd1*, *hyd2* as well as *ldhA* (Bisaillon et al., 2006; Turcot et al., 2008).

However, even strains that have been highly engineered for optimal hydrogen production may benefit from optimization of bioprocess parameters as shown by work with *E. coli* DJT135 strain which carries mutations in uptake hydrogenases (*hyd*1, *hyd*2), lactate dehydrogenase (*ldh*A), and possesses a constitutively active FhlA (Bisaillon et al., 2006; Turcot et al., 2008). Initial work with batch cultures showed that the hydrogen yields of this metabolically engineered strain can be affected by growth conditions. Limitation for some nutrients, glucose and fixed nitrogen, increased yields up to the theoretical maximum, 2 H_2.mol^{-1}glucose, whereas varying the phosphate or sulfate concentration was without effect. The physiological basis for these effects is largely unclear. For glucose limitation, one plausible explanation is that under carbon limiting conditions carbon flow to secondary pathways of metabolism like glycogen synthesis is restricted. This strain was also recently used to show the potential utility of *E. coli* in the conversion of complex substrates to biofuels where it was shown that DJT135 was capable of producing hydrogen from a wide variety of pentose and hexose sugars as well as sugar derivatives (Ghosh and Hallenbeck, 2009). The initial studies of hydrogen production by this metabolically engineered strain used substrate limitation, notably glucose limitation, to demonstrate high yields. Of course, this would be impractical for any real life application. Recently a statistical multi process parameter approach was used to identify optimal conditions for hydrogen production under more realistic conditions (Ghosh and Hallenbeck, 2010). *E. coli* DJT135 gave a molar hydrogen yield of 1.69 mol H_2.mol^{-1} glucose under the optimal conditions of 75 mM glucose, 35^{o}C and pH 6.5.

Of course, the strategies that have been applied to improve hydrogen production by *E. coli* should also increase hydrogen production by other bacteria and other fermentation types, but relatively little work in this regard has been done. Some metabolic engineering studies have been performed using *Enterobacter* species and mutants blocked in alcohol and organic acid producing pathways have been reported to have higher hydrogen yields from glucose (Rachman et al., 1997; Rachman et al., 1998; Kumar and Das, 2000; Kumar et al., 2001).

As noted above and elsewhere (Hallenbeck, 2005; Hallenbeck, 2009), facultative anaerobes can only be expected to give a theoretical maximum of 2 mol H_2.mol^{-1} glucose whereas strict anaerobes, usually *Clostridia*, can theoretically approach 4 mol H_2.mol^{-1} glucose when either acetate or acetone is the fermentation end-product. However, *Clostidium sp.* usually produce a mixture of compounds such as acetate (acetic acid), butyrate (butyric acid), lactate, acetone, butanol and ethanol (Hallenbeck and Benemann, 2002; Chin et al., 2003; Hallenbeck, 2005). Therefore, metabolic engineering could be used in principal to augment hydrogen production by eliminating pathways which give lower yields. However, relatively few studies on engineering strains of these bacteria have been reported to date.

Inactivation of the *ack* gene encoding acetate kinase for acetate formation in *Clostridium tyrobutyricum* was reported to give 1.5-fold enhancement in hydrogen production from glucose (2.2 mol of H_2 per mole of glucose versus 1.4 mol of H_2 per mole of glucose) (Liu et al., 2006) However, the basis for this effect is unclear since acetate formation does not drain the reductant pool and thus should not decrease hydrogen production. In fact, eliminating this pathway should increase acetyl-CoA flux into pathways that would decrease hydrogen production, such as ethanol or butyrate production, since they require reducing power (NADH).

Several studies have specifically targeted hydrogen metabolism in attempts to improve hydrogen yields. As noted above, many organisms contain uptake hydrogenases which can produce suboptimal hydrogen yields by consuming some of the hydrogen produced. Thus when uptake hydrogenase gene expression (*hupCBA* operon) in *Clostridium saccharoperbutylacetonicum* strain N1-4 was down regulated using an antisense RNA strategy, hydrogen uptake activity decreased significantly causing a 3.1-fold increase in hydrogen production (Nakayama et al., 2008). Other studies have examined the effect of overexpressing hydrogenase on hydrogen production rates and yields. *Clostridium acetobutylicum* has been reported to be capable of the heterologous overexpression of various [Fe-Fe] hydrogenases and in this system replacing the native gene *hydA1Ca* with a recombinant one via homologous recombination doubles the protein yield (Abendroth et al., (2008). However, the effects of overexpression on hydrogen production were not examined in that study. In a recent investigation of hydrogenase overexpression in *C. acetobutylicum*, high level expression of [Fe-Fe] hydrogenase was demonstrated by both transcriptional analysis and in vitro enzyme assays (Klein et al., 2010). However, there was no effect on molar hydrogen yields, or, more importantly, volumetric production rates, strongly suggesting that hydrogenase activity does not normally limit hydrogen production. Similar conclusions can be drawn from several other studies with *Clostridia*. The effects of the overexpression of endogenous [Fe-Fe] study hydrogenase on whole cell hydrogen evolution have been examined in *Clostridium paraputrificum* (Morimoto *et al.,* 2005) and *Clostridium tyrobutyricum* (Jo et al., 2010). In both cases, hydrogen yields increased somewhat (less than two-fold) and lactate production decreased significantly. The lactate effect might be explained by the preferential utilization of pyruvate under these conditions for hydrogen evolution. However, this cannot

explain the increased butyric acid production by the over-expressing *C. tyrobutyricum*, which of itself would require significant levels of NADH.

In conclusion, metabolic engineering of *E. coli* has clearly shown that the theoretical maximum of 2 mol H_2 $.mol^{-1}$ glucose can be experimentally achieved. In the future, appropriate manipulations in strict anaerobes like *Clostridia* are likely to increase hydrogen yields beyond this, up to 4 mol $H_2.mol^{-1}$ glucose. However, metabolic constraints dictate that this approach alone cannot achieve a greater than 33% conversion of substrate to hydrogen (given that glucose potentially contains 12 moles of hydrogen). For a number of reasons, these yields are unacceptably low (Hallenbeck and Ghosh, 2009). Thus, investigation has turned towards the incorporation of new metabolic pathways to overcome these metabolic barriers.

3.8.2. Introduction of novel enzymes/pathways: Heterologous expression of hydrogenases and accessory enzymes [3]

Of course, the key player in biological hydrogen production is hydrogenase and a great deal of effort has gone into understanding its synthesis, biochemistry and physiology, often, even early on, with its potential to produce a renewable fuel in mind (Greenbaum and Blankinship, 1993; Hansel and Lindblad, 1998). Hydrogenases catalyze one of the simplest chemical reactions, $2H^+ + 2e^- \leftrightarrow H_2$, yet their structure is very complex (Thauer, 1998; Adams and Stiefel, 1998; Vignais et al., 2001; Vignais et al., 2007; Vignais, 2008; Frey, 2002). Until 2004 hydrogenases were classified according to the metals thought to be at their active sites; three classes were recognized: iron-only [Fe-Fe], nickel-iron [Ni-Fe], and "metal-free". In 2004 it was shown that the metal-free hydrogenases in fact contain iron (Lyon et al., 2004), and they are now called [Fe]-hydrogenases, since they contain only a mononuclear Fe active site and no iron-sulfur clusters, in contrast to the [Fe-Fe]-enzymes. In some [Ni-Fe]-hydrogenases one of the Ni-bound cysteine residues is replaced by selenocysteine (Nicolet et

al., 2000; Fontecilla et al., 2007). Hydrogen production by organisms carrying out a dark fermentation is by either a [Ni-Fe] hydrogenase or a [Fe-Fe] hydrogenase (Hallenbeck, 2009). Biological production of hydrogen is also possible by photosynthetic microorganisms such as anoxygenic phototrophic bacteria and cyanobacteria (Hallenbeck and Ghosh, 2009). In these cases, hydrogen is produced by nitrogenase, except for small amounts of hydrogen produced under some circumstances by the reversible hydrogenase of cyanobacteria. Nitrogenase naturally evolves some hydrogen as a by-product of nitrogen fixation, with much greater amounts being produced by the direct reduction of protons in the absence of other substrates (i.e. N_2).

All of these enzymes contain complex metal centers that are very sensitive to oxygen inactivation. Thus, not only must an organism possess the structural genes for a particular hydrogenase, there is also a requirement for a set of genes for the assembly and proper insertion of the complex metal containing cofactors that form the active site for hydrogen activation or proton reduction (reviewed in Böck et al., 2006; Vignais and Billoud, 2007; Vignais 2008; English et al., 2009). In addition, to be physiologically relevant, there must also be present specific electron carriers to couple hydrogenase activity with the energy generating (proton reduction) or energy requiring (hydrogen oxidation) cellular metabolic reactions. Therefore, attempts to increase fermentative or photosynthetic hydrogen production through metabolic engineering must account for these additional factors if it is to be successful. Before these additional requirements were known, many attempts at heterologous expression of hydrogenases were unsuccessful. With this in mind, reports of the expression of hydrogenase activity in organisms that do not possess the requisite maturation machinery must be viewed with some skepticism. For example, it is difficult to reconcile the report of the

heterologous expression of a functional [Fe-Fe] hydrogenase from the strict anaerobe bacterium *C. pasteurianum* in the cyanobacterium *Synechococcus* sp. PCC7942 (Asada et al., (2000) with the fact that no known cyanobacterium possesses the capacity to mature this class of hydrogenase. Likewise, it has been reported that *hydA*, the [Fe-Fe] hydrogenase gene from *Enterobacter cloacae* IIT-BT-08 can be over expressed in *Escherichia coli* BL-21 with the recombinant *E. coli* having a yield of 3.12 mol of H_2 per mole of glucose (Mishra et al., 2004; Chittibabu et al., 2006), even though *E. coli* does not have *hydE*, *hydF*, and *hydG*, thought to be required for maturation of this enzyme (Posewitz et al., 2004).

The structural genes encoding [Ni-Fe] hydrogenases from many organisms have been cloned and sequenced, and studies have revealed a large number of genes involved in the expression, processing and assembly of functional [Ni-Fe] hydrogenase (Leclerc et al.,1988; Cauvin et al.,1991; Vignais and Toussaint, 1994; Olson et al., 1997; Durmowicz and Maier, 1997; Schwartz et al., 1998; Böck et al., 2006). The situation can be complex, with a given organism possessing the genetic capacity to express multiple hydrogenases. For example, *E. coli* encodes four different [Ni-Fe] hydrogenases. In this case, multiple operons encode the structural genes for the different hydrogenases and some accessory genes, with a common set of genes, *hyp*, being used for core generic functions required for maturation of these [Ni-Fe] hydrogenases. Attempts to express functional [Ni-Fe] hydrogenases in organisms belonging to different genera other than the organism providing the hydrogenase-encoding gene(s) have so far largely failed even when they contain homologs of the required maturation genes, possibly due to the inability of host maturation genes to correctly process and assemble functional heterologous [Ni–Fe] hydrogenases (English et al., 2009, Ghosh et al., unpublished results). Thus, to date, the heterologous expression of a functional [Ni-Fe] hydrogenase has

only been reported for *Desulfovibrio*, where the [Ni–Fe] hydrogenase from *D. gigas* could be expressed in *D. fructosovorans* (Rousset et al., 1998), the expression of a NAD-reducing hydrogenase from the gram positive *Rhodococcus opacus* in the gram-negative *Ralstonia eutropha* (Porthun et al., 2002) or, very recently, the expression of a metagenomically derived [Ni-Fe] hydrogenase in *Thiocapsa* (Maroti et al., 2009). In all other cases where heterologous expression of a [Ni-Fe] hydrogenase was successful, the necessary maturation machinery was introduced to the host along with the structural genes (Bascones et al., 2000; Friedrich et al., 1984; Lenz et al., 2005; Umeda et al., 1986). As yet, there has been no demonstration of increased hydrogen production due to the activity of an introduced [Ni-Fe] hydrogenase. Recently, a plasmid carrying *hoxEFUYH* from the cyanobacterium *Synechocystis* sp. PCC 6803 was introduced into *E. coli* and appeared to improve hydrogen production (Maeda et al., 2007b). Paradoxically, the effect appeared to be due to inhibition of hydrogen uptake activity and the majority of this effect was due to the presence of the auxiliary diaphorase subunit, HoxU. No evidence for the successful maturation of the Ni-Fe center was presented, and the basis for the observed effect remains to be clarified.

[Fe-Fe] hydrogenase structural genes are available from a number of sources and expression of a functional hydrogenase of this type could be expected to increase hydrogen production in a number of systems, most noticeably by replacing the inefficient nitrogenase. This would involve expressing Fe-Fe hydrogenase in cyanobacterial heterocysts, possibly making a more effective biophotolytic system, or replacing nitrogenase in photofermentation reactions carried out by photosynthetic bacteria. However, to be successful, it would, at a minimum, be necessary to co-express the required maturation proteins, HydE, HydF, and HydG (Posewitz et al., 2004; King et al., 2006; Böck et al., 2006; English et al., 2009). At

present no species of cyanobacteria or non-sulfur photosynthetic bacteria has been described as containing genes encoding these functions.

Attempts have been made to express active [Fe-Fe] hydrogenase (*hydA*) from various sources in *E. coli*. However, once again, *E. coli* does not possess the required maturation factors necessitating the coexpression of *hydEFG* for successful production of an active hydrogenase (Posewitz et al., 2004; King et al., 2006; Böck et al., 2006). Although originally this was demonstrated using a compatible set of expression plasmids (Posewitz et al., 2004), it might be more effective to express the required genes as an artificial operon carrying *hydA* and *hydEFG* under the control of a single strong *E. coli* promoter (Akhtar and Jones, 2008). This system was used to introduce a ferredoxin-dependent NAD(P)H:H_2 pathway into an *E. coli* strain expressing a [Fe-Fe] hydrogenase along with the maturation enzymes, and ferredoxin (Veit et al., 2008). This increased hydrogen production to some extent over background levels, but even with this approach levels of [Fe-Fe] hydrogenase activity were quite low. Here, electron flow to hydrogenase was most probably limiting, as shown recently by the introduction of a direct artificial pyruvate to hydrogen pathway where reduced ferredoxin was generated from pyruvate by overexpression of YdbK, an endogenous "cryptic" PFOR (pyruvate:ferredoxin oxidoreductase). High yields (1.8 H_2 / glucose) were achieved in an Δ*icsR* background with TPP (thiamine pyrophosphate) supplementation (Akhtar and Jones, 2009). This highlights the fact that, in addition to over expressing a highly active enzyme producing the desired product, in this case hydrogenase, it is equally important to ensure metabolic coupling to central metabolism.

Thus, introduction of a pyruvate to hydrogen pathway into *E. coli* allows near theoretical hydrogen yields for this organism, 2 H_2 / glucose (Hallenbeck, 2005; Hallenbeck,

2009). No metabolic engineering of this type has yet been attempted with strict anaerobic bacteria, theoretically capable of achieving 4 H_2 / glucose (Hallenbeck, 2005; Hallenbeck, 2009). Further research is needed to surpass the present metabolic limits to hydrogen yields, a necessary breakthrough required for the development of a practical system (Hallenbeck and Ghosh, 2009). Further improvements might include strategies such as increasing the activity of [Fe-Fe] hydrogenase and metabolic engineering to generate higher levels of reduced nucleotides or other reductant for hydrogen production. Computational modeling has been used to offer some suggestions as to how this might be achieved, including using the pentose phosphate pathway for glucose oxidation, generating large amounts of NADPH which could then be converted to NADH by a transhydrogenase (Jones 2008). Finally, although it has been proposed that low levels of respiration might be used to completely dissimilate substrate and produce energy to drive reverse electron flow to give additional hydrogen (more than 4 H_2 / glucose) (Hallenbeck and Benemann, 2002; Hallenbeck, 2005), this has not yet been demonstrated experimentally.

3.8.3. Metabolic engineering for improving photofermentative hydrogen production

Photosynthetic bacteria are mostly classified in to two major classes, the purple bacteria and the green bacteria. The purple bacteria and the green bacteria carry out photosynthesis using a single photo system. Inorganic/organic substrates are oxidized to donate electrons to reduce ferredoxin via Fe-S proteins in green bacteria having the PSI type reaction centre. Finally, reduced ferredoxin serves directly as electron donor for the dark reaction (fixing CO_2) as well as for the H_2 production. In another standpoint, purple bacteria contain a PSII like reaction centre thus is incapable of reducing ferredoxin, but can generate ATP via electron flow cyclic mechanism. The electrons desired for nitrogenase mediated H_2

generation is mediated from inorganic or organic substrate, which directs to the "pool of quinone" via reaction centre bacteriochlorophyll (P870). However, the energy potential of quinone is insufficiently negative to reduce NAD directly. Therefore, the electrons from the quinone pool are forced backward to reduce NAD^+ to NADH. This energy requiring process is postulated as reversed electron flow. No oxygen has been evolved in this process. The total amount of H_2 produced is affected by the activity of uptake hydrogenase (Koku et al., 2002). .

Photofermentative microorganisms, such as purple non sulfur (PNS) bacteria, use electrons generated from the oxidation of organic substrates upon photo heterotrophic growth to produce H_2 via the nitrogenase enzyme. Nitrogenase is a protein complex that consists of two different polypeptide subunits. The smaller dimeric component, known as the iron [Fe] protein and it contains a metal cluster [4Fe–4S]. It functions as an ATP-dependent electron donor to the larger heterotetrameric component, known as the molybdenum–iron [Mo-Fe] protein, which includes the enzyme catalytic site. [Mo-Fe] protein has two (oxidation–reduction) centers, one containing iron in the P cluster (an [8Fe–7S] cluster), and the other containing both iron and molybdenum (the Fe and Mo cofactor). Both of the nitrogenase-component proteins are oxygen sensitive (Moran, 2008).

Theoretically, H_2 is a natural spin-off of the nitrogen fixation reaction, the molybdenum-nitrogenase will reduce protons and produce only H_2 in the absence of N_2 (Rees and Howard, 2000). In the process, H_2 production essentially irreversible as it is entirely dependent on ATP requirement of Mo-nitrogenase. Theoretically (12 mol H_2/mol glucose), purple non-sulfur bacteria convert 100% substrate-derived electrons to H_2, since light energy can drive the complete oxidation of substrate molecules (Harwood, 2008). Even though PNS bacteria can eliminate incompletely oxidized metabolic end products, thus

reducing the availability of reductant for H_2 production (Yilmaz et al., 2010; Huang et al., 2010), although this limitation could potentially be bypassed through metabolic engineering. In the literature, glucose-fed *Rhodobacter sphaeroides* cells can excrete pyruvate (Yilmaz et al., 2010), but introduction of *Rhodospirullum rubrum* genes encoding pyruvate-formate lyase and formate-hydrogen lyase into *Rhodobacter sphaeroides* resulted in an increased H_2 yield (to 4 mol H_2.mol^{-1} glucose) (Kim et al., 2008). The main electron sink for PNS bacteria during growth is biomass assemblage (McKinlay et al., 2010) . Increase in biomass production would free additional reductant and ATP for H_2 production, although a significant fraction of the reductant diverted away from biomass accumulation in non growing cells can instead be excreted in the form of incompletely oxidized metabolites. One possible pathway might be biomass accumulation that directly contends with H_2 production for electrons is CO_2 fixation via the Calvin–Benson–Bassham (CBB) pathway (McKinlay et al., 2010**;** McKinlay et al., 2011). The CBB pathway is used to recycle excess reductant during photo heterotrophic growth by PNS bacteria (McKinlay et al., 2010), and H_2-producing *Rhodopseudomonas palustris* can re-assimilate up to a hundred percent of the CO_2 generated from the oxidation of a highly reduced organic substrate (McKinlay et al., 2011). *R. palustris* and *Rhodospirullum rubrum* mutants lacking CBB pathway activity produce more H_2 than their corresponding parent strains (McKinlay et al., 2010**;** McKinlay et al., 2011; Wang et al., 2010), and the increase in H_2 production by the *Rhodopseudomonas* CBB mutant can be completely accounted for by the electrons normally channeled into the CBB pathway in the wild type strain (McKinlay et al., 2011). Slow growth of CBB pathway

mutants was proposed to result from the slow rate of electron flow to, or turnover by, nitrogenase (McKinlay et al., 2010).

One significant avenue to potentially improve the rate of electron flow into H_2 is to increase expression of the proteins that channel electrons to nitrogenase and/or expression of nitrogenase itself. Hence, overexpression of the Rnf complex (predicted to reduce ferredoxin using the photosynthetically created proton gradient) in *Rhodobacter capsulatus* (Schmehl et al., 1993; Biegel et al., 2011; Jeong et al., 2000) and of NifA (the activator that controls transcription of nitrogenase, its accessory proteins, and electron transport proteins) in *R. sphaeroides* have both been shown to increase H_2 production (Liu et al., 2010) . In contrary, its negative impact on photo heterotrophic H_2 production, the CBB pathway could allow PNS bacteria to produce H_2 using an inorganic electron donor and CO_2 as the primary carbon source. This tentative approach would shift the requirement of providing an organic carbon source into a requirement to provide an inorganic electron source, which may be advantageous if a cheap and plentiful electron source were available. For example, *Rhodopseudomonas palustris* produces H_2 during photoautotrophic growth using electrons provided by oxidation of thiosulfate, a byproduct of some industrial processes (Huang et al., 2010). Photosynthesis by PNS bacteria does not generate O_2, so H_2 production by the O_2-sensitive nitrogenase can proceed concurrently with photoautotrophic growth, unlike hydrogenase mediated direct biophotolysis.

In another approach, it has been reported that *Rhodospirillum rubrum* mutant deleted of *hupL* encoding the large subunit of uptake hydrogenase produced increased amount of H_2 (Ruiyan et al., 2006). To date significant research has been performed to inhibit the uptake

hydrogenase activity using different approaches. A suicide vector approach for site directed mutagenesis of uptake hydrogenase (*hupSL*) was performed in *Rhodobacter sphaeroides*. The authors obtained 20% more H_2 production than wild type (Gokhan et al., 2008). By usage of a *R. sphaeroides* mutant MTP4, 50% more H_2 was produced than its wild type counterpart *R. sphaeroides RV* (Kondo et al., 2002). Another mutant (P3 mutant) with a 2.7 fold decrease in core antennal (LH1) content and 1.6 fold increase in peripheral antennal (LH2) content gave accelerated H_2 production compared to wild type (Vasilyeva et al., 1999). Efficiency of the enzymes is another important factor affecting the rate of hydrogen photo-production. In photosynthetic bacteria, net hydrogen production can be increased by improving the efficiency of nitrogenase. By knocking out *glnB* and *glnK*, genes encoding PII-like proteins, the problem of repression of nitrogenase by ammonium ions has been overcome in *R. sphaeroides* (Kim et al., 2008). In addition, Clostridial *hydA* has been cloned into *Rhodospirullum rubrum* and the native hydrogenase of *R. rubrum* (*hydC*) has been over expressed. In both cases pyruvate is the electron donor for hydrogen production (Kim et al., 2008). Moreover, polyhydroxybutyrate (PHB) synthase deficient mutants (*Rhodobacter sphaeroides* KD131) have been shown to increase hydrogen yields during photofermentation of organic acids since the reducing equivalents that are utilized for PHB synthesis could be harnessed for hydrogen production (Kim et al., 2006)

To this end, metabolic engineering by genetic modification on photofermentative bacteria could be a stable solution to optimize and redirect the flow of reducing equivalents (electrons) to the enzyme and also the accumulation of protons (H^+), to achieve a satisfactory amount of H_2 production towards achieving or surpassing molar hydrogen yield.

4. Objective of the present study

The overall purpose of the research presented in this thesis was to attempt to improve fermentative hydrogen production by using metabolic engineering and the optimization of experimental conditions through the statistical optimization of process variables.

The main aim of the statistical optimization of process variables was to maximize the use of metabolic energy in the generation of additional hydrogen production under optimized conditions for both dark fermentative pathways as well as photofermentative pathways.

One major hypothesis for the metabolic engineering that was employed was that by maximizing the reducing energy (NADH pool) in the cell, substrate conversion of a broad range of organic substrates (pentose and hexoses) could be driven further towards completion for additional hydrogen production through dark fermentation. Thus, the metabolic engineering strategy was to knock out competing pathways; namely the lactate dehydrogenase pathway (*ldhA)*, the alcohol dehydrogenase pathway (*adhE)*, the succinate pathway (*mdh)* and the TCA cycle regulator (*arcA),* respectively. Another strategy used was to introduce a novel metabolic pathway (*Ni-Fe hyd*) to drive this reducing energy to hydrogen in an attempt to surpass the stoichiometric yield with potential NADH recycling.

The photofermentation studies focused on improving hydrogen yields from different carbon sources (glucose and crude glycerol) using two different photofermentative microorganisms *Rhodopseudomonas palustris* CGA009 (naturally uptake hydrogenase deficient strain) and *Rhodobacter capsulatus* JP91 (*hup*-) in batch operation. This presented some technical challenges for the establishment of the proper operational conditions for maximum hydrogen yields.

Finally, this thesis also involved the application of metabolic engineering to the development of a *amtB* mutant strain of *Rhodobacter capsulatus* (DG9). Since AmtB is directly involved in the nitrogenase switch-on-off phenomenon through the ADP-ribosylation of the Fe-protein of nitrogenase, it was hypothesized that this strain would show prolonged nitrogenase activity and thus improve or surpass previously reported photofermentative hydrogen yields.

CHAPTER 1: Fermentative hydrogen yields from different sugars by batch cultures of metabolically engineered *Escherichia coli* DJT135

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Author contribution: The experimental design, wet laboratory experiments and all dataset analysis had been carried out by D.G. All the work was under supervision of Professor Dr. Patrick Hallenbeck.

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Keywords: Biofuels; Biohydrogen; Metabolically engineered; *Escherichia coli*; Spectrum of carbon sources; Hydrogen yields

ABSTRACT

Future sustainable production of biofuels will depend upon the ability to use complex substrates present in biomass if the use of simple sugars derived from food crops is to be avoided. Therefore, organisms capable of using a variety of fermentable carbon sources must be found or developed for processes that could produce hydrogen via fermentation. Here we have examined the ability of a metabolically engineered strain of *Escherichia coli*, DJT135, to produce hydrogen from glucose as well as various other carbon sources, including pentoses. The effects of pH, temperature and carbon source were investigated in batch experiments. Maximal hydrogen production from glucose was obtained at an initial pH of 6.5 and temperature of 35 °C. Kinetic growth studies showed that the μmax was 0.0495 h^{-1} with a Ks of 0.0274 g L^{-1} when glucose was the sole carbon source in M9 (1X) minimal medium. Among the many sugar and sugar derivatives tested, hydrogen yields were highest with fructose, sorbitol and d-glucose; 1.27, 1.46 and 1.51 mol H_2 mol^{-1} substrate respectively.

INTRODUCTION

A great deal of effort is being devoted at present to the development of green renewable technologies for producing alternative biofuels. One possible approach is to use biological means for producing hydrogen [1],[2] and [3], which is actively being investigated for use as a future fuel. It has become obvious that to produce substantial amounts of biofuels without seriously compromising the world food supply will require technologies to utilize biomass resources with their vast amounts of energy stored in lignocellulosic compounds [4], [5] and [6]. Tapping into the available chemical energy of the compounds generated by hydrolysis of such complex substrates requires the fermentative conversion of a variety of hexose and pentose sugars into a biofuel. However, the capacities to both use a wide variety of substrates, and to carry out a rapid and high yielding fermentation, do not necessarily occur in the same organism. Two approaches are commonly used to create a useful organism with the desired characteristics; either to increase the fermentative powers of an organism with an omnivorous appetite, or to engineer a strong fermenter to be able to consume a wider range of substrates. Indeed, for bioethanol production both approaches have been tried. *Escherichia coli*, known for its capacity to degrade a variety of hexoses, pentoses, and sugar derivatives has been engineered to produce greatly increased titers of ethanol [7] and [8]. Conversely, the naturally strong ethanol producers, *Zymomonas mobilis* and *Saccharomyces cerevisiae*, have been engineered to expand their repertoire of possible substrates [9] and [10].

A number of recent studies have reported on hydrogen yields with a variety of newly isolated bacteria that can potentially degrade both six and five carbon substrates. *Thermoanaerobacterium thermosaccharolyticum*, isolated from a biohydrogen

reactor fed with palm oil mill effluent, was reported to be capable of generating significant amounts of hydrogen from a variety of hexoses and pentoses [11]. A new fermentative hydrogen-producing bacterium, *Enterobacter asburiae*, was isolated from a domestic landfill and and shown to produce significant quantities of hydrogen from a variety of six carbon substrates [12]. *Citrobacter* Y19, isolated from an anaerobic wastewater sludge digester gave a high hydrogen yield, 2.5 H_2/glucose, at low glucose concentrations (1 g/l) [13]. As well, *Clostridium beijerinckii* has been shown to use a variety of six carbon substrates as well as xylose [14] and [15]. The hyperthermophile *Caldicellulosiruptor saccharolyticus* can degrade a variety of hexoses and pentoses and was shown to be active on a paper sludge hydrolysate [16]. A novel mesophilic bacterium belonging to clostridial cluster XI, *Sporoacetigenium mesophilum*, isolated from an anaerobic digester, ferments a variety of hexoses and pentoses to hydrogen, CO_2, ethanol and acetate [17].

Here we have examined the yields of hydrogen from different C-6 and C-5 substrates with *E. coli* DJT135, a metabolically engineered strain bearing knockout mutations in the uptake hydrogenases (Δ*hya*, Δ*hya*) and lactate dehydrogenase (*ldh*A), and constitutively expresses *fhlA*, which encodes the positive regulator of the formate hydrogen lyase (FHL), hydrogen evolving pathway. This strain has previously been shown to be highly competent for the conversion of glucose or formate to hydrogen [18], [19] and [20].

MATERIALS AND METHODS

E. coli strain DJT135 [11] was used throughout. All chemicals used were ACS reagent grade or better and were obtained from either Bioshop Canada Inc., Burlington, Ontario, or Fisher Scientific Company, Ottawa, Ontario. Cultures were prepared by overnight aerobic growth (250 rpm agitation of conical flasks) at 37°C in nutrient broth containing

chloramphenicol and used to inoculate a Bioflow (C-30) bioreactor (350 ml liquid working volume). Modified M9 minimal media [18] supplemented with various carbon sources and chloramphenicol was used. The entire setup was made anaerobic by sparging with oxygen free argon gas for 15 min. Hydrogen was measured by injecting a 50 μl aliquot of the headspace gas into a Shimadzu GC8 chromatograph equipped with a thermal conductivity detector (TCD) and a 2 m column containing molecular sieve 5A with argon as the carrier gas. The injector port was maintained at 40°C, the detector at 50°C and 100 mA was supplied to the TCD. Limits of detection were 2 nmol H_2. Reducing sugars in the fermentation broth were determined by the standard di-nitro salicylic acid method [21]. The limit of detection, 50 μM, was well below the amount detected in the culture medium.

RESULTS AND DISCUSSION

The effect of initial pH and temperature on hydrogen production

The initial pH of the fermentation medium had a strong effect on hydrogen production. *E. coli* strain DJT135 grew over a wide range of initial pHs (4 to 9), with hydrogen production observed over essentially the same pH range (Fig. 1A). At low initial pHs (4.5 and 5.5), cultures exhibited a prolonged lag phase. Maximum hydrogen production and little or no lag phase were seen at an initial pH of 6.5. Although the exact optimum pH varies with organism, this value is within the relatively narrow range usually seen [2], [22] and [23]. As well, a prolonged lag phase has previously been reported for cultures where the initial pH is below the optimum [24]. As to be expected, hydrogen production by *E. coli* DJT135 also varied appreciably with temperature (Fig. 1B). Hydrogen production increased from 25°C to 35°C, while afterwards it decreased. Maximum hydrogen production was obtained at 35°C, typical for a mesophilic fermentation. This effect is most

probably related to temperature effects on growth and not on the enzymes involved in hydrogen production since a study of hydrogen production with non-growing cells of this strain showed that the hydrogen production rate at 45°C was essentially the same as that obtained at 37°C [19].

Although higher yields (mol H_2/mol substrate) can be obtained with thermophilic bacteria using reactors operated at higher temperatures, volumetric production rates (l H_2/l reactor/hr) obtained with thermophilic strains are usually much lower than those observed with mesophilic strains [2] and [3]. As well, the additional energy inputs to heat such reactors would be appreciable. Double reciprocal (Lineweaver–Burke) plots were used to obtain the kinetic parameters for cell growth with glucose (1%) as substrate under the optimal conditions determined above. The maximum specific growth rate (μmax) and saturationconstant (Ks) of the cell were found to be 0.050 h^{-1} and 0.027 g L^{-1} (Fig. 2). This maximum specific growth rate is consistent with that seen in some studies with other *E. coli* strains (see for example [25]). Saturation of growth at relatively low carbon concentrations reflects the efficient sugar transport capabilities of *E. coli*.

Hydrogen yields with different carbon sources

Hydrogen yields on different carbon sources, including a variety of hexoses, pentoses, and sugar derivatives were determined (Table 1). Among the different carbon sources examined, glucose, sorbitol and fructose gave the highest hydrogen yields; 1.51, 1.47, and 1.27, respectively. *E. coli* DJT135 also showed relatively good hydrogen yields with l-arabinose, mannitol and trehalose. The vast majority of previous studies, with either pure cultures or mixed consortia, have largely used either complex substrates, or where defined

carbon compounds were used, either glucose or sucrose (see for example [22]). For a bacterium, such as *E. coli*, with an enteric type metabolism, hexoses are degraded through the glycolytic pathway yielding a maximum of $2H_2$/ hexose: 1 hexose → 2 pyruvate → $2H_2 + 2CO_2$ + 2(acetate+ ethanol) [2] and [3].

The relative amounts of acetate versus ethanol are determined by the oxidation state of the six carbon substrate. Efficient bioenergy production from lignocellulosic materials requires the ability to utilize arabinose and xylose since these can constitute 20 to□35% of the sugars present [26]. However, hydrogen production from these pentose sugars has been relatively little studied. Here we show that *E. coli* strain DJT135 is capable of converting substantial amounts of these sugars to hydrogen (1.02, 0.57 mol H_2/mole substrate, respectively (Table 1)).

In general *E. coli* degrades pentoses through the non-oxidative pentose phosphate pathway: 3pentose → 5pyruvate → $5H_2 + 5CO_2$ + 5(acetate + ethanol). The relative amounts of acetate versus ethanol are determined by the oxidation state of the five carbon substrate. Thus one expects a maximum of 1.67 mol H_2/mol pentose, and therefore arabinose and xylose gave 61 and 34% of the theoretical maximum respectively. Previously, mixed cultures have been reported to give yields of either 0.065 [27] or 1.5 [28] mol H_2/mole substrate with arabinose or 0.7 mol H_2/mole substrate with xylose [29]. A Clostridium species isolated from termites was reported to ferment both arabinose and xylose, but yields were low; 0.097 mol H_2/mole arabinose and 0.092 mol H_2/mole xylose [30]. Thus the yields reported in the present study from these substrates are very significant.

An examination of Table 1 shows that there was considerable variation in yields from different substrates. The reason for this is unclear at present, and thus presents a fruitful area

for future research. It certainly suggests that flux through the different pathways is influenced by the nature of the substrate, including its entry point into central metabolism, as well as its oxidation state, which would lead to a different pattern of end products. These factors could also influence the fraction of available carbon going into biomass as well as storage materials such as glycogen. Therefore, optimizing yields from specific substrates might require a detailed investigation of flux through various metabolic pathways thus suggesting means by which to channel more substrate into pathways leading to hydrogen production.

CONCLUSIONS

Here we have demonstrated fermentative hydrogen production from a variety of sugars and sugar derivatives by *E. coli* DJT135 in a batch bioreactor. The maximum molar hydrogen yield with glucose at the optimal conditions of pH 6.5 and 35°C was 1.51 mol H_2 mol^{-1}. As well, the potential of this organism for using cheap raw materials, in particular hydrolysates derived from lignocellulosic materials, was explored by examining the yields obtained from arabinose and xylose. Good yields of hydrogen, the highest yet reported for a pure mesophilic culture, were obtained from these substrates. In addition, this metabolically engineered organism was shown to be capable of significant hydrogen production from a variety of other carbon sources including sorbitol, fructose, trehalose, maltose, lactose, galactose, and sucrose. These observations indicate the potential of suitably modified *E. coli* strains to use various types of carbohydrates present in different wastes for biohydrogen production while at the same time carrying out effective waste treatment.

