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Improvements in Fermentative Hydrogen Production through Physiological Manipulation and Metabolic Engineering

par

Mona Talaat Aly Abo-Hashesh

Département de Microbiologie et Immunologie
Faculté de Médecine

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présentée par:

Mona-Talaat Aly Abo-Hashesh

a été évaluée par un jury composé des personnes suivantes

Dr. George Szatmari, Ph.D.
President-rapporteur

Dr. Patrick C. Hallenbeck, Ph.D.
Directeur de recherche

Dr. France Daigle, Ph.D.
Membre du jury

Dr. Éric Déziel, Ph.D.
Examinateur externe

Dr. Christine Des Rosiers, Ph.D.
Représentant du doyen

RÉSUMÉ

La production biologique d'hydrogène (H_2) représente une technologie possible pour la production à grande échelle durable de H_2 nécessaire pour l'économie future de l'hydrogène. Cependant, l'obstacle majeur à l'élaboration d'un processus pratique a été la faiblesse des rendements qui sont obtenus, généralement autour de 25%, bien en sous des rendements pouvant être atteints pour la production de biocarburants à partir d'autres processus. L'objectif de cette thèse était de tenter d'améliorer la production d'H_2 par la manipulation physiologique et le génie métabolique.

Une hypothèse qui a été étudiée était que la production d'H_2 pourrait être améliorée et rendue plus économique en utilisant un procédé de fermentation microaérobie sombre car cela pourrait fournir la puissance supplémentaire nécessaire pour une conversion plus complète du substrat et donc une production plus grande d'H_2 sans l'aide de l'énergie lumineuse. Les concentrations optimales d'O_2 pour la production de H_2 microaérobie ont été examinées ainsi que l'impact des sources de carbone et d'azote sur le processus. La recherche présentée ici a démontré la capacité de *Rhodobacter capsulatus* JP91 *hup*$^-$ (un mutant déficient d'absorption-hydrogénase) de produire de l'H_2 sous condition microaérobie sombre avec une limitation dans des quantités d'O_2 et d'azote fixé. D'autres travaux devraient être entrepris pour augmenter les rendements d'H_2 en utilisant cette technologie.

De plus, un processus de photofermentation a été créé pour améliorer le rendement d'H_2 à partir du glucose à l'aide de *R. capsulatus* JP91 *hup*$^-$ soit en mode non renouvelé (batch) et / ou en conditions de culture en continu. Certains défis techniques ont été surmontés en mettant en place des conditions adéquates de fonctionnement pour un rendement accru d'H_2. Un rendement maximal de 3,3 mols de H_2/ mol de glucose a été trouvé pour les cultures en batch tandis que pour les cultures en continu, il était de 10,3 mols H_2/ mol de glucose, beaucoup plus élevé que celui rapporté antérieurement et proche de la valeur maximale théorique de 12 mols

H_2/ mol de glucose. Dans les cultures en batch l'efficacité maximale de conversion d'énergie lumineuse était de 0,7% alors qu'elle était de 1,34% dans les cultures en continu avec un rendement de conversion maximum de la valeur de chauffage du glucose de 91,14%. Diverses autres approches pour l'augmentation des rendements des processus de photofermentation sont proposées. Les résultats globaux indiquent qu'un processus photofermentatif efficace de production d'H_2 à partir du glucose en une seule étape avec des cultures en continu dans des photobioréacteurs pourrait être développé ce qui serait un processus beaucoup plus prometteur que les processus en deux étapes ou avec les co-cultures étudiés antérieurement.

En outre, l'expression hétérologue d'hydrogenase a été utilisée comme une stratégie d'ingénierie métabolique afin d'améliorer la production d'H_2 par fermentation. La capacité d'exprimer une hydrogénase d'une espèce avec des gènes de maturation d'une autre espèce a été examinée. Une stratégie a démontré que la protéine HydA orpheline de *R. rubrum* est fonctionnelle et active lorsque co-exprimée chez *Escherichia coli* avec HydE, HydF et HydG provenant d'organisme différent. La co-expression des gènes [FeFe]-hydrogénase structurels et de maturation dans des micro-organismes qui n'ont pas une [FeFe]-hydrogénase indigène peut entraîner le succès dans l'assemblage et la biosynthèse d'hydrogénase active. Toutefois, d'autres facteurs peuvent être nécessaires pour obtenir des rendements considérablement augmentés en protéines ainsi que l'activité spécifique des hydrogénases recombinantes.

Une autre stratégie a consisté à surexprimer une [FeFe]-hydrogénase très active dans une souche hôte de *E. coli*. L'expression d'une hydrogénase qui peut interagir directement avec le NADPH est souhaitable car cela, plutôt que de la ferrédoxine réduite, est naturellement produit par le métabolisme. Toutefois, la maturation de ce type d'hydrogénase chez *E. coli* n'a pas été rapportée auparavant. L'opéron *hnd* (*hndA, B, C, D*) de *Desulfovibrio fructosovorans* code pour une [FeFe]-hydrogénase NADP-dépendante, a été exprimé dans différentes souches d'*E. coli* avec les gènes de maturation *hydE, hydF et hydG* de *Clostridium acetobutylicum*.

L'activité de l'hydrogénase a été détectée *in vitro*, donc une NADP-dépendante [FeFe]-hydrogénase multimérique active a été exprimée avec succès chez *E. coli* pour la première fois. Les recherches futures pourraient conduire à l'expression de cette enzyme chez les souches de *E. coli* qui produisent plus de NADPH, ouvrant la voie à une augmentation des rendements d'hydrogène via la voie des pentoses phosphates.

Mots-clés: Production d'hydrogène, Photofermentation, Microaérobie fermentation sombre, Expression hétérologue des hydrogénases

ABSTRACT

Biological hydrogen (H_2) production represents a possible technology for the large scale sustainable production of H_2 needed for a future hydrogen economy. However, the major obstacle to developing a practical process has been the low yields that are obtained, typically around 25%, well below those achievable for the production of other biofuels from the same feedstock. The goal of this thesis was to improve H_2 production through physiological manipulation and metabolic engineering.

One investigated hypothesis was that H_2 production could be improved and made more economical by using a microaerobic dark fermentation process since this could provide the extra reducing power required for driving substrate conversion to completion and hence more H_2 production might be obtained without using light energy. The optimal O_2 concentrations for microaerobic H_2 production were examined as well as the impact of carbon and nitrogen sources on the process. The research reported here proved the capability of *Rhodobacter capsulatus* JP91 *hup*$^-$ (an uptake-hydrogenase deficient mutant) to produce H_2 under microaerobic dark conditions with limiting amounts of O_2 and fixed nitrogen. Further work should be undertaken to increase H_2 yields using this technology.

In addition, a photofermentation process was established to improve H_2 yield from glucose using *R. capsulatus* JP91 *hup*$^-$ strain either in batch and/or continuous culture conditions. Some technical challenges in establishing the proper operational conditions for increased H_2 yield were overcome. A maximum yield of 3.3 mols of H_2/ mol of glucose was found for batch cultures whereas in continous cultures it was 10.3 mols H_2/ mol glucose, much higher than previously reported and close to the theoretical maximum value of 12 mols H_2/ mol glucose. In batch cultures the maximum light conversion efficiency was 0.7% whereas it was 1.34% in continuous cultures with a maximum conversion efficiency of the heating value of glucose of 91.14%. Various approaches to further increasing yields in photofermentation

processes are proposed. The overall results suggest that an efficient single stage photofermentative H_2 production process from glucose using continuous cultures in photobioreactors could be developed which would be a much more promising alternative process to the previously studied two stage photofermentation or co-culture approaches.

Furthermore, the heterologous expression of hydrogenases was used as a metabolic engineering strategy to improve fermentative H_2 production. The capability of expressing a hydrogenase from one species with the maturation genes from another was examined. One strategy demonstrated that the orphan *hydA* of *R. rubrum* is functional and active when co-expressed in *E. coli* with *hydE, hydF* and *hydG* from different organisms. Co-expression of the [FeFe]-hydrogenase structural and maturation genes in microorganisms that lack a native [FeFe]-hydrogenase can successfully result in the assembly and biosynthesis of active hydrogenases. However, other factors may be required for significantly increased protein yields and hence the specific activity of the recombinant hydrogenases.

Another strategy was to overexpress one of the highly active [FeFe]-hydrogenases in a suitable *E. coli* host strain. Expression of a hydrogenase that can directly interact with NADPH is desirable as this, rather than reduced ferredoxin, is naturally produced by its metabolism. However, the successful maturation of this type of hydrogenase in *E. coli* had not been previously reported. The *Desulfovibrio fructosovorans hnd* operon (*hndA*, *B*, *C*, and *D* genes), encoding a NADP-dependent [FeFe]-hydrogenase, was expressed in various *E. coli* strains with the maturation genes *hydE*, *hydF* and *hydG* of *Clostridium acetobutylicum*. Hydrogenase activities were detected *in vitro*, thus a multi-subunit NADP-dependent [FeFe]-active hydrogenase was successfully expressed and matured in *E. coli* for the first time. Future research could lead to the expression of this hydrogenase in *E. coli* host strains that overproduce NADPH, setting the stage for increased hydrogen yields via the pentose phosphate pathway.

Keywords: Hydrogen production, Photofermentation, Microaerobic dark fermentation, Heterologous expression of hydrogenases

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ABBREVIATIONS

°C	Degrees Celsius
µmol	Micromole
Adh	Alcohol dehydrogenase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
Anti-GMO	Anti-genetically modified organisms
ArcAB	Global regulator
ASKA	A complete set of *E. coli* K12 ORF archive
ATP	Adenosine-5'-triphosphate
BACs	Bacterial artificial chromosomes
BCA	Bicinchoninic acid
bp	Base pair
ca	Approximately
CBB	Calvin-Benson-Bassham
CDW	Cell dry weight
CO_2	Carbon dioxide
CoA	Coenzyme-A
COD	Chemical oxygen demand
CODH	Carbon-monoxide dependent dehydrogenase
Cre	Recombinase enzyme
CSTR	Continuous stirred tank reactor
Cyt c2	Cytochrome *c*2
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
e^-	Electron
Ech	*Escherichia*
EcoCyc	Encyclopedia of *E. coli* metabolism
epPCR	Error-prone polymerase chain reaction
FDH	Formate dehydrogenase

FDHN	Formate dehydrogenase-N
FDHO	Formate dehydrogenase-O
FHL	Formate hydrogen lyase
FhlA	FHL activator protein
FixABC	Membrane protein complex
FLP/FRT	Site-directed recombination system
FNR	Fumarate and nitrate reduction regulator
FRT	Flippase recognition target
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase
GTPase	Guanosine triphosphate hydrolase enzyme
h	Hour
H_2	Hydrogen
H_2ase	Hydrogenase
HRT	Hydraulic retention time
Hup	Uptake hydrogenase
HVCE	Heat value conversion efficiency
HycA	FHL repressor protein
Hyd	Hydrogenase
IHFAB	Global regulator
Int	Integrase
IPTG	Isopropyl-D-thiogalactopyranoside
IscR	Iron-sulfur cluster regulator
kb	kilobase
kDa	kiloDalton
KIO	Knock it off
LCE	Light conversion efficiency
LdhA	Lactate dehydrogenase
Lox	Recombination site
mg	Milligram
mmol	Millimole
mol	Mole

MV	Methyl viologen
N_2ase	Nitrogenase enzyme
NAD^+/H	Nicotinamide adenine dinucleotide (+: oxidized, H: reduced)
$NADP^+$/H	Nicotinamide adenine dinucleotide phosphate (+: oxidized, H: reduced)
NarG	Nitrate reductase 1
NFOR	NADH:ferredoxin oxidoreductase
nmol	Nanomole
O_2	Oxygen
OD	Optical density
OptKnock	Computational framework for identifying gene knockout strategies
OptReg	Computational framework for identifying reaction activation/inhibition or elimination candidates for overproduction in microbial systems
OptStrain	Computational framework for redesign of microbial production systems
Ori	Origin of replication
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
PEP	Phosphoenolpyruvate
PEPC	Phosphoenolpyruvate carboxylase
PFL	Pyruvate formate lyase
PFO	Pyruvate-ferredoxin oxidoreductase
PFOR	Pyruvate ferredoxin oxidoreductase
PHB	Polyhydroxybutyrate
PKS/NRPS	Polyketide synthase/Nonribosomal peptide synthetase
PNS	Purple non-sulphur
PP	Pentose phosphate
PSII	Photosystem II
PVDF	Polyvinyldifluoride
Q	Quinone

QH_2	Quinol
RC	Reaction center
RecA	DNA repair and maintenance protein
RecBCD	Exonuclease V
RNA	Ribonucleic acid
Rnf	RING-type zinc finger protein
RSM	Response surface methodology
Rubisco	Ribulose 1,5-bisphosphate carboxylase
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis
SE	Standard error
Tat	Twin-arginine translocation
TCA	Tricarboxylic acid
TECE	Total energy conversion efficiency
TRMR	Trackable multiplex recombineering
TS	Target substrate
UASB	Up flow anaerobic sludge blanket
VFA	Volatile fatty acid
VSS	Volatile suspended solid
Xis/Int	Site-specific recombination system
λ	Lambda

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 chapter "Abo-Hashesh M., Hallenbeck P.C., 2012. Fermentative hydrogen production in microbial technologies. In: Hallenbeck P.C. (ed.), Advanced biofuels production. Springer. ISBN 978-1-4614-1207-6."

[2]Adapted from my contribution in our published review "Keskin T., Abo-Hashesh M., Hallenbeck P.C., 2011. Photofermentative hydrogen production from wastes. Bioresour. Technol., 102:8557-8568."

[3]Adapted from my contribution in our published review "Abo-Hashesh M., Wang R., and Hallenbeck P.C., 2011. Metabolic engineering in dark fermentative hydrogen production; theory and practice. Bioresour. Technol., 102:8414-8422."

[4]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."

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Mona Abo-Hashesh

INTRODUCTION

1. Renewable energy sources

Fossil fuels represent the most important source of energy supporting current economic activity. However, the world is confronting serious problems with increasing energy demand, rising oil prices and depletion of fossil fuel reserves and, on the other hand, the environmental hazards caused by utilization of those fuels (Figure 1 and 2). Extractable fossil fuel reserves will be depleted sometime in the next fifty years, which, along with their negative impact on the environment, necessitates the development of alternative renewable energy sources that are "eco-friendly" and pose little or no environmental hazards (Abo-Hashesh and Hallenbeck, 2012). Current research is investigating applicable green fuels as alternatives where the feedstock is available, less expensive, and provides clean renewable energy, and helps in bioremediation. Green fuels include those produced by physical means, such as hydroelectricity, solar energy, tidal and wind power, as well as those that depend on biochemical means to produce biofuels (Zhang, 2009). Therefore, biofuels are one example of a renewable energy resource. First generation biofuel production depends on food crops as substrate, for example, biodiesel, bioalcohols, bioethers and biogas. The upgraded biofuels extend to second, third and fourth generation where non food crops are being used as substrate. Biohydrogen as well as biofuels from algae are considered the most recent biofuels (Demirbas, 2009).

2. Biological hydrogen production

Hydrogen gas is one of the sustainable energy resources and is currently being considered as a promising renewable energy carrier. Furthermore, it is widely recognized as a clean and effective energy resource for the future since it has the highest energy content per unit weight (122 kJ/g) in comparison to other known fuels. It is not chemically bound to carbon and produces only water when combusted, thus, hydrogen does not make a contribution to environmental pollution (Ni et al.,

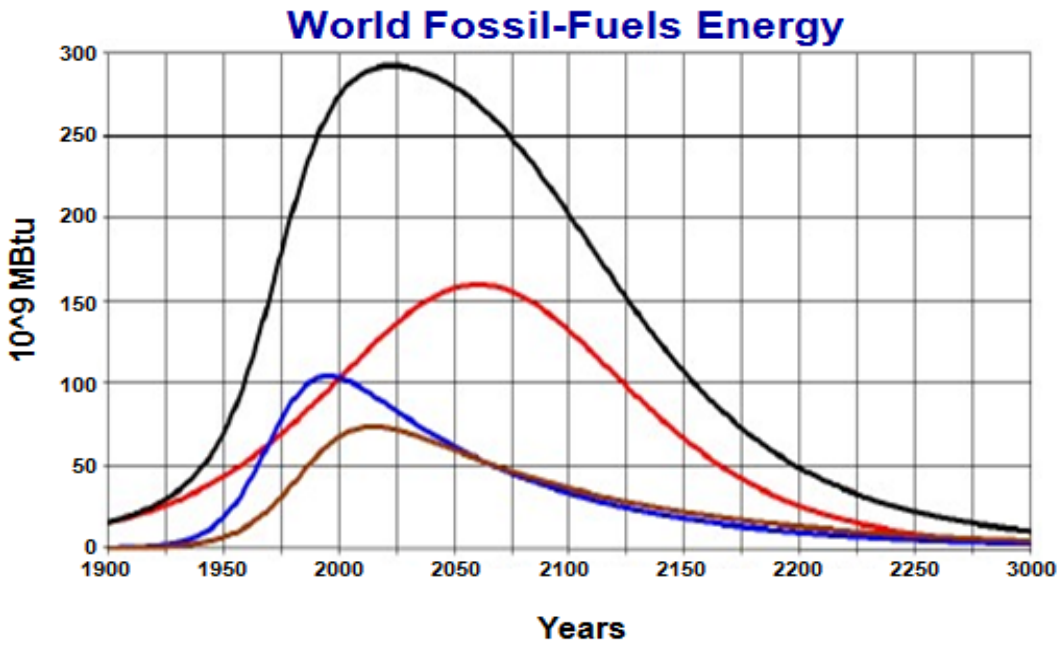

Figure 1. Energy obtained from fossil fuels. Adapted from Roper (2011).

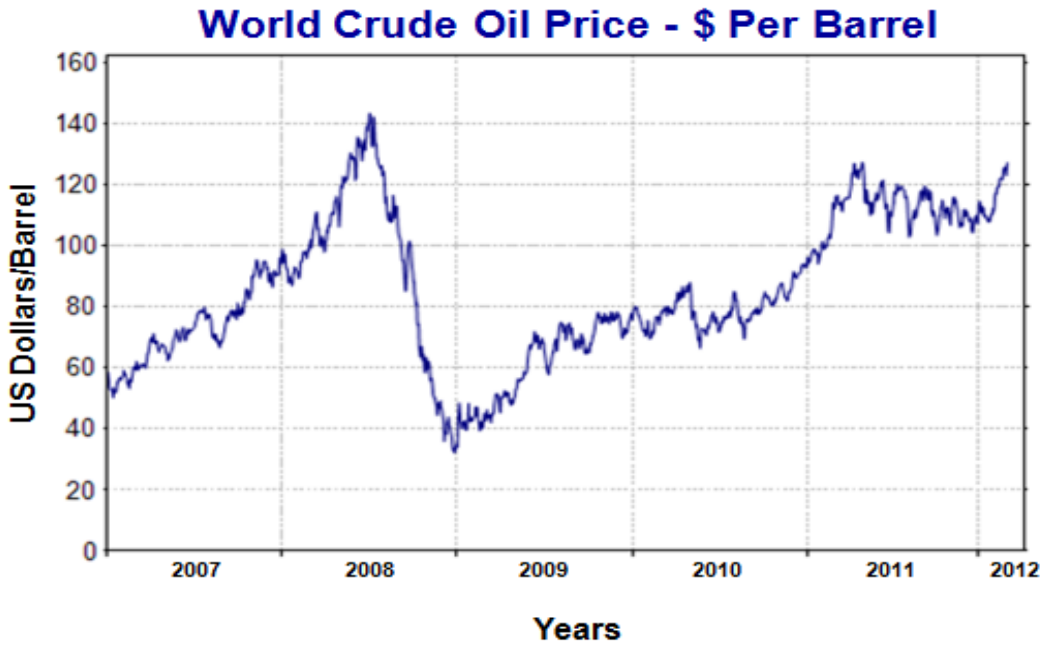

Figure 2. World crude oil prices -$/Barrel. Adapted from Riley (2012).

2005; Hallenbeck et al., 2009). In addition, biohydrogen production can generate renewable energy from wastes thus providing potential waste treatment credits (Hallenbeck, 2011a).

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 (photo-fermentation) of organic compounds using photosynthetic bacteria, dark fermentative hydrogen production using anaerobic (or facultative anaerobic) bacteria and bioelectrohydrogenesis (Hallenbeck et al., 2009; Hallenbeck, 2011a).[1]

2.1 Fermentative hydrogen production

Fermentative 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). However, there are significant technical barriers to the practical realization of these routes, low light conversion efficiencies and the difficulty in constructing low cost photobioreactors (Hawkes et al., 2007; Hallenbeck, 2011a). On the other hand, dark fermentative hydrogen production has the advantages of being simple, commonly requiring mainly anoxic conditions, using a variety of organic wastes as substrate, and giving a high volumetric rate of hydrogen production. Thus, dark fermentative hydrogen production is widely considered as being more feasible than photosynthetic methods. In addition since dark fermentative hydrogen production consumes organic wastes by useful waste treatment is carried out concomitantly with clean energy production. For these

reasons, dark fermentative hydrogen production has gained widespread interest and attention in recent years (Hallenbeck, 2011a,b; Li and Fang, 2007a).[1]

Generally, bacteria ferment various organic substrates mainly carbohydrates, by oxidizing them, generating free energy used to produce ATP, and electrons, which are used to form reduced products (Hallenbeck, 2005; Hallenbeck, 2011a,b). Commonly, volatile fatty acids (VFAs) are produced, but, depending upon the bacterium and the substrate, anaerobic fermentations also lead to the formation of alcohols. These reduced end-products, such as ethanol and butanol, provide one means to regenerate the oxidized electron carriers necessary for continued substrate assimilation and ATP generation. However, as will be seen below, these reduced electron carriers are also needed to drive hydrogen production and therefore, alcohol production results in a correspondingly lower hydrogen yield. In order to maximize the yield of hydrogen, bacterial metabolism must be directed away from alcohols (ethanol and butanol) and reduced acids (Hawkes et al., 2002; Hallenbeck, 2005).[1]

Hydrogen is one of the reduced products made by many different bacteria, and in some ways is an ideal fermentation product since it diffuses away from the producing organism and does not result in medium acidification. Disposal of reducing equivalents generated during fermentation is a major problem in most fermentative bacteria as metabolic redox balance must be achieved for survival. Reducing equivalents can be eliminated via proton reduction, facilitated by hydrogenase and the appropriate electron carriers, leading to the formation of hydrogen. Thus, the activity of hydrogenases, enzymes catalyzing the simplest chemical reaction serves as a means for the disposal of electrons released during the key metabolic oxidations necessary to sustain microbial life in the absence of external electron carriers (Adams and Stiefel, 1998; Hallenbeck and Benemann, 2002).[1]

Several recent reviews exhaustively treat the molecular details of hydrogen producing pathways and how these might be increased using metabolic engineering

(Hallenbeck and Ghosh, 2009; Hallenbeck, 2011a,b; Hallenbeck et al., 2011) and will be reviewed in detail below.

2.2 Photofermentative hydrogen production

Photofermentation is the photosynthetically light-driven fermentative conversion of organic substrates to biohydrogen principally carried out by photosynthetic bacteria. Thus, photofermentation differs from dark fermentation since it requires light, but the use of photosynthetically derived energy permits the use of substrates from which hydrogen cannot be extracted without energy input (organic acids) as well as in principal driving substrate conversion to completion, not thermodynamically possible without energy input. Consequently, light utilization is the key factor in the photofermentation process and developing effective photobioreactors with efficient internal light distribution characteristics is critical, especially for the industrial scale where self-shading inside the fermenter has to be prevented to ensure the efficiency of hydrogen production (Kahn and Durako, 2009).[2]

Photofermentative hydrogen production by purple non-sulfur photosynthetic bacteria was first reported more than sixty years ago (Gest and Kamen, 1949). These alphabacteria appear particularly suited to a photoheterotrophic life style. In this growth mode, they produce energy through photosynthesis, namely generate ATP through the cyclic functioning of their single photosystem. They depend upon reduced fixed carbon compounds, inorganic ions (Fe^{2+}), or hydrogen as source of electrons for their metabolic activities. Although photoheterotrophic growth was through to be primarily carried out by these organisms, a marine cyanobacterium possessing only a single photosystem (it lacks water splitting PSII) has recently been described that leads a photoheterotrophic life in the surface waters of the oceans (Tripp et al., 2010). Thus, this growth mode may be more wide spread than previously thought. Actually, there are several sub-types of purple bacteria; some are sulfur bacteria and non-sulfur, it is the purple non-sulfur bacteria which usually produce hydrogen (Bryant and Frigaard, 2006).[2]

The purple non-sulphur (PNS) bacterial genus *Rhodobacter*, has been the most widely used for biohydrogen production. The PNS can use a broad spectrum of substrates, a versatility that reflects the variety of natural environments in which they are found. All PNS bacteria can grow photo-heterotrophically using reduced carbon compounds as electron donors and carbon source; some species can also grow photo-lithoautotrophically 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 PNS 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).[2]

Many studies have shown that *Rhodobacter* species are highly capable of hydrogen production when they are feeding on organic acids as substrate (Tao et al., 2007). Since they are capable of converting organic acids such as acetic, lactic and butyric to hydrogen (H_2) and carbon dioxide (CO_2) under anaerobic conditions in the presence of light, the organic acids produced during the acidogenic phase of anaerobic digestion of organic wastes can be converted to H_2 and CO_2 by those photosynthetic anaerobic bacteria. Hydrogen gas production capabilities have been reported in some purple photosynthetic bacteria such as *Rhodobacter spheroides* (Koku et al., 2002; Kapdan and Kargi, 2006), *Rhodobacter capsulatus* (He et al., 2005), *Rhodovulum sulfidophilum* W-1S (Maeda et al., 2003) and *Rhodopseudomonas palustris* (Barbosa et al., 2001).[2]

Furthermore, photoproduction of hydrogen has also been reported from CO or other organic acids by carbon-monoxide dependent dehydrogenase (CODH) enzyme containing cultures such as *Rhodospirillum rubrum* and *Rhodopseudomonos palsutris* P4 (Oh et al., 2002; Najafpour et al., 2004).[2]

The optimum growth temperature and pH for the photosynthetic bacteria is in the range of 30-35°C and pH 7.0, respectively (Lee et al., 2002; Kapdan and Kargi, 2006). They appear to prefer organic acids as carbon source such as acetic, butyric (Fang et al., 2005), propionic (Shi and Yu, 2004), lactic (He et al., 2005) and malic acid (Eroğlu et al., 1999), with the highest conversion efficiency is obtained using lactic acid (He et al., 2005). However, some carbohydrates, including the simple sugars glucose, fructose and sucrose (Maeda et al., 2003), and industrial effluents may also be used for hydrogen gas production by photosynthetic bacteria (Eroğlu et al., 2004).[2]

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 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-phosphorylation. Finally, nitrogenase enzyme drives the reduction of protons to hydrogen with the consumption of a great deal of chemical energy (Figure 3) (Hallenbeck, 2011a; Abo-Hashesh and Hallenbeck, 2011).

Indeed, the major problem in using nitrogenase for biohydrogen production is that this enzyme requires a great deal of chemical energy. The equation below (1) displays the considerable amount of energy required for nitrogenase activity (Horton et al., 2007). When the organism lives photo-autotrophically, the hydrogen is

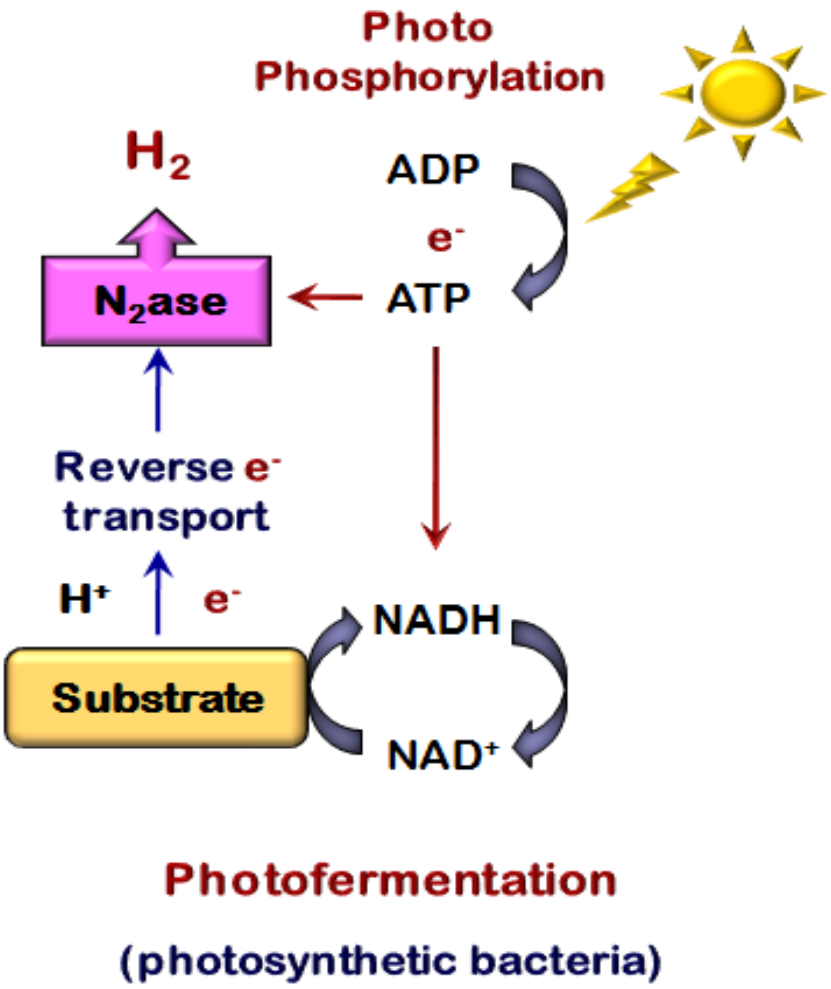

Figure 3. Photo-fermentative hydrogen production via nitrogenase enzyme. ATP produced via photo-phosphorylation is harnessed to drive the reverse electron transport chain required to release more electrons and protons from the feedstock as well as nitrogenase activity for catalyzing the reduction of protons to hydrogen.

produced via N_2 fixing process as a waste product but when it lives photo-heterotrophically it can save the energy needed for fixing N_2 and direct it to hydrogen production. Enzyme activity is inhibited irreversibly by exposure to oxygen and reversibly at high ammonia or N/C ratios (Koku et al., 2003).

$$N_2 + 8H^+ + 8e^- + 16\ ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16\ Pi \qquad (1)$$