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TABLES

Table 1 – Hydrogen yields of *E. coli* DJT135 on different carbon sources.[a]

C-source	H_2 Yield (mol/mol^{-1})
Arabinose	1.02 (±0.007)
Fructose	1.27 (±0.014)
Galactose	0.69 (±0.007)
Gluconate	0.97 (±0.007)
Glucose	1.51 (±0.007)
Lactose	0.73 (±0.014)
Maltose	0.72 (±0.028)
Mannitol	0.95 (±0.028)
Sorbitol	1.47 (±0.007)
Sucrose	0.70 (±0.014)
Trehalose	1.03 (±0.014)
Xylose	0.57 (±0.007)

a Cultures were incubated with the appropriate C-source (1% w/v) at 35 °C and pH 6.5.

FIGURES

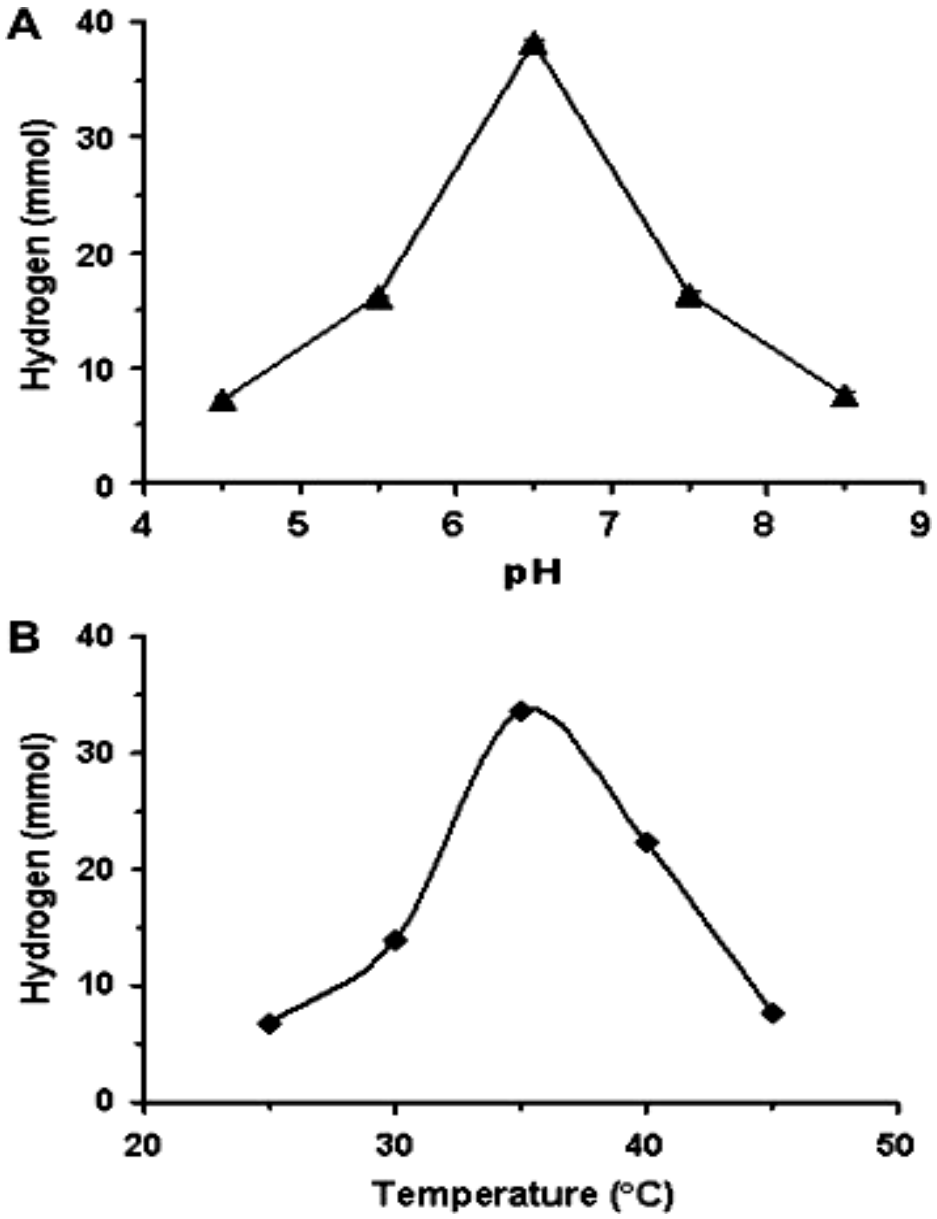

Figure 1. Variation of hydrogen production with pH and temperature. A. Total hydrogen production at different initial pHs. Batch cultures were carried out at the indicated initial pHs with glucose (1% (w/v)) and the amounts of hydrogen produced were determined after 48 h incubation. B. Total hydrogen production at different temperatures. Batch cultures were carried out at the indicated temperatures with glucose (1% (w/v)) were incubated for 48 h and the amounts of hydrogen produced were determined after 48 h incubation.

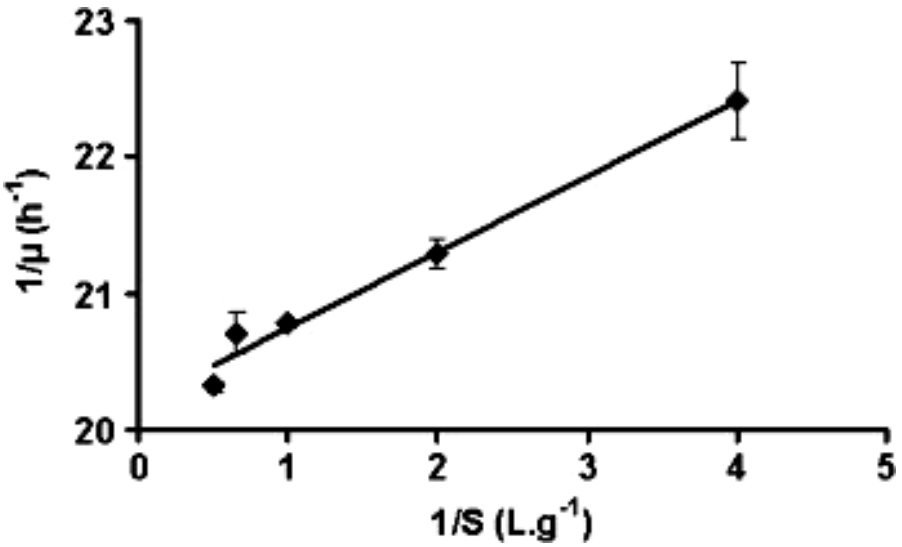

Figure 2. Lineweaver-Burk (double recipricol) plot of the growth kinetics of *E. coli* DJT135 at different concentrations of glucose as sole carbon source. Initial pH was 6.5 and the temperature was maintained at 35^{o}C.

CHAPTER 2: Response surface methodology for process parameter optimization of hydrogen yield by the metabolically engineered strain *Escherichia coli* DJT135

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Author contribution: The experimental design, wet laboratory experiments and all dataset analysis had been carried out by D.G. All the work was under supervision of Professor Dr. Patrick Hallenbeck.

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Response surface methodology for process parameter optimization of hydrogen yield by the metabolically engineered strain *Escherichia coli* DJT135

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Keywords: Hydrogen yield; Response surface methodology (RSM); Metabolic Engineering; Biofuels

ABSTRACT

Metabolically engineered microbial strains can be usefully employed to give higher yields, but this also requires development of a suitable bioprocess. Maximization of product yield during fermentation requires that a number of process parameters, some of which may interact, be optimized. Here we report the effects of different fermentative process conditions; pH, temperature and glucose concentration, on the molar hydrogen yield by a genetically optimized *Escherichia coli* strain, DJT135. In order to simultaneously reduce the number of the experiments, and to obtain the interactions between the variables important for achieving maximum hydrogen production, a 3^K full factorial Box–Behnken design and response surface methodology (RSM) were employed for experimental design and analysis. A maximum molar hydrogen yield of 1.69 mol H_2 mol^{-1} glucose was obtained under the optimal conditions of 75 mM glucose, 35°C and pH 6.5. Thus, RSM with Box–Behnken design is a useful method for achieving higher molar hydrogen yields by metabolically engineered organisms.

INTRODUCTION

Biological hydrogen production is under active investigation as a possible means to renewably produce biofuel, in particular for the transportation sector. Among the different possible technologies (Hallenbeck and Benemann, 2002 and Hallenbeck and Ghosh, 2009), fermentative hydrogen production is attractive as it can potentially use a wide range of waste materials as substrates and could probably be done on a practical level with existing reactor technology (Hallenbeck, 2005, Hallenbeck et al., 2009 and Kapdan and Kargi, 2006). However, the major stumbling block is the low yields obtained (Hallenbeck, 2009 and Hallenbeck and Ghosh, 2009). Various strategies and methods for improving hydrogen production rates and yields have been under investigation for the past few decades. One factor that has been considered for improvement is the metabolic conversion of substrate to hydrogen. Although metagenomics might find naturally occurring organisms with intrinsic higher yield, this is unlikely for a number of reasons (Hallenbeck, 2005 and Hallenbeck et al., 2009), suggesting the need to apply metabolic engineering to this problem to redirect and optimize the flow of reducing equivalents to the hydrogen producing enzymes (nitrogenase or hydrogenase) (Vignais et al., 2006).

Previously, we engineered *Escherichia coli* DJT135 for increased hydrogen production and showed that higher yields, approaching the theoretical maximum for this organism of 2 mol H_2 per glucose, could be obtained in either batch cultures (Bisaillon et al., 2006) or under continuous culture conditions (Turcot et al., 2008). However, maximum yields were obtained under impractical conditions, in particular at very low substrate concentrations. While for practical purposes continuous cultures potentially offer some advantages, batch cultures are attractive since they could be run under non-sterile conditions. However, to

develop a more practical bioprocess, operating conditions need to be optimized. Conventional 'change-one-factor-at-a-time' processes are time-consuming and are incapable of reaching the true optimum since potential interactions among process variables are ignored. On the other hand, an experimental design based on the statistical modeling can be a very useful tool for evaluating the interactions between a set of independent experimental factors and observed responses, while at the same time reducing the number of experiments required to determine optimal conditions. Here we have examined the interactive effects between the physico-chemical parameters (pH, temperature and glucose concentration) and molar hydrogen yield of *E. coli* DJT135 using response surface methodology to maximize the hydrogen yield.

MATERIALS AND METHODS

Microorganisms and pre-inoculum preparation

Metabolically engineered strain *E. coli* DJT135 was used for the multi-process parameter optimization studies. This strain carries mutations in uptake hydrogenase (*Δhya-Km, Δhyb-Km*), lactate dehydrogenase (*ldhA*) and *fhlA*, coding for the regulator of formate hydrogenase (FHL) component synthesis (Bisaillon et al., 2006 and Turcot et al., 2008). Cultures were maintained on nutrient agar slants at 4°C and sub-cultured monthly. Primary inocula were grown to late log phase in nutrient broth at 37°C on a shaker (150 rpm). Inocula to be used directly in the experiments were prepared by inoculating M9 glucose medium (Miller, 1992) with primary inoculum, followed by incubation at 37°C for 18 h. Cells were then harvested and washed with sterile phosphate buffered saline (PBS).

Batch reactor studies

All the experiments were performed in a Bioflow (C-30) reactor with a 350 ml working liquid volume under constant agitation (150 rpm). M9 medium, with the indicated

glucose concentrations, was used throughout. The pH was periodically adjusted as necessary by aseptically adding sterile 5 M NaOH or 5 M H_3PO_4. The reaction vessel was made anaerobic by flushing with oxygen free argon for 15 min.

Analytical methods

Gas composition was analyzed by gas chromatography using a Shimadzu GC equipped with a thermal conductivity detector and with argon as carrier gas. Hydrogen production was calculated from the measurement of headspace gas composition and total volume of gas produced (Jo et al., 2008), at each time interval (2 h) using Eq. (1) based on mass balance:

$$V=V_o\gamma_i+\sum V_i\gamma i \qquad \text{Equation (1)}$$

Where V is the cumulative hydrogen gas volume at the current (i); Vo is the volume of the headspace of the reaction vessel (350 ml); Vi is the gas volume discharged from the reaction vessel at the time intervals (i); γi is the fraction of hydrogen gas discharged from the vessel at the time intervals (i). Glucose was determined by the standard di-nitro salicylic acid method (Miller, 1959).

Optimization study

A 3^K factorial Box–Behnken model was used as the experimental design model to optimize the key process parameters for enhanced hydrogen production. For three factors, the Box–Behnken design offers some advantages in requiring fewer experimental runs and is rotatable if the variance of the predicted response at any point × depends only on the distance of × from the design center point (Box and Behnken, 1960). The 3^K factorial design also

allows efficient estimation of second degree quadratic polynomials and gives the combination of values that optimizes the response within the region of the three dimensional observation space (Annadurai et al., 1999). In developing the regression equation, the relation between the coded values and actual values are described according to the following equation:

$$x_i = (X_i - X_i^*)/\Delta X_i \qquad \text{Equation (2)}$$

Where xi is the coded value of the ith independent variable; Xi is the uncoded value of the ith independent variable; X_i^* is the uncoded value of the ith independent variable at the center point, and ΔXi is the step change value. The levels of the variables and the experimental design are shown in Table 1. Hydrogen yield was associated with simultaneous changes in glucose (25, 75 and 125 mM), temperature (25, 35 and 45°C) and the pH (4.5, 6.5 and 8.5) of the culture medium. A total of 12 experimental runs decided by the 3K factorial Box–Behnken design were carried out, and the center point was replicated three times to estimate experimental errors. For predicting the optimal condition, the quadratic polynomial equation was fitted to correlate the relationship between variables and response (i.e., molar hydrogen yield), and estimated with the following equation:

$$Y = \alpha_0 + \sum_{i=1}^{3} \alpha_i X_i + \sum_{i=1}^{3} \alpha_{ii} X_i^2 + \sum_{i=1}^{3} + \sum_{i<j=2}^{3} \alpha_{ij} X_i X_j \qquad \text{Equation (3)}$$

Where Xi are the input variables, which influence the response variable Y; α0 is the offset term; αi is theith linear coefficient; αij is the ijth interaction coefficient. The input values of X1, X2 and X3 corresponding to the maximum value of Y were solved by setting the partial derivatives of the functions to zero.

RESULTS AND DISCUSSION

Greater hydrogen yields can be sought through metabolic engineering, but realizing the potential increase achieved through genetic intervention requires bioprocess optimization. A number of variables could possibly restrict hydrogen production rates and yields.

However, assuming an adequate medium formulation, the most important key parameters are probably substrate concentration, temperature and pH. First of all, pH is one of the most important factors in hydrogen production due to its effects on metabolic pathways, thus potentially modulating end product distribution, as well as possibly affecting the duration of the lag phase (Bartacek et al., 2007, Davila-Vazquez et al., 2008, Hallenbeck, 2005, Hallenbeck, 2009, Hawkes et al., 2007 and Lay, 2000). Optimum initial pH is probably the resultant sum of a number of factors; it must be within a range that does not inhibit growth and permits high level expression of the requisite fermentation pathways while at the same time a higher initial pH value would delay the onset of acid inhibition (Lee et al., 2002). Initial glucose concentration also plays an important role on the yield and production rate of hydrogen (Fabriano and Perego, 2002). Low initial glucose concentrations result in low rates of fermentation, and total fermentation times increase with high initial substrate concentrations. In addition, temperature affects the maximum specific growth, substrate utilization rate, and the metabolic pathway of microorganisms, resulting in major shifts in end product composition (Van Ginkel et al., 2001). Optimal conditions determined by the one-variable-at-a-time approach can not be directly used to predict the true optimal conditions for a particular bioprocess due to potential interactions between the independent variables. To overcome this problem, a full or fractional factorial design coupled with RSM (response

surface methodology) can be used to advantage (Hallenbeck and Ghosh, 2009 and Wang and Wan, 2009).

Previously, only a few studies have simultaneously examined the effects of substrate concentration, temperature, and pH on hydrogen yield (Jo et al., 2008, Mu et al., 2006, Wang et al., 2005 and Wang and Wan, 2008). Here, using the modified *E. coli* strain DJT135, we have determined the optimal levels of these key factors and the effect of their interactions on molar hydrogen yields using RSM with a Box–Behnken design. *E. coli* may be particularly useful in biofuels production since in general it shows broad substrate specificity and therefore is capable of catabolising a variety of five and six carbon compounds. In fact, we have recently shown that the modified strain used here, *E. coli* DJT135, is capable of producing hydrogen from a variety of hexoses and pentoses with relatively high yields (Ghosh and Hallenbeck, 2009).

The method used here was a Design of Experiments approach where a statistical design is used to choose a series of experimental conditions such that a minimum number will give a robust description and verification of a model (Hanrahan and Lu, 2006). This technique is often applied in engineering and manufacturing, but can be advantageously applied to biological systems in some cases, especially where a bioprocess, such as in this study, is involved (Lee and Gilmore, 2006). We wished to assess three independent variables, their effect on hydrogen yields as well as the degree, if any, of their interaction. The appropriate method for these goals is a Box–Behnken since it uses a minimum of tests, is robust, and can be applied when values at the extremes are uninteresting (Whittinghill, 1998).

Therefore 12 experimental fermentations were run as described in Materials and Methods at the different parameter values indicated by the design and the resultant hydrogen

yield measured (Table 1). The statistical treatment of the test variables along with the measured response values, expressed as hydrogen yield corresponding to each combination, are summarized in Table 1. The summary of the analysis of variance (ANOVA) of the results of the quadratic model fitting are shown in Table 2. ANOVA is essential to test significance and adequacy of the model. The model *F*-value of 42.04 implies that the model is significant.

There is only a 0.04% chance that a "model *F*-value" this large could occur due to noise. Value of "Prob > *F*" less than 0.05 indicates that the model terms are significant. In this case X_1, X_2, X_3, X_1X_2, X_2X_3, X_1X_3, X_1^2, X_2^2 and X_3^2 are significant model terms. The "Lack of fit *F*-value" of 9.15 implies that the lack of fit is not significant relative to the pure error. There is a 10% chance that a "Lack of fit *F*-value" this large could occur due to noise. The squared regression statistic (R^2), the determination coefficient, a measure of the goodness of fit of the model, was very significant at the level of 98%, i.e., the model was unable to explain only 2% of the total variations. Adequate precision measures the signal to noise ratio. A greater ratio greater than 4 is desirable. In this case the ratio of 16.066 indicates an adequate signal. A very high degree of precision and a good deal of reliability of the experimental values were indicated by a low value of the coefficient of variation (CV = 14.3%).

The application of response surface methodology results in an empirical relationship between the hydrogen yield and the process variables. Thus, the following regression equations, analogous to the Eq. (3), shows the relative hydrogen yield (Y) as a function of the test variables X_1 (glucose), X_2 (pH) and X_3 (temperature).

$$Y_{CODED}=1.69-0.43X_1+0.15X_2-0.024X_3-0.21X_1X_2+0.11X_1X_3+0.0007X_2X_3-0.31X_1^2-0.63X_2^2-0.69X_3^2$$ Equation (4a)

$$Y_{ACTUAL}=-14.29734+0.016166X_1+2.27598X_2+0.46263X_3-(2.08\times0.0497)X_1X_2+(2.18\times0.018)X_1X_3+(3.5\times0.018)X_2X_3-(1.26\times0.00483)X_1^2-0.158X_2^2-(6.928\times0.005002)X_3^2$$ Equation (4b)

The first of these (4a), is the equation actually used in development of the response curves, thus is valid for the coded values, i.e., −1, 0, 1, of the variables shown in Table 1. Actual values can be calculated from the second equation (4b).

The two and three dimensional contour plots with glucose concentration and pH, or glucose concentration and temperature (Fig. 1B and C), had an elliptical nature and a clear elongated running diagonal, indicating a significant interactive effect on Y between the two independent variables. On the other hand, the contour plot of Y with respect to temperature and pH had a circular nature, suggesting that temperature and pH were only very slightly interdependent, and that their interactive effects were not significant (Fig. 1A).

Plots of residuals versus responses predicted by the model were randomly distributed around zero without any trends (not shown). This indicates good prediction of maximum response along with constant variance and adequacy of the quadratic models. As shown in Fig. 1, in the design boundary, each response surface plot had a clear peak and the corresponding contour plot had a clear maximum, which means that the maximum hydrogen yield could be achieved inside the design boundaries. Similar results have previously been obtained in optimization studies with mixed cultures (Mu et al., 2006, Wang et al., 2005 and Wang and

Wan, 2008). Hydrogen yield increased with increasing temperatures, initial pH and glucose concentrations to the optimal levels, and then decreased with a further increase in these parameters. The predicted hydrogen yield of 1.69 mol H_2 mol^{-1} is as high, or higher, than that reported previously (Table 3). We verified the predicted maximum, 75 mM glucose, 35°C, pH 6.5, by running three replicates under these conditions and obtained a yield of 1.68 ± 0.01 H_2/glucose, in excellent agreement with the predicted value. In fact, this, taken together with the residual analysis, validates the model in general. Thus, not only can it be used to predict the optimal conditions, it would also be useful in predicting yields and parameters to be used if for some reason one of the operating parameters was constrained at a suboptimal value.

Therefore, response surface optimization can be successfully used to optimize high hydrogen yields with metabolically engineered organisms. However, since this type of treatment treats the bacterium as essentially a black box, thus ignoring the underlying metabolic pathways with their multiple interconnections and dependence upon intracellular metabolite pools, it cannot be used to analyze or predict how metabolic engineering itself could be used to enhance the production of hydrogen, or other metabolites. A more useful approach for this purpose would be metabolic flux modeling and analysis. Rather, its utility is to show how bioprocess operational parameters should be set to obtain maximum performance with strains that have been engineered to achieve maximum metabolic performance. This has been specifically demonstrated in the present study for hydrogen production by an *E. coli* strain, DJT135, which was previously optimized through a series of genetic manipulations.

CONCLUSIONS

The present work focused on the optimization of key process parameters for the maximizing the hydrogen yield using statistical methodology. Experimental results showed that glucose concentration, temperature and pH all had significant influences on the hydrogen yield. Glucose concentration and pH, glucose concentration and temperature were interdependent and had a significant interactive effect on the hydrogen yield. On the other hand, the interactive effect of temperature and pH was insignificant. Accurate prediction of the maximum value of the experimental response and the constant variance of the residuals indicated that the quadratic model adequately described the response surface within the experimental region. The maximum hydrogen yield of 1.69 mol H_2 mol^{-1} was obtained under the optimal conditions of 75 mM glucose concentration, 35°C and pH 6.5. Finally, RSM was shown to be useful optimizing the hydrogen yield and thus improving the bioprocess for hydrogen production by metabolically engineered strain *E. coli* DJT135.

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Table 1
Box-Behnken experimental design with three independent variables.

Trial	Glucose (mM)		pH		Temperature (°C)		Hydrogen yield, Y
	X_1	Code	X_2	Code	X_3	Code	(mol H_2 mol^{-1} glucose)
1	25.0	−1	4.5	−1	35.0	0	0.7714
2	125.0	1	4.5	−1	35.0	0	0.349
3	25.0	−1	8.5	1	35.0	0	1.544
4	125.0	1	8.5	1	35.0	0	0.288
5	25.0	−1	6.5	0	25.0	−1	1.342
6	125.0	1	6.5	0	25.0	−1	0.234
7	25.0	−1	6.5	0	45.0	1	0.906
8	125.0	1	6.5	0	45.0	1	0.2349
9	75.0	0	4.5	−1	25.0	−1	0.179
10	75.0	0	8.5	1	25.0	−1	0.425
11	75.0	0	4.5	−1	45.0	1	0.285
12	75.0	0	8.5	1	45.0	1	0.559
13[a]	75.0	0	6.5	0	35.0	0	1.7234
14[a]	75.0	0	6.5	0	35.0	0	1.6316
15[a]	75.0	0	6.5	0	35.0	0	1.7009

$Y_{CODED} = 1.69 - 0.43X_1 + 0.15X_2 - 0.024X_3 - 0.21X_1X_2 + 0.11X_1X_3 + 0.0007X_2X_3 - 0.31X_1^2 - 0.63X_2^2 - 0.69X_3^2$.
$Y_{ACTUAL} = -14.29734 + 0.016166X_1 + 2.27598X_2 + 0.46263X_3 - (2.08 \times 0.0497)X_1X_2 + (2.18 \times 0.018)X_1X_3 + (3.5 \times 0.018)X_2X_3 - (1.26 \times 0.00483)X_1^2 - 0.158X_2^2 - (6.928 \times 0.005002)X_3^2$.

[a] The center point was replicated three times.

Table 2
ANOVA for hydrogen yield by *E. coli* DJT135.[a]

Factors	Statistics				
	Sum of squares	Degrees of freedom	Mean square	*F*-value	*P*-value
Model	5.10	9	0.57	**42.04**	0.0004
X_1	1.49	1	1.49	110.84	0.0001
X_2	0.19	1	0.19	14.06	0.0133
X_3	0.0047	1	0.0047	0.35	0.5783
X_1X_2	0.17	1	0.17	12.89	0.0157
X_1X_3	0.048	1	0.048	3.54	0.1187
X_2X_3	0.00096	1	0.00096	0.015	0.9087
X_1^2	0.37	1	0.37	27.17	0.0034
X_2^2	1.48	1	1.48	109.47	0.0001
X_3^2	1.76	1	1.76	130.81	0.0001
Residual	0.067	5	0.013		
Lack of fit	0.063	3	0.021	**9.15**	0.1001
Pure error	0.0045	2	0.0022		
Cor. Total	5.17	14			

[a] Coefficient of determination ($R2$) = 0.987.

Table 3
Comparison of hydrogen yields by various metabolically engineered facultative anaerobic bacteria in batch mode.

Microorganism	Relevant genotype or phenotype	Substrate	Hydrogen yield (mol mol^{-1})	References
Escherichia coli BW25113	Δ*hya*B, Δ*hyb*C Δ*hyc*A, Δ*fdo*G, pCA24 N-FhlA	Formate	1.2	Maeda et al. (2007)
E .coli BW25113	Δ*hya*B, Δ*hyb*C Δ*hyc*A, Δ*fdo*G/pCA24 N-FhlA	Glucose	1.3	Maeda et al. (2007)
E. coli SR15	Δ*ldh*A, Δ*frd*BC, Δ*hyc*A, *fhl*A	Glucose	1.8	Yoshida et al. (2006)
E. coli DJT135	Δ*hya*-Km, Δ*hyb*-Km, Δ*ldh*A, *fhl*A-C	Glucose	1.69	Present study
E. coli BL21(DE3)	Δ*isc*R pAF pYdbK	Glucose	1.88	Akhtar and Jones (2009)
Enterobacter aerogenes AY-2	Allyl alcohol resistant mutant	Glucose	1.1	Rachman et al. (1998)

FIGURES

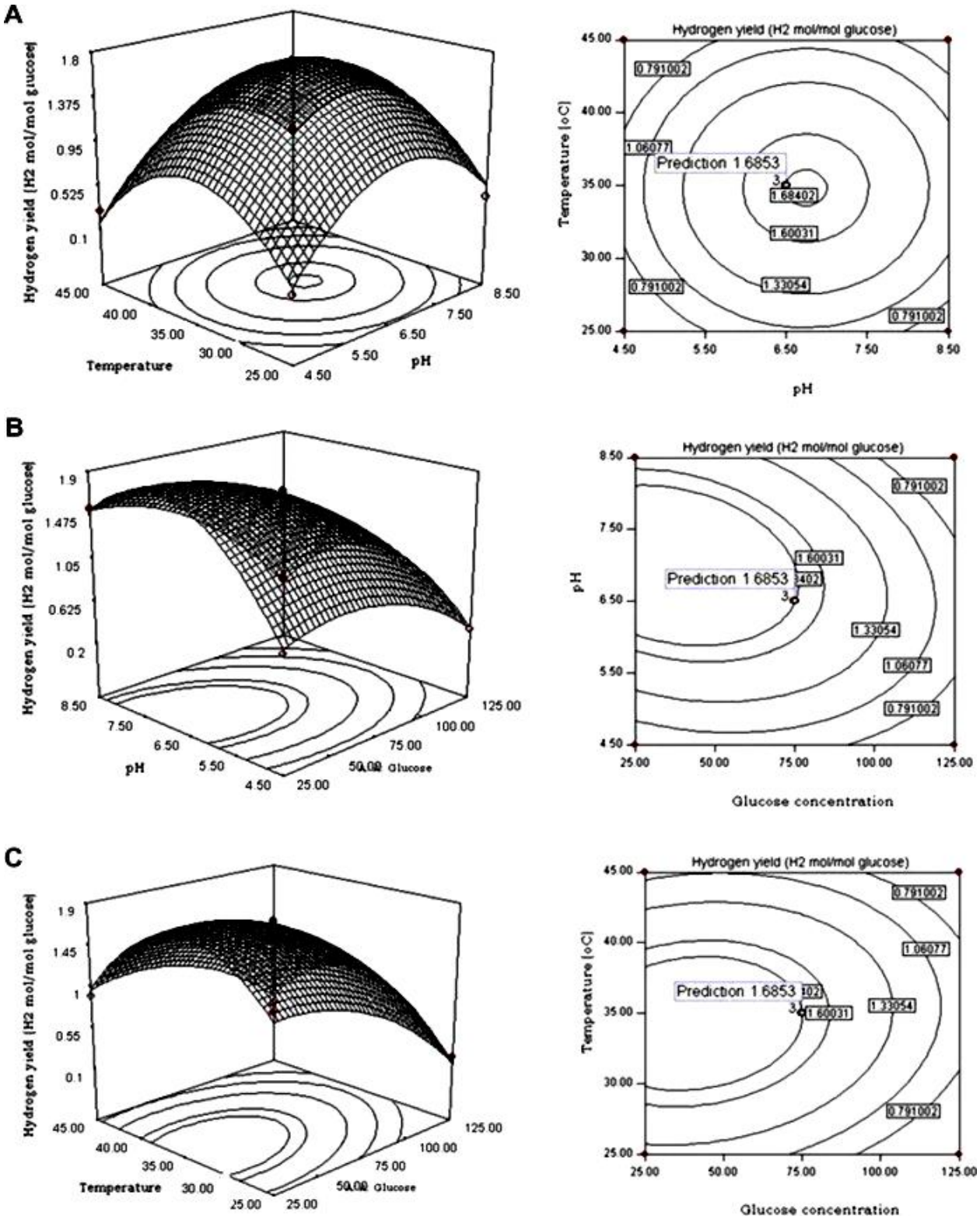
A
Hydrogen yield [H2 mol/mol glucose]
1.8
1.375
0.95
0.525
0.1
45.00
40.00
35.00
30.00
25.00
Temperature
4.50
5.50
6.50
7.50
8.50
pH
Hydrogen yield (H2 mol/mol glucose)
Temperature [oC]
Prediction 1.6853
0.791002
1.06077
1.68402
1.60031
1.33054
pH
B
Hydrogen yield [H2 mol/mol glucose]
1.9
1.475
1.05
0.625
0.2
8.50
7.50
6.50
5.50
4.50
pH
25.00
50.00
75.00
100.00
125.00
Glucose
Hydrogen yield (H2 mol/mol glucose)
Prediction 1.6853
Glucose concentration
C
Hydrogen yield [H2 mol/mol glucose]
1.9
1.45
1
0.55
0.1
45.00
40.00
35.00
30.00
25.00
Temperature
Hydrogen yield (H2 mol/mol glucose)
Prediction 1.6853
Temperature [oC]
Glucose concentration

Fig 1.Two and three dimensional contour plots for the maximum hydrogen yield. RSM plots were generated using the data shown inTable 1. Inputs were the 15 experimental fermentation runs carried out under the conditions established by the Box–Behnken design. (A) Hydrogen yield (H_2/glucose) as a function of temperature and pH. (B) Hydrogen yield (H_2/glucose) as a function of pH and glucose concentration. (C) Hydrogen yield (H_2/glucose) as a function of temperature and glucose concentration.

CHAPTER 3: Increasing the metabolic capacity of *Escherichia coli* for hydrogen production through heterologous expression of the *Ralstonia eutropha* SH operon

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Author contribution: The manuscript was written through contributions of all authors. A.B. did the cloning and some of the original characterization. D.G. created the *E. coli* mutant strains and did final characterization. PCH carried out the experimental design, helped analyse results and wrote the manuscript. All authors have given approval to the final version of the manuscript.

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Increasing the metabolic capacity of *Escherichia coli* for hydrogen production through heterologous expression of the *Ralstonia eutropha* SH operon

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Keywords: Biohydrogen; metabolic engineering; heterologous expression; hydrogen production from NADH

ABSTRACT

Background

Fermentative hydrogen production is an attractive means for the sustainable production of this future energy carrier but is hampered by low yields. One possible solution is to create, using metabolic engineering, strains which can bypass the normal metabolic limits to substrate conversion to hydrogen. *Escherchia coli* can degrade a variety of sugars to hydrogen but can only convert electrons available at the pyruvate node to hydrogen, and is unable to use the electrons available in NADH generated during glycolysis.

Results

Here, the heterologous expression of the soluble [NiFe] hydrogenase from *Ralstonia eutropha* H16 (the SH hydrogenase) was attempted to demonstrate the introduction of a pathway capable of deriving hydrogen from NADH. Successful expression was demonstrated by in vitro assay of enzyme activity. Moreover, expression of SH restored anaerobic growth on glucose to *adhE* strains, normally blocked for growth due to the inability to re-oxidize NADH. Measurement of in vivo hydrogen production showed that several metabolically engineered strains were capable of using the SH hydrogenase to derive 2 mol H_2 per mol of glucose consumed, close to the theoretical maximum.

Conclusion

Here we have shown for the first time substantial in vivo hydrogen production by a heterologously expressed [NiFe] hydrogenase, the NAD-dependent H_2ase of *R. eutropha*. This hydrogenase was able to couple metabolically generated NADH to hydrogen production,

thus rescuing an *adhE* mutant. This enlarges the range of metabolism available for hydrogen production, thus potentially opening the door to the creation of greatly improved hydrogen production. Strategies for further increasing yields should revolve around making additional NADH available.

BACKGROUND

Concerns about climate change and dwindling petroleum reserves are fuelling resurgence in the search for alternative, renewable fuels [1]. Among the possible candidates is hydrogen, and a great deal of active research is underway on hydrogen production, storage and utilization. Hydrogen is an attractive alternative fuel since it has the highest energy content per unit mass of any known fuel (143 $GJ.t^{-1}$), it can easily be converted to electricity by fuel cells, and its combustion produces water as the only by-product. One requirement for a sustainable hydrogen economy is a renewable green technology for producing hydrogen. Biological hydrogen production could possibly be one such process that since it potentially uses renewable energy resources and operates at ambient temperature and atmospheric pressure [2, 3]. Of the different possible approaches, dark fermentative hydrogen production has been the most extensively studied as it is viewed as being closer to near term application and uses readily available waste streams [4, 5]. Hydrogen metabolism is widespread among microbes which have evolved a variety of metabolic networks and biosynthetic machinery to deal with this simple molecule. Many microorganisms can obtain energy by metabolically coupling hydrogen oxidation to a various kinds of electron acceptors such as fumarate, sulphate, carbon monoxide and oxygen. [6] Other organisms, carrying out fermentation in anaerobic environments, reduce protons to hydrogen as a means of disposing of excess reducing equivalents from oxidative metabolic pathways [7].

There are two major enzymes, [FeFe] and [NiFe] hydrogenases, containing complex active sites composed of metal ions and carbon monoxide and cyanide ligands, responsible for the production of hydrogen through the reduction of protons [8]. [NiFe] hydrogenase is widespread in both bacteria and archaea, has broad range of substrate specificity, and has a

relative low and reversible sensitivity to oxygen [9]. Unlike these hydrogenases, [FeFe] hydrogenases are limited to a narrow spectrum of bacteria and a few unicellular eukaryotes and they are extremely sensitive to irreversible oxygen inactivation [10]. [NiFe] hydrogenases consist of a large subunit, containing the Ni-Fe catalytic centre, and a small subunit, which has three Fe-S clusters which transfer electrons from the external electron donor to Ni-Fe active centre to reduce the protons. The molecular assembly of [NiFe] hydrogenase necessary to make it biologically active and functional requires the concerted actions of several maturation systems [11, 12].

Although dark hydrogen fermentation is attractive, there are a number of challenges to its implementation. The major bottleneck is the low yields that are obtained; at most 2 mol H_2 / mol glucose for enteric bacteria, such as *Escherchia coli*, or 4 mol H_2 / mol glucose for strict anaerobes carrying out *Clostridial* type fermentations [2, 4, 7]. Various attempts have previously been made to improve biological hydrogen production in terms of molar hydrogen yields and cumulative hydrogen production rates [13-15]. *E. coli* has been the organism of choice for metabolic engineering given the ease of genetic manipulation in this organism and the large tool box that is available. Moreover, it is intrinsically of interest given its wide substrate specificity; it is capable of producing hydrogen from a variety of six and five carbon sugars and sugar derivatives [16]. Different approaches have been applied and it has been shown that it is possible to attain close to the theoretical yield for this organism of 2 H_2 / mol glucose [17-19].