Another problem is that hydrogen can also be an electron donor for purple bacteria when it is oxidized by a membrane bound [NiFe]-hydrogenase, thus reducing the net hydrogen evolved through nitrogenase activity (Kapdan and Kargi, 2006). Thus, hydrogenase negative mutants of photo-fermentative bacteria can be used to enhance hydrogen gas production and have been reported, under some conditions, to produce 2-3 times more hydrogen (Kim et al., 2006). Other factors as well are known to affect hydrogen production via photofermentation process including; light intensity, carbon source, and the type of microbial culture. Increasing light intensity can have, up to a certain point, a stimulatory affect on hydrogen yield as well as production rate, whereas it has a counter effect on light conversion efficiencies (Barbosa et al., 2001; Shi and Yu, 2005). Light intensity might also affect the consumption rates of organic acids. For example, it has been reported that butyrate consumption requires higher light intensities as compared to acetate and propionate (Shi and Yu, 2005).[2]

2.3 Dark fermentative hydrogen production

Dark fermentation is a promising method of biohydrogen production due to its high rate of H_2 evolution from a diverse range of substrates and its lack of a requirement for a direct input of solar energy. Furthermore, fermentative organisms have a high growth rate and their hydrogenase enzymes are free from the inhibitory effects of oxygen as the process is anaerobic (Hallenbeck and Benemann, 2002; Hallenbeck, 2009). A variety of bacteria such as *E. coli*, *Enterobacter aerogenes*, and Firmicutes are known to ferment sugars and produce hydrogen under dark

fermentative conditions. Fermentative bacteria have very high rates of hydrogen evolution compared with other biological hydrogen production processes. Despite these advantages, there are many obstacles such as hydrogen consumption by uptake hydrogenases, substrate utilization, and overall low production yields due to inefficient metabolic pathways that limit the theoretical maximal yields from substrates (Hallenbeck and Benemann, 2002; Hallenbeck and Ghosh, 2009).[3]

Brief pathways leading to hydrogen production from glucose in Clostridia and Enterobacteriaceae are shown in figure 4. Glycolytic conversion to pyruvate results in the production of the reduced form of nicotinamide adenine dinucleotide (NADH). Pyruvate can then be further converted to acetylcoenzyme A (acetyl-CoA), carbon dioxide, and hydrogen by pyruvate–ferredoxin oxidoreductase and one of many hydrogenases. Alternatively, pyruvate is dissimilated to acetyl-CoA and formate, which is readily converted to hydrogen and carbon dioxide through the action of either a [NiFe]-hydrogenase (Ech type) or an [FeFe]-hydrogenase. Acetyl-CoA is a central intermediate that gives rise to a variety of soluble metabolites such as acetate, butyrate, ethanol, etc. depending upon cellular requirements for ATP and maintaining redox balance (Hawkes et al., 2007; Hallenbeck, 2011a,b).[1]

E. coli is an ideal example of a facultative anaerobe, able to grow under both anaerobic and aerobic conditions. Under aerobic conditions, the pyruvate generated via glycolysis is converted to acetyl-CoA, NADH and CO_2 by pyruvate dehydrogenase (PDH). The acetyl-CoA is then further metabolized through the TCA cycle or into acetate (Wolfe, 2005). In contrast, under anaerobic conditions, the pyruvate generated by glycolysis is converted to formate and acetyl CoA by pyruvate formate lyase (PFL). The acetyl CoA is further broken down into acetate and ethanol and a portion of the pyruvate is converted into lactate under acidic conditions upon induction of lactate dehydrogenase. In addition, the cell produces succinate via succinate pathway. This anaerobic production of organic acids is referred to as mixed acid fermentation (Figure 5) (Zhu and Shimizu, 2004; Hallenbeck, 2005). The reduced fermentation end-products help cells to maintain a constant supply of NAD^+

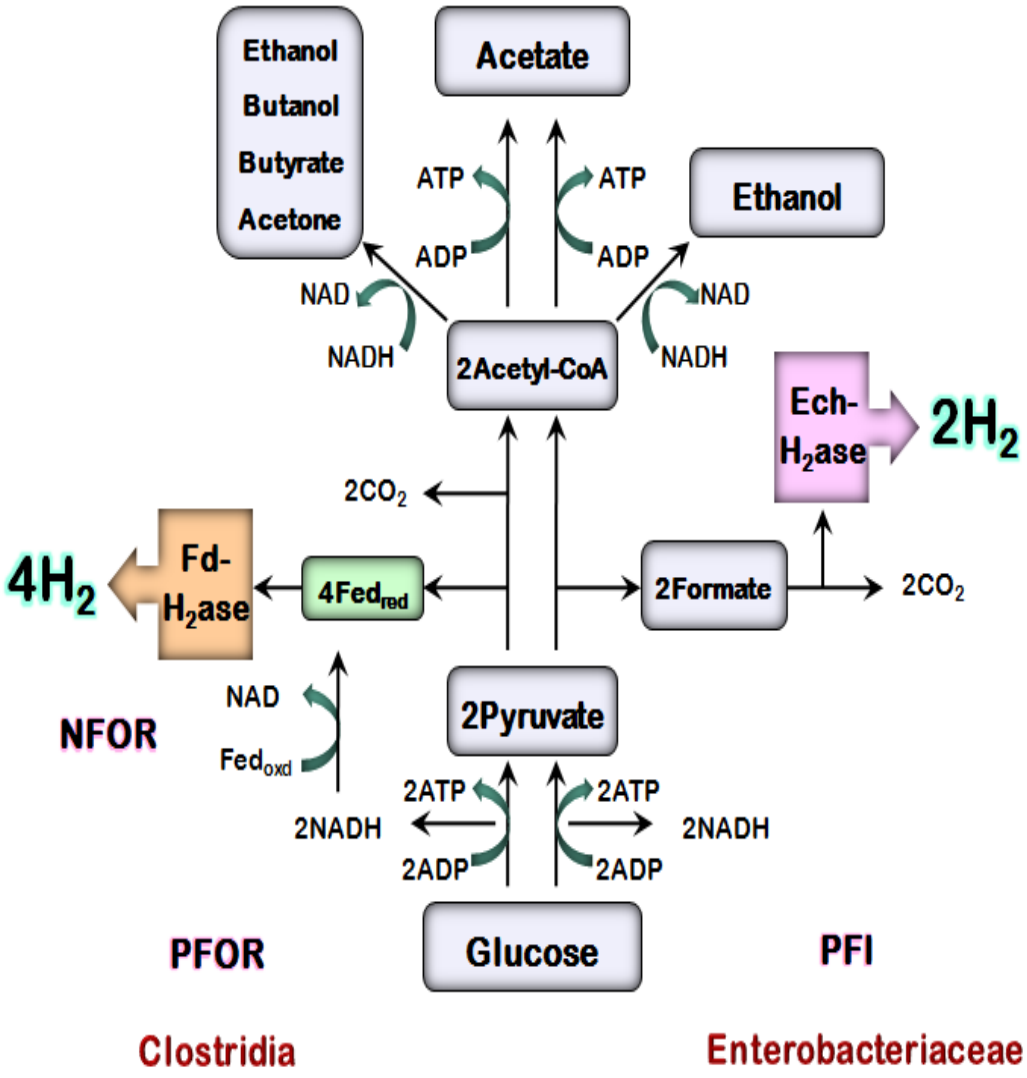

Figure 4. Metabolic pathways relevant for hydrogen production in Clostridia and Enterobacteriaceae. In Clostridia, glycolytic conversion of glucose to pyruvate results in the production of reduced nicotinamide adenine dinucleotide (NADH). Pyruvate then is converted to acetylcoenzyme A (acetyl-CoA), carbon dioxide, and hydrogen via pyruvate-ferredoxin oxidoreductase (PFO) and one of many hydrogenases. On the other hand, in Enterobacteriaceae, pyruvate is dissimilated to acetyl-CoA and formate, which is readily converted to hydrogen and carbon dioxide through the action of either a [NiFe]-hydrogenase (Ech type) or an [FeFe]-hydrogenase. Acetyl-CoA is a central intermediate that gives rise to a variety of soluble metabolites such as acetate, ethanol, butanol, acetone and butyrate.

for glycolysis (Berrıos-Rivera et al., 2002). The formation of lactate, ethanol, and succinate generates NAD^+, while the production of acetate yields ATP via substrate level phosphorylation (Wolfe, 2005). Formate is produced in order to get rid of extra reducing equivalents that would have been lost through the reduction of NAD^+ under aerobic conditions (Sawers, 2005). In addition, formate can be further broken down into hydrogen gas and carbon dioxide under acidic conditions in order to maintain the pH of the fermentation broth and to lower the concentration of formate in the cell (Hakobyan et al., 2005).[3]

E. coli is capable of two types of hydrogen metabolism. The first involves respiratory hydrogen oxidation (uptake) that is combined with quinone reduction (Sawers, 1994; Skibinski et al., 2002). The second is hydrogen evolution during mixed acid fermentation via Formate Hydrogen Lyase pathway (FHL). *E. coli* is capable of producing H_2 only from the catabolic intermediate formic acid during mixed acid fermentation where the maximum of H_2 yield per glucose is 2 mols H_2 (Hallenbeck and Benemann, 2002).[3]

The *E. coli* FHL pathway catalyzes the oxidation of formate into hydrogen and CO_2 in response to acidification brought about by fermentation (Penfold et al., 2003). The FHL enzyme complex is comprised of formate dehydrogenase H (FDH), and a hydrogen-evolving hydrogenase (hydrogenase 3). Formate dehydrogenase, encoded by *fdhF*, is regulated by the presence of hydrogenase 3 (Hyd 3), and four polypeptides which are all encoded on the *hyc* operon (Self et al., 2004).[3]

Hydrogenase 3 is composed of a cytoplasmically oriented large subunit, encoded by *hycE* and a small subunit encoded by *hycG*. The remaining four polypeptides along with the product of *hycG* are integral membrane electron transfer components (Sawers, 2005). Transcription of the FHL complex is activated by the gene product of *fhlA*, called the FHL activator protein (FhlA) that is regulated by Fnr and repressed by HycA (Self et al., 2004). The FHL repressor protein (HycA) is

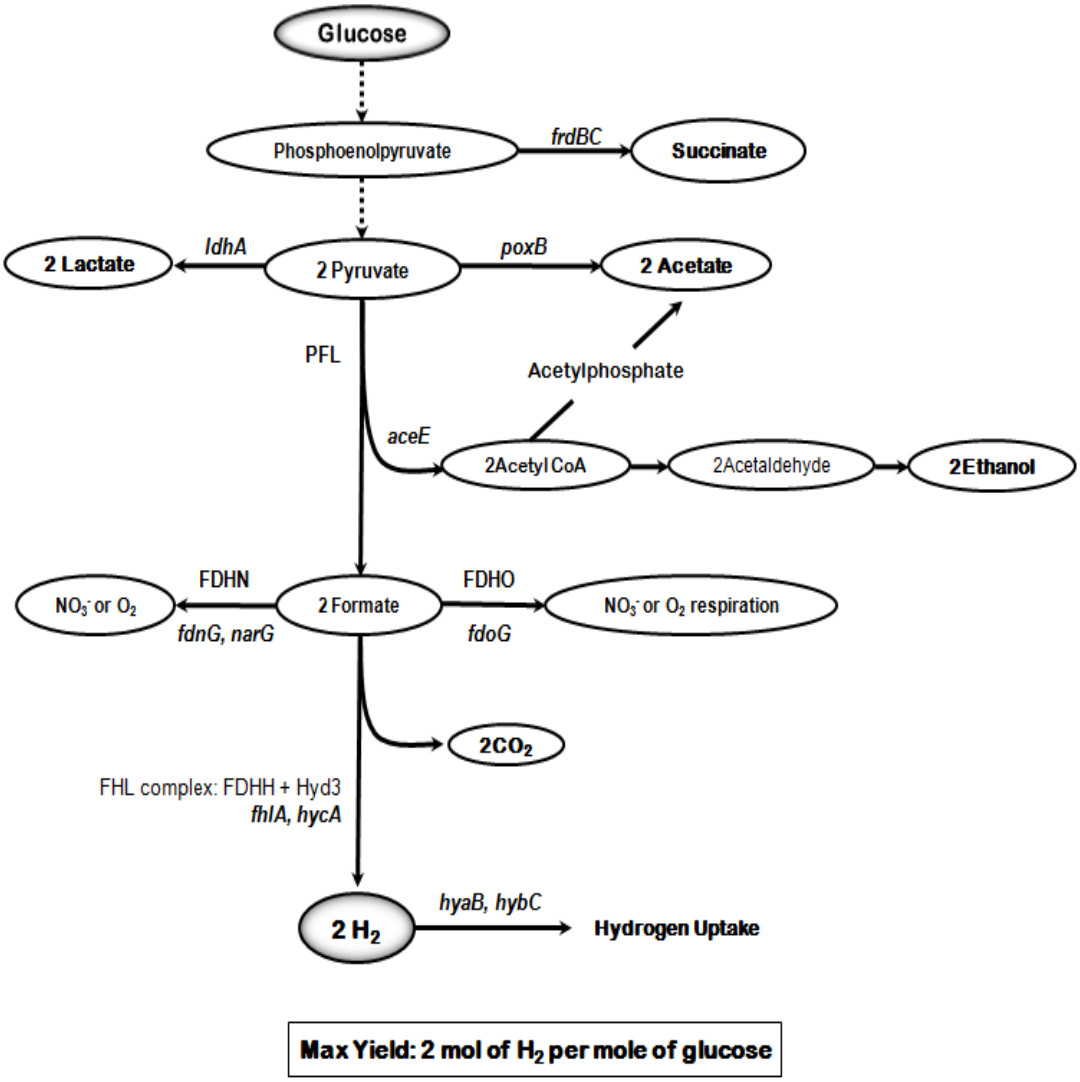

Figure 5. Fermentative hydrogen production from glucose by *E. coli*, a well-studied facultative anaerobic bacterium. H_2 is produced through the action of the FHL complex. The maximum theoretical H_2 yield is 2 mols per mol of glucose. The glucose metabolic pathway yields succinate, lactate, acetate, 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.[4]

encoded by *hycA*, found in the same operon as Hyd3 (Penfold et al., 2003). This repressor binds to FhlA and stops FHL transcription (Boehm et al., 1990). Formate is exported by FocA and/or FocB and is also a substrate for formate dehydrogenase-N (FDHN; FdnG), which is linked with nitrate reductase A (NarG), and formate dehydrogenase-O (FDHO; FdoG). HypABCDEF are maturation proteins for hydrogenases 1, 2, and 3 (Figure 5) (Fan et al., 2009).[3]

Strict anaerobic bacteria, in particular Clostridia, are in theory capable of generating up to 4 mols H_2/ mol glucose (Hallenbeck, 2005; Hallenbeck, 2009). Fermentative hydrogen production pathway from glucose by *C. acetobutylicum*, a strict anaerobic bacterium is shown in figure 6. Hydrogen can be produced through the action of PFOR and NFOR. The glucose metabolic pathway results in lactate, acetate, ethanol, acetone, butanol and butyrate as fermentation end-products. Three different types of [FeFe]-hydrogenases have been described in Clostridia: HydA, the classical clostridial hydrogenase that reacts with reduced ferredoxin, HndABCD, a NADP-dependent hydrogenase, and HydABC a novel bifurcating hydrogenase that reacts simultaneously with NADH and reduced ferredoxin.[4]

However, the 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; Hallenbeck et al., 2011).[4]