However, all metabolic engineering approaches using the native metabolic machinery are restricted to a maximum yield of 2 H_2 / mol glucose since *E. coli* is normally unable to drive

hydrogen production with NAD(P)H. Thus, deriving additional hydrogen from reduced electron carriers (NADH, NADPH) formed during substrate degradation requires development of metabolically engineered strains with the introduction and expression of non-native hydrogen producing pathways including foreign hydrogenases. Moreover, this approach has the potential for increasing other properties of interest including the use of protein engineering to channel electron flow, and to improve oxygen tolerance. In this study, we have attempted to drive hydrogen evolution from NADH generated by cellular metabolism through the expression of the soluble, reversible, NAD-linked [NiFe] hydrogenase (SH-H_2ase) operon from *Ralstonia eutropha* and have examined hydrogen yields in metabolically engineered non-hydrogen producing *E. coli* strains altered so as to potentially produce higher cellular levels of NADH.

RESULTS AND DISCUSSION

Rationale

Anaerobically grown *E. coli* carries out mixed acid type fermentation when grown under anaerobic conditions. Sugars are degraded to pyruvate by the glycolytic pathway, producing ATP and reducing NAD^+ to NADH. The amount of NADH that is produced depends upon the redox state of the substrate, and this in turn controls fermentation product distribution. Pyruvate is principally converted to formate and acetyl-CoA. Under the appropriate conditions, usually acidic pH, formate is broken down via the formate-hydrogen lyase pathway producing CO_2 and H_2. Thus, *E. coli* is only capable of the production of a maximum of 2 H_2 per mole of glucose that enters glycolysis (Figure 1). The NADH that is generated during anaerobic growth on sugars must be oxidized to NAD^+ for glycolytic metabolism to continue since NAD^+ is a necessary cofactor for the oxidation of glyceraldehyde. Although in theory NADH could be oxidized by the reduction of pyruvate to lactate by lactate dehydrogenase, in practice this pathway is only fully expressed under acidic conditions, and does not seem to be sufficient on its own to permit anaerobic growth. Therefore, mutants deleted for alcohol dehydrogenase (*adhE*) cannot grow anaerobically on sugars more reduced than glycerinate [24]. Thus, *adhE* mutants might produce excess levels of NADH when incubated anaerobically. Some NADH can be recycled through the oxidation of oxaloacetate to malate, leading ultimately to the formation of succinate, but again, this side pathway is not sufficient in itself to permit anaerobic growth on sugars. Thus, the goal of this research was to attempt to introduce a heterologous pathway that would allow the production of hydrogen by reoxidizing NADH, thus potentially allowing additional hydrogen production while, depending upon the strain, rescuing growth of some mutant strains (Fig. 1).

Initial overexpression of SH hydrogenase

In order to examine the potential for engineering *E. coli* to produce hydrogen from NADH, we chose to express the SH hydrogenase from *Ralstonia eutropha* H16. The SH operon consists of nine genes; *hoxFUYHWI* and *hypA2B2F2* [25]. HoxHY is the hydrogenase module and HoxFU is a NADH dehydrogenase. *hoxW* encodes an highly specific endopeptidase required for the C-terminal processing of HoxH during hydrogenase maturation [26]. Although SH is usually isolated as a heterotetrameric protein (HoxHYFU), HoxI has been shown to provide a NADPH binding domain to a hexameric form of SH that can be isolated under certain conditions [27]. *hypA2B2F2* are duplicate copies of three of the seven *R. eutropha* hydrogenase maturation genes (*hyp*). Interestingly, they can substitute for *hypA1B1F1* in the maturation of both the SH hydrogenase and the MBH (membrane bound) hydrogenase [28]. A previous attempt to express the SH operon in *E. coli* from its native promoter (PSH) was unsuccessful [29], presumably because there is an absolute requirement for the transcriptional activator HoxA for expression from this promoter [25]. Therefore, we wished to express the SH operon from a promoter active in *E. coli*. Plasmid pJWPH5 which expresses the SH operon in *E. coli* under the control of the inducible trc promoter of the vector pTRC99A was constructed as described in Materials and Methods.

The hydrogen evolution capacity of batch cultures of various strains of *E. coli* were tested (Additional File, Table S1) under anaerobic conditions with LB medium as previously described [17]. Hydrogen was evolved by strain BW535, wild-type for the hydrogenase activities of Hyd1, Hyd2, and Hyd3 [30]. Hydrogen was also evolved by strain JW135, a Hyd1- Hyd2- (*Δhya-Km Δhyb-Km*) derivative of BW535. As expected under the conditions used here, strain FTD147, which lacks the hydrogen evolving Hyd3 as well as hydrogen

consuming Hyd1 and Hyd2 [20], showed no hydrogen evolution (*E. coli* Hyd4 is inactive under these conditions [21]. The hydrogen evolution of strains containing pJWPH5 that had been altered so that they potentially produced more NADH was also tested. *ΔadhE* and *ΔarcA* derivatives of strain FTD147 were examined for hydrogen evolution in anaerobically incubated LB-glucose (0.4%) medium (+ 0.05 mM IPTG) (Additional File, Table S1). As discussed above, *ΔadhE* mutants should have excess NADH levels when incubated anaerobically and might consequently support hydrogen evolution by SH hydrogenase. There was no detectable hydrogen evolution by such a strain (FTAB4). Another strain, FTAB5, potentially able to produce increased levels of NADH under anaerobic conditions, was also examined. Strain FTAB5, which carries pJWPH55, is *arcA* derivative of strain FTD147. ArcA is a two-component regulator that is responsible for the anaerobic repression of synthesis of enzymes of the TCA cycle. Therefore, a strain mutated in ArcA might be expected to express the TCA cycle under anaerobic conditions, potentially permitting the generation of excess NADH from acetyl-CoA. Indeed, in vitro TCA cycle enzyme activities are greatly increased in ArcA mutants grown under anaerobic conditions [1] (Perrenoud and Sauer (2005)), suggesting that additional NADH would become available if it could be more effectively oxidized under these conditions. However, there was no detectable hydrogen evolution by this strain (Additional File, Table S1).

Synthesis of SH hydrogenase proteins was checked by a Western blot (Additional File, Figure S1) of an extract of strain FTD147/pJWPH5 grown under anaerobic conditions (LB + 0.05 mM IPTG). Prominent protein bands at 67 and 55 kDa were observed, corresponding to HoxF and HoxH respectively. Although there was a high level of expressed protein in the supernatant (Additional File, Figure S1, lane 2), there appeared to be some inclusion body

formation since a significant quantity was also recovered in the pellet obtained after centrifugation of the crude extract, produced by sonication, for 15 min at 10,000 rpm (Figure S1, lane 3). As a control, a high-speed supernatant of an extract of *R. eutropha* H16 grown anaerobically in FGN medium mineral salts medium [31] as previously modified [32] was included (Additional File, Figure S1, lane1). Thus, sufficient levels of SH hydrogenase appeared to be synthesized under anaerobic conditions in *E. coli* strain FTD147 strain carrying pJWPH5.

Lack of hydrogen evolution by anaerobically incubated FTD147-derivatives carrying pJWPH5 was therefore not due to lack of SH hydrogenase protein, but might rather be due to inadequate hydrogenase maturation. This was verified by checking the in vitro NAD-linked hydrogenase activity of extracts of anaerobically grown cultures of *R. eutropha* H16, *E. coli* FTD147, and *E. coli* FTAB1 (FTD147/pJWPH5) using a spectrophotometric assay [33]. As expected, high levels of H_2-dependent NAD^+ reduction was observed with the R. eutropha H16 extract, and no reduction was seen with FTD147. However, only a very low level of activity was observed for FTAB1 (Additional File, Table S2). Thus, it appears that indeed maturation of *R. eutropha* SH hydrogenase to a functional hydrogenase is very inefficient in *E. coli* grown anaerobically under these conditions.

Anaerobic growth of *adhE* mutants carrying pJWPH5 (SH hydrogenase) on M9 glucose in the presence of nickel and iron

Since SH is a [NiFe] hydrogenase, we hypothesized that the low activity observed might be due to the insufficient supply of nickel and iron and therefore we assessed the effect of added nickel and iron on the formation of active SH hydrogenase[34]. First this was assayed using a growth test run under conditions such that only strains possessing an SH hydrogenase would

grow. As described above, strains that are mutated in *adhE* have been reported to be impaired for growth under anaerobic conditions. We constructed various *ΔadhE* derivatives of both FTD147 and JW135 (FTGH2, FTJWDC3, JWGH1, DG2, FTDPH10) and verified that they were unable to grow under anaerobic conditions on sorbitol or glucose minimal media (Figure S2). Reasoning that growth could be restored if a means of reoxidizing NADH were introduced, these mutants were tested for the rescue of anaerobic growth on glucose by the introduction of pJWPH5, plasmid carrying the SH operon under the control of the trc promoter. Indeed, under these growth conditions, M9-glucose + IPTG + Ni + Fe, these derivatives were able to grow (not shown).

NAD^+ reduction *in vitro*

The growth results strongly suggested that SH expressed and active in anaerobic cultures grown on nickel and iron supplemented M9-glucose. This was verified by assaying the SH hydrogenase activity of the strains in vitro measuring the capacity of extracts to reduce NAD+ under a hydrogen atmosphere, i.e. to carry out hydrogen oxidation (Table 2). As expected, the positive control, an extract of *R. eutropha* H16 showed significant NAD+ reduction activity whereas the two *E. coli* strains, FTD147 and JW135, gave insignificant levels of activity. However, extracts of strains carrying pJWPH5 all showed varying but significant levels of SH hydrogenase activity in vitro. The parental strains, FTD147 and JW135 had specific activities close to 1 μmol NADH /min /mg protein, or 16% of that of an extract of *R. eutropha* H16. Extracts of strains carrying mutations that could be thought to increase cellular NADH levels, FTGH2, FTJWDC3, JWGH1, DG2, FTDPH10, and DG1, gave even higher specific SH hydrogenase levels, varying from 3.5±0.1μmol NADH /min /mg protein to 7.1±0.31 μmol NADH /min /mg protein. These results (Table 2), obtained with nickel and iron amended M9,

demonstrate the importance of media supplementation with the metals required for cofactor synthesis since, in their absence, very little in vitro activity can be demonstrated (Table S2). The highest activities were observed with JWGH1, a JW135 derivative carrying a mutation in the NADH consuming enzyme lactate dehydrogenase and in ArcA in addition to alcohol dehydrogenase, and FTJWDC3, a FTD147 derivative mutated in malate dehydrogenase in addition to alcohol dehydrogenase. Somewhat lower levels of in vitro activity were observed with extracts of DJ1, a JW135 derived strain additionally mutated in *arcA*, and FTGH2, a FTD147 derivative mutated in both lactate dehydrogenase and malate dehydrogenase. A FTD147 derivative, DG2, carrying the same mutations as the JW135 derived strain JWGH1, gave only about 50% of the in vitro activity of that strain. Finally, FTDPH10, mutated in alcohol dehydrogenase and lactate dehydrogenase, gave only 50% of the highest observed in vitro activity, but even so this was more than three-fold higher than the parental strain, FTD147 carrying pJWPH5. It is clear from these results that, even though transcription is under control of the IPTG inducible trc promoter, higher levels of SH hydrogenase, as measured by in vitro activity, were present in strains in which the ability to reoxidize NADH anaerobically were compromised. The exact mechanism behind this enhancement is unclear, but might be related to general effects on growth. In addition, the results shown in Table 2 suggest that the effect of the introduction of multiple mutations in pathways that oxidize NADH appears to be additive, with abolition of malate dehydrogenase being more effective in a *adhE* strain than eliminating lactate dehydrogenase activity. At any rate, these results demonstrate the first successful heterologous expression in *E. coli* of a multi-subunit [NiFe] hydrogenase capable of interacting with NAD^+/NADH.

In vivo hydrogen production by *E. coli* strains expressing SH hydrogenase

The results of the in vitro activity assays and the growth studies both provided evidence for the active expression of SH hydrogenase carried by pJWPH5. Therefore it was of interest to determine if these strains could produce hydrogen in vivo from glucose, demonstrating the establishment of a novel hydrogen producing pathway in *E. coli*. The different strains were incubated anaerobically in modified M9-glucose. Growth was followed by measuring the OD (600nm) (Figure 2A) and the hydrogen produced was assayed using gas chromatography (Figure 2B). All strains showed significant growth over the experimental period after a variable lag period (Figure 2A). Growth was highest, and at nearly the same level, in strains FTGH2, FTJWDC3, and JWGH1. FTGH2 and FTJWDC3 both carry *adhE* and *mdh*; FTGH2 carries in addition *ldhA*. Final optical densities were appreciably lower in strains DJ1, DG2, and FTDPH10. Nevertheless, the growth of strains carrying *adhE*; FTGH2, FTJWDC3, JWGH1, DG2, and FTDPH10, demonstrates that they were sufficiently capable of reoxidizing NADH to permit growth. Since growth was only observed in strains carrying pJWPH5, NADH oxidation must have been provided by the action of SH hydrogenase.

Hydrogen production by these cultures was also examined (Figure 2B). All strains tested showed appreciable hydrogen evolution activity with final hydrogen levels of between 2000 and 3600 nmol H_2 per vial (2 ml of culture). Strains FTJWDC3 and JWGH1 produced the greatest amount of hydrogen, whereas strains FTGH2 and DG2 produced the least. Interestingly, the two best hydrogen producers were also the strains that were shown to have the highest levels of SH hydrogenase activity in vitro (Table 2). Strain DG2, which gave one of the lowest SH hydrogenase activities in vitro, also produced the least amount of hydrogen. Taken together this suggests that hydrogen production levels are controlled by the amount of

active SH hydrogenase that is present, but further work would be required to firmly establish this point. In addition, alterations in carbon flux through the different metabolic pathways operating in the various strains may have an influence as well.

To measure the efficiency of hydrogen production, the amount of glucose consumed at the end point was determined and used to calculate the hydrogen yields, mol H_2 produced / mol glucose consumed, of the different cultures (Table 3). All strains showed very good hydrogen yields, varying from 1.41 to 2.1 mol H_2 / mol glucose, with strains FTJWDC3 and JWGH1 being the most efficient. The yields observed here are higher than those normally observed with wild type cultures and are as high, or slightly higher, than the theoretical maximum for *E. coli* (see figure 1 and earlier discussion). These yields are also much higher than those obtained in previous studies where heterologous hydrogen producing pathways were introduced into *E. coli*. In several previous studies, ferredoxin-dependent [FeFe] hydrogenase pathways were introduced along with the enzymes necessary to reduce ferredoxin with either NADH or NADPH. However, yields were disappointingly low; 0.025[35], 0.04[36], 0.05[37] mol H_2 / mol glucose. On the other hand, when a [FeFe] hydrogenase was coupled with metabolism by the expression of a pyruvate: ferredoxin oxidoreductase, yields as high as 1.46[38] mol H_2 / mol glucose were obtained. Here we have introduced a [NiFe] hydrogenase dependent pathway and shown that it is capable of higher (44% greater than the highest previously reported) yields than the previously characterized [FeFe] hydrogenase dependent pathways. Others have previously reported the heterologous expression of [NiFe] hydrogenases [34, 39-43], but only in one case [43] was the in vivo hydrogen yield reported. In this report, a cyanobacterial [NiFe] hydrogenase was expressed in an *E. coli* strain which also possessed a native hydrogenase 3. Thus the two hydrogenase

activities are confounded and one cannot say how much was due to the introduced hydrogenase, which might very well have had an indirect effect since its expression increased formate flux through Hyd3. Here we have unequivocally shown that heterologous expression of the [NiFe] SH hydrogenase can give up to 2 mol H_2 / mol glucose since we used a strain devoid of native hydrogenase activity.

CONCLUSIONS

The work reported here shows convincingly for the first time that a pyridine nucleotide dependent [NiFe] hydrogenase can be heterologously expressed in *E. coli* and produce appreciable amounts of hydrogen from NAD(P)H produced by cellular metabolism. This represents a significant advance in the ability to engineer hydrogen producing pathways in *E. coli*. Moving forward, a number of improvements could be made. Increasing flux through the system would be required to increase the rates of hydrogen production. In addition, a practical hydrogen production system would require that greater yields be obtained from the substrate, which could be brought about in several different ways. For one thing, more efficient coupling with the native hydrogen producing system, which produces hydrogen indirectly from pyruvate through the pyruvate:formate lyase system, should further increase yields. Another possibility would be to introduce a mechanism whereby additional NADH could be generated through the further metabolism of pyruvate, for example, through the anaerobic functioning of the citric acid cycle.

MATERIALS AND METHODS

Design and construction of expression system

Plasmid pJWPH5 for expression of the SH operon in *E. coli* under the control of the inducible *trc* promoter of the vector pTRC99A was constructed as follows. A 2.6 kb fragment of the 5' end of the SH operon contained in plasmid pCH455 was PCR amplified using a primer which introduced an upstream XbaI site, and cloned into the XbaI/BamHI sites of pBluescript, giving pAB3. The SH operon was reconstituted by ligating BamHI-HindIII digested pCH455 and pAB3, giving plasmid pAB13. Finally, digestion of pAB13 with XbaI – HindIII gave a 14.2 kb fragment containing the SH operon, minus promoter sequence, which was cloned into pTRC99A, giving pJWPH5. Constructions were verified by restriction digests and PCR reactions.

Preparation of metabolically engineered *E.coli* strains

Escherichia coli strains JW135 and FTD147 were used as the host strains for metabolic pathway alterations. FTD147 lacks the hydrogen evolving Hyd3 as well as hydrogen consuming Hyd1 and Hyd2[20], and shows no hydrogen evolution under the conditions employed in this study (*E. coli* Hyd4 is inactive under these conditions[21]). Various metabolic alterations were made that would potentially increase NADH levels and thus provide substrate for the SH hydrogenase. Thus, different NADH utilizing pathways were blocked by mutating; *adhE*, *ldhA*, and/or *mdh*. Mutations were introduced into the parental strains by P1 bacteriophage transduction (DC1048-Δ*adhE*::Tn*10* TcR, SE1752-*ΔldhA::*Tn*10* TcR, JW3205-1-*Δmdh::*Tn*10 kanR*, QC2575-*ΔarcA::TcR*). Mutants are typically designated

as *E. coli* FTGH2 (Δ*ldhA*, Δ*adhE*, Δ*mdh*); FTJWDC3 (Δ*adhE*, Δ*mdh*); DG2 (Δ*ldhA*, Δ*adhE*, Δ*arcA*), FTDPH10 (Δ*ldhA*, Δ*adhE*) respectively. To make strains carrying multiple mutations, the tetracycline resistance marker was removed by growing them on Maloy Nunn medium [22]. Phenotypes were confirmed by high performance liquid chromatography and growth was scored on minimal M9-Sorbitol medium under anaerobic conditions (Figure S2). pJWPH5 carrying the SH operon was introduced into the various strains by chemical transformation.

Heterologous expression of SH in metabolically engineered *E.coli* strains.

All *E. coli* strains were grown overnight aerobically at 37ºC in 5 ml LB medium with the appropriate antibiotics; ampicillin (100 µg/ml), tetracycline (15 µg/ml), and kanamycin (25 µg/ml). Antibiotic concentrations were used at half their standard concentrations in M9 (1X) minimal medium. Preinocula were prepared by growing the mutants on modified M9-glucose medium containing; ampicillin (50 µg/ml), 100 µM $FeCl_3$, 25 µM $NiSO_4$ and 0.05 mM IPTG under anaerobic conditions. Hydrogen production assays were carried out by inoculating the same medium contained in anaerobic tubes sealed with butyl rubber stoppers and incubating under anaerobic condition with glucose as sole carbon source (0.4% w/v and 0.2% w/v).

Analytical methods

The concentration of hydrogen in the collected gas was determined using a gas chromatograph (Shimadzu GC-8A) equipped with a thermal conductivity detector, a 1 m column packed with molecular sieve 5A and with argon as carrier gas. Bacterial growth was determined by measuring the optical density at 600 nm using a double beam spectrophotometer (Shimadzu).

Glucose concentrations were determined spectrophotometrically at 490 nm using a phenol-sulphuric acid assay [23]

AUTHORS' CONTRIBUTIONS

The manuscript was written through contributions of all authors. AB did the cloning and some of the original characterization. DG created the *E. coli* mutant strains and did final characterization. PCH carried out the experimental design, helped analyse results and wrote the manuscript. All authors have given approval to the final version of the manuscript.

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FIGURES

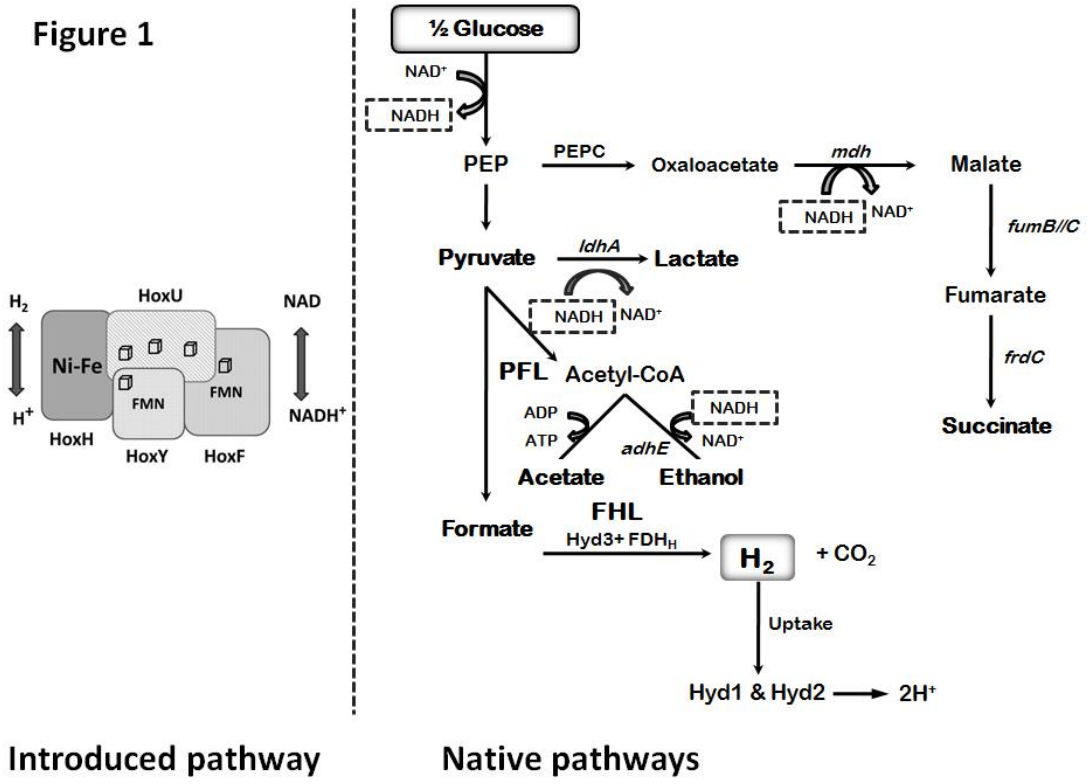

Figure 1. Native and engineered metabolic pathways involved in hydrogen production by *E. coli*. On the right is shown the main multiple pathways of mixed acid fermentation. Key enzymes and enzyme complexes are indicated by either the genetic nomenclature or the commonly used pathway abbreviation: Phosphoenolpyruvate (PEP); Phosphoenolpyruvate carboxylase (PEPC); Fumarate reductase (*frdC*); Lactate dehydrogenase (*ldhA*); Pyruvate formate lyase (PFL); Formate hydrogen lyase (FHL); Hydrogenase 3 (Hyd 3); formate dehydrogenase-H (FDHH); Uptake hydrogenases; hydrogenase 1 (Hyd 1) and hydrogenase 2 (Hyd 2); fumarase (*fumB*); fumurate reductase (*frdC*). Points where NADH is produced or consumed are noted. On the left is a schematic of the SH hydrogenase, which, if functional, might consume NADH, reducing protons to hydrogen.

Figure 2A

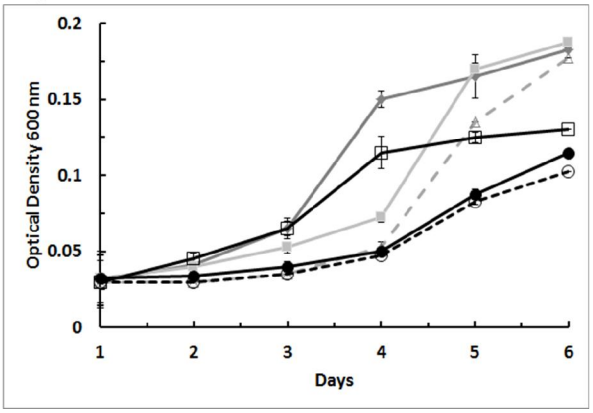

Figure 2B

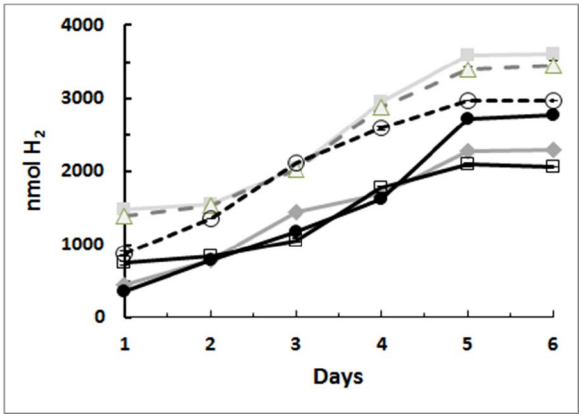

Figure 2. Growth and *in vivo* hydrogen production by strains expressing SH hydrogenase cultures, pregrown under the same conditions, were incubated at 37º C in anaerobic vials containing modified M9 glucose (+IPTG, Ni and Fe). Samples were taken periodically to measure OD (Figure 2A) and hydrogen (Figure 2B).
FTGH2/pJWPH5 ( ); FTJWDC3/pJWPH5 ( ); JWGH1/pJWPH5 ( );

DJ1/pJWPH5( ); DG2/ pJWPH5 ( ); FTDPH10/ pJWPH5 ( )

Tables

Table 1. Strains used

Strain	Genotype	Reference/construction
FTD147	Δ*hyaB* Δ*hybC* Δ*hycE*	Skibinski et al 2002
JW135	Δ*hya-Km,* Δ*hyb-Km*	Menon et al 1991
FTAB1	FTD147/ pJWPH5*	This study
FTAB4	FTD147, *ΔadhE, zch::*Tn*10*, pJWPH5	P1 DC1048 (*ΔadhE::*Tn*10*),
FTAB5	FTD147 *ΔarcA ::*Tn*10*, pJWPH5	P1 QC2575 (*ΔarcA::*Tn*10*),
FTDPH10	FTD147/pJWPH5 *adhE, ldhA*	P1 DC1048 (*ΔadhE::*Tn*10*), SE1752 (*ΔldhA::*Tn*10*)
DG2	FTD147/pJWPH5 *adhE, ldhA, arcA*	P1 DC1048 (*ΔadhE::*Tn*10*), SE1752 (*ΔldhA::*Tn*10*), QC2575 (*ΔarcA::*Tn*10*)
FTJWDC3	FTD147/pJWPH5 *adhE, mdh*	P1 DC1048 (*ΔadhE::*Tn*10*), JW3205-1 (*Δmdh::*Tn*5*)
FTGH2	FTD147/pJWPH5 *adhE, ldhA, mdh*	P1 DC1048 (*ΔadhE::*Tn*10*), SE1752 (*ΔldhA::*Tn*10*), JW3205-1 (*Δmdh::*Tn*5*)
DJ1	JW135/pJWPH5 *arcA*	P1 QC2575 (*ΔarcA::*Tn*10*)
JWGH1	JW135/pJWPH5 *adhE, ldhA, arcA*	P1 DC1048 (*ΔadhE::*Tn*10*), SE1752 (*ΔldhA::*Tn*10*), QC2575 (*ΔarcA::*Tn*10*)

* Plasmid containing the *Ralstonia eutropha* SH operon under the control of the *trc* promoter in pTrc99A

Table 2. In vitro NAD+ reduction activity of various strains

	Strain	μmol NADH min^{-1} mg^{-1}
	R. eutropha H16	6.1±0.4
	FTD147	0
	JW135	0.04±0.009
+ pJWPH5	FTD147	0.94±0.16
	JW135	1.09±0..003
	FTGH2	3.9±0.06
	FTJWDC3	6.4±0.10
	JWGH1	7.1±0.31
	DJ1	4.45±0.003
	DG2	3.56±0.11
	FTDPH10	3.5±0.1

Table 3 In vivo hydrogen yields of strains expressing SH hydrogenase

Strain			Yield[a]
	Background	**Mutations**	
FTDPH10	FTD147 (*ΔhyaB ΔhybC ΔhycE*) /pJWPH5	*adhE, ldhA*	1.41
DG2		*adhE, ldhA, arcA*	1.46
FTJWDC3		*adhE, mdh*	2.08
FTGH2		*adhE, ldhA, mdh*	1.49
DJ1	JW135/ pJWPH5	*arcA*	1.55
JWGH1		*adhE, ldhA, arcA*	2.11

[a]mol H_2/mol glucose consumed

ADDITIONAL FILES

Additional_file_1 as PDF

Additional file 1 Figure S1 Western blot analysis of expression of SH hydrogenase **Figure S2** *adhE* strains are unable to grow anaerobically on reduced sugars **Table S1** Hydrogen evolution by various strains of *E. coli* incubated under anaerobic conditions **Table S2** *in vitro* H_2-dependent NAD reduction by various extracts

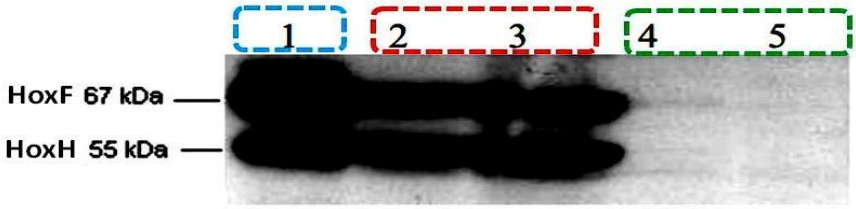

Figure S1. Western blot analysis of expression of SH hydrogenase.

FTD147/pJWPH5) was cultured overnight at 30° C in LB medium with 0.05 mM IPTG under anaerobic conditions. The culture was harvested by centrifugation, sonicated, and centrifuged (15 min, 10,000 rpm). 25-35 µg pellet (lane 2) and supernatant (lane 3) were electrophoresed on 12% SDS-polyacrylamide gels (Laemmli and Favre (1973)), transferred to PVDF membrane, developed with primary anti-serum to SH hydrogenase, and revealed by chemiluminescence as previously described (Yakunin and Hallenbeck (1998)). A similarly cultured and prepared extract of FTD147 was also analyzed (supernatant, lane 4, pellet, lane 5) and found to be devoid of these protein bands. As a positive control, an aliquot of the supernatant of a 45 min 100,00 g centrifugation of a sonicated extract of *R. eutropha* H16 grown anaerobically overnight on NB medium at 30° C was also loaded (lane 1).

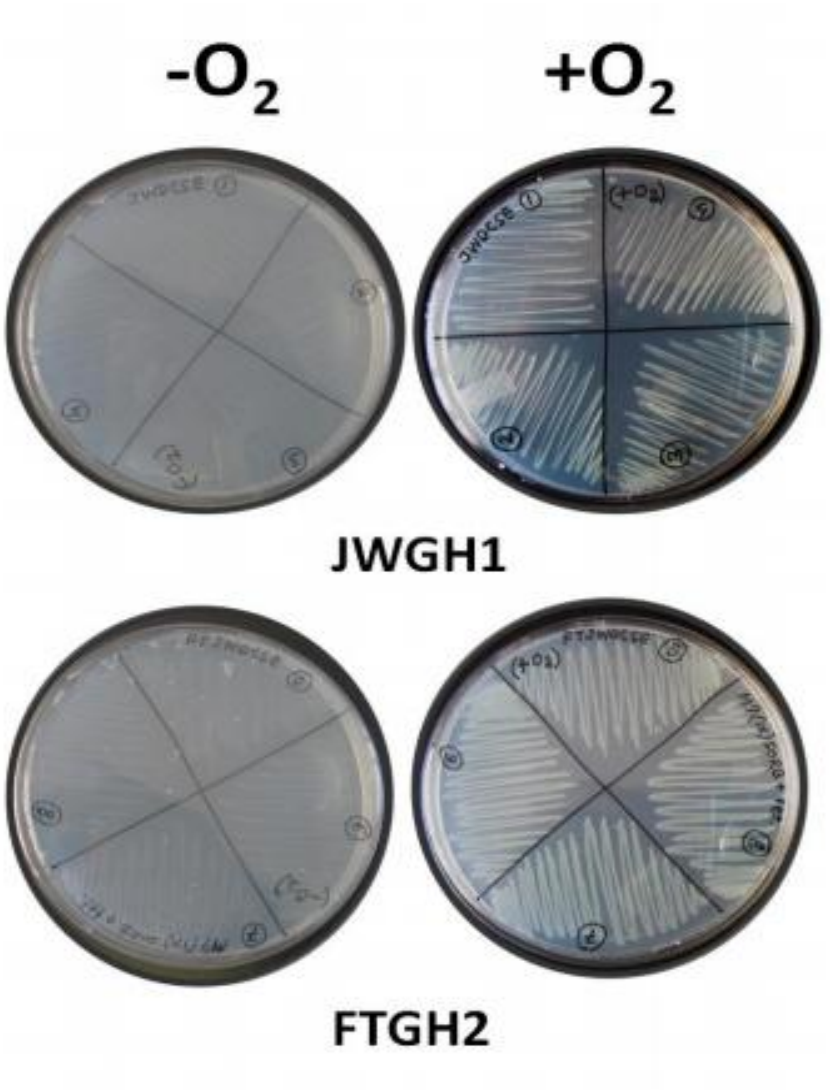

Figure S2. *adhE* strains are unable to grow anaerobically on reduced sugars

The constructed strains, DG2 and FTGH2 were tested for anaerobic growth on reduced sugars by streaking M9-sorbitol (supplemented with Ni^{+}, Fe^{2+} and IPTG) and incubating at 37º C in anaerobic jars (left). As a positive control, these strains were shown to grow on the same medium incubated aerobically (Right)

Table S1. Hydrogen evolution by various strains of *E. coli* incubated under anaerobic conditions.

***E. coli* Strain**	**Relevant genotype**	**H_2**
BW545	Wild type	+
JW135	△*hya*-Km △*hyb*-Km	+
FTD147	△*hyaB* △*hybC*, △*hycE*	-
FTAB4	FTD147, △*adhE, zch::*Tn*10*, pJWPH5	-
FTAB5	FTD147 △*arcA ::*Tn*10*, pJWPH5	-

Table S2. *In vitro* H_2-dependent NAD reduction by various extracts

Strain	**µmol NADH min^{-1} mg^{-1}**
R. eutropha H16	5.87 ± 0.99
E. coli FTD147	0.00
E. coli FTAB1	0.39 ± 0.19

* The in vitro NAD-linked hydrogenase activity of extracts of anaerobically grown cultures of *R. eutropha* H16, *E. coli* FTD147, and E. coli FTAB1 were assayed spectrophotometrically (Schneider and Schlegel (1976)). Twenty µg of extract were incubated in a stoppered cuvette containing 1.9 ml of hydrogen-saturated Tris buffer (50 mM, pH 8, 30°C) that had been flushed with hydrogen. The reaction was initiated by the addition of NAD to 0.8 mM, and the reduction of NAD followed at 365 nm.

CHAPTER 4: Near Stoichiometric reforming of biodiesel derived crude glycerol to hydrogen by Photofermentation

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Author contribution: The experimental design and wet laboratory experiments had been carried out by D.G. and A.T. partially helping D.G. on this project to make his internship under my supervision. D.G. performed all dataset analysis. All the work was under supervision of Professor Dr. Patrick Hallenbeck.

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Near Stoichiometric reforming of biodiesel derived crude glycerol to hydrogen by Photofermentation.

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Keywords: Biohydrogen; Photofermentation; Photosynthetic bacteria; Biodiesel-derived glycerol

ABSTRACT

Biodiesel manufacture produces crude glycerol as a major byproduct. At the scale estimated for future biodiesel production, extensive quantities of crude glycerol fraction will be generated, creating a large waste stream with potentially significant environmental impacts. The magnitude of projected future crude glycerol supplies suggests that its conversion to a biofuel is the only viable route to producing a product that does not cause market saturation. Previously it was shown that crude glycerol could be converted to hydrogen, a possible future clean energy carrier, by photofermentation using *Rhodopseudomonas palustris* through photofermentation. Here, the effects of nitrogen source and different concentrations of crude glycerol on this process were assessed. At 20 mM glycerol, 4 mM glutamate, 6.1 mol hydrogen/mole of crude glycerol were obtained under optimal conditions, a yield of 87% of the theoretical, and significantly higher than what was achieved previously.

INTRODUCTION

Increasing crude oil costs, limited fossil fuel reserves and climate change effects have led to a search for alternative energy carriers. The production of biodiesel, a first generation biofuel, is increasing each year, with a total world production of about 15 billion liters in 2009. A crude glycerol fraction, 1 kg for each 10 l of biodiesel, is obtained as a result of the transesterification reaction used to produce biodiesel. Thus, with the greatly increased production of biodiesel a glut of glycerol has been created, causing a collapse in the value of glycerol, with glycerol prices plummeting over 10-fold in the past few years, forcing traditional crude glycerol refineries to close [1]. Hence there is an urgent need to develop a practical process for converting waste glycerol to useful products, a need that will only grow with the expected future enormous quantities of glycerol that will be generated as biodiesel production increases even more. However, the crude glycerol fraction contains a number of impurities [2], making its purification for use as a fine chemical too costly [1].

Given the enormous quantities of crude glycerol likely to be produced in the near future with ramped up biodiesel production, it would only be practical to convert it to a high volume, low value commodity. One possibility is its conversion by fermentation to hydrogen, under development as a future fuel. However, traditional fermentation processes yield at most 1 mol of hydrogen per mole of glycerol. For example, a number of studies have shown that *Escherchia coli*, even after genetic modification, converts glycerol to hydrogen (1 mol/mol) and ethanol (1 mol/mol) [3], [4], [5], [6], [7], [8] and [9]. Similar yields have been obtained with *Enterobacter aerogenes* after medium optimization [10]. Conversion with mixed cultures results in even lower yields, 0.20–0.37 mol H_2/mol glycerol [11]. Recently, it has been shown that the biohydrogen can be produced from crude glycerol using the

photosynthetic bacterium, *Rhodopseudomonas palustris*[12], in a photofermentation process [13] and [14] known for nearly 60 years [15]. Photofermentation can be used to convert sugars, organic acids and wastes to hydrogen as varying stoichiometires [16] and [17]. The theoretical stoichiometric yield from glycerol is 7 mol hydrogen, but the previous study on production by photofermentation only achieved 4 mol hydrogen/mol of crude glycerol [12]. Thus, we considered it desirable to attempt to increase hydrogen yields. In the present study, 6.1 mol hydrogen/mole of crude glycerol were obtained under optimal conditions, a yield of 87%.

EXPERIMENTAL

Bacterial strain and culture conditions

The photosynthetic purple bacterium *R. palustris* (CGA009) was maintained regularly in screw cap sealed tubes (1.6 cm by 12.5 cm) completely filled with RCV succinate amended with biotin (15 μg/l) and para-amino-acid benzoic acid (200 μg/l) incubated at 30 °C in an environmental chamber Biotronette Mark III (Lab-line Instruments) equipped with three 150 W incandescent bulbs.

Photofermentation of glycerol

Hydrogen production from crude glycerol was measured using 125 ml serum bottles containing RCV medium, containing either succinate or a crude glycerol fraction derived from biodiesel manufacture as carbon source, and ammonium sulphate or glutamate as nitrogen source at the indicated concentrations. Inoculation into 80 ml of RCV was made with 5 ml of bacterial culture in the late log phase of growth. The bottles were fitted with rubber stoppers, purged for 15 min with oxygen-free argon and then submerged in a water bath maintained at

30^{o}C. The intensity of light provided by a panel of six 50 W halogen bulbs was 200 W/m ^{2} at the surface of the bottles. The hydrogen produced was measured by water displacement using graduated cylinders connected to the serum bottles by relatively hydrogen impermeable FEP 890 tubing.

Analytical procedures

The concentration of hydrogen in the collected gas was determined using a gas chromatograph (Shimadzu GC-8A) equipped with a thermal conductivity detector and a 1 m column packed with molecular sieve 5A with Ar as carrier gas. Glycerol concentrations were determined spectrophotometrically at 410 as 3, 5-diacetyl-1, 4-dihydrolutidine, produced by periodate oxidation and reaction with acetylacetone in the presence of ammonium acetate [18].

RESULTS AND DISCUSSION

Effect of different sources of carbon and nitrogen on growth of *R. Palustris*

In order to develop active inocula for examining hydrogen production from crude glycerol, we examined growth on two different nitrogen sources, glutamate and ammonium, with two different carbon sources, glycerol and succinate. Glycerol was compared with succinate since succinate is the most common substrate used with *R. palustris* which is known to actively grow with this substrate. Therefore, the effects of different carbon, succinate (4 mM) and crude glycerol (10 mM), and nitrogen, (4 mM) and ammonium sulphate (4 mM), sources on bacterial growth were assessed (Fig. 1). Although glutamate is often used at higher concentrations, an intermediate concentration of 4 mM glutamate was chosen since this concentration gave a good balance between hydrogen and biomass production (not shown). Combinations of succinate with ammonium sulphate and crude glycerol with glutamate

appeared to be optimal for bacterial growth.With final optical densities well above 1.0 absorbance units (1.41 and 1.32 respectively at 600 nm) they clearly exceeded the optical densities of other combinations. Moreover, it is interesting to note that cultures growing on succinate and ammonium sulphate grew the fastest, achieving an optical density greater than 1.0 by the second day. Given these results, innocula were subsequently prepared by first obtaining active cultures on succinate/ammonium medium before several transfers in glycerol/glutamate prior to the experimental run.

Effect of different concentrations of crude glycerol on cumulative hydrogen production

Cultures acclimated to crude glycerol and glutamate were used to assess the effects of different crude glycerol concentrations on hydrogen production. A wide range of concentrations (20 mM‑160 mM) was selected to observe the effect of varying concentrations of the crude glycerol fraction on the rate of hydrogen production, and to establish if there was a threshold concentration above which hydrogen production was inhibited by the presence of toxic substances in the crude glycerol fraction. Total cumulative H_2 production and overall rates of H_2 production were highest at an initial concentration of 80 mM (Fig. 2). At 160 mM glycerol there was a significant drop in hydrogen production, possibly due an increased concentration of inhibitors.

Thus, there appears to be a balance between the ability of crude glycerol to drive hydrogen evolution and possible inhibition due to impurities. Some inhibition was also noted at concentrations between 20 and 40 mM, suggesting that there might be several levels of inhibition: a threshold at low concentrations, between 20 and 40 mM crude glycerol, and another at high concentrations between 80 mM and 160 mM raw glycerol. A possible

explanation for this phenomenon is that there is a balance between the ability of crude glycerol to drive hydrogen evolution and possible inhibition due to impurities at certain concentrations of crude glycerol. Full understanding of this complex response would require further study. For example, it might be instructive to measure the actual light intensity within the culture which is a complex function of cell biomass, initial glycerol concentration and incident light intensity. At any rate, examination of glycerol consumption showed a direct relationship between glycerol consumption and hydrogen productivity. Cultures with good productivity, 20 mM and 80 mM were also active in glycerol consumption, consuming the majority of added glycerol (90% and 57% respectively), whereas cultures exhibiting poor hydrogen productivity also were weak glycerol consumers, with 20% or less of added glycerol being consumed.

Hydrogen yields

Different processes could be used to convert glycerol to hydrogen, including in addition to photofermentation, studied here, fermentation, electrohydrogenesis, steam reforming and autoreforming. In all cases the goal should be to derive as much hydrogen as possible from the substrate with minimal production of side products. Thus, the ultimate objective is the complete chemical conversion of glycerol to hydrogen, as given in the equation below:

$$C_3H_8O_3 + 3H_2O \rightarrow 3CO_2 + 7H_2$$

The yields obtained at different initial crude glycerol concentrations were calculated on the basis of the glycerol consumed, as determined by spectrophotometric analysis. Glutamate was assumed to not contribute to hydrogen production since separate experiments showed that cultures incubated in media containing 4 mM glutamate as sole carbon (and nitrogen) source

did not produce any hydrogen, an effect that has been well established over the last three decades [19]. On this basis it could be determined that hydrogen yields decrease in inverse proportion to the initial concentration of crude glycerol (Fig. 3). A maximum yield of 6.1 H_2/crude glycerol (mol/mol), or 87% of the theoretical maximum value was obtained with an initial concentration of 20 mM, the lowest concentration studied.

This is significantly higher than previously reported values from any of the possible conversion processes (Table 1). The yields of standard dark fermentation metabolisms appear to be limited to 1–2 mol H_2/mole glycerol at most, a yield much lower than that obtained here, and with the consequent production [11], [20], [21], [22], [23], [24], [25] of large amounts of side products . Electrohydrogenesis is a biological route that can, in principal, achieve higher yields than a simple fermentation. However, the one study that has been done with this method obtained a yield of 3.9 [26], only 56% of the theoretical maximum and 64% of what we have found here with photofermentation. Steam reforming of crude glycerol, another possible process, requires high temperatures, 300–850^{o}C and a catalyst, sometimes made from noble metals. Although relatively high yields can be obtained, 65–75%, production is short lived due to severe coking, and, more importantly, significant quantities of CO are produced which would therefore require that the hydrogen stream be highly purified to prevent fuel cell poisoning [27] and [28]. Auto thermal reforming might be another option, but the required addition of O_2 would lead to increased production of CO with a consequent decrease in H_2 yields.

The present study has shown that a crude glycerol fraction from biodiesel manufacture can be converted to hydrogen at near stoichiometric yields. However, development of a

practical process based on this photofermentative capacity of photosynthetic bacteria would require a number of changes and improvements. The crude glycerol fraction is relatively poor in nitrogen. The addition of glutamate, used in the present study, would be impractical, and a low-cost substitute would have to be used, possibly in conjunction with a strain that had been modified such that its nitrogenase, the enzymatic system that produces hydrogen in the photofermentation process, was no longer subject to ammonia inhibition.

CONCLUSIONS

In the present study, we achieved a maximum yield of 6.1 mol of hydrogen per mole of crude glycerol, which is equivalent to 87% of the theoretical maximum, significantly higher than previously reported values (Table 1). Experimentally, it has been shown that the initial concentration of crude glycerol has a profound effect on both the cumulative production and yield of hydrogen. It has also been shown that glutamate is a better source of nitrogen than ammonium sulphate hydrogen production, quite possibly due to inhibitory effects of ammonium on nitrogenase. Even though we have shown that it is possible to achieve high yield conversions, there are still a number of limiting factors that must be overcome to increase the yields and rates of hydrogen production. These limitations include low light conversion efficiencies, and the need to develop low-cost, hydrogen impermeable photo bioreactors.

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TABLES

Table 1 – Hydrogen yields from glycerol by different reforming methods.

Method/Microorganism	H_2 yield (mol/mol)	Reference
Fermentation		
Pure glycerol		
Halanaerobium saccharolyticum	2.16	[20]
Klebsiella sp.	0.35	[21]
Enterobacter aerogenes	0.89	[22]
Biodiesel-derived glycerol		
Mixed culture	0.18	[11]
Klebsiella pneumoniae	0.53	[23]
Enterobacter aerogenes	0.77	[24]
Thermotoga neapolitna	2.76	[25]
Photofermentation		
Rhodopseudomonas palustris	4.0	[12]
Rhodopseudomonas palustris	6.1	Present study
Electrohydrogenesis		
Mixed culture	3.9	[26]
Steam/Autothermal Reforming		
Ni/CeO_2 600 °C	5.25	[27]
Ni-based 804 °C	4.5	[28]

FIGURES

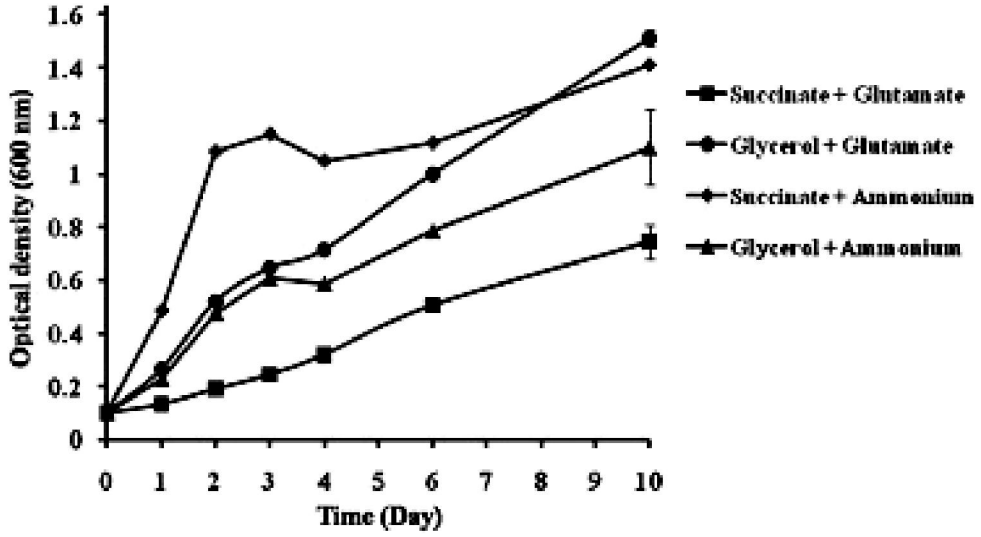

Figure1. Growth of *Rhodopseudomonas palustris* on different nitrogen and carbon sources. Growth of *R. palustris* (CGA009) was measured using the same experimental set-up used to measure hydrogen production.

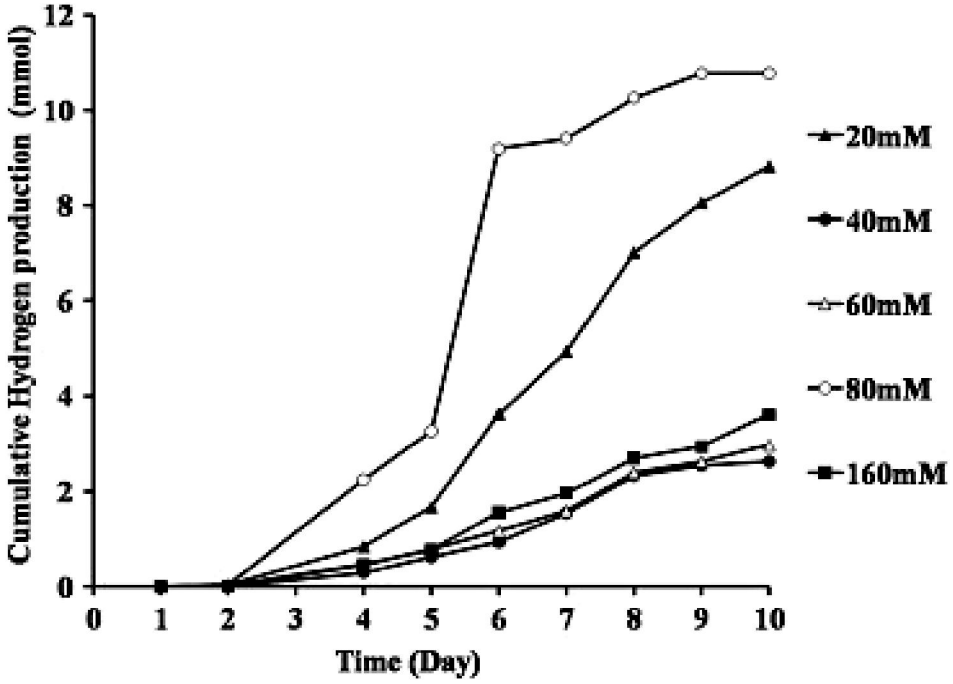

Figure 2. Effect of crude glycerol concentration on total hydrogen production. Hydrogen production from crude glycerol was measured using 125 ml serum bottles containing RCV medium with 4 mM glutamate.

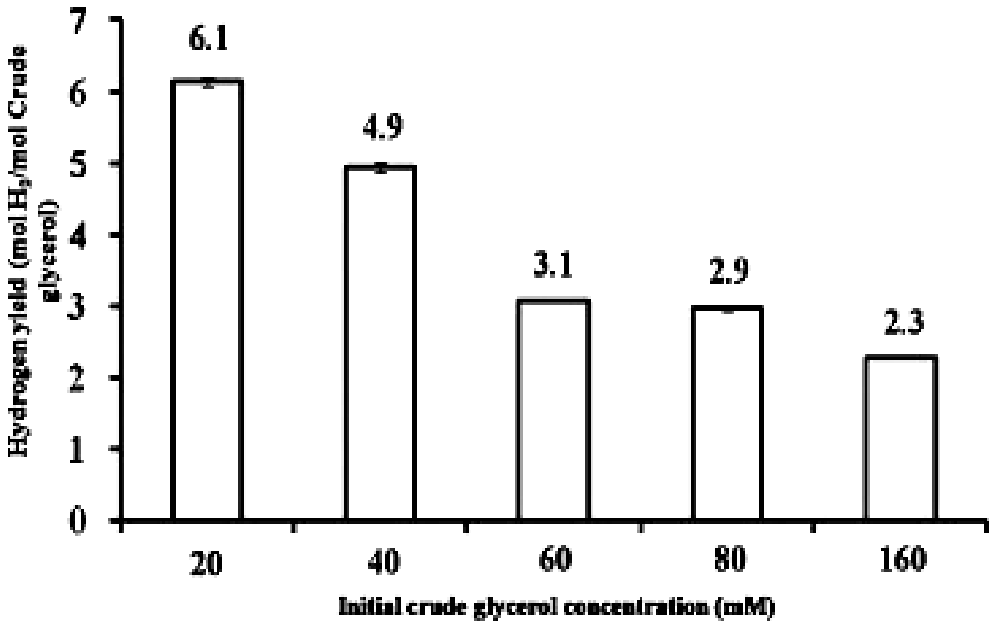

Figure 3. Effect of crude glycerol concentration on hydrogen yields. Hydrogen was determined using gas chromatography and glycerol concentrations were measured by reaction with acetylacetone

CHAPTER 5: 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

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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

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Keywords: Biohydrogen; Photofermentation; Photosynthetic bacteria; Biodiesel-derived glycerol

ABSTRACT

Photofermentation by the photosynthetic bacterium *Rhodopseudomonas palustris* has been used to convert the crude glycerol fraction from biodiesel production to hydrogen as a means of converting this large resource to useful energy. In the present study response surface methodology was applied to investigate the interactive effects among several important process parameters; light intensity, and the concentrations of crude glycerol and glutamate, on the stoichiometric conversion of crude glycerol to hydrogen. Under optimal conditions, a light intensity of 175 W/m^2, 30 mM glycerol, and 4.5 mM glutamate, 6.69 mol hydrogen/mole of crude glycerol were obtained, a yield 96% of theoretical. Determination of nitrogenase activity and expression levels showed that there was relatively little variation in levels of nitrogenase protein with changes in process variables whereas nitrogenase activity varied considerably, with maximal nitrogenase activity (228 nmol of C_2H_4/ml/min) at the optimal central point.

INTRODUCTION

Biodiesel manufacturing produces extensive amounts of crude glycerol as the major side product since the currently used technology, base-catalyzed *trans*-esterification of oil, generates about 10 kg of crude glycerol per 100 l of biodiesel produced. Biodiesel production, and consequently that of the main side product, crude glycerol, are increasing annually. Even at current production levels, the crude glycerol produced during biodiesel manufacture has become a waste disposal problem (Johnson and Taconi, 2007). At the scale estimated for future biodiesel production, the enormous quantities of crude glycerol will create a waste stream with potentially significant environmental impacts. Thus there is an urgent need for the development of practical processes for converting these large quantities of waste crude glycerol to useable products. The magnitude of projected future supplies suggests that its conversion to a biofuel is the only feasible avenue to creating a product that will not overwhelm demand. Several studies have shown that this fraction can be used to feed anaerobic digesters to produce methane, but achieving significant yields and operational stability requires co-digestion with a nitrogen rich waste stream due to the low nitrogen content of biodiesel waste (Astals et al., 2011 and Siles et al., 2010). One of the more promising ways could be its conversion to biohydrogen by photofermentation. It was recently reported that crude glycerol could be successfully converted to biohydrogen by photofermentation using *Rhodopseudomonas palustris* (CGA009) with a molar hydrogen yield of 3 mol H_2/mol of crude glycerol (Sabourin-Provost and Hallenbeck, 2009). However, improvement of this process is desirable since the theoretical stoichiometric yield is 7 mol H_2/mol of glycerol.

Bioprocess development involving photofermentation is complicated since a variety of nutritional and physico-chemical process variables including light intensity, carbon and nitrogen sources, might be expected to affect the hydrogen yield. The C:N ratio is particularly important due to its potential influence over nitrogenase expression, the enzyme responsible for the observed hydrogen evolution (Keskin et al., 2011 and Hallenbeck, 2011). Bioprocess optimization using a conventional 'change-one-factor-at-a-time' process is very time-consuming, and, moreover, this method may not find the true optimum since interactions between process variables are neglected. On the other hand, experimental design based on statistical modeling can be a very useful tool to evaluate the interactions between a set of independent experimental factors and observed responses, and, moreover, is a time saving method since it requires the least number of experiments (Annadurai et al., 1999). One such method is Box–Behnken statistical design which, as an independent quadratic design, does not contain an embedded factorial or fractional factorial design and uses a relatively small number of observations to estimate the process parameters. Thus, for analysis of three process parameters, Box–Behnken offers the advantage of requiring the least number of experimental runs (Whittinghill, 1998). In the present study response surface methodology was used to maximize the molar hydrogen yield from glycerol during photofermentation by *R. palustris* (CGA009). This method permitted the examination of the effect of three important process parameters; light intensity, glycerol concentration and glutamate concentration, and their interaction on the molar hydrogen yield.

MATERIALS AND METHODS

Bacterial strain and culture conditions

The photosynthetic purple bacterium *R. palustris* (CGA009) was maintained regularly in screw cap sealed tubes (1.6 cm by 12.5 cm) completely filled with RCV succinate amended with biotin (15 μg/l) and para-amino-acid benzoic acid (200 μg/l) and incubated at 30°C in an environmental chamber Biotronette Mark III (Lab-line Instruments) equipped with three 150 W incandescent bulbs. A crude glycerol fraction was obtained from Rothsay Biodiesel, a large, high volume, Canadian biodiesel manufacturer. Methanol represents one of the single largest cost factors in biodiesel manufacture from waste products and is routinely recovered from the glycerol fraction by large scale manufacturers as a cost effective procedure. Thus the crude glycerol fraction is typically <0.5% methanol.

Photofermentation of glycerol

Hydrogen production from crude glycerol was measured using 125 ml serum bottles containing RCV medium made up with either succinate or a crude glycerol fraction derived from biodiesel manufacture as carbon source, and ammonium sulfate or glutamate as nitrogen source at the indicated concentrations. Inoculation into 80 ml of RCV was made with 5 ml of bacterial culture in the late log phase of growth. The bottles were fitted with rubber stoppers, purged for 15 min with oxygen-free argon, and then submerged in a 30°C water bath. The intensity of light provided by a panel of six 50 W halogen bulbs was 200 W/m^2 at the surface of the bottles. The hydrogen produced was measured by water displacement using graduated cylinders connected to the serum bottles by relatively hydrogen impermeable FEP 890 tubing.

Analytical procedures

The concentration of hydrogen in the collected gas was determined using a gas chromatograph (Shimadzu GC-8A) equipped with a thermal conductivity detector and a 1 m

column packed with molecular sieve 5A with as carrier gas. Glycerol concentrations were determined spectrophotometrically at 410 as 3,5-diacetyl-1,4-dihydrolutidine, produced by periodate oxidation and reaction with acetylacetone in the presence of ammonium acetate (Bondioli and Della Bella, 2005). The crude biodiesel fraction had a glycerol content of 85%.

In vivo nitrogenase activity was measured by the acetylene reduction assay (Hallenbeck et al., 1982) with 5 ml culture samples. Solutions were made anaerobic by sparging with oxygen free argon which had been passed through a heated copper catalyst to remove any traces of oxygen. At the indicated times, 50 µl of aliquots of the gas phase and the culture were separately withdrawn from the vials for the analysis of ethylene by GC–FID and Western blot analysis of Fe-protein expression. To monitor Fe-protein expression, the culture samples were treated with SDS–PAGE sample buffer (Laemmli and Favre, 1973) and immediately incubated in a boiling-water bath for 5 min. Equal amounts of protein (3 µg/well) were analyzed by Laemmli sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12.5% total acrylamide, 30:0.2 acrylamide/bis-acrylamide (Kanemoto and Ludden, 1984) and with subsequent immunoblotting chemiluminescence detection (LumiGlo chemiluminescence substrate system assay (Mandel Scientific)). Western blots were scanned using a Multi Image machine and the Alpha image 2200 and Image Quant TL v2005 software were used to perform densitometric analysis of the labeled bands. Protein concentrations were determined by the Bradford method (Bradford, 1976) after 2 min of sonication of whole cell culture.

Optimization study

A 3^K Box–Behnken model was used as the experimental design model to optimize key process parameters for enhanced hydrogen production. For three factors, the Box–Behnken

design offers some advantages in requiring fewer experimental runs and is rotatable if the variance of the predicted response at any point x depends only on the distance of x from the design center point (Box and Behnken, 1960). The 3^K factorial design also allows efficient estimation of second degree quadratic polynomials and obtains the combination of values that optimizes the response within the region of the three dimensional observation space (Annadurai et al., 1999). In developing the regression equation, the relation between the coded values and actual values can be described by the following equation:

$$x_i = (X_i - X_i^*)/\Delta X_i \qquad \text{Equation (1)}$$

where xi is the coded value of the ith independent variable, Xi is the uncoded value of the ith independent variable, X_i^* is the uncoded value of the ith independent variable at the center point, and ΔXi is the step change value.

The levels of the variables and the experimental design are shown in Table 1. Hydrogen yield was associated with simultaneous changes in light intensity (100, 175 and 250 W/m^2), glycerol concentration (10, 30 and 50 mM) and the glutamate concentration (1.0, 4.5 and 8.0) of the culture medium. A total of fifteen experimental runs decided by the 3K factorial Box–Behnken design were carried out, and the center point was replicated three times to estimate experimental errors. For predicting the optimal conditions, the quadratic polynomial equation was fitted to correlate the relationship between variables and response (i.e. molar hydrogen yield, nitrogenase activity), and estimated with the following equation:

$$Y = \alpha_0 + \sum_{i=1} \alpha_i X_i + \sum_{i=1} \alpha_{ii} X_i^2 + \sum_{i=1} \sum_{i<j=2} \alpha_{ij} X_i X_j \qquad \text{Equation (2)}$$

where Xi are the input variables, which influence the response variable Y, α0 the offset term, αi the ith linear coefficient, αij is the ijth interaction coefficient. The input values of X1, X2 and X3 corresponding to the maximum value of Y were solved by setting the partial derivatives of the functions to zero.

RESULTS AND DISCUSSION

Influence of process parameters on hydrogen yield and nitrogenase activity

R. palustris produces hydrogen from glycerol in a nitrogenase dependent reaction. Therefore, greater hydrogen yields could possibly be obtained through bioprocess optimization of nitrogenase activity. A number of variables could possibly restrict effective nitrogenase activity and thus hydrogen yields. Assuming an adequate medium formulation, the most important key parameters are probably glycerol concentration, glutamate concentration and light intensity. Light intensity is one of the most important factors influencing hydrogen production due to its effects on nitrogenase protein expression and on supporting its activity through the supply of high energy electrons and ATP (Jouanneau et al., 1985). Light intensity will also have an effect on overall growth kinetics, including the duration of the lag phase and doubling times (Fang et al., 2005).

The initial C:N ratio in the production medium also plays an important role in determining the yield and rate of hydrogen production. Low initial C:N ratios result in low rates of photofermentation and lower hydrogen yields. On the other hand, total fermentation times increase with higher initial C:N ratios. Of course, the C:N balance can have several direct effects on nitrogenase, affecting both its synthesis and, through the presence of excess NH_4^+ ions, its activity. Thus, various amino acids which have only minor inhibitory effects on nitrogenase expression are often used in media formulations for photofermentation

in combination with different carbon sources (Gabrielyan et al., 2010). Glutamate usually is chosen as the most suitable nitrogen source to support photofermentative hydrogen production. Thus, optimization of both initial glycerol and glutamate concentrations should increase molar hydrogen yields as well as nitrogenase activity. A factorial design approach is appropriate in this case due to potential interactions between the independent variables. This should be especially apparent with the key process parameters, initial glycerol and glutamate concentrations since, as described above, they will interact by establishing a particular C:N. To overcome this problem, a full or fractional factorial design coupled with RSM (response surface methodology) can be used to advantage (Wang and Wan, 2009).

Here, using the *R. palustris* (CGA009), we have determined the optimal levels of these key factors and the effect of their interactions on molar hydrogen yields and nitrogenase activity using RSM with a Box‐Behnken design. The statistical treatment of the test variables along with the measured response values, expressed as hydrogen yield and nitrogenase activity at each combination, are summarized in Table 1. ANOVA is essential to test the significance and adequate fit of experimental data to the model. The summary of the analysis of variance (ANOVA) of the results of the quadratic model fitting are shown in Table 2 for hydrogen yield. The various ANOVA parameters; model F, coefficient of variation, squared regression, etc. all indicated a good fit of experimental values. The summary of the analysis of variance (ANOVA) of the results of the quadratic model fitting are shown in Table 3 for nitrogenase activity. Again, the ANOVA values showed a highly significant fit to the model.

The application of response surface methodology results in an empirical relationship between hydrogen yield, nitrogenase activity and the process variables. Thus, the following regression equations ((3a) for coded values and (3b) for actual experimental values),

analogous to the Eq. (1), give the relative hydrogen yield ($Y1$) as a function of the test variables $X1$ (light intensity), $X2$ (glycerol concentration) and $X3$ (temperature). Two other regression equations ((4a) for coded values and (4b) for actual experimental values), analogous to Eq. (2), give the relative nitrogenase activity ($Y2$) as a function of the test variables $X1$ (light intensity), $X2$(crude glycerol concentration) and $X3$ (temperature).

$$Y1_{CODED} = 6.35 + 0.24X_1 + 0.17X_2 - 0.03X_3 - 0.35X_1X_2 + 0.08X_1X_3 + 0.04X_2X_3 - 3.24X_1^2 - 3.09X_2^2 - 3.10X_3^2 \quad \text{Equation (3a)}$$

$$Y1_{ACTUAL} = -23.31 + 0.20X_1 + 0.46X_2 + 2.25X_3 - 0.03X_1X_2 + 0.01X_1X_3 + 0.13X_2X_3 - 0.12X_1^2 - 0.39X_2^2 - 0.25X_3^2 \quad \text{Equation (3b)}$$

$$Y2_{CODED} = 204.84 + 45.71X_1 - 54.98X_2 - 0.40X_3 - 29.57X_1X_2 - 64.00X_1X_3 = 0.58X_2X_3 - 43.10X_1^2 - 47.27X_2^2 + 30.30X_3^2 \quad \text{Equation (4a)}$$

$$Y2_{ACTUAL} = 404.18 + 4.98X_1 - 7.75X_2 - 20.04X_3 - 0.02X_1X_2 - 0.25X_1X_3 = 0.42X_2X_3 - 0.38X_1^2 - 0.12X_2^2 + 2.47X_3^2 \quad \text{Equation (4b)}$$

Two and three dimensional contour plots of the variation of hydrogen yield with light intensity and glycerol concentration; glycerol and glutamate concentration; and light intensity and glutamate concentration (Fig. 1A–C), were elliptical and had clear elongated diagonals indicating significant interactive effects on hydrogen yield (Y1) between the three independent variables. The two and three dimensional contour plots concerning of the variation of nitrogenase activity with light intensity and glycerol concentration (Fig. 2A), had an elliptical nature and a clear elongated running diagonal, indicating a significant interactive effect on nitrogenase activity (Y2) between the two independent variables. On the other hand, two and three dimensional contour plots (Fig. 2B and C) representing the variation of nitrogenase activity with glutamate concentration and light intensity; glycerol concentration and glutamate concentration, clearly have saddle points indicating that interactions between light intensity and glutamate concentration, and glutamate concentration and glycerol concentration, have a less direct influence on nitrogenase activity. Saddle points, such as those seen in Fig. 2B and C, of RSM plots represent stationary points, that is points of zero gradient, with positive curvature on one side and negative curvature on the other side. In this case they indicate a relative lack of interaction of either light intensity and glutamate concentration, or glutamate concentration and glycerol concentration on nitrogenase activity. Thus, under the conditions used here, light intensity and glycerol concentration are the most important variables in terms of optimizing nitrogenase activity.

The residual plots of the model were randomly distributed without any trends (not shown). This indicates good prediction of maximum response along with constant variance and adequacy of the quadratic models. As shown in Fig. 1 and Fig. 2, within the design boundary each response surface plot had a clear peak and the corresponding contour plot had a

clear maximum, demonstrating that maximum hydrogen yield and nitrogenase activity could be achieved inside the design boundaries. Moreover, the predicted hydrogen yield of 6.35 mol H_2/mol glycerol is higher than the previously reported yield of 6.1 mol H_2/mol glycerol (Ghosh et al., 2011). Validation experiments were conducted *R. palustris* in duplicate to experimentally confirm the predicted optimum. The mean hydrogen yield was 6.69 mol H_2/mol crude glycerol, very close to the predicted value of 6.35 mol H_2/mol crude glycerol. As well, the mean nitrogenase activity was 228 nmol of C_2H_4/ml/min, very close to the predicted value of 205 nmol of C_2H_4/ml/min. These results therefore show that response surface optimization can be successfully applied to obtain maximum hydrogen yields and nitrogenase activity.

Nitrogenase protein expression and its relationship to activity

Finally, nitrogenase protein expression at the 10th day of incubation was assessed for each experimental run. Samples from the different cultures were directly submitted to SDS-PAGE electrophoresis and Western blotting with anitsera to either the nitrogenase Fe-protein or the Mo-Fe protein followed by densitometric analysis. The results clearly show that nitrogenase Fe-protein expression was nearly the same (the difference between the lowest and highest amounts was ~30%) under all the different process values represented by the experimental sets (Fig. 3). Very similar results were obtained for Mo-Fe protein expression (not shown). However, there was much greater variation in nitrogenase activity with bioprocess parameters. This would suggest that the limiting factor for nitrogenase activity, and therefore presumably hydrogen production, is not the quantity of enzyme present, but how effectively it is supplied with high energy electrons and/or ATP. This is in agreement with

previous studies that have suggested that under some conditions nitrogenase enzyme is in excess and therefore that total activity reflects the rate of nitrogenase reduction rather than the quantity of enzyme present (Hallenbeck et al., 1998 and Yakunin et al., 1999). This merits future study as it indicates that further increases in production could possibly be achieved through maximization of these factors.

CONCLUSIONS

Response surface methodology with a Box–Behnken design was successfully used to improve the photofermentative molar hydrogen yield from crude glycerol by identifying the optimal conditions. The three independent variables studied; crude glycerol concentration, glutamate concentration, and light intensity all had significant interactive effects on hydrogen yield and nitrogenase activity with a maximum hydrogen yield (6.69 mol H_2/mol) at the optimal point (30 mM glycerol, 4.5 mM glutamate, 175 W/m^2). Thus, RSM was able to define conditions allowing the essentially stoichiometric (7 mol H_2/mol glycerol) conversion of waste glycerol generated during biodiesel manufacture to hydrogen by photofermentation with *R. palustris*.

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

Trial	Light intensity		Glycerol conc.		Glutamate Conc.		H_2 yield *Y1*	Nitrogenase activity *Y2*
	X_1	Code	X_2	Code	X_3	Code		
1	100	−1	10.0	−1	4.50	0	0.019	139
2	250	1	10.0	−1	4.50	0	0.047	25
3	100	−1	50.0	1	4.50	0	0.003	35
4	250	1	50.0	1	4.50	0	0.002	31
5	100	−1	30.0	0	1.00	−1	0.007	61
6	250	1	30.0	0	1.00	−1	0.012	317
7	100	−1	30.0	0	8.00	1	0.006	195
8	250	1	30.0	0	8.00	1	0.012	195
9	175	0	10.0	−1	1.00	−1	0.232	220
10	175	0	50.0	1	1.00	−1	0.188	162
11	175	0	10.0	−1	8.00	1	0.044	212
12	175	0	50.0	1	8.00	1	0.175	157
13[a]	175	0	30.0	0	4.50	0	5.802	228
14[a]	175	0	30.0	0	4.50	0	6.568	192
15[a]	175	0	30.0	0	4.50	0	6.692	195

$$Y1_{CODED} = 6.35 + 0.24X_1 + 0.17X_2 - 0.03X_3 - 0.35X_1X_2 + 0.08X_1X_3 + 0.04X_2X_3 - 3.24X_1^2 - 3.09X_2^2 - 3.10X_3^2$$

$$Y1_{ACTUAL} = -23.31 + 0.20X_1 + 0.46X_2 + 2.25X_3 - 0.03X_1X_2 + 0.01X_1X_3 + 0.13X_2X_3 - 0.12X_1^2 - 0.39X_2^2 - 0.25X_3^2$$

$$Y2_{CODED} = 204.84 + 45.71X_1 - 54.98X_2 - 0.40X_3 - 29.57X_1X_2 - 64.00X_1X_3 = 0.58X_2X_3 - 43.10X_1^2 - 47.27X_2^2 + 30.30X_3^2$$

$$Y2_{ACTUAL} = 404.18 + 4.98X_1 - 7.75X_2 - 20.04X_3 - 0.02X_1X_2 - 0.25X_1X_3 = 0.42X_2X_3 - 0.38X_1^2 - 0.12X_2^2 + 2.47X_3^2$$

[a] The center point was replicated three times.