3. Hydrogen producing enzymes

Three classes of enzymes are capable of hydrogen production: nitrogenases (Masukawa et al., 2002), alkaline phosphatases (Yang and Metcalf, 2004) and hydrogenases (Vignais et al. 2001; Vignais and Colbeau, 2004; Meyer, 2007; Heinekey, 2009). Hydrogenases are the most efficient catalysts for hydrogen

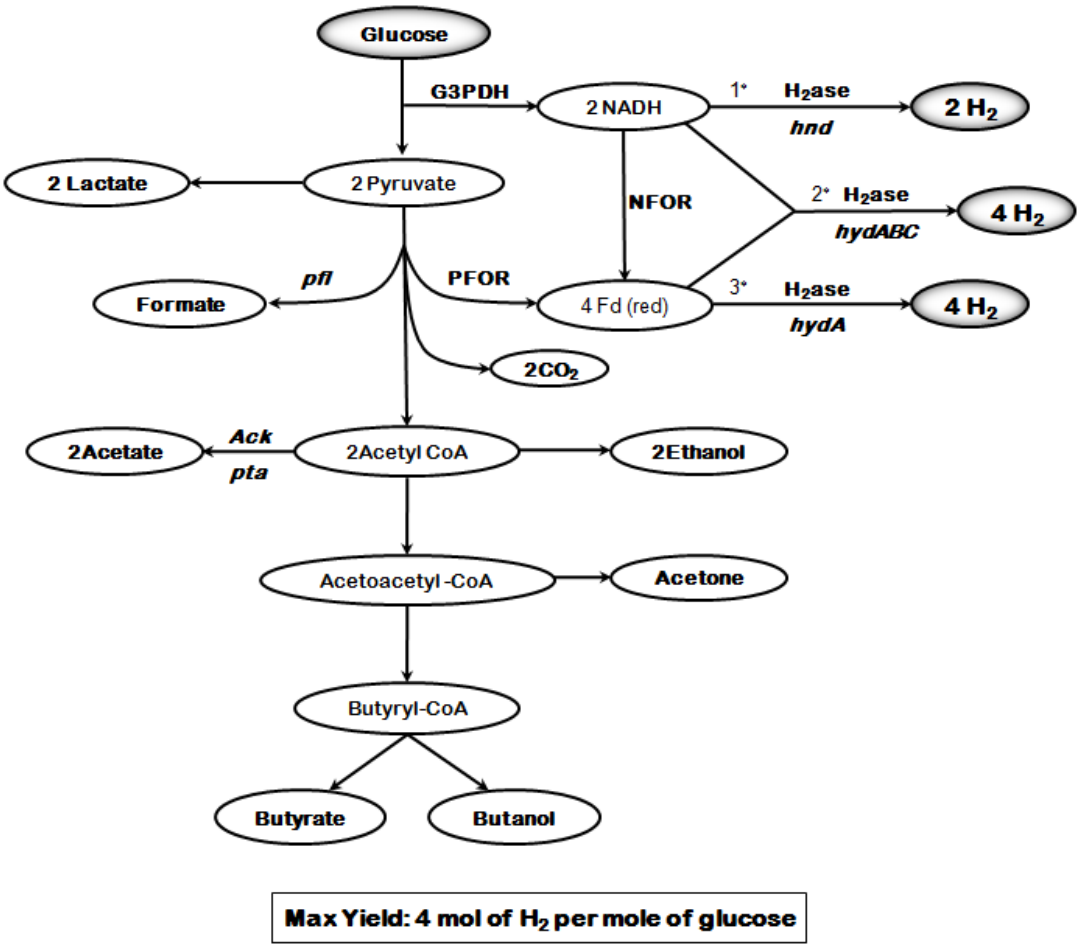

Figure 6. Fermentative hydrogen production from glucose by *C. acetobutylicum*, a strict anaerobic bacterium. H_2 can be produced through the action of PFOR and NFOR. The maximum theoretical H_2 yield is 4 mols per mol of glucose. The glucose metabolic pathway results in lactate, acetate, ethanol, acetone, 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 [FeFe]-hydrogenases have been described in Clostridia. HydA, the classical clostridial hydrogenase that reacts with reduced ferredoxin, HndABCD, a NADP-dependent hydrogenase, and HydABC a novel bifurcating hydrogenase that reacts simultaneously with NADH and reduced ferredoxin.[4]

production with high rates 1000 times higher than nitrogenases (Hallenbeck and Benemann, 2002). They belong to an iron–sulfur [FeS] protein family that possess active sites consisting of inorganic sulfide and iron atoms bound by cysteinyl sulfur atoms to the polypeptide chain (Heinekey, 2009).[3]

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 as nitrogen.

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.

3.1 Hydrogenases

Hydrogenases (H_2ases) were first identified by Stephenson and Stickland in 1931 in colon bacteria. They catalyze the simplest redox reaction: $2H^+ + 2e^- \leftrightarrow H_2$ and perform a functional role in microbial energy metabolism (Thauer, 1998; Adams and Stiefel, 1998; Vignais et al., 2001; Vignais et al., 2007; Vignais, 2008; Frey, 2002). The majority of these enzymes are found in Archaea and bacteria, but a few

are found in the Eukaryota (Vignais et al., 2001). H_2ases are divided into three main groups based on their metallo-center composition: [NiFe]-hydrogenases, [FeFe]-hydrogenases and [Fe]-hydrogenases (Figure 7). The latter have only been described in methanogenic archaea and contain neither nickel nor iron-sulfur clusters but only a mononuclear Fe active site (Lyon et al., 2004; Pilak et al., 2006; Shima et al., 2008). Hydrogen production by organisms carrying out a dark fermentation is by either a [NiFe]-hydrogenase or a [FeFe]-hydrogenase (Hallenbeck, 2009). Both [NiFe]- and [FeFe]-hydrogenases have an active site and a few Fe-S clusters that are buried in the protein. The active site, where catalyzis occurs, is also a metallocluster, and each metal is combined to carbon monoxide (CO) and cyanide (CN^-) ligands (Fontecilla-Camps et al., 2007).

Most of H_2ases catalyze either hydrogen uptake or evolution *in vivo* based on the needs of the host organism and the oxidation state of the H_2ase redox partner and active site. Thus, H_2ase redox partners may act as electron donors or acceptors and include; NAD^+, cytochromes, coenzyme F420 or ferredoxins. Both [NiFe]-hydrogenases and [FeFe]-hydrogenases can be involved in biohydrogen evolving organisms where electrons coming from a electron carrier and hydrogen ions coming from the cellular environment react together at the buried H-cluster active site of hydrogenase to produce H_2 (Figure 8) (Vignais et al., 2001; Abo-Hashesh et al., 2011a). The functional core of hydrogen evolving enzymes is an active site formed of a complex metallo-clusters whose synthesis and insertion in a special protein maturation process requires a number of accessory gene products (Casalot and Rousset, 2001; Hallenbeck and Benemann, 2002; Hallenbeck, 2005).

The most efficient hydrogen producing enzymes are [FeFe]-hydrogenases, which can have an activity about 10–100 times higher than that of [NiFe]-hydrogenases (Horner et al., 2002). The essential activity of these hydrogenases is carried on a single subunit which possesses the [2Fe] catalytic core, but they can be multimeric in addition to being monomeric and can have a variety of electron donors and/or acceptors (Hallenbeck, 2011b). [FeFe]-hydrogenases usually function to

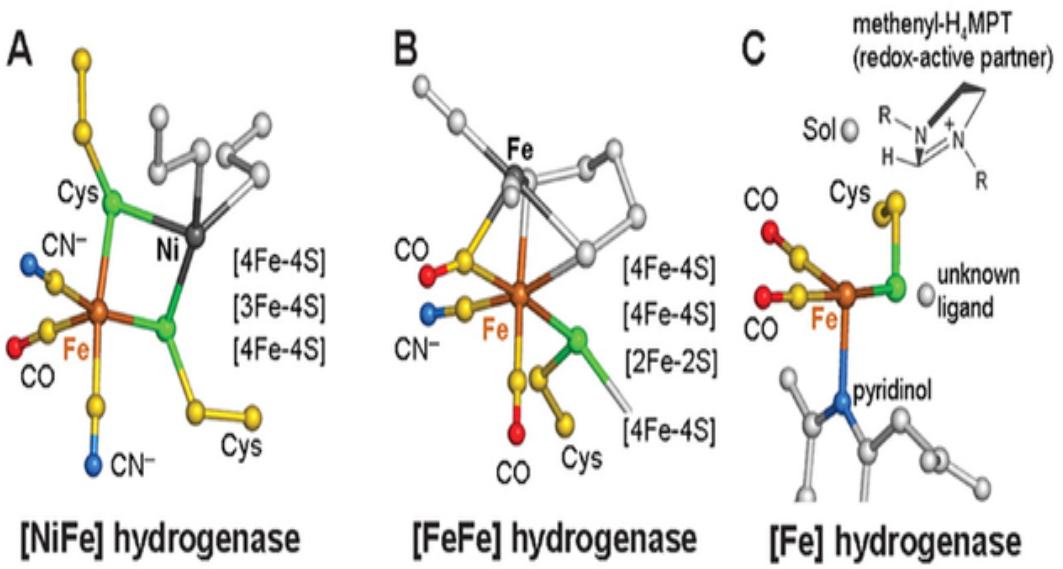

Figure 7. Structure of active sites of the three classes of hydrogenases. [NiFe]-hydrogenases and [FeFe]-hydrogenases have some prevalent features in their structures since each enzyme has two metals in their active sites (either an iron and a nickel atom or two iron atoms) and they have CN^- ligand and a few Fe-S clusters that are buried in protein. [Fe]-hydrogenase has a single iron atom at the active site. As well, the three kinds have some structural similarities since an iron atom linked to a CO group at the active sites (Adapted from Shima et al., 2008).

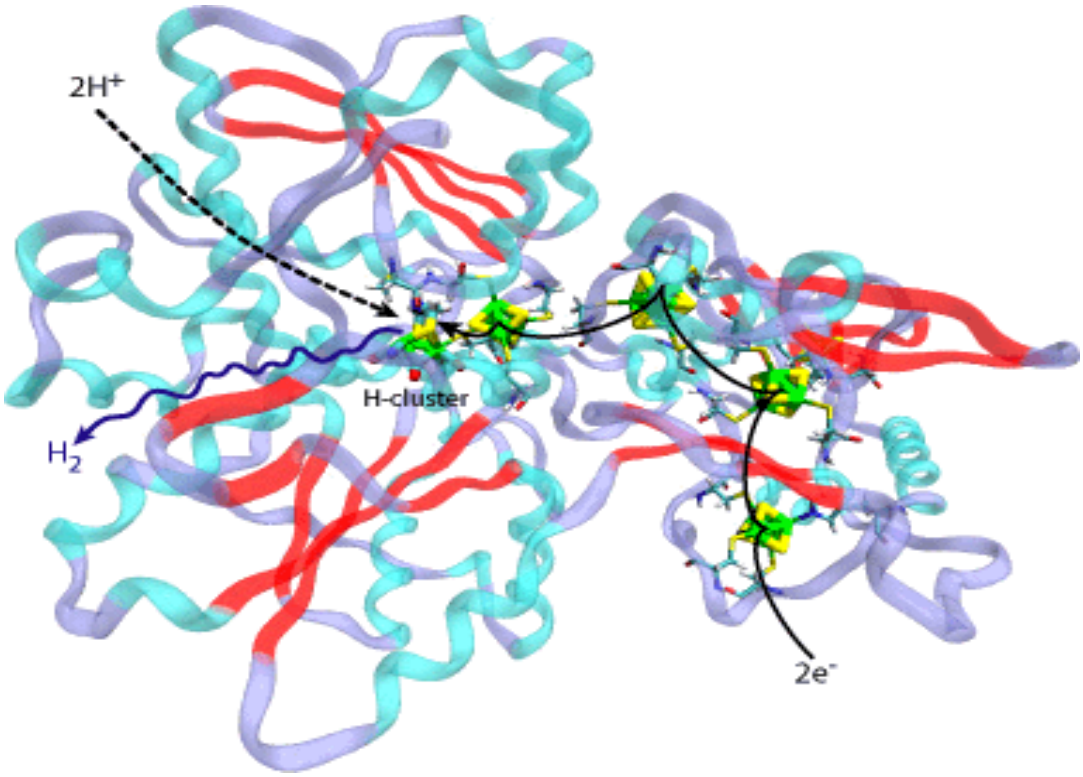

Figure 8. Hydrogen production via hydrogenase enzyme. Electrons come from a carrier and the hydrogen ions come from the cell environment react together at the hydrogenase's buried H-cluster active site to produce H_2 (Adapted from Cohen, 2006).

evolve hydrogen, but depending upon cellular circuitry can consume hydrogen (Hallenbeck, 2009; Abo-Hashesh et al., 2011a).

Many organisms have been found to contain [Ni-Fe] or [Ni-Fe-Se] where one of the Ni-bound cysteine residues is replaced by selenocysteine (Nicolet et al., 2000; Fontecilla-Camps et al., 2007). Those hydrogenases mainly act as an "uptake" hydrogenase, and whose typical metabolic function is to derive reductant from H_2. However, some can probably function to evolve H_2 (Hallenbeck, 2005). An example of this would be hydrogenase 3 of the formate hydrogen lyase complex of enteric bacteria, which evolves hydrogen (Dubini et al., 2002; Hakobyan et al., 2005). The catalytic core of the [NiFe]-hydrogenases is composed of a heterodimeric protein (Vignais et al., 2001). The large subunit contains the Ni–Fe active site and the small subunit usually contains Fe–S clusters which serve as electron transfer points to the electron acceptor or donor (Vardar-Schara et al., 2008; Abo-Hashesh et al., 2011a).

These various characteristics of H_2ases are generally also related to different cellular locations. For instance, hydrogen evolution is most typically cytoplasmic, whereas hydrogen uptake is usually periplasmic or membrane-bound. On the other hand, cytoplasmic bidirectional H_2ases also may catalyze H_2 uptake. Some bacteria may have two or more distinct H_2ases, found in different cellular locations which in turn reflect the value of H_2 in their central metabolism and meet the energetic requirements under different growth conditions (Vignais et al., 2001). All these enzymes are generally sensitive to oxygen and must be either spatially or temporally separated from it in order to produce hydrogen at optimal rates (Tamagnini et al., 2007).

Maturation of [FeFe]-hydrogenase requires a series of three other metalloproteins, HydE, HydF and HydG for accomplishing the synthesis and assembly of a di-iron center with a dithiolate bridging ligand as well as CO and CN ligands (McGlynn et al., 2007; Nicolet et al., 2010; Nicolet and Fontecilla-Camps, 2012).

X-ray crystallographic and infrared spectroscopic studies have shown that the [FeFe]-hydrogenase active site, the so-called buried H-cluster, consists of a di-iron center that shares four cysteine ligands. Three of the 4 cysteines are non protein ligands; CO, CN, and a small dithiolate [Fe]-bridging molecule, and one protein ligand with a regular [4Fe-4S] subcluster. Each Fe atom in the di-iron center has one CO and one CN ligand and connects to the other Fe through an additional bridging CO ligand. Also, [FeFe]-hydrogenases contain additional [4Fe–4S] and [2Fe–2S] ferredoxin clusters that work as electron transfer centers and connect the active site electrically to the protein surface (Pierik et al., 1998; Nicolet et al., 2001; Nicolet et al., 2010).