Table 2
ANOVA for hydrogen yield by *R. palustris* from biodiesel waste.

Factors	Statistics[a]				
	Sum of squares	Degrees of freedom	Mean square	*F*-value	*P*-value
Model	95.1	9	10.6	112	0.0001
X_1	0.036	1	0.036	0.096	0.967
X_2	0.061	1	0.061	0.192	0.977
X_3	0.257	1	0.257	0.055	0.825
X_1X_2	0.039	1	0.039	0.103	0.966
X_1X_3	0.0055	1	0.0055	0.045	0.998
X_2X_3	0.384	1	0.384	0.081	0.787
X_1^2	38.8	1	38.8	410.94	0.0001
X_2^2	35.3	1	35.3	373.65	0.0001
X_3^2	35.5	1	35.5	375.72	0.0001
Residual	0.47	5	0.095		
Lack of fit	0.391	3	0.130	0.011	0.998
Pure error	0.46	2	0.23		
Cor total	95.6	14			

[a] Coefficient of determination (R^2) = 0.99. The model *F* value of 112 implies that the model is significant. There is only a 0.01% chance that a "Model *F*-value" this large could occur due to noise. Value of "Prob > *F*" less than 0.05 indicates that the model terms are significant. In this case X_1, X_2, X_3, X_1X_2, X_2X_3, X_1X_3, X_1^2, X_2^2 and X_3^2 are significant model terms. The "Lack of fit *F*-value" of 0.011 implies that the lack of fit is not significant relative to the pure error. The squared regression statistic (R^2), the determination coefficient, a measure of the goodness of fit of the model, was very significant at the level of 99%, i.e. the model was unable to explain only 1% of the total variations. Adequate precision measures the signal to noise ratio. A greater ratio greater than 4 is desirable. In this case the ratio of 25.40 indicates an adequate signal. A very high degree of precision and a good deal of reliability of the experimental values were indicated by a low value of the coefficient of variation (C.V. = 23.27%).

Table 3
ANOVA for nitrogenase activity by *R. palustris* producing hydrogen from biodiesel waste.

Factors	Statistics[a]				
	Sum of squares	Degrees of freedom	Mean square	*F*-value	*P*-value
Model	79,900	9	8800	4.80	0.049
X_1	16,700	1	16,000	9.10	0.030
X_2	24,000	1	24,000	13.0	0.015
X_3	1.30	1	1.30	0.14	0.980
X_1X_2	3500	1	3500	1.90	0.227
X_1X_3	16,400	1	16,400	8.90	0.031
X_2X_3	1.37	1	1.40	0.148	0.980
X_1^2	6860	1	6860	3.70	0.110
X_2^2	8250	1	8250	4.50	0.088
X_3^2	3390	1	3390	1.80	0.230
Residual	9200	5	1840		
Lack of fit	8403.29	3	2801.10	7.01	0.1274
Pure error	799.54	2	399.77		
Cor total	89,062.30	14			

[a] Coefficient of determination (R^2) = 0.97. The model *F* value of 4.82 implies that the model is significant. There is only a 4.90% chance that a "Model *F*-value" this large could occur due to noise. Value of "Prob > *F*" less than 0.05 indicates that the model terms are significant. In this case X_1, X_2, X_3, X_1X_2, X_2X_3, X_1X_3, X_1^2, X_2^2 and X_3^2 are significant model terms. The "Lack of fit *F*-value" of 7.01 implies that the lack of fit is not significant relative to the pure error. The squared regression statistic (R^2), the determination coefficient, a measure of the goodness of fit of the model, was very significant at the level of 97%, i.e. the model was unable to explain only 3% of the total variations. Adequate precision measures the signal to noise ratio. A greater ratio greater than 4 is desirable. In this case the ratio of 7.39 indicates an adequate signal. A very high degree of precision and a good deal of reliability of the experimental values were indicated by a low value of the coefficient of variation (C.V. = 24.83%).

FIGURES

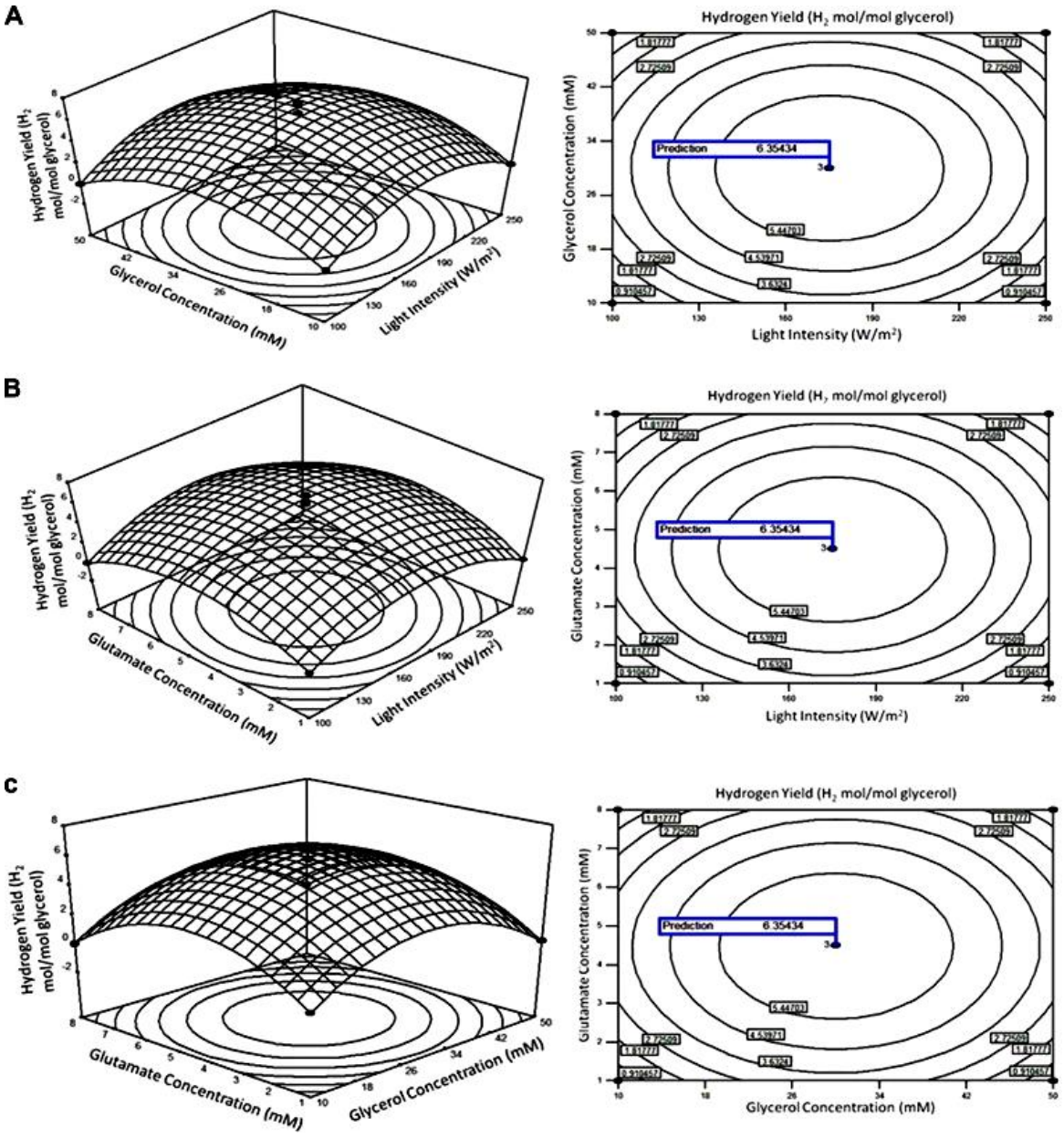

Figure1. Two and three dimensional contour plots for the maximum hydrogen yield. RSM plots were generated using the data shown in Table 1: Inputs were the 15 experimental photo fermentative runs carried out under the conditions established by the Box–Behnken design. (A) Hydrogen yield (H_2 mol/mol glycerol) as a function of glycerol concentration and light intensity. (B) Hydrogen yield (H_2 mol/mol glycerol) as a function of glutamate concentration and light intensity. (C) Hydrogen yield (H_2 mol/mol glycerol) as a function of glutamate concentration and glycerol concentration.

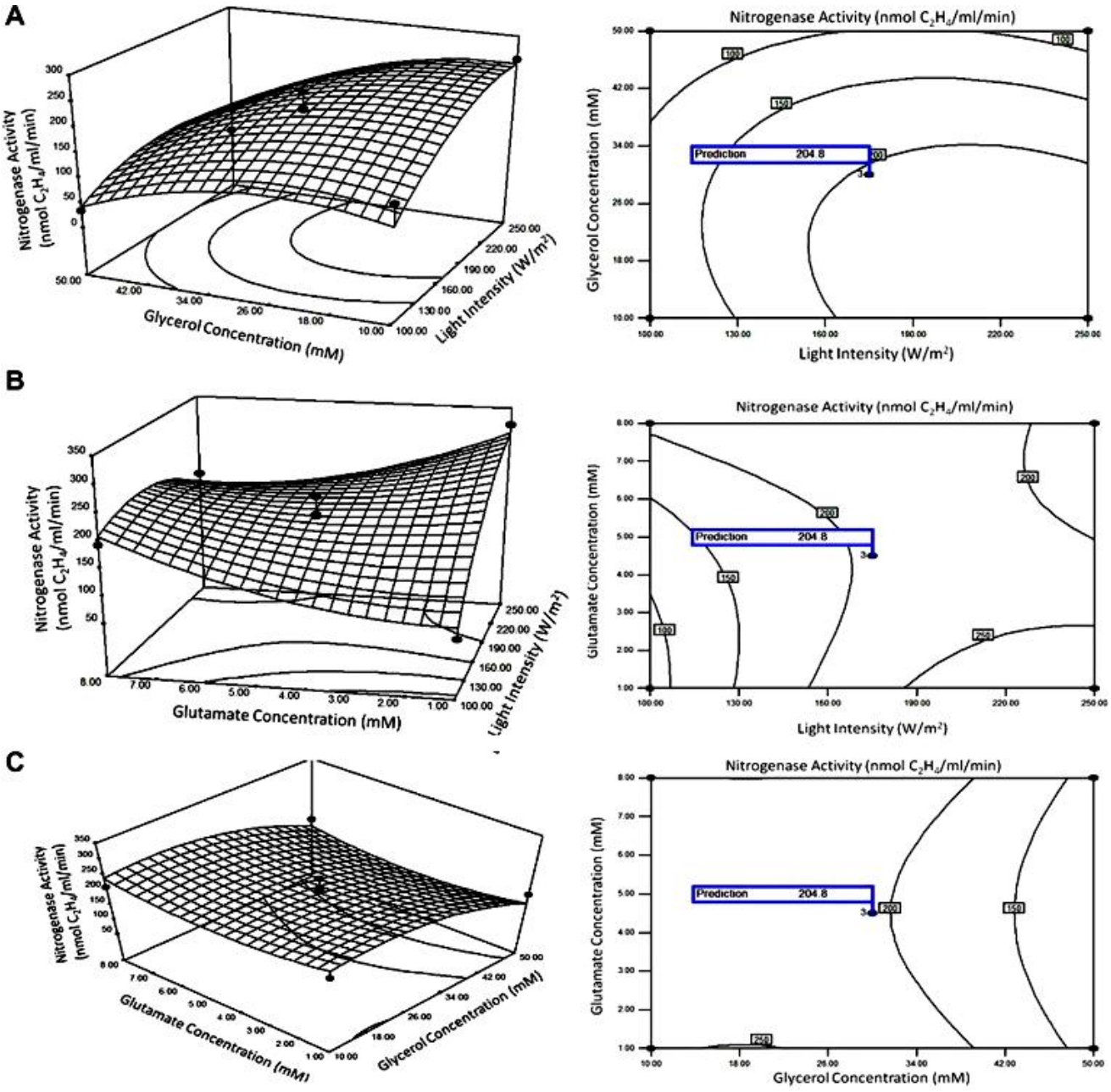

Figure 2. Two and three dimensional contour plots for the maximum nitrogenase activity. RSM plots were generated using the data shown in Table 1: Inputs were the 15 experimental photo fermentative runs carried out under the conditions established by the Box–Behnken design. (A) Nitrogenase activity (nmol of C_2H_4/ml/min) as a function of glycerol concentration and light intensity. (B) Nitrogenase activity (nmol of C_2H_4/ml/min) as a function of glutamate concentration and light intensity. (C) Nitrogenase activity (nmol of C_2H_4/ml/min) as a function of glutamate concentration and glycerol concentration.

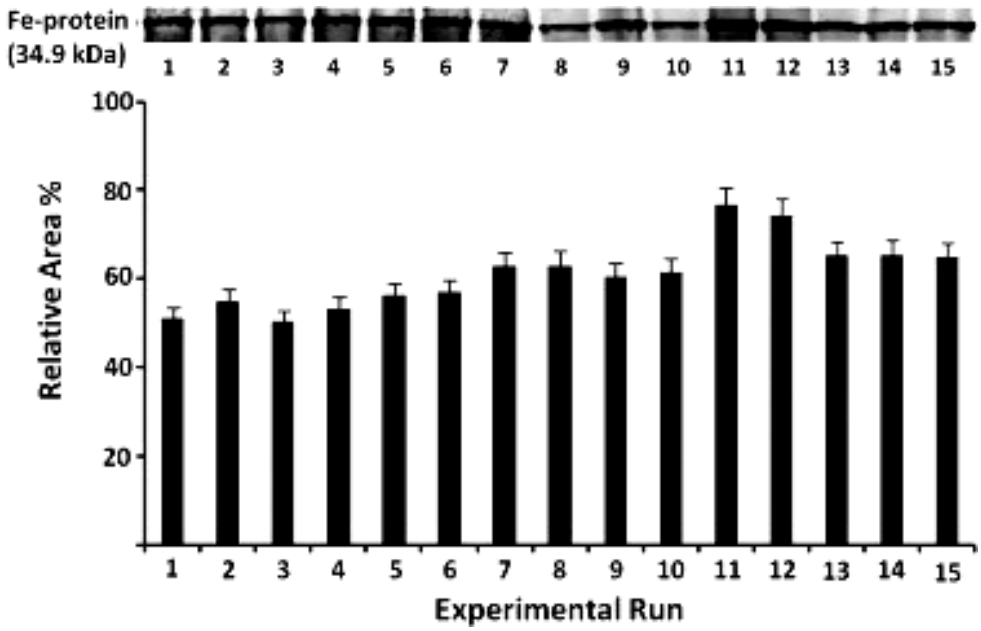

Figure 3. Densitometric analysis of nitrogenase Fe-protein levels in *R. palustris* **grown under the different experimental conditions.**

CHAPTER 6: Optimization of the Hydrogen Yield from Single-Stage Photofermentation of Glucose by *Rhodobacter capsulatus* JP91 using Response Surface Methodology

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Author contribution: The experimental design and wet laboratory experiments had been carried out by D.G. and I.F.S. partially helping D.G. on this project to make her internship under my supervision. D.G. performed all dataset analysis. All the work was under supervision of Professor Dr. Patrick Hallenbeck.

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Optimization of the Hydrogen Yield from Single-Stage Photofermentation of Glucose by *Rhodobacter capsulatus* JP91 using Response Surface Methodology

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Keywords: Biohydrogen; Photofermentation; Photosynthetic bacteria; H_2 Yields

ABSTRACT

Hydrogen production from glucose via single-stage photofermentation was examined with the photosynthetic bacterium *Rhodobacter capsulatus* JP91 (*hup*-). Response surface methodology with Box–Behnken design was used to optimize the independent experimental variables of glucose concentration, glutamate concentration and light intensity, as well as examining their interactive effects for maximization of molar hydrogen yield. Under optimal condition with a light intensity of 175 W/m^2, 35 mM glucose, and 4.5 mM glutamate, a maximum hydrogen yield of 5.5 (±0.15) mol H_2/mol glucose, and a maximum nitrogenase activity of 246 (±3.5) nmol C_2H_4/ml/min were obtained. Densitometric analysis of nitrogenase Fe-protein expression under different conditions showed significant variation in Fe-protein expression with a maximum at the optimized central point. Even under optimum conditions for hydrogen production, a significant fraction of the Fe-protein was found in the ADP-ribosylated state, suggesting that further improvement in yields might be possible

INTRODUCTION

Biological hydrogen production is a possible means of producing a clean burning, carbon-neutral fuel. A number of different avenues are being considered (Hallenbeck, 2011). Fermentative hydrogen production is attractive for initial adoption of a biohydrogen technology since it can target various waste streams, and fuel production can thus potentially be coupled to a waste treatment process with the attendant waste treatment credits increasing the overall economics. However, presently known organisms and metabolic pathways allow, at best, a maximum conversion of only 33% (Hallenbeck and Ghosh, 2009), too low to be practical in terms of fuel production and, since the byproducts represent an additional waste stream, too inefficient to be used in waste treatment.

One way to potentially circumvent this problem is to develop a two-stage hybrid system where additional energy is extracted from the byproducts of the hydrogen-producing first stage, and a number of systems have been proposed (Hallenbeck, 2011). Of these, conversion of the organic acid fermentation byproducts to hydrogen by photofermentation with photosynthetic bacteria has been particularly well studied since it holds the promise of complete substrate (organic acids) conversion to hydrogen (Keskin and Hallenbeck, 2012). A five-year project, Hyolution, carried out a series of studies of a potential two-stage process involving a second stage photofermentation and demonstrated that additional hydrogen could be obtained in this way, with a combined yield for the two stages of 5 to 7 mol of hydrogen per mole of hexose (Claassen and de Vrije, 2006, Claassen et al., 2010 and Özgür et al., 2010a). Nevertheless, these yields are below what are needed for a system and, moreover, several problems with the direct use of first stage effluents were observed, including inhibition by acidic pH and excessive concentrations of substrate and fixed nitrogen. Overcoming these

problems requires extensive substrate pretreatment, such as neutralization, dilution, nutrient addition, as well as sterilization and centrifugation for some substrates. Thus, although simple in theory, in practice such a two-stage system would appear to require extensive additional processing, increasing energy utilization and process costs.

Photofermentation by photosynthetic bacteria is catalyzed by nitrogenase and thus occurs when the ratio of fixed carbon to fixed nitrogen is high, i.e. at low levels of easily assimilated fixed nitrogen (Masepohl and Hallenbeck, 2010 and Androga et al., 2011). For example, ammonium suppresses photofermentation (Koku et al., 2002) whereas various amino acids are permissive (He et al., 2006 and Gabrielyan et al., 2010). Thus, although organic acids are usually used to demonstrate hydrogen production by photofermentation, any carbon source which allows growth should in theory be a substrate for hydrogen evolution under low fixed nitrogen conditions. A number of studies have examined photofermentative hydrogen production from a variety of waste streams (Keskin et al., 2011 and Wu et al., 2012) and high-yield photofermentative production of hydrogen from waste glycerol produced during biodiesel manufacture has been demonstrated (Ghosh et al., 2012). This suggests that a single-stage system where photosynthetic bacteria directly convert the sugar substrate to hydrogen might be feasible.

An initial study using *Rhodobacter capsulatus* JP91 has shown that photofermentative hydrogen production from glucose is feasible with a maximum reported hydrogen yield of 3 mol H_2/mol of glucose (Abo-Hashesh et al., 2011). In that study, only a single glucose concentration at two different glutamate concentrations and a single light intensity were investigated, suggesting that a more thorough investigation of the various process parameters could lead to higher hydrogen yields. Here, in a continuation of that previous work, the effects

of variations in the concentrations of glucose and glutamate, as well as different light intensities on the molar hydrogen yield were systematically studied using response surface methodology with Box Behnken design. Many studies have shown that the optimization of nutritional and environmental conditions plays an important role in developing bioprocesses and improving their performance. A conventional, change-one-factor-at-a-time process is time-consuming and is incapable of reaching the true optimum due to neglecting of the interaction among process variables. Design of experiments (DOE) based on the statistical modeling is a very useful tool for evaluating interactions between a set of independent experimental factors and observed responses, and is a time-saving method requiring the least number of experiments (Annadurai et al., 1999).

A suitable method is provided by Box Behnken statistical design, an independent quadratic design devised for response surface methodology (Box and Behnken, 1960). This methodology was applied in the present study to define the optimal operational conditions for maximizing the hydrogen yield from glucose during single-stage photofermentation. Operation under the defined optimal conditions increased yields 85% to 5.5 ($\pm$0.15) mol H_2 per mole of glucose.

METHODS

Bacterial strain and culture conditions

The purple non-sulfur photosynthetic bacterium *R. capsulatus* (JP91), a markerless *hup*- derivative of B10, was kindly provided by Dr. John Willison, and was maintained regularly in screw cap sealed tubes (1.6 cm by 12.5 cm) completely filled with RCV glucose medium amended with thiamine-HCl (1 mg/l) and incubated at 30 °C in an Biotronette Mark

III (Labline Instruments) environmental chamber equipped with three 150 W incandescent bulbs.

Batch reactor studies

Photofermentation experiments were carried out with 100-ml cultures, inoculated (5% (v/v)) from a culture in the late log phase of growth, in RCV-glucose medium in serum bottles sealed with butyl rubber stoppers and rendered anaerobic by sparging with oxygen-free argon for 15 min. Cultures were incubated for 6 days in a glass-sided temperature controlled (30^{o}C) water bath. The cultures were illuminated with a bank of six 50-W halogen bulbs placed 25 cm from the culture vessels and the light intensity was adjusted using a potentiometer to give the required light intensities. The amount of gas evolved was measured by water displacement using graduated cylinders filled with water and partially submerged in a tub of water connected to the serum bottles by relatively hydrogen impermeable FEP 890 tubing.

Analytical methods

The concentration of hydrogen in the collected gas was determined using a gas chromatograph (Shimadzu GC-8A) equipped with a thermal conductivity detector, a 1 m column packed with molecular sieve 5A and with argon as carrier gas. Bacterial growth was determined by measuring the optical density at 600 nm using a double-beam spectrophotometer (Shimadzu). Glucose concentrations were estimated spectrophotometrically at 490 nm with a phenol‑sulphuric acid assay (Dubois et al., 1956). Light intensities were measured using a Delta OHM photo/radiometer (HD 2102.1).

In vivo nitrogenase activity was measured by the acetylene reduction assay (Hallenbeck et al., 1982) with 5 ml culture samples. After initial trials to set up the

experimental design to examine growth profile, cumulative hydrogen production and hydrogen yield at different time intervals, it was found that day 6 was the time point where hydrogen production was maximal. For that reason day 6 was chosen as the optimal time point for sampling for nitrogenase activity and the determination of its modification status. Solutions were made anaerobic by sparging with oxygen-free argon which had been passed through a heated copper catalyst to remove any traces of oxygen. The *in vivo* nitrogenase activity of the samples was detected under saturating light conditions in a shaking water bath maintained at 30^{o}C. The concentration of ethylene present in the gas phase was determined using gas chromatography by withdrawal of 50 μl of the gas phase at a predetermined time for the analysis of ethylene by GC-FID. To monitor Fe-protein expression, aliquots of the liquid phase of the culture were withdrawn, brought to 1 X sodium dodecyl sulfate sample buffer concentration and immediately incubated in a boiling-water bath for 5 min. Equal amounts of total protein (3 μg/well) were analyzed by sodium dodecyl-polyacrylamide gel electrophoresis (12.5% total acrylamide) with low crosslinker (prepared as previously described (Yakunin et al. 1999) and subsequent immunoblotting with chemiluminescence detection using the LumiGlo chemiiluminescence substrate system (Mandel Scientific). The developed immunoblots were scanned using a Multi Image machine and quantified using ImageQuant TL v2005 software. To minimize artifacts introduced during analysis, bands on the same batch of gels were compared and the results plotted on a relative scale. Protein concentrations were determined by the Bradford method (Bradford, 1976) after 2 min sonication of the whole cell culture.

Optimization study

A 3^K factorial Box–Behnken model was used as the experimental design model to optimize the key process parameters for enhanced hydrogen production. For three factors, the Box–Behnken design offers some advantages in requiring fewer experimental runs and is rotatable if the variance of the predicted response at any point x depends only on the distance of x from the design center point (Box and Behnken, 1960). The 3^K factorial design also allows efficient estimation of second-degree quadratic polynomials and obtains the combination of values that optimizes the response within the region of the three dimensional observation space (Annadurai et al., 1999). In developing the regression equation, the relation between the coded values and actual values are described according to the following equation:

$$x_i = (X_i - X_i^*)/\Delta X_i \qquad \text{Equation (1)}$$

where xi is the coded value of the ith independent variable, Xi is the uncoded value of the ith independent variable, Xi is the uncoded value of the ith independent variable at the center point, and ΔXi is the step change value. A total of fifteen experimental runs decided by the 3^K factorial Box–Behnken design were carried out, and the center point was replicated three times to estimate experimental errors. For predicting the optimal condition, the quadratic polynomial equation was fitted to correlate the relationship between variables and response (i.e., molar hydrogen yield, nitrogenase activity), and estimated with the following equation:

$$Y = \alpha_0 + \sum_{i=1}^{3} \alpha_i X_i + \sum_{i=1}^{3} \alpha_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{i<j=2}^{3} \alpha_{ij} X_i X_j \qquad \text{Equation (2)}$$

Where Xi are the input variables, which influence the response variable Y, $\alpha 0$ the offset term, α i the ith linear coefficient, α ij is the ijth interaction coefficient. The input values of X1, X2 and X3 corresponding to the maximum value of Y were solved by setting the partial derivatives of the functions to zero. MATLAB and Minitab software, standard software for mathematical modeling and multiprocess parameter optimization, were used for DOE and for data analysis. Three runs were made for the validation experiments and two runs were made and averaged for the other points.

RESULTS AND DISCUSSION

Response surface methodology and the range of values chosen

Modeling of complex biological systems requires a set of assumptions and simplification at the outset, which can be provided by an empirical statistical model. As applied to the area of research of the present study, a Box Behnken model could provide information on both hydrogen yield horizons as well as suggest how the regulation of existing and inserted metabolic pathways needs to be altered in order to obtain the maximum hydrogen yield. In this design the treatment combinations are at the center and the midpoints of edges of the process space, giving a geometry which suggests a sphere within the process space. Here this method was used to determine the optimal values of three important process variables in maximizing hydrogen yield. Thus, hydrogen yield was related to simultaneous changes in light intensity (100, 175 and 250 W/m^2), glucose concentration (20, 35 and 50 mM) and the glutamate concentration (1.0, 4.5 and 8.0 mM) of the culture medium. The values chosen were based on a number of factors, including preliminary results (not shown), previous work on single-stage photofermentation of glucose (Abo-Hashesh et al. 2011), a study on the RSM

optimization of the photofermentative production of hydrogen from glycerol (Ghosh et al. 2012), and previous studies on photofermentation of sugars (Jung-Yeol et al., 2012, Androga et al., 2011, Akroum-Amrouche et al., 2011, Keskin et al., 2011 and Keskin and Hallenbeck, 2012). The levels of the variables and the experimental design are shown in Table 1.

Optimization of process parameters for nitrogenase activity

R. capsulatus JP91 produces hydrogen from glucose in a nitrogenase-dependent reaction. Therefore it is very likely that greater hydrogen yields can be achieved by maximizing nitrogenase activity through bioprocess optimization. A number of variables could possibly restrict effective nitrogenase activity and thus hydrogen yields; however, assuming an adequate medium formulation, the most important key parameters are probably glucose concentration, glutamate concentration and light intensity. First of all, light intensity is one of the most important factors in hydrogen production due to its potential effects on nitrogenase enzyme regulation and flux through the electron transport chain (Jouanneau et al., 1985). In turn, light intensity could potentially modulate endproduct distribution, as well as the duration of the lag phase and growth kinetics.

The initial ratio of carbon to nitrogen in the production medium also plays an important role. A low initial carbon to nitrogen ratio results in lower rates of fermentation and lower hydrogen yields, whereas total fermentation times increase with a higher initial carbon to nitrogen ratio. In hydrogen production by photofermentation, this effect is mainly due to the influence of the carbon to nitrogen ratio on nitrogenase synthesis with nitrogenase repression occurring when there is excessive fixed nitrogen in the medium. However, since hydrogen production by nitrogenase is mainly observed in actively growing cells, fixed nitrogen must be

supplied to support cell growth. On way around this problem experimentally is to supply fixed nitrogen in the form of an amino acid, many of which have only minor effects on nitrogenase repression when supplied at moderate concentrations. Glutamate has been found to be the most suitable amino acid for photofermentative hydrogen production (Gabrielyan et al., 2010). In the present study, glutamate was used in combination with glucose.

The application of response surface methodology resulted in an empirical relationship between nitrogenase activity and the process variables. Two regression equations ((3a) for coded values and (3b) for actual experimental values), analogous to the Eq. (2), show the relative nitrogenase activity (Y2) as a function of the test variables X_1 (light intensity), X_2 (glucose concentration) and X_3 (temperature).

$$Y2_{CODED} = 221.99 + 7.01X_1 + 5.47X_2 + 20.81X_3 - 25.75X_1X_2 - 38.54X_1X_3 + 11.06X_2X_3 - 78.31X_1^2 - 90.70X_2^2 - 45.23X_3^2 \quad \text{Equation (3a)}$$

$$Y2_{ACTUAL} = 1051.49 + 6.43X_1 + 31.64X_2 + 57.49X_3 - 0.02X_1X_2 - 0.15X_1X_3 + 0.21X_2X_3 - 0.01X_1^2 - 0.40X_2^2 - 3.69X_3^2 \quad \text{Equation (3b)}$$

Statistical analysis, such as analysis of variance (ANOVA), is essential to test significance and adequacy of the model. The summary of the (ANOVA) of the results of the quadratic model fitting are shown in Table 2 for nitrogenase activity. The two- and three-dimensional contour plots of nitrogenase activity versus light intensity and glucose concentration, light intensity and glutamate concentration, glucose concentration and

glutamate concentration (Fig. 1A–C), were elliptical and had elongated running diagonals, indicating a significant interactive effect on nitrogenase activity (Y2) between the three independent variables. The relative amount of interaction can be judged by the degree of ellipticity. Thus, the greatest interactive effects on nitrogenase activity were seen for light intensity and glucose concentration whereas the least interaction was seen for glucose and glutamate concentrations. Therefore, in general, light intensity had the greatest influence on the effects of glucose or glutamate concentrations on nitrogenase activity.

The residual plots of the model were randomly distributed without any trends (not shown), indicating the adequacy of the quadratic model in predicting the maximum response. In general, the reproducibility was very good, although some replicate-to-replicate variation can be noted, for example when comparing run 13 with runs 14 and 15, carried out under the same conditions. The reason for this variation is not known, perhaps a difference in inoculums or other factors that are hard to control. Nonetheless, these three biological replicates gave a mean of 222 ($\pm$43) nmol C_2H_4/ml/min, so the low value in run 13 is only 19% less than the mean and well above the values obtained with the other runs under sub optimal conditions. As shown in Fig. 1, each response surface plot had a clear peak and the corresponding contour plot had a clear maximum, which means that the maximum response (nitrogenase activity) could be achieved inside the design boundaries. The mean nitrogenase activity, determined by validation experiments (performed in triplicate) carried out under the determined optimal conditions, was 246 ($\pm$3.5) nmol C_2H_4/ml/min, very close to the predicted value of 222 ($\pm$43) nmol C_2H_4/ml/min.

Optimization of hydrogen yields

The hypothesis of the present study was that maximum hydrogen yields would be obtained under conditions that optimize nitrogenase activity. This was checked by modeling and measuring hydrogen yields under the same conditions used for nitrogenase activity. Thus, the following regression equations ((4a) for coded values and (4b) for actual experimental values), analogous to the Eq. (2), were developed which give the relative hydrogen yield (Y1) as a function of the test variables X_1 (light intensity), X_2 (glucose concentration) and X_3 (glutamate concentration).

$$Y1_{CODED} = 5.30 + 0.04X_1 - 0.17X_2 - 0.05X_3 + 0.17X_1X_2 - 0.08X_1X_3 + 0.39X_2X_3 - 2.63X_1^2 - 2.65X_2^2 - 2.58X_3^2 \quad \text{Equation (4a)}$$

$$Y1_{ACTUAL} = -27.92 + 0.17X_1 + 0.82X_2 + 1.92X_3 - 0.01X_1X_2 - 0.06X_1X_3 + 0.03X_2X_3 - 0.09X_1^2 - 0.01X_2^2 - 0.21X_3^2 \quad \text{Equation (4b)}$$

ANOVA was used to test the significance and adequacy of the quadratic model fitting. A summary of the results is shown in Table 3 for hydrogen yield. The two- and three-dimensional contour plots presenting hydrogen yield versus light intensity and glucose concentration, glucose and glutamate concentration, light intensity and glutamate concentration (Fig. 2A–C), were elliptical with elongated diagonals indicating a significant interactive effect between the independent variables and hydrogen yields (Y1). As noted for effects on nitrogenase activity, the strength of the interaction between the variables can be judged by the degree of ellipticity. Interaction between the variables appears stronger in the

case of hydrogen yields (Fig. 2) than for nitrogenase activity (Fig. 1). It is noteworthy that the interactive effects between glucose and glutamate were much stronger when hydrogen yields were examined (Fig. 2C) than when nitrogenase activity was examined (Fig. 1C). This suggests that hydrogen yields may not be directly proportional to nitrogenase activity, a point that is further discussed below.

Residual plots of the model were randomly distributed without any trends (not shown), again indicating the predictive power of the quadratic models. As was seen for nitrogenase activity (shown in Fig. 1), the surface plots for hydrogen yield, shown in Fig. 2, had clear peaks and the corresponding contour plots had clear maxima, indicating that maximum hydrogen yield could be achieved inside the design boundaries. The hydrogen yield predicted by the model, 5.29 mol H_2/mol glucose, is higher than the previously reported molar hydrogen yield of 3 mol H_2/mol glucose (Abo-Hashesh et al., 2011) and higher than the vast majority of previous studies which examined single-stage fermentation of glucose (Table 4). Validation experiments (carried out in triplicate) were conducted to confirm the predicted optimal conditions, and gave a mean hydrogen yield of 5.5 (+0.15) mol H_2/mol glucose, very close to the predicted value (5.29 mol H_2/mol glucose).

From a practical point of view, volumetric productivity is also an important process parameter. Under the optimal conditions, volumetric hydrogen productivity in this study was high (1.4 l/l/day), substantially greater than previously found for a two-stage sequential system (0.27 and 0.63 l/l/day) capable of achieving similar yields (Özgür et al., 2010b), and higher than what is typically found for hydrogen production from organic acids by photosynthetic bacteria (0.34 l/l/day (Akroum-Amrouche et al., 2011).

Although in special cases where immobilized cultures are used, dark fermentative volumetric productivities were much higher (Table 1, Hallenbeck and Ghosh, 2009), in general, the rates observed in the present study compare favorably with those obtained in a variety of studies of dark fermentative hydrogen production with non-immobilized cultures (Hawkes et al., 2007 and Hallenbeck and Ghosh, 2009).

Analysis of nitrogenase protein expression

Densitometric analysis of nitrogenase protein expression was performed on the sixth day of incubation for all combinatorial experimental runs. Since hydrogen production was maximal at day 6, it was chosen as the optimal time point for sampling for nitrogenase activity and the determination of the relative amount of nitrogenase (Fe protein) and its modification status. The results show that nitrogenase Fe-protein expression was highest at the optimal point (Fig. 3), perhaps accounting, at least in part, for the maximum nitrogenase activity and hydrogen yield observed under these conditions. However, it should be noted that there was only a maximum of a 2.6-fold variation in the amount of Fe-protein over the range of conditions used (Fig. 3), whereas nitrogenase activity and hydrogen yield varied by more than twenty-fold (Fig. 1 and Fig. 2; and Table 1). This strongly suggests that factors other than the total amount of enzyme present are controlling activity and hydrogen production. These factors are probably related to flux restriction of either high-energy electrons or ATP at the level of metabolic supply to nitrogenase, as previously suggested by studies of the relationship between nitrogenase protein content, nitrogenase activity, and nitrogen supply (Yakunin et al.1999).