Examination of amino acid sequences of the maturation proteins reveals a great deal about their structure and function. Both HydE and HydG contain the conserved CysX3CysX2Cys sequence which is characteristic of the so-called ‘‘Radical-SAM’’ superfamily and they are involved in the synthesis of the ligands (Sofia et al., 2001; Nicolet and Drennan, 2004). HydF contains a [4Fe–4S] cluster and all the characteristics of a nucleotide-binding protein, displaying GTPase activity. It is thought to act as a scaffold protein in which the di-iron center is assembled before insertion into hydrogenase (Posewitz et al., 2004; Nicolet et al., 2010; King et al., 2006).

3.2 Nitrogenases

Nitrogenase is the key enzyme which 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 (Figure 9) (Hillmer and Gest, 1977; Masepohl and Hallenbeck, 2010). Nitrogenase synthesis is repressed by high concentrations of fixed nitrogen, especially ammonium, and oxygen. Active enzyme requires molybdenum,

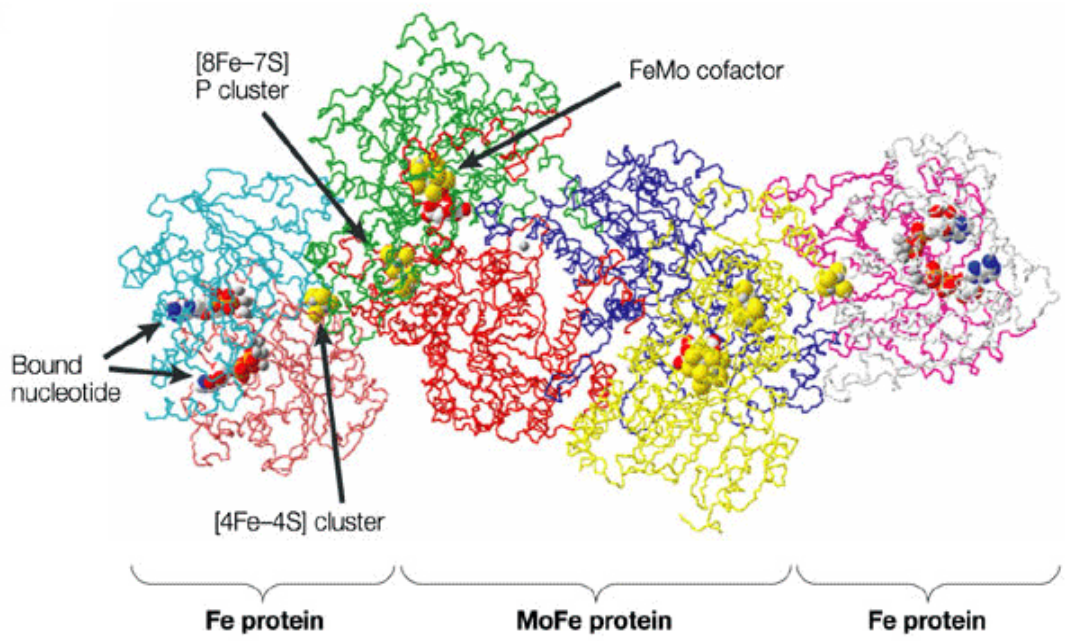

Figure 9. Structure of nitrogenase enzyme. Nitrogenase is a protein complex that consists of two different polypeptide subunits. The smaller dimeric component, known as the iron [Fe] protein and it contains one metal cluster [4Fe–4S]. It functions as an ATP-dependent electron donor to the larger heterotetrameric component, known as the molybdenum–iron [MoFe] protein, which includes the enzyme catalytic site. [MoFe] 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 FeMo cofactor). Both of the nitrogenase-component proteins are oxygen sensitive (Adapted from Moran, 2008).

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 (Takabatake et al., 2004). 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 (Oh et al., 2004; Takabatake et al., 2004). 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 (Oh et al., 2004). The inhibitory action of ammonium is reversible, and hydrogen production activity can be recovered after ammonia is consumed (Takabatake et al., 2004; Masepohl and Hallenbeck, 2010).[2]

Photoheterotrophic growth of these bacteria under nitrogen limiting conditions leads to the nearly stoichiometric conversion of various organic acids to hydrogen through the action of their nitrogenase enzyme. Hydrogen production under these conditions by nitrogenase appears to be a response to the metabolic need to maintain redox balance (Hallenbeck, 2009). Assimilation of the different carbon compounds generates excess electrons as NADH which must be disposed of to permit continued growth and metabolic activity. Therefore, under nitrogen deficient conditions, hydrogen production allows the reoxidation of NADH. Under nitrogen replete conditions, this need is met by CO_2 fixation by Rubisco, an otherwise seemingly paradoxical fixation of carbon dioxide during the assimilation of an organic carbon source. Thus, as much as 68% of the CO_2 given off during growth on acetate is refixed through the Calvin cycle (McKinlay and Harwood, 2010). Indeed, the metabolic requirement for this electron valve is so great that in strains lacking Rubisco mutants expressing nitrogenase under normally repressing conditions (presence of fixed nitrogen) are readily obtained (Laguna et al., 2010; Hallenbeck, 2009).[2]

As noted above, two energetic requirements for nitrogenase activity, highly reducing electrons, and ATP, must be met by cellular metabolism. In both cases, the needed energy is ultimately derived from the photosynthetic activity of the organism, primarily by generation of a proton gradient created during electron flow through the photosynthetic apparatus driven by captured light energy. This gradient is then used to generate ATP, through the action of ATP synthase, and high energy electrons, through some form of reverse electron flow (Hallenbeck, 2009). During cyclic anoxygenic photosynthesis, a photon stimulates the excitation of bacteriochlorophyll in the reaction center (RC) and this energy causes the release of an electron which subsequently reduces the membrane quinone (Q) pool. The quinone cycle works to release protons to the periplasmic space and to reduce the cytochrome *bc1* complex, which reduces cytochrome *c*2. Cyt *c*2 in turn reduces the oxidized primary electron donors in the RC, thus closing the cycle. The protons which have accumulated in the periplasm form an electrochemical gradient which is used by the ATP-synthase to generate ATP. The electrochemical gradient is also used to drive the reduction of ferredoxin by NADH, necessary for nitrogenase activity, by mechanisms which are little understood, but apparently involve either the Rnf or FixABC complexes (Hallenbeck, 2011a).[2]

4. Improvement of fermentative hydrogen production

Microbial fermentative hydrogen production is considered to be a promising energy generating technology as it is technically a simple process over the other biological processes and has relatively many advantages. Various efforts are underway in order to make fermentative biohydrogen an economically feasible alternative to other means of hydrogen production, including optimizing culture conditions, nutritional studies, artificial environments, heterologous expression of specific enzymes, and gene knockouts (Mathews and Wang, 2009).

There are several technical barriers to developing a practical fermentative hydrogen production process. The major obstacle is a low hydrogen yield obtained

from the feedstock which is typically well below the theoretical value. In addition, low yields also lead to the generation of side products whose large scale production would generate a waste disposal problem. Various strategies are being developed to overcome these obstacles and improve fermentative hydrogen production through physiological manipulation, metabolic engineering and engineering efficient hydrogenases.

4.1 Physiological manipulation

Fermentative hydrogen production process is influenced by various parameters such as inoculum, substrate type, reactor configuration, temperature, pH, and media components, including especially nitrogen, phosphate, and metal ions (Li and Fang, 2007a). Possible means of increasing hydrogen production through manipulation of these parameters are described below.

4.1.1 Inoculum

Many different culture types have been used to produce hydrogen from various substrates, and both pure and mixed cultures have been used. *Clostridium* and *Enterobacter* are the most widely used organisms for fermentative hydrogen production in pure cultures. Due to the metabolic pathways involved, the yield of hydrogen from *Clostridium* species is generally higher than that from facultative anaerobic *Enterobacter* sp. (Hallenbeck, 2009). The metabolic pathways used in the two bacterial types are shown in figures 5 and 6. The critical difference is whether or not additional hydrogen can be extracted from the NADH that is produced during glycolysis. *Clostridium*, depending upon the organism, has a variety of pathways leading to hydrogen production since there are different hydrogenases that can accept electrons either directly from NADH, or indirectly, from ferredoxin reduced by NADH (Figure 6). This leads to a theoretical maximum yield of 4 mols of hydrogen per mol of glucose. On the other hand, Enterobacteriaceae, such as *Escherchia coli*, lacks this ability and therefore can, at maximum, produce only 2 mols of hydrogen

per mol of glucose. Hence, when mixed cultures are to be used, it is advantageous to select for Clostridia species in the inoculum. However, Clostridia have several disadvantages, such as their sensitivity to inhibition by oxygen, and their need for specific nutrient and other environmental requirements for spore germination, if sporulation occurs in response to unfavorable environmental conditions such as lack of nutrients (Hawkes et al., 2002).[1]

Although many studies have been conducted using glucose with batch cultures of pure organisms, a practical process probably necessitates the use of continuous operation with mixed cultures since the substrate to be used would be organic wastes, or, eventually deconstructed ligno-cellulosic biomass, and substrate sterilization would be too energy intensive and costly. In addition, the use of inexpensive feedstocks (i.e. wastes) allows for the generation of waste treatment credits. However, full utilization of such materials requires wide ranging hydrolytic capacities, something much more likely to be found with mixed cultures (Hallenbeck et al., 2009). Inocula for mixed culture studies are commonly obtained from sewage sludge, compost, or soil (Zhu and Beland, 2006; Wang and Wan, 2008). However, mixed cultures need to be pretreated by one of several methods in order to suppress as much hydrogen-consuming bacterial activity as possible while still preserving the activity of the hydrogen producing bacteria (Wang and Wan, 2008, Wang and Wan, 2009).[1]

The pretreatment methods reported for enriching hydrogen producing bacteria from mixed cultures include; heat-shock, exposure to acid or base, aeration, freezing and thawing, chloroform, sodium 2-bromoethanesulfonate or 2-bromoethanesulfonic acid and iodopropane (Mohan et al., 2008; Sompong et al., 2009). Heat-shock has been the most widely used pretreatment method for enriching hydrogen producing bacteria for inocula. However, it is not always as effective in comparison with other methods as it may reduce the activity of some hydrogen producing bacteria (Li and Fang, 2007a; Wang and Wan, 2008). As an alternative, pretreatment of mixed cultures with base may prevent a shift in the microbial population to non-hydrogen

producing acidogens, thus having benefits for fermentative hydrogen production (Kim and Shin, 2008). Microbial analysis methods such as Polymerase chain reaction (PCR)-Denaturing gradient gel electrophoresis (DGGE) have been used to determine the community structure of mixed cultures and the changes that may occur after certain pretreatment (Kim and Shin, 2008; Shin et al., 2004).[1]

4.1.2 Feedstocks

Many studies have examined the hydrogen production potential of different carbon sources varying from simple sugars such as glucose to more complex substrates such as biomass (reviewed in: Hallenbeck et al., 2009). In general, carbohydrates are the preferred organic carbon source for hydrogen producing fermentations. Glucose, either as a hexose, or derived from polymers such as starch and cellulose, gives a maximum yield of $4H_2$ per glucose when *Clostridium* type organisms are present and when acetic acid is the by-product (Equation 2), while half of this yield per glucose is obtained if butyrate is the fermentation end-product (Equation 3) (Hawkes et al., 2002, Hallenbeck, 2005; Hallenbeck, 2009).[1]

$$C_6H_{12}O_6 \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (2)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (3)$$

Glucose and sucrose are the most commonly used pure substrates in both batch and continuous processes because of their relatively simple structures, ease of biodegradability, as well as their presence in several industrial effluents (Kapdan and Kargi, 2006). In addition, they can be readily obtained from the polymers present in many agricultural and biomass wastes. In the future, to meet the demand for renewable energy and for a truly sustainable process, more sustainable feedstocks will need to be utilized. These may be in the form of sugar-containing crops such as sweet sorghum and sugar beet, starch based crops such as corn or wheat, or ligno-cellulosics such as fodder grass and *Miscanthus* (Hawkes et al., 2002). The practical

maximum yield, based on 4 hydrogen per glucose, by mixed or pure cultures operated at or near atmospheric pressures and mesophilic temperatures is in the range of 45–60% for either pure substrates or wastes (Fang and Liu, 2002).[1]

Hawkes et al. (2002) reported that dilute substrates typically 1% target substrate (TS), e.g. 10 g/l glucose, have been used for hydrogen production, as dilute substrates have been shown to give the best yields. At high substrate concentration levels, hydrogen production is often observed to decrease in proportion to further increasing levels of the substrate, and yields are usually reduced (Lay, 2001).[1]

Some complex substrates may not be ideal for direct use in fermentative hydrogen production due to their recalcitrance to degradation caused by their structure. Therefore, pretreatment may be required before they can easily be used by hydrogen producing bacteria. For example, the hydrogen yield from cornstalk wastes after acidification pretreatment was much larger than that from cornstalk wastes without any pretreatment (Zhang et al., 2007a). Likewise, activated sludge from wastewater treatment plants contains high levels of organic matter and thus is a potential substrate for hydrogen production. The ability of hydrogen producing bacteria to produce hydrogen from it can be improved after appropriate pretreatment such as; ultra-sonication, acidification, freezing and thawing, sterilization, and exposure to microwaves (Wang et al., 2003; Ting et al., 2004). To maximize hydrogen production from a particular waste stream, it is recommended to carryout preliminary studies of different pretreatment methods.[1]

4.1.3 Nutrients

Careful consideration needs to be given to the possible requirement for the addition of inorganic nutrients for optimal hydrogen production from a particular feedstock. Nitrogen is an essential component of proteins, nucleic acids and other cellular components and therefore its availability can greatly affect the growth of hydrogen producing bacteria, as well as, it seems, directly affecting fermentative

hydrogen production (Bisaillon et al., 2006). Fixed nitrogen in the form of an ammonium salt is the most widely used nitrogen source for fermentative hydrogen production. Some authors recommend studying the effects of nitrogen sources other than ammonia on the process of hydrogen production (Wang and Wan, 2008).[1]

Phosphate plays a dual role in supporting hydrogen production due to it being an essential element in microbial nutrition as well as offering buffering capacity. Increasing phosphate concentration in an appropriate range can increase the ability of hydrogen producing bacteria to produce hydrogen during the fermentative process. However, excessive phosphate concentrations can adversely affect hydrogen production (Lay et al., 2005; Bisaillon et al., 2006). In continuous studies with *Clostridium pasteurianum* under phosphate limitation, ethanol, butanol and 1, 3-propandiol were the major products from glucose (Dabrock et al., 1992). Many studies have shown that appropriate C/N and C/P are essential for effective fermentative hydrogen production (Wang and Wan, 2008). For comparison, the C:P recommended for anaerobic digestion is about 130:1, corresponding to a chemical oxygen demand (COD):P of 350:1 for carbohydrate.[1]

Iron is an essential component of hydrogenase enzymes and, consequently limitation for iron results in decreased hydrogenase activity. Ferrous [Fe^{+2}] has the most widely investigated metal ion for fermentative hydrogen production (Wang and Wan, 2008). Batch studies with mixed cultures on sucrose found that low iron concentrations favored ethanol and butanol production, whilst maximum hydrogen yields were observed with the addition of 800 mg of $FeCl_2$/l to the growth medium (Lee et al., 2001). Reduced fermentation end-products, such as lactate, ethanol and butanol, require reductant that could otherwise be used for hydrogen production so their production must be minimized in H_2 producing cultures if H_2 yields are to be maximized. Therefore, iron must be presented at greater than limiting levels. Thus, for example, batch experiments with glucose have shown that iron concentrations of less than 0.56 mg/l are growth limiting for *C. pasteurianum* and cause significant lactic acid production (Dabrock et al., 1992). Once again, although trace levels of

iron are required for fermentative hydrogen production, at high concentrations, iron may inhibit the activity of hydrogen producing bacteria (Chen and Line, 2003).[1]