Interestingly, Fe-protein was partially ADP-ribosylated under some of the experimental conditions. ADP-ribosylation of Fe-protein is a unique molecular mechanism for controlling nitrogenase activity in many purple non-sulfur photosynthetic bacteria and is related to the NH_4^+-induced "switch off" effect (Masepohl and Hallenbeck, 2010). Depending upon the conditions, this can have the effect of reducing overall nitrogenase activity. Since hydrogen evolution is catalyzed exclusively by nitrogenase during photofermentation, overall hydrogen production and possibly yields might be improved by reducing or eliminating modification of nitrogenase.

However, the relationship between Fe protein modification and the experimental variables studied here, the concentration of glucose and glutamate, and the light intensity, are obscure and require additional study. Further investigation is also required to understand the factors controlling nitrogenase modification during photofermentation of glucose and how this might affect overall hydrogen productivity. Moreover, in the present study, the culture pH remained in the alkaline range, suggesting that the formation of organic acid by products was minimal. However, in this initial study a metabolite analysis was not performed. The logical extension of this research therefore would be to carry out an identification and analysis of metabolic end products to guide metabolic engineering for strain improvement and to more fully understand the possible role of ADP-ribosylation in restricting hydrogen production.

CONCLUSIONS

Response surface methodology using a Box–Behnken design was used to optimize substrate (glucose), fixed nitrogen, and light intensity to maximize the hydrogen yield during the single stage photofermentation of glucose by the photosynthetic bacterium *R. capsulatus*.

The three independent variables studied, glucose, glutamate, and light intensity all had significant interactive effects on hydrogen yield and nitrogenase activity. As well, levels of nitrogenase expression were also shown to be influenced by different combinations of these process variables. The optimized yield obtained through RSM is 85% higher than that previously achieved and is similar to what was previously shown for two-stage systems. Thus, single stage photofermentation of glucose is promising. Further improvements might be achieved through metabolic engineering.

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

Trial	Light intensity		Glucose conc.		Glutamate conc.		H_2 yield *Y1*	N_2ase activity *Y2*
	X_1	Code	X_2	Code	X_3	Code		
1	100.00	−1	20.00	−1	4.50	0	0.01	10.42
2	250.00	1	20.00	−1	4.50	0	0.02	58.36
3	100.00	−1	50.00	1	4.50	0	0.01	99.08
4	250.00	1	50.00	1	4.50	0	0.03	44.03
5	100.00	−1	35.00	0	1.00	−1	0.03	24.30
6	250.00	1	35.00	0	1.00	−1	0.32	132.97
7	100.00	−1	35.00	0	8.00	1	0.03	141.01
8	250.00	1	35.00	0	8.00	1	0.01	95.52
9	175.00	0	20.00	−1	1.00	−1	0.11	82.94
10	175.00	0	50.00	1	1.00	−1	0.08	45.56
11	175.00	0	20.00	−1	8.00	1	0.05	104.45
12	175.00	0	50.00	1	8.00	1	0.04	111.30
13[a]	175.00	0	35.00	0	4.50	0	4.83	171.74
14[a]	175.00	0	35.00	0	4.50	0	5.53	245.47
15[a]	175.00	0	35.00	0	4.50	0	5.53	248.76

$Y1_{CODED} = 5.30 + 0.04X_1 - 0.17X_2 - 0.05X_3 + 0.17X_1X_2 - 0.08X_1X_3 + 0.39X_2X_3 - 2.63X_1^2 - 2.65X_2^2 - 2.58X_3^2$.
$Y1_{ACTUAL} = -27.92 + 0.17X_1 + 0.82X_2 + 1.92X_3 - 0.01X_1X_2 - 0.06X_1X_3 + 0.03X_2X_3 - 0.09X_1^2 - 0.01X_2^2 - 0.21X_3^2$.
$Y2_{CODED} = 221.99 + 7.01X_1 + 5.47X_2 + 20.81X_3 - 25.75X_1X_2 - 38.54X_1X_3 + 11.06X_2X_3 - 78.31X_1^2 - 90.70X_2^2 - 45.23X_3^2$.
$Y2_{ACTUAL} = -1051.49 + 6.43X_1 + 31.64X_2 + 57.45X_3 - 0.02X_1X_2 - 0.15X_1X_3 + 0.21X_2X_3 - 0.01X_1^2 - 0.40X_2^2 - 3.69X_3^2$.

[a] The center point was replicated three times, the other points were replicated twice. The hydrogen values given for these points are the average of the two runs.

Table 2
ANOVA for Nitrogenase Activity of *Rhodobacter capsulatus* JP91 with glucose as substrate.

Factors	Statistics[a]				
	Sum of squares	Degrees of freedom	Mean square	*F*-value	*P*-value
Model	66562.59	9	7395.84	**6.38**	0.0275
X_1	392.98	1	392.98	0.34	0.5856
X_2	239.80	1	239.80	0.21	0.6682
X_3	3465.70	1	3465.70	2.99	0.1443
X_1X_2	2651.74	1	2651.74	2.29	0.1907
X_1X_3	5941.33	1	5941.33	5.13	0.0729
X_2X_3	489.07	1	489.07	0.42	0.5445
X_1^2	22645.81	1	22645.81	19.55	0.0069
X_2^2	30376.41	1	30376.41	26.22	0.0037
X_3^2	7551.88	1	7551.88	6.52	0.0511
Residual	5793.02	5	1158.60		
Lack of fit	2000.02	3	666.67	**0.35**	0.7971
Pure error	3793.01	2	1896.50		
Cor total	72355.62	14			

[a]Coefficient of determination (R^2) = 0.97.
The model F value of 6.38 implies that the model is significant. There is only a 2.75% chance that a "Model F-value" this large could occur due to noise. Value of "Prob > F" less than 0.05 indicates that the model terms are significant. In this case X_1, X_2, X_3, X_1X_2, X_2X_3, X_1X_3, X_1^2, X_2^2 and X_3^2 are significant model terms. The "Lack of fit F-value" of 0.35 implies that the lack of fit is not significant relative to the pure error. The squared regression statistic (R^2), the determination coefficient, a measure of the goodness of fit of the model, was very significant at the level of 97%, i.e., the model was unable to explain only 3% of the total variations. Adequate precision measures the signal to noise ratio. A greater ratio greater than 4 is desirable. In this case the ratio of 7.46 indicates an adequate signal. A very high degree of precision and a good deal of reliability of the experimental values were indicated by a low value of the coefficient of variation (C.V. = 31.60%).

Table 3
ANOVA for hydrogen yield by *Rhodobacter capsulatus* JP91 with glucose as substrate.

Factors	Statistics[a]				
	Sum of squares	Degrees of freedom	Mean square	*F*-value	*P*-value
Model	65.90	9	7.32	**105.65**	0.0001
X_1	0.010	1	0.010	0.15	0.7145
X_2	0.062	1	0.062	0.07	0.9723
X_3	0.023	1	0.023	0.33	0.5930
X_1X_2	0.019	1	0.019	0.026	0.9952
X_1X_3	0.023	1	0.023	0.34	0.5859
X_2X_3	0.048	1	0.047	0.173	0.9554
X_1^2	25.51	1	25.51	368.07	0.0001
X_2^2	25.98	1	25.98	374.80	0.0001
X_3^2	24.48	1	24.48	353.27	0.0001
Residual	0.35	5	0.069		
Lack of fit	0.014	3	0.229	**0.027**	0.9921
Pure error	0.33	2	0.17		
Cor Total	66.25	14			

[a]Coefficient of determination (R^2) = 0.99.

The model F value of 105.65 implies that the model is significant. There is only a 0.01% chance that a "Model F-value" this large could occur due to noise. Value of "Prob > F" less than 0.05 indicates that the model terms are significant. In this case X_1, X_2, X_3, X_1X_2, X_2X_3, X_1X_3, X_1^2, X_2^2 and X_3^2 are significant model terms. The "Lack of fit F-value" of 0.027 implies that the lack of fit is not significant relative to the pure error. The squared regression statistic (R^2), the determination coefficient, a measure of the goodness of fit of the model, was very significant at the level of 99%, i.e., the model was unable to explain only 1% of the total variations. Adequate precision measures the signal to noise ratio. A greater ratio greater than 4 is desirable. In this case the ratio of 24.76 indicates an adequate signal. A very high degree of precision and a good deal of reliability of the experimental values were indicated by a low value of the coefficient of variation (C.V. = 23.74%).

Table 4
Comparison of yields for single stage conversion of glucose to hydrogen.

Yield mol H_2/mol	Organism	Reference
5.0	*Rhodobacter sphaeroides*[a]	Macler et al. (1979)
0.45	*Rhodobacter sphaeroides*[b]	Margaritis and Vogrinetz (1983)
0.82	*Rhodobacter sphaeroides*	Jeong et al. (2008)
0.9	*Rhodobium marinum*	Ike et al. (1999)
0.9	***Rubrivivax gelatinosus***	Li and Fang (2008)
1.0	*Rhodobacter sphaeroides*	Fang et al. (2006)
1.53	*Rhodobacter sphaeroides*	Kim et al. (2006)
1.81	*Rhodobacter sphaeroides*	Lee et al. (2011)
1.70	*Rhodobacter sphaeroides*[c]	Kim et al. (2006)
4.2	*Rhodobacter capsulatus*[d]	Ooshima et al. (1998)
6.5	*Rhodobacter sphaeroides*	Tao et al. (2008)
3.0	*Rhodobacter capsulatus*[d]	Abo-Hashesh et al. (2011)
5.5	*Rhodobacter capsulatus*[d]	This study

[a] Glc-.
[b] VM81 (from Glc-).
[c] (Hup-, Phb-).
[d] (Hup-).

FIGURES

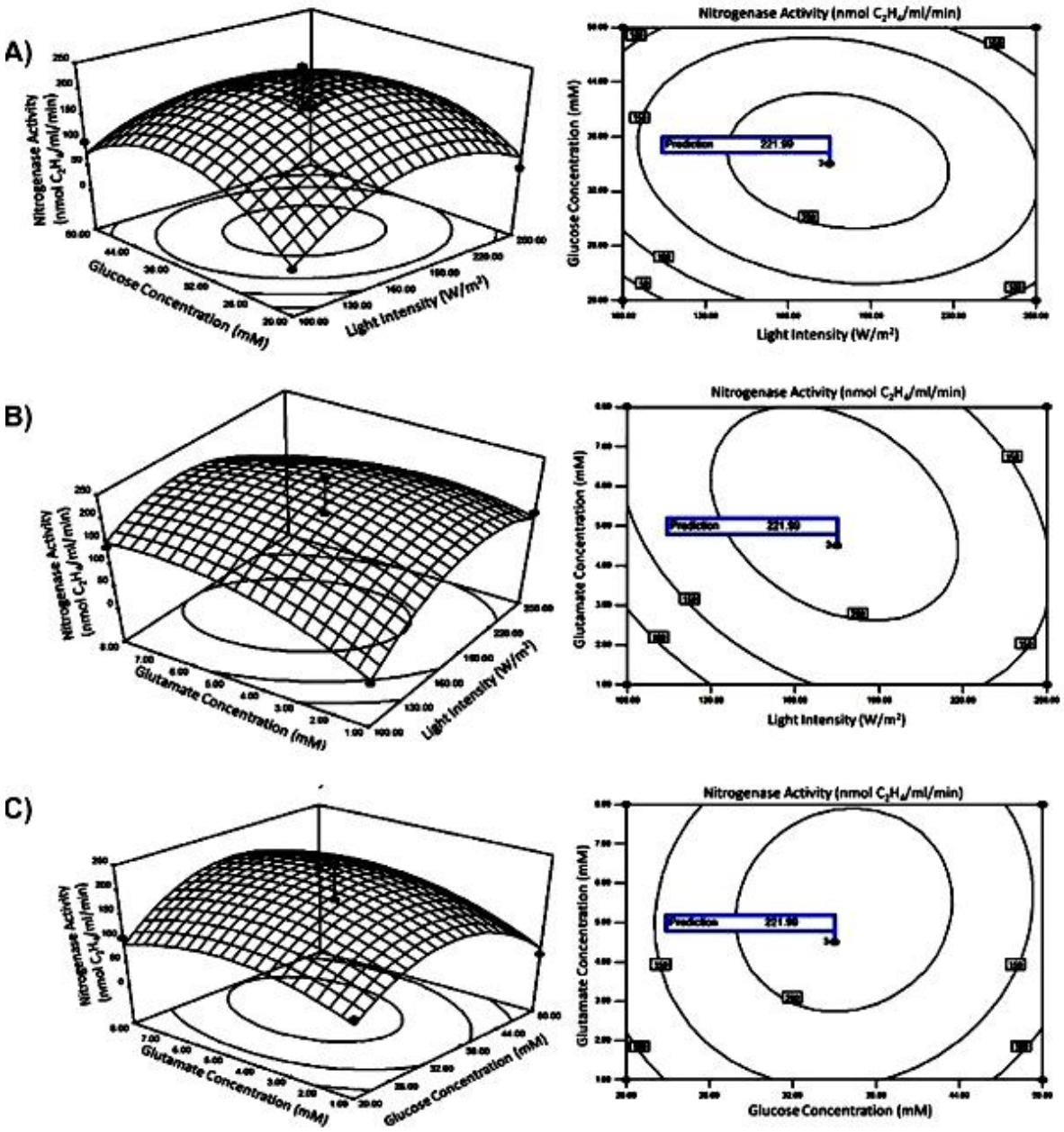

Figure 1. Two and three dimensional contour plots for the maximum nitrogenase activity. RSM plots were generated using the data shown in Table 1: Inputs were the 15 experimental photofermentative runs carried out under the conditions established by the Box–Behnken design. (A) Nitrogenase activity (nmol of C_2H_4/ml/min) as a function of glucose concentration and light intensity. (B) Nitrogenase activity (nmol of C_2H_4/ml/min) as a function of glutamate concentration and light intensity. (C) Nitrogenase activity (nmol of C_2H_4/ml/min) as a function of glutamate concentration and glucose concentration.

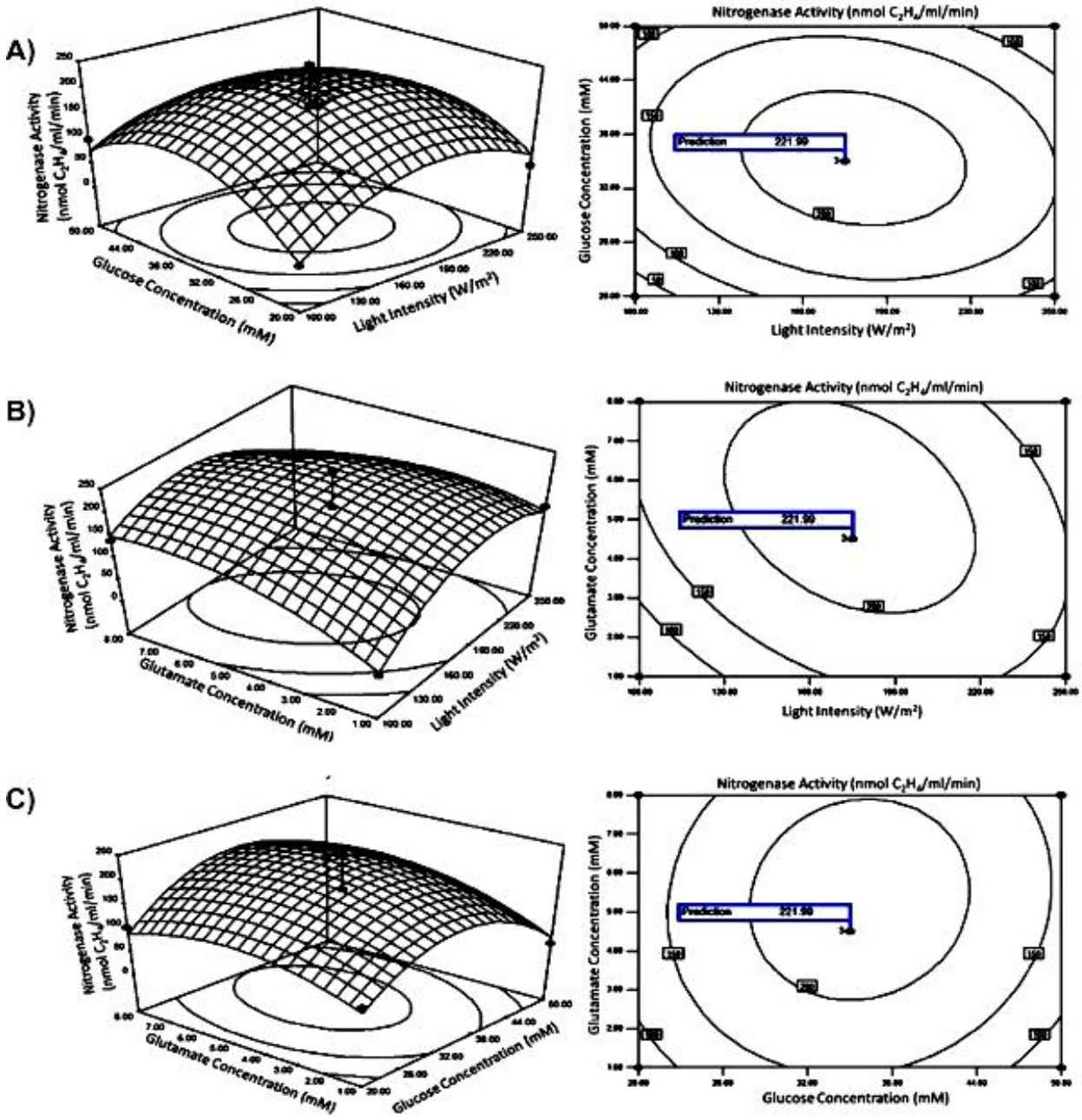

Figure 2. Two and three dimensional contour plots for the maximum hydrogen yield. RSM plots were generated using the data shown in Table I: Inputs were the 15 experimental photofermentative runs carried out under the conditions established by the Box–Behnken design. (A) Hydrogen yield (H_2 mol/mol glucose) as a function of glucose concentration and light intensity. (B) Hydrogen yield (H_2 mol/mol glucose) as a function of glutamate concentration and light intensity. (C) Hydrogen yield (H_2 mol/mol glucose) as a function of glutamate concentration and glucose concentration.

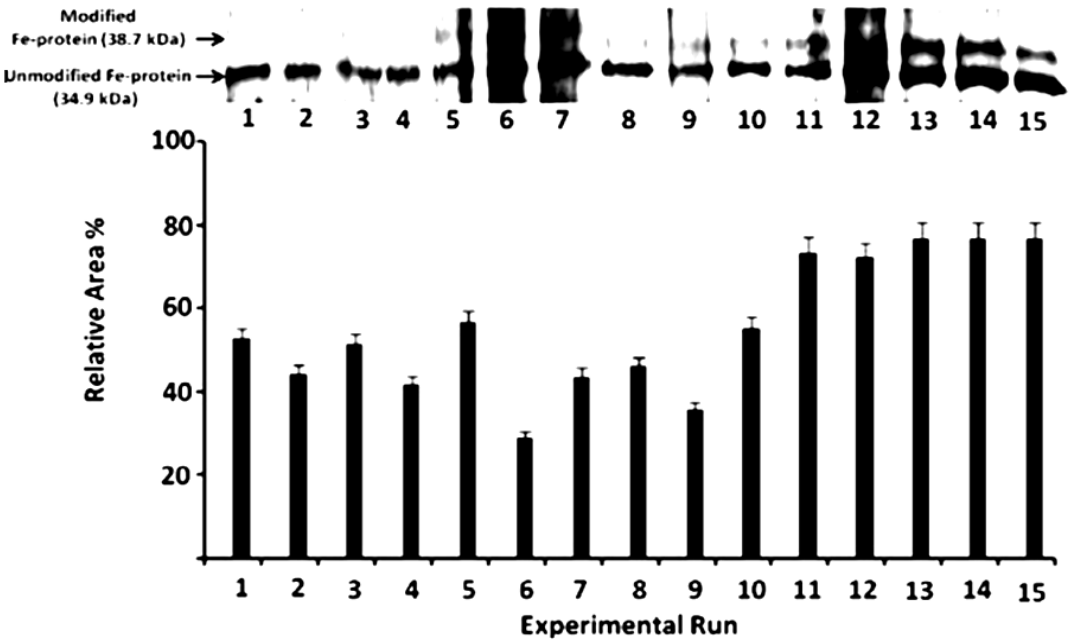

Figure 3. Nitrogenase expression and densitometric analysis of nitrogenase Fe-protein in *Rhodobacter capsulatus* strain growing under different experimental conditions (glucose concentration, glutamate concentration and light intensity). On the immunoblots, the lower band represents unmodified or un-ribosylated Fe-protein monomer, while the upper, slower migrating band is an ADP-ribosylated subunit. Densitometric analysis were done with two replicas of SDS–PAGE gels with the same amount of total protein (3 μg) loaded per well. Error bars represent the standard deviation from the mean.

CHAPTER 7: Amelioration of single-stage Biohydrogen yields using the metabolically engineered photosynthetic bacterium *Rhodobacter capsulatus* DG9

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

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Amelioration of single-stage Biohydrogen yields using the metabolically engineered photosynthetic bacterium *Rhodobacter capsulatus* DG9

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ABSTRACT

An outstanding challenge in biohydrogen production from sugars is the relatively low yields that are obtained, with a maximum of 4 moles of H_2 per mole of glucose (out of 12 possible) by dark fermentation. Previously, photosynthetic bacteria have been used, either in co-culture or in two stage systems, to increase yields by conversion of some of the by-products of dark fermentation to hydrogen. We have been developing a single-stage system where sugars are converted to hydrogen in one step through photofermentation. However, even under optimal conditions a significant fraction of the Fe-protein of nitrogenase was found to be modified, suggesting that further improvements in yields might be obtained if ADP-ribosylation could be prevented. Here we have investigated this hypothesis. An AmtB-derivative of *Rhodobacter capsulatus* JP91 (*hup*-) was created by conjugating in *amtB::Km* using the suicide vector pSUP202. Preliminary experimental results show that the newly engineered strain, *R. capsulatus* DG9, produces 8.2 (±0.06) mol hydrogen/mole of glucose under optimal conditions in batch cultures (light intensity, 175 W/m^2; 35 mM glucose, and 4.5 mM glutamate). Western blot analyses of the ADP-ribosylation status of the nitrogenase Fe-protein were investigated on metabolically engineered strain *R. capsulatus* DG9.

INTRODUCTION

Increasing conflicts between the energy requirement of industry and an inability to replace needs from limited energy resources have resulted in an immerse increase in fossil fuel utilization. Even increasing levels of greenhouse gases from the combustion of fossil fuels in turn provoked the risks of global warming. Moreover, the limited availability of global oil reserves and concerns about climate change from greenhouse gas emissions instigated marked interest in the development of clean and renewable energy alternatives to satisfy growing energy demands. Therefore, diversification of energy resources is a foremost requirement in the current global scenario. Hydrogen (H_2) gas is an important and promising energy carrier that could play a significant role in the reduction of greenhouse gas emissions. On combustion, H_2 produces water and hence is as considered a clean and carbon-neutral energy carrier. With a high energy yield of 122 kJ/g, which is 2.75-fold greater than that of hydrocarbon fuels, H_2 is regarded as an ideal energy. Hydrogen gas is an attractive future energy carrier due to its potentially higher efficiency of conversion to usable power, low to nonexistent generation of pollutants, and high energy density. It is therefore considered a promising clean, renewable source of alternative energy in the realm of fossil fuel depletion and environmental pollution (Christopher and Dimitrios, 2012). Biological hydrogen production is more advantageous than other methods since it is potentially less energy intensive, being carried out at ambient temperature and pressure (Kraemer and Bagley, 2007; Nishio and Nakashimada, 2004). A variety of biological technologies for hydrogen production are currently available, including: biophotolysis of water using algae/cyanobacteria, photo-decomposition (photofermentation) of organic compounds using photosynthetic bacteria, dark fermentative hydrogen production using anaerobic (or facultative anaerobic) bacteria and bioelectrohydrogenesis (Hallenbeck et

al., 2009; Hallenbeck, 2011). Biological hydrogen production including both photosynthetic and dark fermentative hydrogen production has been investigated over the last three decades. Photosynthetic hydrogen production is inherently attractive since its promise is to convert abundantly available solar energy into hydrogen, using either water (biophotolysis cyanobacteria or green algae) or organic waste streams (photofermentation-photosynthetic bacteria). Photofermentative hydrogen production by purple non-sulfur photosynthetic bacteria was first reported more than sixty years ago (Gest and Kamen, 1949). The purple non-sulphur bacterial genus *Rhodobacter*, has been the most widely used for biohydrogen production. This bacteria can use a broad spectrum of substrates, a versatility that reflects the variety of natural environments in which they are found. All purple non-sulphur bacteria can grow photo-heterotrophically using reduced carbon compounds as electron donors and carbon source; some species can also grow photolithoautotrophically using S^{2-}, H_2 or Fe^{2+} as electron donors and CO_2 as the sole carbon source (Larimer et al., 2004). Depending upon the species, purple non-sulphur bacteria are able to use a wide variety of organic carbon compounds; pyruvate, acetate and other organic acids, amino acids, alcohols and carbohydrates. Some species can also use one-carbon atom compounds such as methanol and formate, while some other species grow using aromatic organic compounds such as benzoate, cinnamate, chlorobenzoate, phenylacetate or phenol (Harwood, 2008). Several studies have also shown that *Rhodobacter* species are highly capable of hydrogen production when they are feeding on organic acids as substrate (Tao et al., 2007). Hydrogen gas production ability have been depicted in some purple photosynthetic bacteria such as *Rhodobacter spheroides* (Koku et al., 2002), *R. capsulatus* (He et al., 2005), *Rhodovulum sulfidophilum* W-1S (Maeda et al., 2003) and *Rhodopseudomonas palustris* (Barbosa et al., 2001). To this end, industrial effluents

including carbohydrates, simple sugars glucose, fructose and sucrose may also be used for hydrogen gas production by photosynthetic bacteria to remove the conflict on food crops competition. The photosynthetic bacteria produce chemical energy from sunlight via photo-phosphorylation and this reducing energy is harnessed to release the electrons and protons from feedstock. Additional energy is produced via substrate level phosphorylation (Hallenbeck, 2011). Finally, Nitrogenase catalyzes the biological reduction of protons to hydrogen and dinitrogen to ammonia. In this biological process, reduced ferredoxin provides the strong reducing agent necessary for reduction of protons to hydrogen. Considerable chemical energy is required for substrate reduction in the form of ATP with 2 ATP/e^-, or 4 ATP/H_2. In the absence of N_2, all the flux through the enzyme is used to reduce protons to hydrogen giving ADP, inorganic phosphate, and hydrogen as final products. Hydrogen production under these conditions is apparently a response to the metabolic need to maintain redox balance (Masepohl and Hallenbeck, 2010). The key enzyme Nitrogenase catalyzes the production of hydrogen gas in photosynthetic bacteria, and the particular requirements for its regulation, biosynthesis, and enzyme activity set specific conditions which must be met for successful, efficient hydrogen production. It is an iron-sulfur molybdenum enzyme that is highly sensitive to oxygen (Hillmer and Gest, 1977; Masepohl and Hallenbeck, 2010). Even though, Nitrogenase synthesis is repressed by high concentrations of fixed nitrogen, especially ammonium, and oxygen. Active enzyme requires molybdenum, which is also a regulatory factor and large quantities of iron (Masepohl and Hallenbeck, 2010). Substrate reduction requires high energy electrons and chemical energy input in the form of ATP with 2 ATP/e-, or 4 ATP/H_2. Enzyme activity is inhibited by presence of oxygen, ammonia or at high N/C ratios (Koku et al., 2003). Hence, the process requires anoxic conditions and ammonium to be

present in only very limited amounts. Therefore, nitrogen for cell growth that is supplied in the form of proteins, glutamate or yeast extract enhance hydrogen gas production much better than ammonium. Metabolism shifts to the utilization of organic compounds for cell synthesis or storage (polyhydroxybutyrate) rather than hydrogen production in the presence of high nitrogen concentrations resulting in excess biomass growth and reduction in light diffusion. The inhibitory action of ammonium is reversible, and hydrogen production activity can be recovered after ammonia is consumed (Oh et al., 2004; Takabatake et al., 2004; Masepohl and Hallenbeck, 2010)

An initial study using *Rhodobacter capsulatus* JP91 has shown that photofermentative hydrogen production from glucose is feasible with a maximum reported hydrogen yield of 3 molH_2/ mol of glucose (Abo-Hashesh et al., 2011). In that study, only a single glucose concentration at two different glutamate concentrations and a single light intensity were investigated, suggesting that a more thorough investigation of the various process parameters could lead to higher hydrogen yields. In a continuation of that previous work, the effects of variations in the concentrations of glucose and glutamate, as well as different light intensities on the molar hydrogen yield were systematically studied using response surface methodology with Box Behnken design. Many studies have shown that the optimization of nutritional and environmental conditions plays an important role in developing bioprocesses and improving their performance. A conventional, change-one factor-at-a-time process is time-consuming and is incapable of reaching the true optimum due to neglecting of the interaction among process variables. Design of experiments (DOE) based on the statistical modeling is a very useful tool for evaluating interactions between a set of independent experimental factors and observed responses, and is a time-saving method requiring the least number of experiments

(Annadurai et al., 1999). In this continuation study, hydrogen production from glucose via single-stage photofermentation was examined with the photosynthetic bacterium *Rhodobacter capsulatus* JP91 (*hup*-) and a maximum hydrogen yield of 5.5 (± 0.15) mol H_2/mol glucose was obtained in optimum condition with a light intensity of 175 W/m^2, 35 mM glucose, and 4.5 mM glutamate using response surface methodology. The optimized yield obtained through RSM is 85% higher than that previously achieved and is similar to what was previously shown for two-stage systems having hightest Fe-protein expression and nitrogenase activity (Ghosh et al. 2012). However, it should be noted that there was only a maximum of a 2.6 fold variation in the amount of Fe-protein over the range of conditions used, whereas nitrogenase activity and hydrogen yield varied by more than twenty-fold. This strongly suggests that factors other than the total amount of enzyme present are controlling activity and hydrogen production. These factors are probably related to flux restriction of either high-energy electrons or ATP at the level of metabolic supply to nitrogenase, as previously suggested by studies of the relationship between nitrogenase protein content, nitrogenase activity, and nitrogen supply (Yakunin et al.1999). Interestingly, Fe-protein was partially ADP-ribosylated under some of the experimental conditions. ADP-ribosylation of Fe-protein is a unique molecular mechanism for controlling nitrogenase activity in many purple non-sulfur photosynthetic bacteria and is related to the NH_4^+-induced "switch off" effect (Masepohl and Hallenbeck, 2010). Depending upon the conditions, this can have the effect of reducing overall nitrogenase activity. Since hydrogen evolution is catalyzed exclusively by nitrogenase during photofermentation, overall hydrogen production and possibly yields might be improved by reducing or eliminating modification of nitrogenase. However, the relationship between Fe protein modification and the experimental variables, the concentration of glucose and

glutamate, and the light intensity, are obscure and require additional study. Further investigation is also required to understand the factors controlling nitrogenase modification during photofermentation of glucose and how this might affect overall hydrogen productivity. (Ghosh et al. 2012). However, even under optimal conditions a significant fraction of the Fe-protein of nitrogenase was found to be modified, suggesting that further improvements in yields might be obtained if ADP-ribosylation could be prevented.

Based on this hypothesis in this present study, an AmtB- derivative of *Rhodobacter capsulatus* JP91 (*hup*-) was created by conjugating in *amtB::Km* using the suicide vector pSUP202. Preliminary experimental results show that the newly engineered strain, *R. capsulatus* DG9, produces 8.2 (±0.06) mol hydrogen/mole of glucose under optimal conditions in batch cultures (light intensity, 175 W/m^2; 35 mM glucose, and 4.5 mM glutamate). Western blot analyses of ADP-ribosylation status of the nitrogenase Fe-protein were investigated on metabolically engineered strain *R. capsulatus* DG9.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions

The purple non-sulfur photosynthetic bacterium *R. capsulatus* (JP91), a markerless *hup* derivative of B10 (Table 1), was kindly provided by Dr. John Willison, and was maintained regularly in screw cap sealed tubes (1.6 cm by 12.5 cm) completely filled with RCV glucose medium amended with thiamine-HCl (1 mg/l) and incubated at 30^oC in an Biotronette Mark III (Labline Instruments) environmental chamber equipped with three 150 W incandescent bulbs.

E. coli cells (Table 1) were grown aerobically overnight in 5 ml LB (Luria-Bertani) with ampicillin (100 µg/ml) and incubated at 37°C in a shaker (Thermo scientific,

model MAXQ 5000). LB plates, also used for culturing *E. coli*, were incubated in a 37°C incubator (Canlab) overnight. Liquid LB medium consists of 10 g bacto-tryptone, 5g yeast extract and 10 g NaCl, which are mixed in 1 L of distilled water and autoclaved (58). If LB plates were needed, 15 g agar was added before autoclaving. Appropriate antibiotics were added after cooling down. Around 25 ml of LB-agar was poured into Petri dishes (Fisher brand) in a laminar flow hood (Environmental air control, INC) and left for 30 minutes to solidify and then kept at 4°C for storage.

Strain construction and screening

In order to make chemically competent cells, 100 µl of glycerol stock of the desired cells, from -80°C, were inoculated in 500 ml of LB without antibiotic. Cells were incubated in a 37°C shaker until they reached an OD_{600} of 0.3 to 0.4. Cells were harvested by centrifugation at 5000 rpm at 4°C for 10 minutes in a Sorval GSA rotor, in 250 ml centrifuge bottles. The supernatant was discarded and the pellets were placed on ice and resuspended in 1/4 volume of ice cold 100 mM $MgCl_2$ (125ml). Cells were centrifuged at 4000 rpm for 10 minutes in the same conditions. The supernatant was decanted and the cell pellets were placed on ice and resuspended in 1/20 volume of ice cold 100mM $CaCl_2$ (25 ml). An additional 9/20 volume of $CaCl_2$ (225 ml) was added to the suspension and kept on ice for 20 minutes. The cell suspension was centrifuged at 4000 rpm and the cell pellets were resuspended in 1/50 volume of ice cold sterile 85 mM $CaCl_2$ in 15% glycerol W/V (10 ml), dispensed in 100 µl aliquots, and frozen at -80°C.

pAY41 (Table 1) was maintained in strain DH5α. QIAprep spin miniprep kit and Spin Smart™ Plasmid miniprep DNA purification kit, were used to isolate pAY41 from DH5α. The concentration of DNA was measured using a NanoDrop spectrophotometer (ND-1000).

Finally purified pAY41 was transformed in *E.coli* 17.1 to make the conjugative donor. Bacterial conjugations were carried out by the method suggested by Leclerc research group (Leclerc et al., 1988). *R. capsulatus* strain JP91, was cultured in YPS overnight at 30°C in 17ml screw-cap tubes phototrophically and pAY41/ *E.coli* 17.1 were grown in LB with ampicillin (100µg/ml) at 37°C in shaking mode (150 rpm). After growth, 50 µl of each culture was mixed together and spread on 2 cm^2 of the center of a YPS plate with no antibiotics and incubated overnight in the dark at 30°C. A bacterial suspension was then recovered with 1 ml RCV and no antibiotics. Serial dilutions were made and were spread on RCV plates with kanamycin (10µg/ml) and tetracycline (1.5 µg/ml). Plates were incubated anaerobically (gas pack) at 30 °C and incubated in the presence of light in the Biotronette Mark III Environmental Chamber (Labline instruments) until the appearance of red colonies on the plates. Afterwards, these colonies were grown in YPS tubes with tetracycline (1.5µg/ml). After growth, the culture was transferred to RCV+30mM $(NH_4)_2SO_4^+$ tubes with tetracycline and kept at 30°C with light for further experiments. The *amtB* mutant positive transconjugants (*R. capsulatus* DG9) were examined by growing in RCV agar medium supplemented with thiamine-HCl (1 mg/l) and antibiotics (kanamycin and tetracycline). QIAamp DNA Mini Kit was used to extract the genomic DNA from both wild (*R. capsulatus* JP91) and mutant type (*R. capsulatus* DG9).

Finally, the positive transconjugants were screened by PCR using designed screening primers, *amtB*-upstream (5'-CCGTTCAAGCTCGAGGAGGTCCG-3'); *amtB*-downstream (5'-TCAGCGGGAATGATGAGCGGCGC-3'). Primers were designed using *amtB* sequence of *Rhodobacter capsulatus* SB1003 (>gi: 294675557).

Batch Reactor Studies

Photofermentation experiments were carried out with 100-ml cultures, inoculated (5% (v/v)) from a culture in the late log phase of growth, in RCV-glucose medium in serum bottles sealed with butyl rubber stoppers and rendered anaerobic by sparging with oxygen free argon for 15 min. Cultures were incubated for 6 days in a glass-sided temperature controlled ($30^{o}C$) water bath. The cultures were illuminated with a bank of six 50-W halogen bulbs placed 25 cm from the culture vessels and the light intensity was adjusted using a potentiometer to give the required light intensities. The amount of gas evolved was measured by water displacement using graduated cylinders filled with water and partially submerged in a tub of water connected to the serum bottles by relatively hydrogen impermeable FEP 890 tubing.

Analytical Methods

The concentration of hydrogen in the collected gas was determined using a gas chromatograph (Shimadzu GC-8A) equipped with a thermal conductivity detector, a 1m column packed with molecular sieve 5A and with argon as carrier gas. Glucose concentrations were estimated spectrophotometrically at 490 nm with a phenol-sulphuric acid assay (Dubois et al., 1956). Light intensities were measured using a Delta OHM photo/radiometer (HD 2102.1). To monitor Fe-protein expression, aliquots of the liquid phase of the culture were withdrawn, brought to 1 X sodium dodecyl sulfate sample buffer concentration and immediately incubated in a boiling-water bath for 5 min. Equal amounts of total protein (3 μg/ well) were analyzed by sodium dodecyl-polyacrylamide gel electrophoresis (12.5% total acrylamide) with low crosslinker concentration (prepared as previously described (Yakunin et al., 1999) and subsequent immunoblotting with chemiluminescence detection using the LumiGlo chemiiluminescence substrate system (Mandel Scientific). Protein concentrations

were determined by the Bradford method (Bradford et al., 1976) after 2 minutes sonication of the whole cell culture.

RESULTS AND DISCUSSION

Construction and screening of *Rhodobacter capsulatus* DG9

After conjugation, the strain was screened for correct chromosomal integration of the *amtB-kanR* cassette in the *amtB* operon in *R. capsulatus* JP91 after curing of plasmid pAY41. This was done using YPS screening plates with kanamycin and tetracycline (Details in Methodology section). The physiological screening is shown in Figure 1a. Finally the metabolically engineered *R. capsulatus* DG9 strains were screened by PCR using specific designed primers using extracted genomic DNA from both *R. capsulatus* JP91 and DG9 strains. The molecular characterization clearly showed that genetic integration occurred in the *amtB* gene in the chromosome of *R. capsulatus* DG9 (Figure 1b)**.**

Hydrogen gas production by *Rhodobacter capsulatus* DG9

Preliminary experiments with *Rhodobacter capsulatus* DG9 which had been acclimatized to RCV-glucose were carried out at central optimized concentration 35 mM of glucose, 4.5 mM glutamate as nitrogen source in RCV minimal medium (containing Thiamine-HCl) at light intensity of 175 W/m^2. Therefore, we carried out a more detailed analysis on total gas production and cumulative hydrogen production on a daily basis until day 6. The total hydrogen production (Figure 2a**)** and cumulative hydrogen production is shown in Figure 2b.

Expression of Nitrogenase (Fe-protein)

Analysis of nitrogenase protein expression was performed on the sixth day. Since the molar hydrogen yield was maximal at day 6, it was chosen as the optimal time point for

sampling for determination of the relative amount of nitrogenase (Fe protein) and its modification status. Interestingly, Fe-protein of the metabolically engineered *R capsulatus* DG9 strain was not ADP-ribosylated under previously optimized experimental conditions in Figure 3. Further investigation is also required to understand the factors (likely GlnK, DraG protein expression) controlling nitrogenase Fe protein regulation during photofermentation of glucose and how this might affect overall hydrogen productivity at the molecular level.

CONCLUSIONS

In this study we have shown that the metabolically engineered *R. capsulatus* DG9 strain (*amtB*-, *hup*-) shows promise for the development of a single stage photofermentation process for converting glucose, and possibly other sugars, to hydrogen. Maximum yields, 8.2 mol of H_2 per mole of glucose was obtained in batch operative mode. As for our hypothesis it has also been proven that this newly developed engineered strain does not have any ADP-ribosylation phenomenon on Fe-protein of nitrogenase enzyme system; which indirectly recovers the NH_4^+ switch-off mechanism to provide higher hydrogen yield in batch operation. In a recent study, *R. capsulatus* JP91 was shown to produce hydrogen yield of 10.3 molH_2/ mol glucose in continuous mode (Abo-Hashesh et al., 2013). Thus the future direction will be looking at improving hydrogen yield using this metabolically engineered strain *R. capsulatus* DG9 to surpass the stoichiometric yield of 12 mol H_2/ mol glucose followed by continuous bioprocess development. Hydrogen profile, nitrogenase activity and ADP-ribosylation of Fe-protein are also currently being studied using different amino acids as nitrogen source. Moreover it could also be done by revealing the clear mechanism of hydrogen yield and nitrogenase regulation with NH_4^+ switch-off mechanism considering expression studies on GlnK and DraG protein expression upon different nitrogen regimes.

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TABLES

Table 1: Relevant characteristics of the strains

Strains/ Plasmid	Relevant characteristics	Reference
E. coli		
DH5α	***F-φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17(rk-, mk+) phoA supE44 λ- thi-1 gyrA96 relA1***	**Invitrogen**
S17.1	***RP4-2-Tc::Mu-Km::Tn7, pro, res$^-$, mod$^+$, Tp^r, Sm^r***	**Simon et al.,1983**
Rhodobacter capsulatus		
JP91	***hup-* derivative of B10 wild type; *kan^S, tet^S***	**Willison et al., 1987**
DG9	***hup-, amtB-, kan^R, tet^S* derivative of JP91**	**This study**
Plasmid		
pAY41	***2.6 kb amtB-kan^R fragment from pAY37 (Non polar kan^R cassette) cloned (As EcoRI fragment) into EcoRI site of pSUP202; kan^R, tet^R, amp^R***	**Yakunin et al., 2002**

Table 2: Comparison of yields for single stage conversion using *R. capsulatus* strains in batch mode

Strains	Reference	Yield (mol H_2/mol glucose)
Rhodobacter capsulatus (*hup*-)	Ooshima et al. 1998	4.2
Rhodobacter capsulatus JP91 (*hup*-)	Abo-Hashesh et al. 2011	3.0
Rhodobacter capsulatus JP91 (*hup*-)	Ghosh et al., 2012	5.5
Rhodobacter capsulatus DG9 (*hup*-, *amtB*-)	This study	8.2

FIGURES

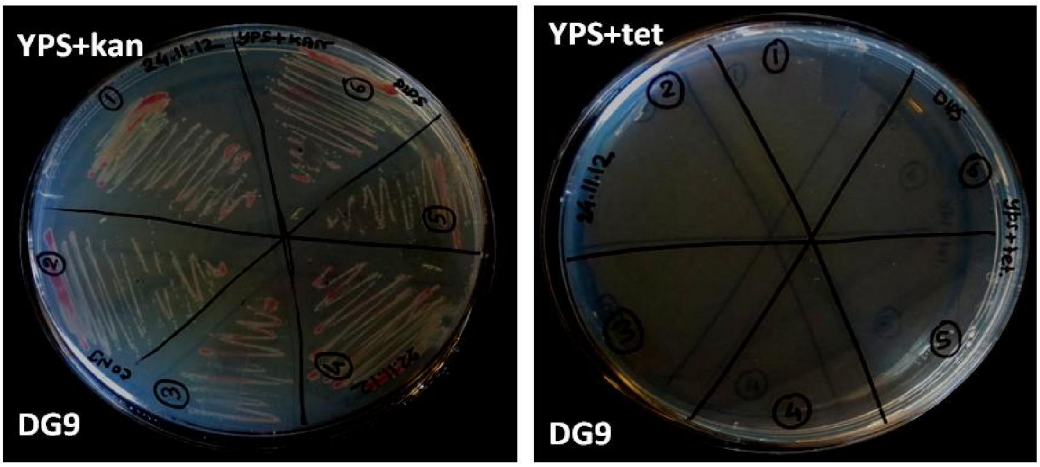

Figure 2a: Physiological screening of metabolically engineered *Rhodobacter capsulatus* DG9. [Screening plates were YPS-Kanamycin (kan) and YPS-tetracycline (tet); screening plates were incubated at 30^{o}C for overnight]

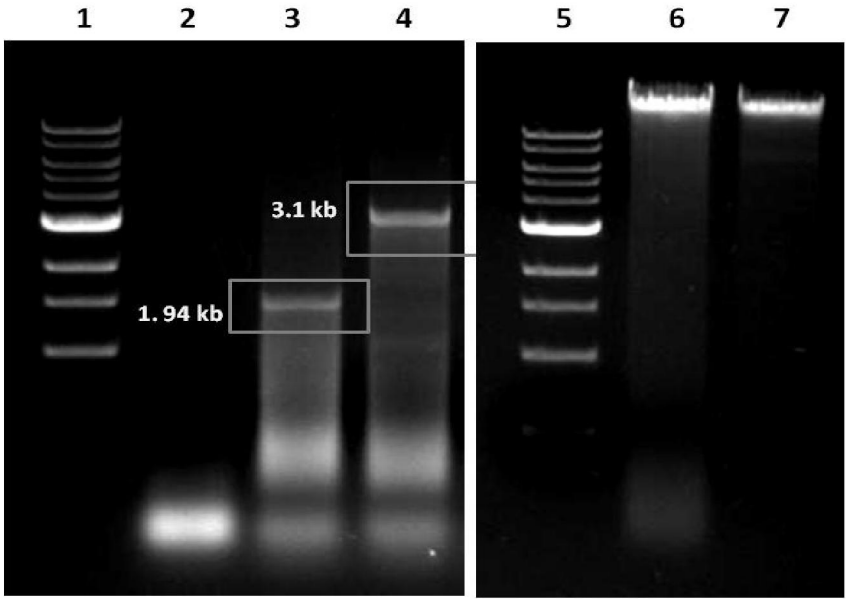

Figure 2b: Molecular screening of metabolically engineered *Rhodobacter capsulatus* DG9. Where Lane1: 1kb molecular weight marker; Lane2: PCR control; Lane3: *amtB* amplicon (JP91); Lane4: *amtB::Kan*^{R} amplicon (DG9); Lane5: 1kb molecular weight marker; Lane 6: genomic DNA of *Rhodobacter capsulatus* JP91; Lane7: genomic DNA of *Rhodobacter capsulatus* DG9; primers annealing temperature were kept at 66^{o}C; and 5% (w/v) DMSO were used upon PCR reaction as *Rhodobacter capsulatus* genomic DNA has high GC%.

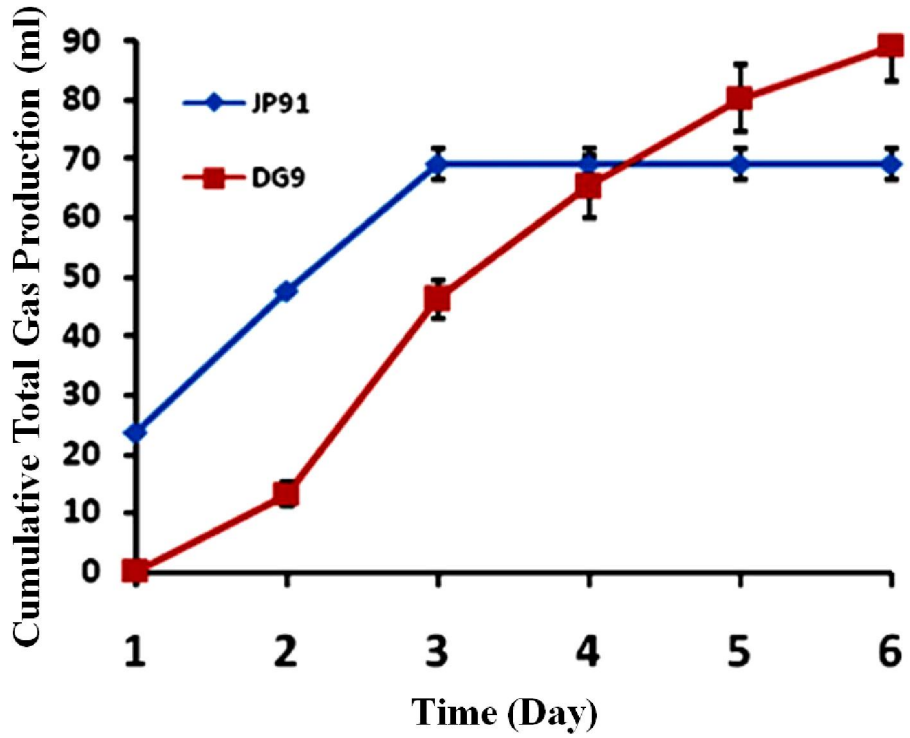

Figure 2a: Cumulative total gas production using *Rhodobacter capsulatus* DG9

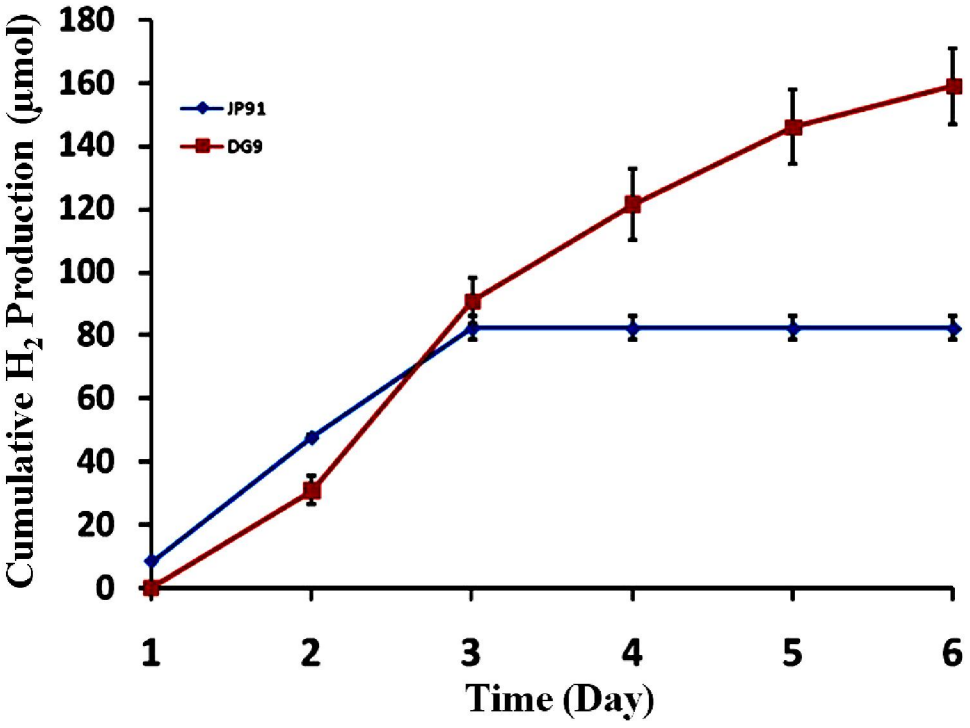

Figure 2b: Cumulative H_2 gas production using *Rhodobacter capsulatus* DG9

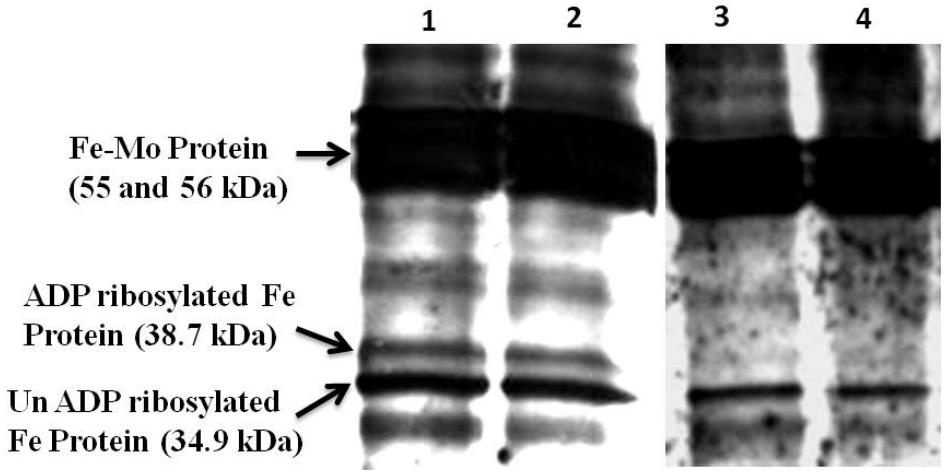

Figure 3: **Nitrogenase Fe Protein expression under optimized condition (light intensity, 175 W/m^2; 35 mM glucose, and 4.5 mM glutamate).** [Lane1 and Lane 2 (repetition): ADP-Ribosylated Fe Protein expression from *Rhodobacter capsulatus* JP91; Lane3 and 4 (repetition): Un-ADP-ribosylated Fe Protein expression from *R. capsulatus* DG9; on the immunoblots, the 34.9 band represents Un-ADP ribosylated Fe protein monomer, while the 38.7 kDa band is an ADP-ribosylated subunit; upper dense band of 55 and 56 kDa bands represent nitrogenase Fe-Mo protein]

DISCUSSION

This thesis examined various means to improve the molar hydrogen yield with special emphasis of metabolic engineering with interactive effect with bioprocess independent variable followed by Response surface methodology.

1. Improving hydrogen yield from different sugars by metabolically engineered *Escherichia coli* DJT135 in batch mode

Future sustainable production of biofuels will depend upon the ability to use complex substrates present in biomass if the use of simple sugars derived from food crops is to be avoided. Therefore, organisms capable of using a variety of fermentable carbon sources must be found or developed for processes that could produce hydrogen via fermentation. Here we have examined the ability of a metabolically engineered strain of *Escherichia coli*, DJT135, to produce hydrogen from glucose as well as various other carbon sources, including pentoses. The initial pH of the fermentation medium had a strong effect on hydrogen production. *E. coli* strain DJT135 grew over a wide range of initial pHs (4 to 9), with hydrogen production observed over essentially the same pH range. At low initial pHs (4.5 and 5.5), cultures exhibited a prolonged lag phase. Maximum hydrogen production and little or no lag phase were seen at an initial pH of 6.5. Although the exact optimum pH varies with organism, this value is within the relatively narrow range usually seen. As well, a prolonged lag phase has previously been reported for cultures where the initial pH is below the optimum. As to be expected, hydrogen production by *E. coli* DJT135 also varied appreciably with temperature. Hydrogen production increased from 25°C to 35°C, while afterwards it decreased. Maximum hydrogen production was obtained at 35°C, typical for mesophilic fermentation. This effect is most probably related to temperature effects on growth and not on the enzymes involved in

hydrogen production since a study of hydrogen production with non-growing cells of this strain showed that the hydrogen production rate at 45°C was essentially the same as that obtained at 37°C. Double reciprocal (Lineweaver–Burke) plots were used to obtain the kinetic parameters for cell growth with glucose (1%) as substrate under the optimal conditions determined above. The maximum specific growth rate (μmax) and saturation constant (Ks) of the cell were found to be 0.050 h^{-1} and 0.027 g L^{-1}. *E. coli* DJT135 also showed relatively good hydrogen yields with l-arabinose, mannitol and trehalose. For a bacterium, such as *E. coli*, with an enteric type metabolism, hexoses are degraded through the glycolytic pathway yielding a maximum of $2H_2$/ hexose. 1hexose → 2pyruvate → $2H_2 + 2CO_2 + 2$ (acetate+ ethanol). The relative amounts of acetate versus ethanol are determined by the oxidation state of the six carbon substrate. Efficient bioenergy production from lignocellulosic materials requires the ability to utilize arabinose and xylose since these can constitute 20 to 35% of the sugars present. However, hydrogen production from these pentose sugars has not been well studied. Here we show that *E. coli* strain DJT135 is capable of converting substantial amounts of these sugars to hydrogen (1.02, 0.57 mol H_2/mole substrate, respectively. In general *E. coli* degrades pentoses through the non-oxidative pentose phosphate pathway: 3pentose → 5pyruvate → $5H_2 + 5CO_2 + 5$ (acetate + ethanol). The relative amounts of acetate versus ethanol are determined by the oxidation state of the five carbon substrate. Thus one expects a maximum of 1.67 mol H_2/mol pentose, and therefore arabinose and xylose gave 61 and 34% of the theoretical maximum respectively. A *Clostridium* species isolated from termites was reported to ferment both arabinose and xylose, but yields were low; 0.097 mol H_2/mole arabinose and 0.092 mol H_2/mole xylose. Thus the yields reported in the present study from these substrates are very significant. Moreover, there was considerable

variation in yields from different substrates. The reason for this is unclear at present, and thus presents a fruitful area for future research. It certainly suggests that flux through the different pathways is influenced by the nature of the substrate, including its entry point into central metabolism, as well as its oxidation state, which would lead to a different pattern of end products. These factors could also influence the fraction of available carbon going into biomass as well as storage materials such as glycogen. Therefore, optimizing yields from specific substrates might require a detailed investigation of flux through various metabolic pathways thus suggesting means by which to channel more substrate into pathways leading to hydrogen production.

2. Response surface methodology for process parameter optimization on *E. coli* DJT135 to reach near stoichiometric yield

Greater hydrogen yields can be obtained through metabolic engineering, but realizing the potential increase achieved through genetic intervention requires bioprocess optimization. A number of variables could possibly restrict hydrogen production rates and yields. However, assuming an adequate medium formulation, the most important key parameters are probably substrate concentration, temperature and pH. First of all, pH is one of the most important factors in hydrogen production due to its effects on metabolic pathways, thus potentially modulating end product distribution, as well as possibly affecting the duration of the lag phase. However, assuming an adequate medium formulation, the most important key parameters are probably substrate concentration, temperature and pH. First of all, pH is one of the most important factors in hydrogen production due to its effects on metabolic pathways, thus potentially modulating end product distribution, as well as possibly affecting the duration of the lag phase (Bartacek et al., 2007, Davila-Vazquez et al., 2008, Hallenbeck,

2005, Hallenbeck, 2009, Hawkes et al., 2007 and Lay, 2000). Optimum initial pH is probably the resultant sum of a number of factors; it must be within a range that does not inhibit growth and permits high level expression of the requisite fermentation pathways while at the same time a higher initial pH value would delay the onset of acid inhibition (Lee et al., 2002). Initial glucose concentration also plays an important role on the yield and production rate of hydrogen (Fabriano and Perego, 2002). Low initial glucose concentrations result in low rates of fermentation, and total fermentation times increase with high initial substrate concentrations. In addition, temperature affects the maximum specific growth, substrate utilization rate, and the metabolic pathway of microorganisms, resulting in major shifts in end product composition (Van Ginkel et al., 2001). Optimal conditions determined by the one-variable-at-a-time approach can not be directly used to predict the true optimal conditions for a particular bioprocess due to potential interactions between the independent variables. To overcome this problem, a full or fractional factorial design coupled with RSM (response surface methodology) can be used to advantage (Hallenbeck and Ghosh, 2009 and Wang and Wan, 2009).

Previously, only a few studies have simultaneously examined the effects of substrate concentration, temperature, and pH on hydrogen yield (Jo et al., 2008, Mu et al., 2006, Wang et al., 2005 and Wang and Wan, 2008). Here, using the modified *E. coli* strain DJT135, we have determined the optimal levels of these key factors and the effect of their interactions on molar hydrogen yields using RSM with a Box–Behnken design. *E. coli* may be particularly useful in biofuels production since in general it shows broad substrate specificity and therefore is capable of catabolising a variety of five and six carbon compounds. In fact, we have recently shown that the modified strain used here, *E. coli* DJT135, is capable of producing

hydrogen from a variety of hexoses and pentoses with relatively high yields (Ghosh and Hallenbeck, 2009).

The method used here was a Design of Experiments approach where a statistical design is used to choose a series of experimental conditions such that a minimum number will give a robust description and verification of a model (Hanrahan and Lu, 2006). This technique is often applied in engineering and manufacturing, but can be advantageously applied to biological systems in some cases, especially where a bioprocess, such as in this study, is involved (Lee and Gilmore, 2006). We wished to assess three independent variables, their effect on hydrogen yields as well as the degree, if any, of their interaction. The appropriate method for these goals is a Box–Behnken since it uses a minimum of tests, is robust, and can be applied when values at the extremes are uninteresting (Whittinghill, 1998).

Therefore 12 experimental fermentations were run at the different parameter values indicated by the design and the resultant hydrogen yield measured. The summary of the analysis of variance (ANOVA) of the results of the quadratic model has shown that the model *F*-value of 42.04 implies that the model is significant. There is only a 0.04% chance that a "model *F*-value" this large could occur due to noise. Value of "Prob > *F*" less than 0.05 indicates that the model terms are significant. In this case X_1, X_2, X_3, X_1X_2, X_2X_3, X_1X_3, $X21$, $X22$ and $X23$ are significant model terms. The "Lack of fit *F*-value" of 9.15 implies that the lack of fit is not significant relative to the pure error. There is a 10% chance that a "Lack of fit *F*-value" this large could occur due to noise. The squared regression statistic (R^2), the determination coefficient, a measure of the goodness of fit of the model, was very significant at the level of 98%, i.e., the model was unable to explain only 2% of the total variations. Adequate precision measures the signal to noise ratio. A greater ratio greater than 4 is

desirable. In this case the ratio of 16.066 indicates an adequate signal. A very high degree of precision and a good deal of reliability of the experimental values were indicated by a low value of the coefficient of variation (CV = 14.3%).

Plots of residuals versus responses predicted by the model were randomly distributed around zero without any trends (not shown). This indicates good prediction of maximum response along with constant variance and adequacy of the quadratic models. Similar results have previously been obtained in optimization studies with mixed cultures (Mu et al., 2006, Wang et al., 2005 and Wang and Wan, 2008). Hydrogen yield increased with increasing temperatures, initial pH and glucose concentrations to the optimal levels, and then decreased with a further increase in these parameters. The predicted hydrogen yield of 1.69 mol $H_2.mol^{-1}$ is as high, or higher, than that reported previously.

We verified the predicted maximum, 75 mM glucose, 35°C, pH 6.5, by running three replicates under these conditions and obtained a yield of 1.68 ± 0.01 H_2/glucose, in excellent agreement with the predicted value. In fact, this, taken together with the residual analysis, validates the model in general. Thus, not only can it be used to predict the optimal conditions, it would also be useful in predicting yields and parameters to be used if for some reason one of the operating parameters was constrained at a suboptimal value.

Therefore, response surface optimization can be successfully used to optimize high hydrogen yields with metabolically engineered organisms. However, since this type of treatment treats the bacterium as essentially a black box, thus ignoring the underlying metabolic pathways with their multiple interconnections and dependence upon intracellular metabolite pools, it cannot be used to analyze or predict how metabolic engineering itself could be used to enhance the production of hydrogen, or other metabolites. A more useful

approach for this purpose would be metabolic flux modeling and analysis. Rather, its utility is to show how bioprocess operational parameters should be set to obtain maximum performance with strains that have been engineered to achieve maximum metabolic performance. This has been specifically demonstrated in the present study for hydrogen production by an *E. coli* strain, DJT135, which was previously optimized through a series of genetic manipulations.

3. Heterologous expression of the *Ralstonia eutropha* SH operon in metabolically engineered *E.coli* to surpass stoichiometric hydrogen yield

Anaerobically grown *E. coli* carries out mixed acid type fermentation when grown under anaerobic conditions. Sugars are degraded to pyruvate by the glycolytic pathway, producing ATP and reducing NAD^+ to NADH. The amount of NADH that is produced depends upon the redox state of the substrate, and this in turn controls fermentation product distribution. Pyruvate is principally converted to formate and acetyl-CoA. Under the appropriate conditions, usually acidic pH, formate is broken down via the formate-hydrogen lyase pathway producing CO_2 and H_2. Thus, *E. coli* is only capable of the production of a maximum of 2 H_2 per mole of glucose that enters glycolysis. The NADH that is generated during anaerobic growth on sugars must be oxidized to NAD^+ for glycolytic metabolism to continue since NAD^+ is a necessary cofactor for the oxidation of glyceraldehyde. Although in theory NADH could be oxidized by the reduction of pyruvate to lactate by lactate dehydrogenase, in practice this pathway is only fully expressed under acidic conditions, and does not seem to be sufficient on its own to permit anaerobic growth. Therefore, mutants deleted for alcohol dehydrogenase (*adhE*) cannot grow anaerobically on sugars. Thus, *adhE* mutants might produce excess levels of NADH when incubated anaerobically. Some NADH

can be recycled through the oxidation of oxaloacetate to malate, leading ultimately to the formation of succinate, but again, this side pathway is not sufficient in itself to permit anaerobic growth on sugars. In order to examine the potential for engineering *E. coli* to produce hydrogen from NADH, we chose to express the SH hydrogenase from *Ralstonia eutropha* H16. The SH operon consists of nine genes; *hoxFUYHWI* and *hypA2B2F2* [25]. HoxHY is the hydrogenase module and HoxFU is a NADH dehydrogenase. *hoxW* encodes an highly specific endopeptidase required for the C-terminal processing of HoxH during hydrogenase maturation (Thiemermann et al., 1996). Although SH is usually isolated as a heterotetrameric protein (HoxHYFU), HoxI has been shown to provide a NADPH binding domain to a hexameric form of SH that can be isolated under certain conditions (Burgdorf et al., 2005). *hypA2B2F2* are duplicate copies of three of the seven *R. eutropha* hydrogenase maturation genes (*hyp*). Interestingly, they can substitute for *hypA1B1F1* in the maturation of both the SH hydrogenase and the MBH (membrane bound) hydrogenase (Wolf et al., 1998). A previous attempt to express the SH operon in *E. coli* from its native promoter (PSH) was unsuccessful (Tran-Betcke et al., 1990), presumably because there is an absolute requirement for the transcriptional activator HoxA for expression from this promoter (Schwartz et al., 1998).Therefore, we wished to express the SH operon from a promoter active in *E. coli*. Plasmid pJWPH5 was used for expression of the SH operon in *E. coli* under the control of the inducible *trc* promoter of the vector pTRC99A.

The hydrogen evolution capacity of batch cultures of various strains of *E. coli* were tested under anaerobic conditions with LB medium as previously described (Bisaillon et al., 2006). Hydrogen was evolved by strain BW535, wild-type for the hydrogenase activities of Hyd1, Hyd2, and Hyd3 [30]. Hydrogen was also evolved by strain JW135, a Hyd1- Hyd2-

(*Δhya-Km Δhyb-Km*) derivative of BW535. As expected under the conditions used here, strain FTD147, which lacks the hydrogen evolving Hyd3 as well as hydrogen consuming Hyd1 and Hyd2 [20], showed no hydrogen evolution (*E. coli* Hyd4 is inactive under these conditions (Self et al., 2004)). The hydrogen evolution of strains containing pJWPH5 that had been altered so that they potentially produced more NADH was also tested. *ΔadhE* and *ΔarcA* derivatives of strain FTD147 were examined for hydrogen evolution in anaerobically incubated LB-glucose (0.4%) medium (+ 0.05 mM IPTG). As discussed above, *ΔadhE* mutants should have excess NADH levels when incubated anaerobically and might consequently support hydrogen evolution by SH hydrogenase. There was no detectable hydrogen evolution by such a strain (FTAB4). Another strain, FTAB5, potentially able to produce increased levels of NADH under anaerobic conditions, was also examined. Strain FTAB5, which carries pJWPH55, is a *ΔarcA* derivative of strain FTD147. ArcA is a two-component regulator that is responsible for the anaerobic repression of synthesis of enzymes of the TCA cycle. Therefore, a strain mutated in ArcA might be expected to express the TCA cycle under anaerobic conditions, potentially permitting the generation of excess NADH from acetyl-CoA. Indeed, in vitro TCA cycle enzyme activities are greatly increased in ArcA mutants grown under anaerobic conditions (Hallenbeck, 2012). Even additional NADH would become available if it could be more effectively oxidized under these conditions. However, there was no detectable hydrogen evolution by this strain (Perrenoud and Sauer, 2005).

Synthesis of SH hydrogenase proteins was verified by a Western blot of an extract of strain FTD147/pJWPH5 grown under anaerobic conditions (LB + 0.05 mM IPTG). Prominent protein bands at 67 and 55 kDa were observed, corresponding to HoxF and HoxH respectively. Although there was a high level of expressed protein in the supernatant, there

appeared to be some inclusion body formation since a significant quantity was also recovered in the pellet obtained after centrifugation of the crude extract, produced by sonication, for 15 min at 10,000 rpm. As a control, a high-speed supernatant of an extract of *R. eutropha* H16 grown anaerobically in FGN medium mineral salts medium (Schlegel et al., 1961) as previously modified (Friedrich et al., 1981) was included. Thus, sufficient levels of SH hydrogenase appeared to be synthesized under anaerobic conditions in *E. coli* strain FTD147 strain carrying pJWPH5.

Lack of hydrogen evolution by anaerobically incubated FTD147-derivatives carrying pJWPH5 was therefore not due to lack of SH hydrogenase protein, but might rather be due to inadequate hydrogenase maturation. This was verified by checking the in vitro NAD-linked hydrogenase activity of extracts of anaerobically grown cultures of *R. eutropha* H16, *E. coli* FTD147, and *E. coli* FTAB1 (FTD147/pJWPH5) using a spectrophotometrically assay (Schneider et al., 1976). As expected, high levels of H_2-dependent NAD^+ reduction were observed with the *R. eutropha* H16 extract, and no reduction was seen with FTD147. However, only a very low level of activity was observed for FTAB1. Thus, it appears that indeed maturation of *R. eutropha* SH hydrogenase to a functional hydrogenase is very inefficient in *E. coli* grown anaerobically under these conditions. Furthermore, anaerobic growth of *adhE* mutants carrying pJWPH5 (SH hydrogenase) were examined on M9 glucose in the presence of nickel and iron. As described above, strains that are mutated in *adhE* have been reported to be impaired for growth under anaerobic conditions. We constructed various *ΔadhE* derivatives of both FTD147 and JW135 (FTGH2, FTJWDC3, JWGH1, DG2, FTDPH10) and verified that they were unable to grow under anaerobic conditions on sorbitol or glucose minimal media. Reasoning that growth could be restored if a means of reoxidizing

NADH were introduced, these mutants were tested for the rescue of anaerobic growth on glucose by the introduction of pJWPH5, plasmid carrying the SH operon under the control of the *trc* promoter. Indeed, under these growth conditions, M9-glucose + IPTG + Ni + Fe, these derivatives were able to grow (not shown). The growth results strongly suggested that SH expressed and active in anaerobic cultures grown on nickel and iron supplemented M9-glucose. This was verified by assaying the SH hydrogenase activity of the strains in vitro measuring the capacity of extracts to reduce NAD+ under a hydrogen atmosphere. As expected, the positive control, an extract of *R. eutropha* H16 showed significant NAD+ reduction activity whereas the two *E. coli* strains, FTD147 and JW135, gave insignificant levels of activity. However, extracts of strains carrying pJWPH5 all showed varying but significant levels of SH hydrogenase activity in vitro. The parental strains, FTD147 and JW135 had specific activities close to 1 μmol NADH /min /mg protein, or 16% of that of an extract of *R. eutropha* H16. Extracts of strains carrying mutations that could be thought to increase cellular NADH levels, FTGH2, FTJWDC3, JWGH1, DG2, FTDPH10, and DG1, gave even higher specific SH hydrogenase levels, varying from 3.5±0.1μmol NADH /min /mg protein to 7.1±0.31 μmol NADH /min /mg protein. These results, obtained with nickel and iron amended M9, demonstrate the importance of media supplementation with the metals required for cofactor synthesis since, in their absence, very little in vitro activity can be demonstrated.

The highest activities were observed with JWGH1, a JW135 derivative carrying a mutation in the NADH consuming enzyme lactate dehydrogenase and in ArcA in addition to alcohol dehydrogenase, and FTJWDC3, a FTD147 derivative mutated in malate dehydrogenase in addition to alcohol dehydrogenase. Somewhat lower levels of in vitro

activity were observed with extracts of DJ1, a JW135 derived strain additionally mutated in *arcA*, and FTGH2, a FTD147 derivative mutated in both lactate dehydrogenase and malate dehydrogenase. A FTD147 derivative, DG2, carrying the same mutations as the JW135 derived strain JWGH1, gave only about 50% of the in vitro activity of that strain. Finally, FTDPH10, mutated in alcohol dehydrogenase and lactate dehydrogenase, gave only 50% of the highest observed in vitro activity, but even so this was more than three-fold higher than the parental strain, FTD147 carrying pJWPH5. It is clear from these results that, even though transcription is under control of the IPTG inducible trc promoter, higher levels of SH hydrogenase, as measured by in vitro activity, were present in strains in which the ability to reoxidize NADH anaerobically were compromised. The exact mechanism behind this enhancement is unclear, but might be related to general effects on growth. In addition, the results suggest that the effect of the introduction of multiple mutations in pathways that oxidize NADH appears to be additive, with abolition of malate dehydrogenase being more effective in a *adhE* strain than eliminating lactate dehydrogenase activity. At any rate, these results demonstrate the first successful heterologous expression in *E. coli* of a multi-subunit [NiFe] hydrogenase capable of interacting with NAD^+/NADH.