Several authors have also investigated the toxic effects of some heavy metals on fermentative hydrogen production. For example, it was reported that the relative toxicity of six electroplating metals was in the order: Cu > Ni > Zn > Cr > Cd > Pb (Li and Fang, 2007b), while another study reported that the relative toxicity of three heavy metals for fermentative hydrogen production was in the following order: Zn > Cu > Cr (Lin and Shei, 2008).[1]

4.1.4 Bioreactor configuration

Most of the studies on fermentative hydrogen production have been conducted using batch mode due to its simplicity and ease of control. However, large-scale operations would require continuous production processes for practical engineering reasons. The continuous stirred tank reactor (CSTR) has widely been used for continuous fermentative hydrogen production (Hawkes et al., 2002; Hallenbeck et al., 2009). However, CSTR have some disadvantages as they are very sensitive to fluctuating environmental conditions, such as changes in pH and hydraulic retention time (HRT) and washout of biomass can occur during operation at a high dilution rates (Lin et al., 2007; Hallenbeck et al., 2009). In general, fermentative hydrogen production is somewhat sensitive to HRT, and, in an appropriate range, increasing HRT increases hydrogen production while HRT at much higher levels could decrease hydrogen production proportionally (Chen et al., 2008).[1]

Immobilized-cell reactors provide an attractive alternative to conventional CSTR because they are capable of maintaining higher biomass concentrations and can operate at short HRTs without risk of biomass washout. Biomass immobilization can be achieved through formation of granules, biofilms, or by using gel-entrapment (Li and Fang, 2007b). For example, the formation of granular sludge permitted the

attainment of biomass concentrations as high as 32.2 g VSS/L and enhanced hydrogen production (Zhang et al, 2007b). When compared with a biofilm-based reactor, the granule-based reactor was better for continuous fermentative hydrogen production due to its higher capacity for biomass retention (Zhang et al., 2008). A particular type of immobilized reactor, upflow anaerobic sludge blanket (UASB) reactors, have proven to be popular options for continuous fermentative hydrogen production due to their high treatment efficiency, short HRT, and excellent process stability characteristics. Establishing a stable granular bed may require protracted periods of time, which however, can be at least partially compensated for by the long period of stable operation that can be obtained (Gavala et al., 2006; Hallenbeck and Ghosh, 2009).[1]

4.1.5 Temperature

Temperature is one of the most important factors that influence the activities of hydrogen producing bacteria and hence fermentative hydrogen production. In an appropriate range, increasing temperature could increase hydrogen production while much higher temperature levels temperature can suppress it (Wang and Wan, 2008). Of course, the optimal temperature for fermentative hydrogen production depends upon the organism or microbial community being used and successful hydrogen fermentations have been reported both in the mesophilic range (around 37°C) and the thermophilic range (around 55ºC) (Li and Fang, 2007a). Temperature may also affect product distribution. For example, one study found that the concentration of either ethanol or acetic acid in batch tests increased with increasing temperature from 20ºC to 35ºC, but it decreased with further increases in temperature from 35ºC to 55ºC. Moreover, the concentration of propionic acid and butyric acid decreased with increasing temperatures from 20ºC to 55ºC to the extent that they were undetectable (Wang and Wan, 2008). The changes in soluble metabolites observed with increasing temperature using mixed cultures suggests changes in metabolic pathways caused by a shift in which bacteria are dominant at each temperature. As noted before, in general hydrogen yields should increase with higher temperatures as the

thermodynamics of hydrogen formation become more favorable (Hallenbeck, 2005) and indeed this seems to be the case with yields approaching four mols of hydrogen per mol of glucose in the extreme case (Zeidan and van Niel, 2010).[1]

4.1.6 pH

pH is another important factor that greatly influences hydrogen production with either pure cultures or mixed communities through affecting the activities of hydrogen producing bacteria. The direct effect on the bacterium seems to be to affect the metabolic pathways which are used. With mixed cultures there is an indirect effect since the activity of hydrogen consuming methanogens is inhibited at low pHs. Although it is often suggested that pH may affect hydrogenase activity, this has never been directly shown and is difficult to rationalize since the internal pH should remain constant independent of the pH control of the medium. pH control is important as the generation of hydrogen by fermentative bacteria is also accompanied by the formation of organic acids as metabolic end-products (e.g. lactate, acetate, and butyrate), lowering the pH. These acids accumulate during fermentation causing a sharp drop in culture pH and subsequent inhibition of bacterial hydrogen production (Fabiano and Perego, 2002; Oh et al., 2003).[1]

In an appropriate range, increasing pH could increase the ability of hydrogen producing bacteria to produce hydrogen during fermentative hydrogen production while higher pH could adversely affect the results (Wang and Wan, 2008). Up to now, most studies have been conducted in batch mode without pH control, and therefore only the effect of initial pH on fermentative hydrogen production has been investigated (Khanal et al., 2004, for example). It is reasonable to suppose, given the rather marked effects of pH that have been observed that pH control of continuous cultures should lead to higher hydrogen productivity, better control over end-product distribution of soluble metabolites, and more stable operation in general (Karadag et al., 2010). Although the specific pH values required may differ with species or culture, some generalizations based on previous reported studies, can be made (Collet

et al., 2004). A recent study with a pure culture of *Clostridium butyricum* showed that butyrate and acetate formation were favored at low pHs, Lactate and formate formation at relatively high pHs (6 and above), and hydrogen production was maximum at moderately acidic pHs (pH 5) (Masset et al., 2010). Similarly, fermentation of swine manure supplemented with glucose with a mixed culture showed that acidic conditions favor butyrate formation, while more basic conditions allow methane formation (Wu et al., 2010).[1]

4.1.7 Hydrogen partial pressure:

Hydrogen partial pressure in the liquid phase is one of the key factors affecting hydrogen production. Since hydrogen production by hydrogenases is a reversible process, build up of the product, H_2, depending upon the thermodynamics involved, decrease further conversion of substrate to hydrogen. The effect is different depending upon the hydrogenase reduction pathway involved. Hydrogen production from reduced ferredoxin is much more favorable than hydrogen production from NADH, so it is less sensitive to this effect. Hydrogen production driven by NADH only proceeds at relatively low hydrogen partial pressures, so as hydrogen partial pressures increase, H_2 synthesis decreases and metabolic pathways shift towards production of other reduced substrates, such as lactate, ethanol, acetone and butanol (Hallenbeck, 2005).[1]

Thus a decrease in hydrogen partial pressure should give an enhanced hydrogen yield. Gas sparging has been found to be one useful technique for reducing hydrogen partial pressure in the liquid phase thereby enhancing overall hydrogen yields. For example, lowering dissolved hydrogen by sparging with nitrogen gave a 68% increase in hydrogen yields with a reactor containing enriched mixed microflora operating with 10 g/l glucose-mineral salts at pH 6.0 (Mizuno et al., 2000). In another technique, the partial pressure of hydrogen was effectively reduced using a hollow fiber/silicone rubber membrane, resulting in a 10% improvement in the rate of

hydrogen production and a 15% increase in hydrogen yield (Liang et al., 2002; Wu et al., 2010).[1]

Of course, in nature mixed anaerobic cultures accumulate very little hydrogen as its production is normally balanced by rapid hydrogen consumption by methanogens. Thus mixed microbial cultures must be operated under conditions that simultaneous favor the activity of the hydrogen producers and reduce the activity of hydrogen consumers. While in theory it would appear that one way to prevent interspecies hydrogen transfer would be to remove hydrogen as fast as it was produced, this is hardly possible in practice given the relatively high affinity of hydrogen consumers for hydrogen and the relatively slow mass transfer of hydrogen from the liquid to gas phase. At any rate, a process that affects hydrogen partial pressures by dilution, i.e. sparging, would create a dilute hydrogen stream, adding prohibitive hydrogen concentration costs to the production system.[1]

4.1.8 Metabolic control

Most fermentative bacteria cannot tolerate pH values less than 5.0 and therefore some possess natural homeostatic mechanisms that attempt to limit extreme acidification. For example, in bacteria such as *E. coli*, a drop in pH will trigger hydrogen production from formic acid, thus removing one acid, while at the same time channeling more metabolic flux into lactate, which improves the situation since only one acid (lactic) is produced from pyruvate rather than potentially two (acetic and formic). Of course, lactate production is to be avoided since it decreases net hydrogen production. As well, as noted above, acid pHs favor butyric acid production over acetic acid in *Clostridium* sp., and thus less hydrogen is formed. Thus, the microbial metabolism can be controlled to favor hydrogen production by controlling the pH. This of course entails costly addition of alkali. Hence, it has been suggested that acid tolerant facultative anaerobes possessing a lower pH limit for hydrogen production be used in order to reduce alkali consumption (Fabiano and Perego, 2002; Fang et al., 2002). Another approach to improve hydrogen yields is to block the

formation of these acids, through redirection of metabolic pathways (Hallenbeck et al., 2011).[1]

4.1.9 Population control

In practice, high hydrogen yields are usually associated with acetate production while lower yields are found when butyrate is the main fermentation product, as noted above. This indicates that for optimum hydrogen yield, acetate should be favored as the fermentation end-product. Fermentation by mixed cultures generally results in the formation of a spectrum of products including reduced end-products, lactic acid, alcohols, and propionic acid. Unfortunately, many researchers erroneously contend that the observed propionic acid comes at the expense of the produced hydrogen according to equation (4).

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \qquad (4)$$

This fallacy, now often repeated, is based on a single modeling study which assumed that this reaction takes place (Vavilin et al., 1995). In fact, this has never been actually demonstrated. The only organisms known to produce propionate, the Propionibacteriaceae, ferments lactate to propionate. A blast search shows that not a single member of this group contains genes encoding either a [FeFe] or [NiFe] hydrogenase. Thus, propionate cannot be produced at the expense of hydrogen already generated by a mixed culture. However, the presence of propionate does mean that reducing equivalents that could have been used for hydrogen production have been lost from the system since the true substrate for propionate formation, lactate is made from pyruvate that otherwise could have been used to generate molecules capable of driving proton reduction.[1]

Regardless, controlling the makeup of the bacterial population in a mixed culture is a desirable end, although difficult, if not impossible, to achieve in practice. Pretreatments that select for spore forming fermenters, for example heat treatment,

will mainly select Firmicutes, excellent hydrogen producers. Other techniques can include the addition of long chain fatty acids for example (Ray et al., 2010 a,b). Genetic techniques are currently used to demonstrate the complex nature of the bacterial population in hydrogen producing microflora and the existence of shifts in this population. For example, gene profiling techniques have been used to demonstrate the complexity of a microbial community producing hydrogen from glucose, and to show that the microbial community changed with pH in the range studied (pH 4.0–7.0) (Fang and Lui, 2002; Fang et al., 2002). Similarly, a study on the effect of changes in operating pH (from 5.0 to 8.0) on organic acid production by continuous reactors inoculated with anaerobic digester sludge and fed with a glucose-yeast extract medium showed that acid production switched from butyrate to propionate as the pH increased. This was attributed to a change in the dominant microbial population rather than a metabolic pathway change within the same bacterial population, which would be expected to occur more quickly than the observed transitional period of around 120 h (Horiuchi et al., 2002). One very useful and powerful technique that can be used in general to follow the composition and evolution of bacterial populations is PCR coupled with (DGGE) analysis. Usually, 16S DNA is targeted, since this is the usual case in environmental studies where phylogenetic characterization of widely different organisms is required, but in fact, for the study of hydrogen producing fermentations by mixed cultures, a functional probe (i.e. *hydA*) is probably much more useful (Quéméneur et al., 2011).[1]

4.2 Metabolic engineering

Metabolic engineering offers some powerful tools for remodeling microbes to increase production rates and hydrogen molar yields through the design of optimized bacterial strains via engineering of modified metabolic pathways as well as the engineering of more efficient hydrogenases.[3]

Recent years have seen a great deal of research being carried out on dark hydrogen production, with many improvements in bioprocess parameters leading to

impressive improvements in volumetric production rates. Recent developments in genome sequencing and expression analysis increase the ability to engineer microorganisms for specific metabolic tasks. Metabolic engineering to overcome some of the limiting factors in hydrogen production and to design and construct an efficient microorganism with increased H_2 yields as well as an expanded range of substrate utilization.[3]

4.2.1 Engineering modified metabolic pathways

The modification of metabolic pathways to increase the production of natural products, to allow the production of unnatural products, or to promote the degradation of unfavorable products is referred to as metabolic engineering. Metabolic engineering links systematic and quantitative analysis of pathways using molecular biology, modern analytical techniques, and genomic approaches (Stephanopoulos, 1998; Wiechert, 2002). Metabolic engineering could be used to overcome limiting factors for biohydrogen production in various systems by increasing the flow of electrons to hydrogen producing pathways, increasing substrate utilization, and engineering more efficient and/or oxygen-resistant hydrogen-evolving enzymes (Buhrke et al., 2005). In terms of dark fermentation, metabolic engineering could be used at several different levels for process improvement (Hallenbeck et al., 2011b). For example, it could be used to extend the range of substrates used by a given organism, necessary in many cases if abundant lignocellulosic substrates are to be used as feedstock. Thus, organisms could be given the capacity to directly degrade lignocellulosics, or to use the mixture of pentoses and hexoses available after enzymatic conversion of this feedstock. These modifications are in common with almost any microbial process for producing a biofuel from lignocellulosics. Finally, metabolic engineering could be used to increase the rates and/or yields of hydrogen once the sugars are converted to pyruvate, the key intermediate. Two approaches could be taken, modification of existing pathways or introduction of novel hydrogen producing pathways. As described below, a variety of tools for achieving these types of modifications is now available.[3]

Manipulating microbial metabolism to favor the production of desired products is almost as old as bacterial genetics. Initially, random mutagenesis either spontaneous, chemically or ultraviolet radiation induced, or transposon mediated, was useful to knockout critical enzyme activities, if the proper selective pressure could be found. Disruption of chromosomal genes by this method has been one of the most commonly used methods for performing metabolic engineering (Alam and Clark, 1989; Cameron and Chaplen, 1997; Chartrain et al., 2000). For example, allyl alcohol can be used to select alcohol dehydrogenase (*adh*) mutants since functional Adh converts it to a very toxic aldehyde derivative. However, these methods, in particular chemical mutagenesis, can result in the production many nonspecific mutations. Less frequently, it was possible to select for increased activity in a given metabolic pathway due to mutations causing increased promoter activity, activation of transcription of cryptic genes, or naturally occurring genomic region duplications mediated by multiple ribosomal operons. However, despite the elaboration of many clever selection schemes, efforts in this area were severely constrained by the inability to either introduce the desired mutations at will or to carryout high throughput screening (reviewed in: Abo-Hashesh et al. 2011a).[3]