Regarding in vivo hydrogen production by *E. coli* strains expressing SH hydrogenase, all strains showed significant growth over the experimental period after a variable lag period. Growth was highest, and at nearly the same level, in strains FTGH2, FTJWDC3, and JWGH1. FTGH2 and FTJWDC3 both carry *adhE* and *mdh*; FTGH2 carries in addition *ldhA*. Final optical densities were appreciably lower in strains DJ1, DG2, and FTDPH10. Nevertheless, the growth of strains carrying *adhE*; FTGH2, FTJWDC3, JWGH1, DG2, and FTDPH10, demonstrates that they were sufficiently capable of reoxidizing NADH to permit growth.

Since growth was only observed in strains carrying pJWPH5, NADH oxidation must have been provided by the action of SH hydrogenase.All strains tested showed appreciable hydrogen evolution activity with final hydrogen levels of between 2000 and 3600 nmol H_2 per vial (2 ml of culture). Strains FTJWDC3 and JWGH1 produced the greatest amount of hydrogen, whereas strains FTGH2 and DG2 produced the least. Interestingly, the two best hydrogen producers were also the strains that were shown to have the highest levels of SH hydrogenase activity in vitro. Strain DG2, which gave one of the lowest SH hydrogenase activities in vitro, also produced the least amount of hydrogen. Taken together this suggests that hydrogen production levels are controlled by the amount of active SH hydrogenase that is present, but further work would be required to firmly establish this point. In addition, alterations in carbon flux through the different metabolic pathways operating in the various strains may have an influence as well.

To measure the efficiency of hydrogen production, the amount of glucose consumed at the end point was determined and used to calculate the hydrogen yields, mol H_2 produced / mol glucose consumed, of the different cultures. All strains showed very good hydrogen yields, varying from 1.41 to 2.1 mol H_2 / mol glucose, with strains FTJWDC3 and JWGH1 being the most efficient. Interestingly, since hydrogen yield and total hydrogen production are not always correlated, these were also the strains that produced the greatest amount of hydrogen. The yields observed here are higher than those normally observed with wild type cultures and are as high, or slightly higher, than the theoretical maximum for *E. coli*. These yields are also much higher than those obtained in previous studies where heterologous hydrogen producing pathways were introduced into *E. coli*. In several previous studies, ferredoxin-dependent [Fe-Fe] hydrogenase pathways were introduced along with the enzymes

necessary to reduce ferredoxin with either NADH or NADPH. However, yields were disappointingly low; 0.025 (Kim et al., 2011), 0.04(Cho et al., 2011), 0.05 (Veit et al., 2008) mol H_2 / mol glucose. On the other hand, when a [Fe-Fe] hydrogenase was coupled with metabolism by the expression of a pyruvate: ferredoxin oxidoreductase yields as high as 1.46 (Akhtar et al., 2008) mol H_2 / mol glucose were obtained. Here we have introduced a [Ni-Fe] hydrogenase dependent pathway and shown that it is capable of higher (44% greater than the highest previously reported) yields than the previously characterized [Fe-Fe] hydrogenase dependent pathways. Others have previously reported the heterologous expression of [Ni-Fe] hydrogenases (Sun et al., 2010; Kim et al., 2011; Kim et al., 2012; Wells et al., 2011; Weyman et al., 2011; Zheng et al., 2012), but only in one case [43] was the in vivo hydrogen yield reported. In this report, a cyanobacterial [Ni-Fe] hydrogenase was expressed in an *E. coli* strain which also possessed a native hydrogenase 3. Thus the two hydrogenase activities are confounded and one cannot say how much was due to the introduced hydrogenase, which might very well have had an indirect effect since its expression increased formate flux through Hyd3. Here we have unequivocally shown that heterologous expression of the [Ni-Fe] SH hydrogenase can give upto 2 mol H_2 / mol glucose since we used a strain devoid of native hydrogenase activity.

4. Photofermentation of biodiesel derived crude glycerol to hydrogen

In order to develop active inocula for examining hydrogen production from crude glycerol, we examined growth on two different nitrogen sources, glutamate and ammonium, with two different carbon sources, glycerol and succinate. Glycerol was compared with succinate since succinate is the most common substrate used with *R. palustris* which is known to actively grow with this substrate. Therefore, the effects of different carbon, succinate

(4 mM) and crude glycerol (10 mM), and nitrogen, (4 mM) and ammonium sulphate (4 mM), sources on bacterial growth were assessed. Although glutamate is often used at higher concentrations, an intermediate concentration of 4 mM glutamate was chosen since this concentration gave a good balance between hydrogen and biomass production (not shown). Combinations of succinate with ammonium sulphate and crude glycerol with glutamate appeared to be optimal for bacterial growth. With final optical densities well above 1.0 absorbance units (1.41 and 1.32 respectively at 600 nm) they clearly exceeded the optical densities of other combinations. Moreover, it is interesting to note that cultures growing on succinate and ammonium sulphate grew the fastest, achieving an optical density greater than 1.0 by the second day. Given these results, inocula were subsequently prepared by first obtaining active cultures on succinate/ammonium medium before several transfers in glycerol/glutamate prior to the experimental run.

Cultures acclimated to crude glycerol and glutamate were used to assess the effects of different crude glycerol concentrations on hydrogen production. A wide range of concentrations (20 mM–160 mM) was selected to observe the effect of varying concentrations of the crude glycerol fraction on the rate of hydrogen production, and to establish if there was a threshold concentration above which hydrogen production was inhibited by the presence of toxic substances in the crude glycerol fraction. Total cumulative H_2 production and overall rates of H_2 production were highest at an initial concentration of 80 mM. At 160 mM glycerol there was a significant drop in hydrogen production, possibly due an increased concentration of inhibitors.

Thus, there appears to be a balance between the ability of crude glycerol to drive hydrogen evolution and possible inhibition due to impurities. These impurities are

predominantly methanol, salts (in the form of potassium chloride or sulfate), and non-transesterified fatty acids. Salt and methanol were found to have no negative effects on the growth and metabolism of the bacteria on glycerol. The fatty acid with a higher degree of unsaturation, linoleic acid, was found to have strong inhibitory effect on the utilization of glycerol by the bacteria. Some inhibition was also noted at concentrations between 20 and 40 mM, suggesting that there might be several levels of inhibition: a threshold at low concentrations, between 20 and 40 mM crude glycerol, and another at high concentrations between 80 mM and 160 mM raw glycerol. A possible explanation for this phenomenon is that there is a balance between the ability of crude glycerol to drive hydrogen evolution and possible inhibition due to impurities at certain concentrations of crude glycerol. Full understanding of this complex response would require further study. For example, it might be instructive to measure the actual light intensity within the culture which is a complex function of cell biomass, initial glycerol concentration and incident light intensity. At any rate, examination of glycerol consumption showed a direct relationship between glycerol consumption and hydrogen productivity. Cultures with good productivity, 20 mM and 80 mM were also active in glycerol consumption, consuming the majority of added glycerol (90% and 57% respectively), whereas cultures exhibiting poor hydrogen productivity also were weak glycerol consumers, with 20% or less of added glycerol being consumed.

Different processes could be used to convert glycerol to hydrogen, including in addition to photofermentation, studied here, fermentation, electrohydrogenesis, steam reforming and auto reforming. In all cases the goal should be to derive as much hydrogen as possible from the substrate with minimal production of side products. Thus, the ultimate

objective is the complete chemical conversion of glycerol to hydrogen, as given in the equation below:

$$C_3H_8O_3 + 3H_2O \rightarrow 3CO_2 + 7H_2$$

The yields obtained at different initial crude glycerol concentrations were calculated on the basis of the glycerol consumed, as determined by spectrophotometric analysis. Glutamate was assumed to not contribute to hydrogen production since separate experiments showed that cultures incubated in media containing 4 mM glutamate as sole carbon (and nitrogen) source did not produce any hydrogen, an effect that has been well established over the last three decades (Hillmer et al., 1977). On this basis it could be determined that hydrogen yields decrease in inverse proportion to the initial concentration of crude glycerol. A maximum yield of 6.1 H_2/crude glycerol (mol/mol), or 87% of the theoretical maximum value was obtained with an initial concentration of 20 mM, the lowest concentration studied.

This is significantly higher than previously reported values from any of the possible conversion processes. The yields of standard dark fermentation metabolisms appear to be limited to 1–2 mol H_2/mole glycerol at most, a yield much lower than that obtained here, and with the consequent production (Sharma et al., 2011; Kivisto et al., 2011; Wu et al., 2011; Markov et al., 2011; Liu et al., 2007; Sakai et al., 2007; Ngo et al., 2011) of large amounts of side products. Electrohydrogenesis is a biological route that can, in principal, achieve higher yields than a simple fermentation. However, the one study that has been done with this method obtained a yield of 3.9 (Selembo et al., 2009), only 56% of the theoretical maximum and 64% of what we have found here with photofermentation. Steam reforming of crude glycerol, another possible process, requires high temperatures, 300–850 °C and a catalyst, sometimes

made from noble metals. Although relatively high yields can be obtained, 65–75%, production is short lived due to severe coking, and, more importantly, significant quantities of CO are produced which would therefore require that the hydrogen stream be highly purified to prevent fuel cell poisoning (Adhikari et al., 2008) and (Douette et al., 2007). Auto thermal reforming might be another option, but the required addition of O_2 would lead to increased production of CO with a consequent decrease in H_2 yields.

The present study has shown that a crude glycerol fraction from biodiesel manufacture can be converted to hydrogen at near stoichiometric yields. However, development of a practical process based on this photofermentative capacity of photosynthetic bacteria would require a number of changes and improvements. The crude glycerol fraction is relatively poor in nitrogen. The addition of glutamate, used in the present study, would be impractical, and a low-cost substitute would have to be used, possibly in conjunction with a strain that had been modified such that its nitrogenase, the enzymatic system that produces hydrogen in the photofermentation process, was no longer subject to ammonia inhibition.

5. Response surface methodology for process parameter optimization upon increasing hydrogen yield from crude glycerol

R. palustris produces hydrogen from glycerol in a nitrogenase dependent reaction. Therefore, greater hydrogen yields could possibly be obtained through bioprocess optimization of nitrogenase activity. A number of variables could possibly restrict effective nitrogenase activity and thus hydrogen yields. Assuming an adequate medium formulation, the most important key parameters are probably glycerol concentration, glutamate concentration and light intensity. Light intensity is one of the most important factors influencing hydrogen production due to its effects on nitrogenase protein expression and on supporting its activity

through the supply of high energy electrons and ATP (Jouanneau et al., 1985). Light intensity will also have an effect on overall growth kinetics, including the duration of the lag phase and doubling times (Fang et al., 2005).

The initial C:N ratio in the production medium also plays an important role in determining the yield and rate of hydrogen production. Low initial C:N ratios result in low rates of photofermentation and lower hydrogen yields. On the other hand, total fermentation times increase with higher initial C:N ratios. Of course, the C:N balance can have several direct effects on nitrogenase, affecting both its synthesis and, through the presence of excess NH_4^+ ions, its activity. Thus, various amino acids which have only minor inhibitory effects on nitrogenase expression are often used in media formulations for photofermentation in combination with different carbon sources (Gabrielyan et al., 2010). Glutamate usually is chosen as the most suitable nitrogen source to support photofermentative hydrogen production. Thus, optimization of both initial glycerol and glutamate concentrations should increase molar hydrogen yields as well as nitrogenase activity. A factorial design approach is appropriate in this case due to potential interactions between the independent variables. This should be especially apparent with the key process parameters, initial glycerol and glutamate concentrations since, as described above, they will interact by establishing a particular C:N. To overcome this problem, a full or fractional factorial design coupled with RSM (response surface methodology) can be used to advantage (Wang and Wan, 2009). Here, using the *R. palustris* (CGA009), we have determined the optimal levels of these key factors and the effect of their interactions on molar hydrogen yields and nitrogenase activity using RSM with a Box–Behnken design. The various ANOVA parameters; model F, coefficient of variation,

squared regression, etc. all indicated a good fit of experimental values for both hydrogen yield and nitrogenase activity.

Two and three dimensional contour plots of the variation of hydrogen yield with light intensity and glycerol concentration; glycerol and glutamate concentration; and light intensity and glutamate concentration, were elliptical and had clear elongated diagonals indicating significant interactive effects on hydrogen yield (Y1) between the three independent variables. The two and three dimensional contour plots concerning of the variation of nitrogenase activity with light intensity and glycerol concentration), had an elliptical nature and a clear elongated running diagonal, indicating a significant interactive effect on nitrogenase activity (Y2) between the two independent variables. On the other hand, two and three dimensional contour plots representing the variation of nitrogenase activity with glutamate concentration and light intensity; glycerol concentration and glutamate concentration, clearly have saddle points indicating that interactions between light intensity and glutamate concentration, and glutamate concentration and glycerol concentration, have a less direct influence on nitrogenase activity. Saddle points upon RSM plots represent stationary points that are points of zero gradients, with positive curvature on one side and negative curvature on the other side. In this case they indicate a relative lack of interaction of either light intensity and glutamate concentration, or glutamate concentration and glycerol concentration on nitrogenase activity. Thus, under the conditions used here, light intensity and glycerol concentration are the most important variables in terms of optimizing nitrogenase activity.

The residual plots of the model were randomly distributed without any trends (not shown). This indicates good prediction of maximum response along with constant variance and adequacy of the quadratic models. As shown in experimental results, within the design

boundary each response surface plot had a clear peak and the corresponding contour plot had a clear maximum, demonstrating that maximum hydrogen yield and nitrogenase activity could be achieved inside the design boundaries. Moreover, the predicted hydrogen yield of 6.35 mol H_2/mol glycerol is higher than the previously reported yield of 6.1 mol H_2/mol glycerol (Ghosh et al., 2011). Validation experiments were conducted *R. palustris* in duplicate to experimentally confirm the predicted optimum. The mean hydrogen yield was 6.69 mol H_2/mol crude glycerol, very close to the predicted value of 6.35 mol H_2/mol crude glycerol. As well, the mean nitrogenase activity was 228 nmol of C_2H_4/ml/min, very close to the predicted value of 205 nmol of C_2H_4/ml/min. These results therefore show that response surface optimization can be successfully applied to obtain maximum hydrogen yields and nitrogenase activity.

Finally, nitrogenase protein expression at the 10th day of incubation was assessed for each experimental run. Samples from the different cultures were directly submitted to SDS–PAGE electrophoresis and Western blotting with anitsera to either the nitrogenase Fe-protein or the Mo–Fe protein followed by densitometric analysis. The results clearly show that nitrogenase Fe-protein expression was nearly the same (the difference between the lowest and highest amounts was ~30%) under all the different process values represented by the experimental sets. Very similar results were obtained for Mo–Fe protein expression (not shown). However, there was much greater variation in nitrogenase activity with bioprocess parameters. This would suggest that the limiting factor for nitrogenase activity, and therefore presumably hydrogen production, is not the quantity of enzyme present, but how effectively it is supplied with high energy electrons and/or ATP. This is in agreement with previous studies that have suggested that under some conditions nitrogenase enzyme is in excess and therefore

that total activity reflects the rate of nitrogenase reduction rather than the quantity of enzyme present (Hallenbeck et al., 1998 and Yakunin et al., 1999). This merits future study as it indicates that further increases in production could possibly be achieved through maximization of these factors.

6. Optimization of the Hydrogen Yield from Single-Stage Photofermentation of Glucose by *Rhodobacter capsulatus* JP91 with emphasis on statistical analysis

Modeling of complex biological systems requires a set of assumptions and simplification at the outset, which can be provided by an empirical statistical model. As applied to the area of research of the present study, a Box Behnken model could provide information on both hydrogen yield horizons as well as suggest how the regulation of existing and inserted metabolic pathways needs to be altered in order to obtain the maximum hydrogen yield. In this design the treatment combinations are at the center and the midpoints of edges of the process space, giving a geometry which suggests a sphere within the process space. Here this method was used to determine the optimal values of three important process variables in maximizing hydrogen yield. Thus, hydrogen yield was related to simultaneous changes in light intensity (100, 175 and 250 W/m^2), glucose concentration (20, 35 and 50 mM) and the glutamate concentration (1.0, 4.5 and 8.0 mM) of the culture medium. The values chosen were based on a number of factors, including preliminary results (not shown), previous work on single-stage photofermentation of glucose (Abo-Hashesh et al., 2011), a study on the RSM optimization of the photofermentative production of hydrogen from glycerol (Ghosh et al. 2012), and previous studies on photofermentation of sugars (Jung-Yeol et al., 2012, Androga et al., 2011, Akroum-Amrouche et al., 2011, Keskin et al., 2011 and Keskin and Hallenbeck, 2012).

R. capsulatus JP91 produces hydrogen from glucose in a nitrogenase-dependent reaction. Therefore it is very likely that greater hydrogen yields can be achieved by maximizing nitrogenase activity through bioprocess optimization. A number of variables could possibly restrict effective nitrogenase activity and thus hydrogen yields; however, assuming an adequate medium formulation, the most important key parameters are probably glucose concentration, glutamate concentration and light intensity. First of all, light intensity is one of the most important factors in hydrogen production due to its potential effects on nitrogenase enzyme regulation and flux through the electron transport chain (Jouanneau et al., 1985). In turn, light intensity could potentially modulate end product distribution, as well as the duration of the lag phase and growth kinetics.

The initial ratio of carbon to nitrogen in the production medium also plays an important role. A low initial carbon to nitrogen ratio results in lower rates of fermentation and lower hydrogen yields, whereas total fermentation times increase with a higher initial carbon to nitrogen ratio. In hydrogen production by photofermentation, this effect is mainly due to the influence of the carbon to nitrogen ratio on nitrogenase synthesis with nitrogenase repression occurring when there is excessive fixed nitrogen in the medium. However, since hydrogen production by nitrogenase is mainly observed in actively growing cells, fixed nitrogen must be supplied to support cell growth. On way around this problem experimentally is to supply fixed nitrogen in the form of an amino acid, many of which have only minor effects on nitrogenase repression when supplied at moderate concentrations. Glutamate has been found to be the most suitable amino acid for photofermentative hydrogen production (Gabrielyan et al., 2010). In the present study, glutamate was used in combination with glucose. Statistical analysis, such as analysis of variance (ANOVA), is essential to test significance and adequacy of the

model. It has been shown that the model is adequately significant for both hydrogen yield and nitrogenase activity.

The two- and three-dimensional contour plots of nitrogenase activity versus light intensity and glucose concentration, light intensity and glutamate concentration, glucose concentration and glutamate concentration, were elliptical and had elongated running diagonals, indicating a significant interactive effect on nitrogenase activity (Y2) between the three independent variables. The relative amount of interaction can be judged by the degree of ellipticity. Thus, the greatest interactive effects on nitrogenase activity were seen for light intensity and glucose concentration whereas the least interaction was seen for glucose and glutamate concentrations. Therefore, in general, light intensity had the greatest influence on the effects of glucose or glutamate concentrations on nitrogenase activity.

The residual plots of the model were randomly distributed without any trends (not shown), indicating the adequacy of the quadratic model in predicting the maximum response. In general, the reproducibility was very good, although some replicate-to-replicate variation can be noted, for example when comparing run 13 with runs 14 and 15, carried out under the same conditions. The reason for this variation is not known, perhaps a difference in inocula or other factors that are hard to control. Nonetheless, these three biological replicates gave a mean of 222 (±43) nmol C_2H_4/ml/min, so the low value in run 13 is only 19% less than the mean and well above the values obtained with the other runs under sub optimal conditions. Each response surface plot had a clear peak and the corresponding contour plot had a clear maximum, which means that the maximum response (nitrogenase activity) could be achieved inside the design boundaries. The mean nitrogenase activity, determined by validation experiments (performed in triplicate) carried out under the determined optimal conditions, was

246 (±3.5) nmol C_2H_4/ml/min, very close to the predicted value of 222 (±43) nmol C_2H_4/ml/min.

The two- and three-dimensional contour plots presenting hydrogen yield versus light intensity and glucose concentration, glucose and glutamate concentration, light intensity and glutamate concentration were elliptical with elongated diagonals indicating a significant interactive effect between the independent variables and hydrogen yields (Y1). As noted for effects on nitrogenase activity, the strength of the interaction between the variables can be judged by the degree of ellipticity. Interaction between the variables appears stronger in the case of hydrogen yields than for nitrogenase activity. It is noteworthy that the interactive effects between glucose and glutamate were much stronger when hydrogen yields were examined than when nitrogenase activity was examined. This suggests that hydrogen yields may not be directly proportional to nitrogenase activity, a point that is further discussed below.

Residual plots of the model were randomly distributed without any trends (not shown), again indicating the predictive power of the quadratic models. As was seen for nitrogenase activity, the surface plots for hydrogen yield had clear peaks and the corresponding contour plots had clear maxima, indicating that maximum hydrogen yield could be achieved inside the design boundaries. The hydrogen yield predicted by the model, 5.29 mol H_2/mol glucose is higher than the previously reported molar hydrogen yield of 3 mol H_2/mol glucose (Abo-Hashesh et al., 2011) and higher than the vast majority of previous studies which examined single-stage fermentation of glucose. Validation experiments (carried out in triplicate) were conducted to confirm the predicted optimal conditions, and gave a mean hydrogen yield of 5.5 (+0.15) mol H_2/mol glucose, very close to the predicted value (5.29 mol H_2/mol glucose).

From a practical point of view, volumetric productivity is also an important process parameter. Under the optimal conditions, volumetric hydrogen productivity in this study was high (1.4 l/l/day), substantially greater than previously found for a two-stage sequential system (0.27 and 0.63 l/l/day) capable of achieving similar yields (Özgür et al., 2010b), and higher than what is typically found for hydrogen production from organic acids by photosynthetic bacteria (0.34 l/l/day (Akroum-Amrouche et al., 2011).

Although in special cases where immobilized cultures are used, dark fermentative volumetric productivities were much higher (Hallenbeck and Ghosh, 2009), in general, the rates observed in the present study compare favorably with those obtained in a variety of studies of dark fermentative hydrogen production with non-immobilized cultures (Hawkes et al., 2007 and Hallenbeck and Ghosh, 2009).

Densitometric analysis of nitrogenase protein expression was performed on the sixth day of incubation for all combinatorial experimental runs. Since hydrogen production was maximal at day 6, it was chosen as the optimal time point for sampling for nitrogenase activity and the determination of the relative amount of nitrogenase (Fe protein) and its modification status. The results show that nitrogenase Fe-protein expression was highest at the optimal point, perhaps accounting, at least in part, for the maximum nitrogenase activity and hydrogen yield observed under these conditions. However, it should be noted that there was only a maximum of a 2.6-fold variation in the amount of Fe-protein over the range of conditions used, whereas nitrogenase activity and hydrogen yield varied by more than twenty-fold. This strongly suggests that factors other than the total amount of enzyme present are controlling activity and hydrogen production. These factors are probably related to flux restriction of either high-energy electrons or ATP at the level of metabolic supply to nitrogenase, as

previously suggested by studies of the relationship between nitrogenase protein content, nitrogenase activity, and nitrogen supply (Yakunin et al.1999).

Interestingly, Fe-protein was partially ADP-ribosylated under some of the experimental conditions. ADP-ribosylation of Fe-protein is a unique molecular mechanism for controlling nitrogenase activity in many purple non-sulfur photosynthetic bacteria and is related to the NH_4^+-induced "switch off" effect (Masepohl and Hallenbeck, 2010). Depending upon the conditions, this can have the effect of reducing overall nitrogenase activity. Since hydrogen evolution is catalyzed exclusively by nitrogenase during photofermentation, overall hydrogen production and possibly yields might be improved by reducing or eliminating modification of nitrogenase.

However, the relationship between Fe protein modification and the experimental variables studied here, the concentration of glucose and glutamate, and the light intensity, are obscure and require additional study. Further investigation is also required to understand the factors controlling nitrogenase modification during photofermentation of glucose and how this might affect overall hydrogen productivity. Moreover, in the present study, the culture pH remained in the alkaline range, suggesting that the formation of organic acid by products was minimal. However, in this initial study a metabolite analysis was not performed. The logical extension of this research therefore would be to carry out an identification and analysis of metabolic end products to guide metabolic engineering for strain improvement and to more fully understand the possible role of ADP-ribosylation in restricting hydrogen production.

7. Amelioration of single-stage Biohydrogen yields using the metabolically engineered photosynthetic bacterium *Rhodobacter capsulatus* DG9

In this study we have shown that the *R. capsulatus* DG9 metabolically engineered strain (*amtB*-, *hup*-) shows promise for the development of a single stage photofermentation process for converting glucose, and possibly other sugars, to hydrogen. Maximum yields, 8.2 mol of H_2 per mole of glucose were obtained in batch operative mode. Fermentative studies showed that maximum yields, 8.2 mol of H_2 per mole of glucose in batch operative mode were obtained without any ADP-ribosylation phenomenon on Fe-protein of nitrogenase enzyme system; which indirectly recovers the NH_4^+ switch-off mechanism to provide higher hydrogen yield in batch operation. Moreover the preliminary experiments revealed that the newly developed metabolically engineered strain *Rhodobacter capsulatus* DG9 has a longer lag time period to start the rapid hydrogen production in comparison to *Rhodobacter capsulatus* JP91. The probable reason might be that an indirect huge mutation load has been generated on amino acid metabolism which in turns slows bacterial growth (data not shown; dataset under final calculation steps) and hydrogen production in metabolically engineered strain *Rhodobacter capsulatus* DG9. But it is interesting to note that the cumulative hydrogen production was elevated so quickly after the 3rd day and continued until late stationary phase. Whereas *R. capsulatus* JP91 reached stationary phase earlier and stopped producing hydrogen due to NH_4^+ switch off phenomenon on the Fe protein nitrogenase enzyme system. In this current scenario, two intermediately involved pathways related to GlnK and DraG protein expression are in investigation for revealing the relationship between photofermentative hydrogen production and NH_4^+ switch-on-off mechanism.

CONCLUSIONS AND PERSPECTIVES

E. coli DJT135, a metabolically engineered strain bearing knockout mutations in the uptake hydrogenases (*Δhya, Δhya*) and lactate dehydrogenase (*ldhA*), and that constitutively expresses *fhlA*, which encodes the positive regulator of the formate hydrogen lyase (FHL) were examined on yields of hydrogen from different C-6 and C-5 substrates. Here we have demonstrated fermentative hydrogen production from a variety of sugars and sugar derivatives by *E. coli* DJT135 in a batch bioreactor. The maximum molar hydrogen yield with glucose at the optimal conditions of pH 6.5 and 35°C was 1.51 mol H_2 mol^{-1}. As well, the potential of this organism for using cheap raw materials, in particular hydrolysates derived from lignocellulosic materials, was explored by examining the yields obtained from arabinose and xylose. Good yields of hydrogen, the highest yet reported for a pure mesophilic culture, were obtained from these substrates. In addition, this metabolically engineered organism was shown to be capable of significant hydrogen production from a variety of other carbon sources including sorbitol, fructose, trehalose, maltose, lactose, galactose, and sucrose. These observations indicate the potential of suitably modified *E. coli* strains to use various types of carbohydrates present in different wastes for biohydrogen production while at the same time carrying out effective waste treatment.

As a continuation, the optimization of key process parameters for the maximizing the hydrogen yield using statistical methodology were emphasized using *E.coli* DJT135. Experimental results showed that glucose concentration, temperature and pH all had significant influences on the hydrogen yield. Glucose concentration and pH, glucose concentration and temperature were interdependent and had a significant interactive effect on the hydrogen yield. On the other hand, the interactive effect of temperature and pH was

insignificant. Accurate prediction of the maximum value of the experimental response and the constant variance of the residuals indicated that the quadratic model adequately described the response surface within the experimental region. The maximum hydrogen yield of 1.69 mol H_2 mol^{-1} was obtained under the optimal conditions of 75 mM glucose concentration, 35°C and pH 6.5. Finally, RSM was shown to be useful optimizing the hydrogen yield and thus improving the bioprocess for hydrogen production by metabolically engineered strain *E. coli* DJT135.

Fermentative hydrogen production is an attractive means for the sustainable production of this future energy carrier but is hampered by low yields. One possible solution is to create, using metabolic engineering, strains which can bypass the normal metabolic limits to substrate conversion to hydrogen. *E. coli* can degrade a variety of sugars to hydrogen but can only convert electrons available at the pyruvate node to hydrogen, and is unable to use the electrons available in NADH generated during glycolysis. To this end, metabolic engineering was applied to surpass the molar hydrogen yield in *E.coli* by knocking out the naive metabolic pathways and construction of novel metabolic pathway. The work reported here shows convincingly for the first time that a pyridine nucleotide dependent [Ni-Fe] hydrogenase can be heterologously expressed in *E. coli* and produce appreciable amounts of hydrogen from NAD(P)H produced by cellular metabolism. This represents a significant advance in the ability to engineer hydrogen producing pathways in *E. coli*. Moving forward, a number of improvements could be made. Increasing flux through the system would be required to increase the rates of hydrogen production. In addition, a practical hydrogen production system would require that greater yields be obtained from the substrate, which could be brought about in several different ways. For one thing, more efficient coupling with the native hydrogen

producing system, which produces hydrogen indirectly from pyruvate through the pyruvate: formate lyase system should further increase yields. Another possibility would be to introduce a mechanism whereby additional NADH could be generated through the further metabolism of pyruvate, for example, through the anaerobic functioning of the citric acid cycle.

On the other hand, near stoichiometric reforming of biodiesel derived crude glycerol to hydrogen by photofermentation was carried out by using *Rhodopseudomonas palustris*. In this present study, we achieved a maximum yield of 6.1 mol of hydrogen per mole of crude glycerol, which is equivalent to 87% of the theoretical maximum, significantly higher than the previously reported value. Experimentally, it has been shown that the initial concentration of crude glycerol has a profound effect on both the cumulative production and yield of hydrogen. It has also been shown that glutamate is a better source of nitrogen than ammonium sulphate for hydrogen production, quite possibly due to inhibitory effects of ammonium on nitrogenase. Even though we have shown that it is possible to achieve high yield conversions, there are still a number of limiting factors that must be overcome to increase the yields and rates of hydrogen production. These limitations include low light conversion efficiencies, and the need to develop low-cost, hydrogen impermeable photo bioreactors. Moreover, response surface methodology with a Box–Behnken design was successfully used to improve the photofermentative molar hydrogen yield from crude glycerol by identifying the optimal conditions. The three independent variables studied; crude glycerol concentration, glutamate concentration, and light intensity all had significant interactive effects on hydrogen yield and nitrogenase activity with a maximum hydrogen yield (6.69 mol H_2/mol crude glycerol) at the optimal point (30 mM glycerol, 4.5 mM glutamate, 175 W/m^2). Thus, RSM was able to define conditions allowing the essentially stoichiometric (7 mol H_2/mol glycerol) conversion of waste

glycerol generated during biodiesel manufacture to hydrogen by photofermentation with *R. palustris*.

Single-Stage photofermentation of glucose by *Rhodobacter capsulatus* JP91 was also examined for its better hydrogen productivity through response surface methodology approach. Response surface methodology using a Box–Behnken design was used to optimize substrate (glucose), fixed nitrogen, and light intensity to maximize the hydrogen yield during the single stage photofermentation of glucose by the photosynthetic bacterium *R. capsulatus*. The three independent variables studied, glucose, glutamate, and light intensity all had significant interactive effects on hydrogen yield and nitrogenase activity. As well, levels of nitrogenase expression were also shown to be influenced by different combinations of these process variables. The optimized yield obtained through RSM is 85% higher than that previously achieved and is similar to what was previously shown for two-stage systems. Thus, single stage photofermentation of glucose is promising. Further improvements might be achieved through metabolic engineering.

However, it should be noted that there was only a maximum of a 2.6 fold variation in the amount of Fe-protein over the range of conditions used, whereas nitrogenase activity and hydrogen yield varied by more than twenty-fold. This strongly suggests that factors other than the total amount of enzyme present are controlling activity and hydrogen production. These factors are probably related to flux restriction of either high-energy electrons or ATP at the level of metabolic supply to nitrogenase, as previously suggested by studies of the relationship between nitrogenase protein content, nitrogenase activity, and nitrogen supply (Yakunin et al., 1999). Interestingly, Fe-protein was partially ADP-ribosylated under some of the experimental conditions. ADP-ribosylation of Fe-protein is a unique molecular mechanism for controlling

nitrogenase activity in many purple non-sulfur photosynthetic bacteria and is related to the NH_4^+-induced "switch off" effect (Masepohl and Hallenbeck, 2010). Based on this hypothesis in this present study, an AmtB- derivative of *Rhodobacter capsulatus* JP91 (*hup*-) was created by bacterial conjugation; *R. capsulatus* DG9 *(amtB-, hup-)*.

In preliminary trials it has been shown that *R. capsulatus* DG9 metabolically engineered strain (*amtB*-, *hup*-) shows promise for the development of a single stage photofermentation process for converting glucose, and possibly other sugars, to hydrogen. Maximum yields, 8.2 mol of H_2 per mole of glucose in batch operative mode were obtained. It was also shown this newly developed engineered strain does not have any ADP-ribosylation phenomenon on Fe-protein of nitrogenase enzyme system; which indirectly recovers the NH_4^+ switch-off mechanism to provide higher hydrogen yield in batch operation. *R. capsulatus* JP91 had been shown to produce a hydrogen yield of 10.3 molH_2/ mol glucose in continuous mode (Abo-Hashesh et al., 2013) in a previous study. To this end the future direction will be improving hydrogen yield using this metabolically engineered strain *R. capsulatus* DG9 to surpass the stoichiometric yield of 12 mol H_2/ mol glucose followed by continuous bioprocess development. Hydrogen profile, nitrogenase activity and ADP-ribosylation of Fe-protein are also underway of calculation on different amino acid as nitrogen source. Moreover it could also be done by revealing the clear mechanism of hydrogen yield and nitrogenase regulation with NH_4^+ switch-off mechanism considering expression studies on GlnK and DraG protein expression upon different nitrogen regimes.

In overall, the project looks very impressive and has potential feasibility towards scale up studies for following possibilities. The first segment of the project has clearly shown that a combined approach of metabolic engineering and response surface methodology is a potential driving force towards commercialization. In addition, the huge metabolic pathway alterations have drastically affected the normal growth kinetics of metabolically engineered *E. coli* strains towards biohydrogen production (stochiometric maxima 2 mol H_2/mol glucose equivalent). Even though metabolically engineered *E. coli* strains produce hydrogen through reduced equivalent (NADH) they still suffer from a major bottleneck of growth retardation which indirectly may partially limit the scale up studies. Future studies on engineered *E. coli* strains could be done to study the different growth factors that can accelerate the growth kinetics by lowering the long lag phase to way forward. To this end another microbial platform (photosynthetic bacteria) has been applied having higher stochiometric maxima (12 mol H_2/mol glucose equivalent). This higher stoichiometric maximum could be economically more viable in terms of generation of more biohydrogen, higher substrate and light conversion efficiency. To conceptualize this approach response surface methodology has been first applied to determine the statistically designed central optimum point with special emphasis on the interactive effect on process variables for improving hydrogen yield considering carbon source, organic nitrogen source and light intensity. This interactive effect has helped indirectly in deducing a regression equation which is the backbone of large scale biohydrogen production towards industrialization. Finally, a metabolic engineering approach has been applied on photosynthetic bacteria to further improve the biohydrogen yield at previously attained central optimized point. Therefore, this dual approach has successfully allowed us to achieve higher biohydrogen yields which are almost near to the stochiometric yield. As far as

industrialization the next probable approach would be to follow a continuous mode of operation to attain the steady state kinetics for consistent biohydrogen production rate with higher biohydrogen yields. Instead of these major advantages, photosynthetic bacteria have several limitations : oxygen inactivation of nitrogenase activity (responsible for biohydrogen production), energy intensive processes (require light as energy source), and a narrow range of substrate specificity. In the future, future experiments should be done to engineer photosynthetic microorganisms towards broad substrate regimes and to design novel bioreactors for improvising the feasibility of photo fermentative hydrogen production technology for industrialization.

As far industrial application, large scale production of biohydrogen gas can be utilized for conversion of heavy petroleum fractions into lighter ones via hydrocracking and aromatization. It may be used for production of ammonia which is a major component of most fertilizers. Biohydrogen may be used in fuel cells for local electricity generation, making it possible for biohydrogen to be used as a transportation fuel for electric vehicles. Although requiring expensive technologies, biohydrogen can be cooled, compressed and purified for use in other processes on site by establishing biohydrogen gas stations or sold to a customer via pipeline, cylinders or trucks.

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