Recently, this changed with the availability of gene or even entire genome sequences as well as the development of modern molecular microbiology techniques which permitted the development of methods for site-directed mutagenesis. In the most common variation of site-directed mutagenesis, this involves the delivery of the desired mutation into the chromosome through a forced homologous recombination with a mutant allele ferried by a non-replicating carrier (i.e. suicide mutagenesis) (Martin et al., 1995; Li et al., 2008). Typically, a knockout is created by flanking an antibiotic resistance marker with enough target DNA sequence (~200bp for *E. coli* and many others) to allow for efficient recombination by RecA protein (essential protein for the repair and maintenance of DNA) (Murphy, 1998). Selection for the appropriate antibiotic resistance selects for the desired recombinant if the shuttle plasmid is incapable of replicating; for example, is either temperature sensitive for replication in *E. coli*, or has a narrow host range *ori* (origin of replication) and thus

cannot replicate when transferred from *E. coli* to a distantly related host. Additional foreign genes could be brought into a host using a variety of different plasmids. Although multiple genes, or even operons, can be knocked out in this way using a series of different antibiotic resistance cassettes, and several different heterologous genes can be simultaneously expressed using different compatible plasmids, these approaches quickly run out of options. In addition, cultures must often be grown with expensive antibiotics to maintain their constructed genetic capacity, the antibiotics themselves present a waste disposal problem, and the constructed strains run afoul of anti-GMO sentiments or regulations (reviewed in: Abo-Hashesh et al. 2011a).[3]

New techniques have been introduced that now allow, at least in principal, an almost limitless series of marker-less knockouts to be made in a single strain, as well as the introduction into the chromosome of very large amounts of foreign DNA. While proof of principle has usually been with *E. coli*, many of these can, and have, been extended to other gram negative and even gram positive bacteria, heralding a new age in metabolic engineering. The key to making strains with multiple specific deletions is the use of phage encoded site-specific recombination systems; FLP/FRT, Cre/Lox, or λ Xis/Int. The introduction of a specific short recombination site (FRT, Lox) allows subsequent manipulation, usually excision, when the cognate excisase is expressed. This technology is quite powerful and even allows *in vitro* cloning in the absence of restriction enzymes or ligase (Hartley et al., 2000) and is the basis for the Gateway™ technology. A very useful additional tool is the use of special recombinases, for example, Red from λ phage along with inhibition of host RecBCD. This permits transformation with PCR products that have an antibiotic resistance marker flanked by recognition sites and as little as 40 to 50 bp of homology with the host genome at each end (Datsenko and Wanner, 2000; Yu et al., 2000). The power and simplicity of this method, called recombineering, which allows insertional inactivation without intermediate cloning steps, are demonstrated by the construction of a library (the Keio collection) of *E. coli* where all non-essential genes have been interrupted (Baba et al., 2006). This system has been well developed for *E. coli* and closely related organisms (Sharan et al., 2009). This technology has recently been

taken to the next level by the demonstration of trackable multiplex recombineering (TRMR) where thousands of genetic modifications, virtually covering the entire genome, can be made and assessed simultaneously, essentially allowing the complete remodeling of a microbial genome in a week (Warner et al., 2010).[3]

However, even though phage functions related to the Red recombinase are found in some other bacteria (Datta et al., 2008), this system does not seem widely applicable to a variety of organisms. Nonetheless, site-specific recombination using a phage derived site-specific recombinase and the cognate palindromic recognition site has proved to be a powerful genetic tool. For example the Cre-Lox system, paradoxically widely applied in plant and animal systems first (Copeland et al., 2001), is now being shown to be useful in a variety of gram negative and gram positive organisms for gene inactivation or the generation of large chromosomal rearrangements (Lambert et al., 2007; Leibig et al., 2008; Yan et al., 2008; Ullrich and Schüler 2010; Fontaine et al., 2010). A possible disadvantage of these systems is that multiple rounds of inactivation/excision can introduce a degree of genetic instability since recombination will leave behind either reconstituted recognition sites, allowing for another round of binding by the recombinase and/or multiple scars, regions long enough to permit homologous recombination. This can be avoided to a large extent with the Cre-Lox system since the Lox sites can be made such that Lox71 is generated by Cre action and Lox71 shows greatly decreased binding by Cre.[3]

In addition to metabolic pathway manipulation through gene inactivation, it is often desirable to add new genetic capabilities. While sometimes this can be done by transformation with a plasmid expressing the gene of interest, or even multiple plasmids (if they have compatible origins of replication), or a plasmid with a synthetic operon for the pathway of interest (Akhtar and Jones, 2008), this approach has many limiting factors including problems with plasmid maintenance and, eventually, size restrictions. One way around this problem would be the use of BACs (Bacterial artificial chromosomes), which can carry large amounts (ca. 300kb) of

foreign DNA while at the same time being stably maintained as a single copy in the bacterial cell (Shizuya et al., 1992). However, BACs are based on the F-factor, and thus their use is restricted to *E. coli* or closely related species. An alternative is the genetic manipulation of large pieces of DNA, with large, or multiple operons, using the recombination technology discussed above, followed by introduction into a foreign genome through homologous recombination after transfer by transformation or conjugation. If an interspecies system of conjugation is available this is probably the preferred route for the transfer of large DNA fragments. The heterologous DNA can be introduced at so-called neutral sites provided by phage attachment sites, cryptic prophages, IS elements, etc.. However, this approach depends upon the ability of the host cell to efficiently perform homologous recombination. Recently, the transfer by transposition of large DNA fragments (58kb), essentially polyketide synthase/nonribosomal peptide synthetase (PKS/NRPS) gene clusters for secondary metabolite synthesis, a process that does not require homologous recombination, has been described (Fu et al., 2008).[3]

Finally, to be successful, metabolic engineering efforts should be intelligently guided. For initial simple modifications, alterations are usually obvious and straight forward. Even then, unforeseen consequences can arise due to activation of alternative pathways, metabolic shifts brought about by changes in metabolite pools, or unexpected activities of known enzymes. Efforts to affect major changes in existing pathways or to introduce of novel pathways could greatly benefit from knowledge of the existing metabolic fluxes and metabolimics (Baran et al., 2009, Zamboni and Sauer, 2009). This information combined with the increasingly available genomics information will allow the building of more accurate metabolic models (Durot et al., 2009) permitting the subsequent rational design of organisms with the desired characteristics (Tyo et al., 2010). Many computational frameworks, such as OptKnock, OptStrain, and OptReg, have been developed in order to find the optimum modulation and deletion strategies for the overproduction of fuels and chemicals (Burgard et al., 2003; Pharkya et al., 2004; Pharkya and Maranas, 2006). In what follows, we examine how some of these various metabolic engineering tools

have been applied to hydrogen production through dark fermentation and suggest ways in which the latest generation of technologies can be brought to bear on surpassing present limitations.[3]

In the past, many studies have attempted to optimize hydrogen production through metabolic engineering of *E. coli*. Now, some powerful new tools have become available for metabolic engineering studies with *E. coli*. As discussed above, Datsenko and Wanner (2000) developed a simple and highly efficient method to disrupt chromosomal genes in *E. coli* in which PCR primers provide the homology to the targeted gene(s) and introduce specific gene knockouts. Using this technique, Baba et al. (2006) developed a set of precisely defined single-gene deletion mutants of all nonessential genes in *E. coli* K-12 using P1 mediated transduction. The constructed mutants the 'Keio collection' provide a new resource not only for systematic analyses of unknown gene functions and gene regulatory networks but also for genome-wide testing of mutational effects in a common background. Likewise, the 'ASKA' library contains a complete set of *E. coli* ORF clones and provides a valuable resource for metabolic engineering studies (Kitagawa et al., 2006).[3]

Different metabolic engineering strategies for increasing biohydrogen production under dark fermentative conditions have been described (Table 1). As well, schemas of metabolic engineering possibilities to enhance hydrogen production in *E. coli* and *Clostridium* are given in figures 10 and 11, respectively. Some of these involve the disruption of lactate and/or succinate production. These studies show that hydrogen production is increased by directing carbon flow into making formate. Yoshida et al. (2005) developed an FHL overexpressing *E. coli* strain SR13 that had been deleted for the FHL repressor gene (*hycA*) and has a plasmid that overexpresses *fhlA*. The hydrogen yield was increased from 1.08 to 1.82 mols/ mol glucose in the *E. coli* SR15 strain lacking lactate and succinate production (Yoshida et al., 2006; Yoshida et al., 2007). Likewise, Penfold et al. (2003) developed a *hycA* null *E. coli*

Table 1. Different metabolic engineering strategies for increasing biohydrogen production [3]

Organism	Description	References
E. coli (Δ*hycA fhlA*$^{+}$)	Truncating the N-terminus of FHLA to activate the *hyc* operon.	Self et al. (2001)
E. coli HD701 (Δ*hycA*)	Repressor knockout increase H_2 yields especially at lower glucose concentrations.	Penfold et al. (2003)
E. coli HD701	Expression of invertase enzyme encoded in pUR400 plasmid allows for sucrose utilization.	Penfold and Macaskie (2004)
E. coli SR13 (Δ*hycA fhlA*$^{+}$)	Genetic recombination together with process manipulation was employed to elevate the efficiency of hydrogen production.	Yoshida et al. (2005)
E. coli DJT135 (Δ*hyaB* Δ*hyb,* Δ*ldhA, fhlA**	Uptake H_2ase, increased expression of formate regulon, lactate dehydrogenase deficient, near stoichiometric yields at limiting glucose	Bisaillon et al. (2006); Turcot et al. (2008)
E. coli MC4100 (Δ*tatC* Δ*tatA-E)*	Inactivation of the Tat system prevents correct assembly of the uptake hydrogenases and formate dehydrogenases.	Penfold et al. (2006)
E. coli strains	Modification of glycerol metabolic pathway in the absence of electron acceptors enhances glycerol fermenting pathway.	Murarka et al. (2007)
E. coli SR14 (Δ*hycA fhlA*$^{+}$Δ*ldhA* Δ*frdBC)*	Increase induction of the formate hydrogen lyase (FHL).	Yoshida et al. (2007)
E. coli BW25113 (*fhlA*$^{+}$Δ*hyaB* Δ*hybC* Δ*hycA* Δ*focA* Δ*frdC* Δ*fdoGC* Δ*ldhA)*	Overexpress inducer, eliminate uptake hydrogenases, knock out repressor, prevent formate export, inactivate the succinate and lactate synthesis pathways.	Maeda et al. (2007a)
E. coli K-12 BW25113 (Δ*hyaB* Δ*hybC* Δ*hycE)*	Protein engineering of hydrogenase HycE by an error-prone polymerase chain reaction (epPCR).	Maeda et al. (2008a)
E. coli ZF1(Δ*focA)*, *E. coli* ZF3 (Δ*narL)*	Modification of global transcriptional regulators that modulate the biosynthesis of formate hydrogen lyase.	Fan et al. (2009)
Enterobacter aerogenes IAM1183-O (Δ*hybO)*	Genetic knockout of the small subunit of the uptake hydrogenase encoded by *hybO* improved the hydrogen production.	Zhao et al. (2009)

strain HD701 which has 14-fold increase in hydrogen production over wild type, especially when it had been grown in low glucose concentrations. Overexpression of the FHL activator *fhlA* in this mutant increased the hydrogen production yield by 2.8-fold compared to the wild-type when formate was used as a substrate. Similarly, a strain, DJT135, in which *ldhA* has been inactivated and that activates the formate regulon since it expresses a N-terminally truncated FhlA, has been shown to be capable of producing nearly 2 mols H_2/ mol glucose (Bisaillon et al., 2006, Turcot et al., 2008). Thus, the overexpression of the FHL complex leads to greater hydrogen production and this can be achieved by either eliminating the repressor HycA or truncating the N-terminus of FhlA making it formate-independant and causing it to activate the *hyc* operon (Self et al., 2001).

Maeda et al. (2007a) performed successive rounds of P1 transduction from the Keio *E. coli* K-12 library to introduce multiple, stable mutations into a single bacterium to direct metabolic flux towards hydrogen production. The regulation of FHL was altered by inactivating the repressor *hycA* and by overexpressing the activator encoded by *fhlA*, eliminating hydrogen uptake activity by deleting *hyaB* (hydrogenase 1) and *hybC* (hydrogenase 2), redirecting glucose metabolism to formate using mutations in *fdnG*, *fdoG*, *narG*, *focA*, *focB*, *poxB*, and *aceE*, and inactivating the succinate and lactate synthesis pathways by deleting *frdC* and *ldhA*, respectively. The best of the metabolically engineered strains, BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE*, showed a 4.6-fold increased hydrogen production from glucose with a 2-fold increased hydrogen yield (0.65 to 1.3 mols H_2/ mol glucose). Likewise, a similar strain construction gave a mutant with 141-fold increased hydrogen production from formate, achieving the theoretical yield for hydrogen from formate (Maeda et al., 2008a). In addition, the hydrogen yield from glucose was increased by 50%, and there was threefold higher hydrogen production from glucose with this mutant.[3]

Until now, most of the modifications have been at the level of single genes, or at most operons and little is known about how changes in global regulatory circuits

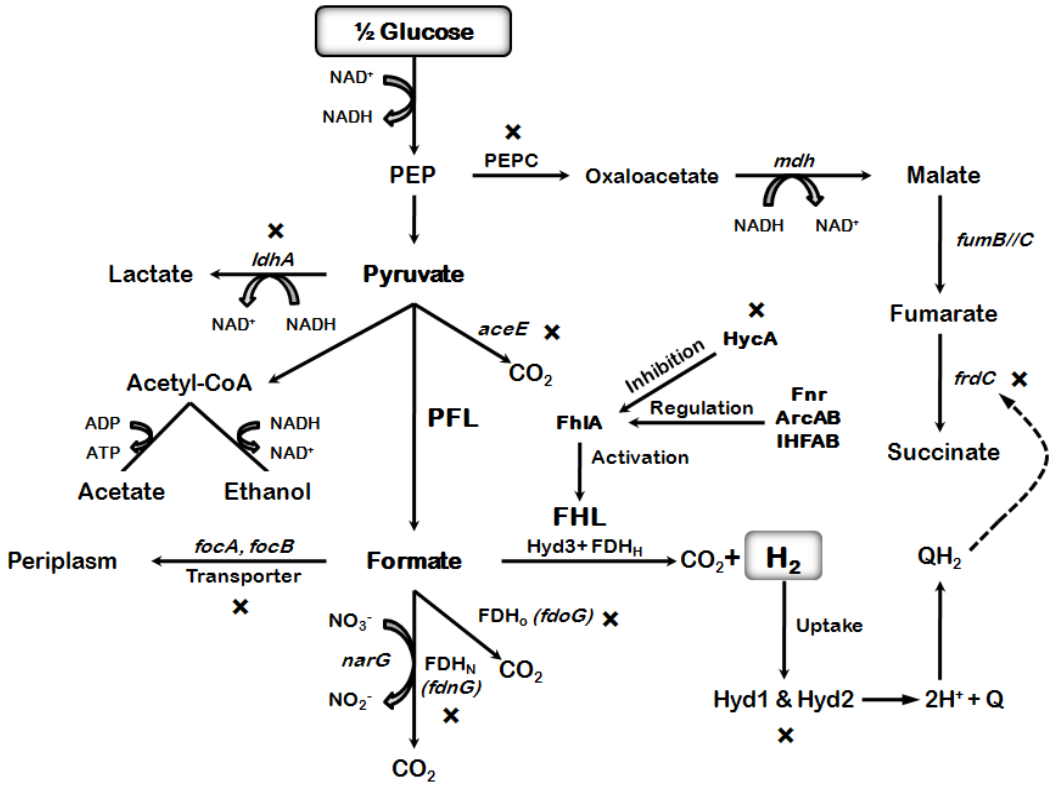

Figure 10. Metabolic engineering possibilities to enhance hydrogen production in *E. coli*. Formate is the key product in hydrogen production in facultative anaerobes such as *E. coli*. The maximum theoretical hydrogen yield is 2 mol of H_2 per mol of glucose. The main fermentative end-products are hydrogen, carbon dioxide, acetate, ethanol as well as lactate and succinate at low pH. Hydrogen production might be enhanced by gene knockout (X), or by activation of a particular pathway. Phosphoenolpyruvate (PEP); Phosphoenolpyruvate carboxylase (PEPC); Fumarate reductase (*frdC*); Lactate dehydrogenase (*ldhA*); Pyruvate dehydrogenase (*aceE*); Pyruvate formate lyase (PFL); Formate hydrogen lyase (FHL); Hydrogenase 3 (Hyd 3); formate dehydrogenase-H (FDHH); FHL activator (*fhlA*); FHL repressor HycA; Global regulators (Fnr, ArcAB, IHFAB); Uptake hydrogenases; hydrogenase 1 (Hyd 1) and hydrogenase 2 (Hyd 2); Formate exporters (*focA* and/or *focB*); Formate is metabolized by formate dehydrogenase- N (FDHN; *fdnG*) which is linked with nitrate reductase 1 (NarG) and formate dehydrogenase-O (FDHo; *fdoG*); Q, quinone pool; QH_2, quinol. (X) Crosses indicate possible chromosomal gene knockouts for enhancing hydrogen production.[3]

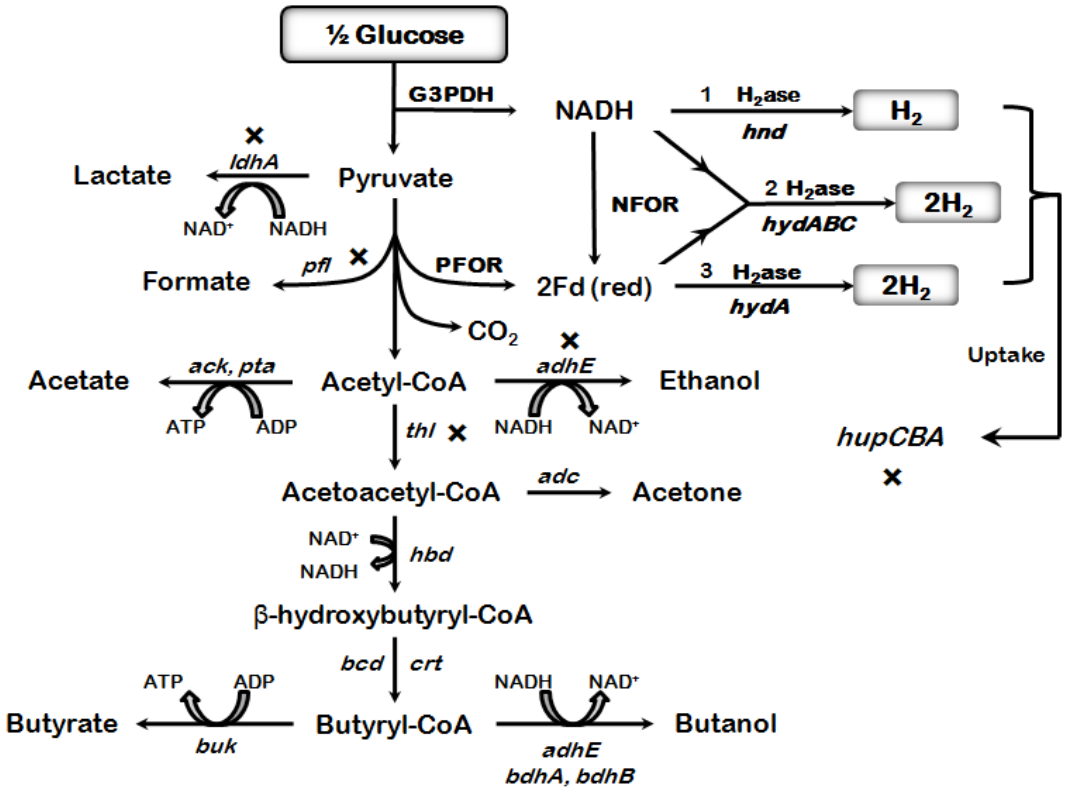

Figure 11. Metabolic engineering possibilities to enhance hydrogen production in Clostridia. Depending upon the species, hydrogen can be produced via PFOR and NFOR pathways or PFL. The maximum theoretical hydrogen yield is 4 mols of H_2 per mol of glucose. Glucose degradation potentially results in lactate, acetate, ethanol, acetone, butanol and butyrate as fermentation end-products in addition to H_2 and CO_2. G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PFOR, pyruvate ferredoxin oxidoreductase; NFOR, NADH:ferredoxin oxidoreductase. Three different types of (FeFe) hydrogenases have been described in Clostridia. HydA, Fd-dependent hydrogenase, HndABCD, a NADP-dependent hydrogenase, and HydABC, a novel bifurcating hydrogenase that reacts simultaneously with NADH and reduced ferredoxin; L-Lactate dehydrogenase *(ldhA)*; aldehyde- alcohol dehydrogenase *(adhE)*; phosphotransacetylase *(pta)*, thiolase *(thl)*; acetoacetate decarboxylase *(adc)*; β-hydroxybutyryl-CoA dehydrogenase *(hbd)*; crotonase *(crt)*; butyryl-CoA dehydrogenase *(bcd)*; butanol dehydrogenase A and B *(bdhA* and *bdhB)*; butyrate kinase *(buk)*. (X) Crosses indicate possible chromosomal gene knockouts for enhancing hydrogen production.[3]

could affect hydrogen production. The modification of global transcriptional regulators to modulate the expression of multiple operons required for the biosynthesis of formate hydrogen lyase represents one approach to improving hydrogen production. The engineered *E. coli* strains ZF1 (Δ*focA*; disrupted in a formate transporter gene) and ZF3 (Δ*narL*; disrupted in a global transcriptional regulator gene) produced 14.9 and 14.4 mmols of H_2/ mg of dry cell weight, respectively, compared to 9.8 mmols of H_2/ mg of dry cell weight generated by wild-type *E. coli* strain W3110. The H_2 yield for strain ZF3 was 0.96 mols H_2/ mol glucose, compared to 0.54 mols H_2/ mol glucose for the wild-type *E. coli* strain. The expression of the global transcriptional regulator protein FNR at levels above natural abundance had a synergistic effect on increasing the H_2 yield in the Δ*focA* genetic background (Fan et al., 2009).[3]

Finally, although one of the advantages of *E. coli* is its ability to produce hydrogen from a wide range of substrates (Ghosh and Hallenbeck, 2009), including sugars typically derived from deconstruction of lignocellulose, increasing its substrate range further would make accessible a number of potentially abundant substrates. Thus, transformation of the pUR400 plasmid encoding invertase activity to an *E. coli* strain allowed it to utilize sucrose (available in crude form as a waste product of the sugar refining industry), resulting in 1.38 ml H_2 per mg cell dry weight (Penfold and Macaskie, 2004). Likewise, Dharmadi et al. (2006) reported that glycerol can be utilized in dark fermentations by *E. coli*, with hydrogen as a major product. The abundance of glycerol as a waste by-product of biodiesel production and its highly reduced state make it an attractive substrate for future biohydrogen production in dark fermentation processes (Dharmadi et al., 2006; Murarka et al., 2007; Gonzalez et al., 2008).[3]

4.2.2 Engineering efficient hydrogenases

One of the most common approaches that has been tried in order to enhance hydrogen productivity in microorganisms is engineering the production of a stable

hydrogenase through expression in a heterologous host. Some examples are presented here. Recombinant strains can be developed through genetic engineering, which leads to higher levels of hydrogen production. The hydrogen yield is low in many organisms containing uptake hydrogenases, as some of the produced hydrogen is consumed. Therefore, knocking out the genes encoding uptake hydrogenases is one way to enhance hydrogen production. Different examples of the heterologous expression of hydrogenase are given in Table 2.[3]

Escherichia coli is an ideal microorganism commonly used in genetic engineering due to its well-characterized genome, well known metabolism, and its ability to utilize a wide range of carbon sources including hexoses and pentoses. In addition, a metabolic pathway database, *EcoCyc*, is available, allowing for metabolic pathway prediction (Keseler et al., 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 as well as tolerance to oxygen (Hallenbeck et al., 2011).[3]

E. coli possess four [Ni-Fe]-hydrogenases. Two hydrogenases, hydrogenase 1 and 2, are involved in periplasmic hydrogen uptake and they are multi-subunit, membrane-bound, nickel-containing Fe/S proteins encoded by the *hya* and *hyb* operons, respectively (Richard et al., 1999). Two others, hydrogenases 3 and 4, are part of cytoplasmically oriented formate hydrogenase complexes (Sawers, 1994; Vignais et al., 2001; Self et al., 2004; Sawers, 2005). Hydrogenase 3, encoded by the *hyc* operon, produces hydrogen from formate as a part of the formate hydrogen lyase complex (FHL-1), which is active during mixed-acid fermentation at slightly acidic pH. However, hydrogenase 3 has been reported to not only have hydrogen evolution activity but also hydrogen oxidation activity under proper conditions (Sauter et al., 1992; Bagramyan and Trchounian, 2003; Maeda et al., 2007b). Hydrogenase 4, encoded by the *hyf* operon, has been reported to produce hydrogen as part of the FHL-2 complex at slightly alkaline pH (Andrews et al., 1997; Bagramyan and

Trchounian, 2003). However, under normal circumstances hydrogenase 4 appears to be cryptic with insignificant expression in the wild-type strain (Self et al., 2004).[3]

Maeda et al. (2007c) reported enhanced hydrogen production in *E. coli* cells expressing the bidirectional hydrogenase (encoded by *hoxEFUYH*) from the cyanobacterium *Synechocystis* sp. PCC 6803. It was suggested that up to 41- fold increase in hydrogen yields was caused by suppression of the hydrogen uptake activity of hydrogenase 1 and hydrogenase 2, although it is difficult to see by what mechanism this might occur. One straightforward means to increase hydrogen yields by decreasing hydrogen uptake activities is to create strains carrying a double deletion of hydrogenase 1 and hydrogenase 2 (reviewed in: Mathews and Wang, 2009). Likewise, improvements in H_2 accumulation can be sought by disrupting the maturation of uptake hydrogenases. Penfold et al. (2006) investigated the effect of deleting the genes (*tatC* and *tatA-E*) encoding the twin-arginine translocation (Tat) system on H_2 production by *E. coli* strain MC4100 and its formate hydrogenlyase upregulated mutant (Δ*hycA*). Inactivation of the Tat system prevents correct assembly of the uptake hydrogenases and formate dehydrogenases in the cytoplasmic membrane and it is postulated that the subsequent loss of basal levels of respiratory-linked hydrogen and formate oxidation accounts for the observed increases in formate-dependent hydrogen evolution.[3]

The first random protein engineering of a hydrogenase was achieved by Maeda et al. (2008b). They engineered HycE for increased hydrogen evolution by an error-prone polymerase chain reaction (epPCR) and DNA shuffling using a host that lacked hydrogenase activity since it carried mutations in *hyaB*, *hybC*, and *hycE*. HycE, The large subunit (569 amino acids) of *E. coli* hydrogenase 3 produces hydrogen from formate via its Ni–Fe site. Seven enhanced HycE variants were obtained due to a 74-amino acid carboxy-terminal truncation. Saturation mutagenesis at T366 of HycE also led to increased hydrogen production via a truncation at this position; hence, deletion of 204 amino acids at the carboxy terminus increased hydrogen production by 30-fold.[3]

The small subunit of the uptake hydrogenase encoded by *hybO*, was targeted for genetic knockout in the *Enterobacter aerogenes* genome for improving hydrogen production. The pYM-Red recombination system was adopted to form an insertional mutation in the *Enterobacter aerogenes* genome, thereby creating mutant strain IAM1183-O (Δ*hybO*). Hydrogen production experiments with this mutant (Δ*hybO*), showed that the maximum specific hydrogen productivity of IAM1183-O was 2747± 13 ml h^{-1} g^{-1}dry cell weight, higher than that of the wild-type strain, 2321± 15 ml h^{-1} g^{-1}dry cell weight. The H_2 yield of the IAM1183-O mutant was 0.78 mols H_2/ mol glucose, somewhat higher than the wild-type, 0.65 mols H_2/ mol glucose (Zhao et al. 2009).[3]

Of course, another way to potentially increase hydrogen production would be through the heterologous expression of hydrogenase. Many early attempts to express a [FeFe]-hydrogenase in *E. coli* by overexpressing *hydA* from organisms such as *Clostridium* were unsuccessful and remained unreported (Posewitz et al., 2004). Later, it was shown that in order to successfully express functional [FeFe]-hydrogenase it is also necessary to co-express three maturation genes, *hydE*, *hydF*, and *hydG*, that are required for H-cluster maturation and insertion if the organism does not possess these genes (i.e. *E. coli*) (King et al., 2006). Thus, reports of hydrogenase activity where these additional genes were not expressed (Karube et al., 1983; Asada et al., 2000; Chittibabu et al., 2006) are highly suspect.[3]

A stable [FeFe]-hydrogenase expression system has been achieved in *E. coli* by cloning and coexpression of *hydE*, *hydF*, and *hydG* from the bacterium *Clostridium acetobutylicum*. Coexpression of the *C. acetobutylicum* maturation proteins with various algal and bacterial [FeFe]-hydrogenases in *E. coli* resulted in purified enzymes with specific activities that were similar to those of the enzymes purified from native sources. In the case of structurally complex [FeFe]-hydrogenases, maturation of the catalytic sites could occur in the absence of an accessory iron-sulfur cluster domain. Initial investigations of the structure and function of the maturation proteins HydE, HydF, and HydG showed that the highly

conserved radical-SAM domains of both HydE and HydG and the GTPase domain of HydF were essential for achieving biosynthesis of active [FeFe]-hydrogenases. Together, these results demonstrate that the catalytic domain and a functionally complete set of Hyd maturation proteins are fundamental to achieving biosynthesis of catalytically active [FeFe]-hydrogenases in *E. coli* (King et al., 2006).[3]

On the other hand, heterologous expression of *hydA* is simpler and possible without the heterologous expression of the accessory genes if these are encoded by the genome of the host bacterium. Thus, the hydrogenase gene from *Enterobacter cloacae* (IIT-BT 08) was amplified and inserted into a prokaryotic expression vector to create a recombinant plasmid (pGEX-4T-2-Cat/hydA). The recombinant plasmid was transformed into a hydrogen producing strain of *Enterobacter aerogenes* (ATCC13408), increasing the hydrogen yield from 440 ml/g glucose in the wild-type to 860 ml/g glucose in the recombinant (Zhao et al., 2010).[3]

Although *E. coli* is perhaps the most useful organism as a target for metabolic engineering, lack of NADH-dependent hydrogenase is one major hurdle for the engineering of hydrogen metabolism in this organism. Several types of NAD(P)H dependent hydrogenases are known, from soluble [NiFe]-hydrogenases (*Ralstonia*) to [FeFe]-hydrogenases. One such [FeFe]-hydrogenase was identified by Malki et al. (1995) who cloned and sequenced a genomic DNA fragment from *Desulfovibrio fructosovorans*, which strongly hybridized with the *hydAB* genes from *D. vulgaris* Hildenborough. The fragment was found to contain four genes, *hndA*, *hndB*, *hndC*, and *hndD* on a *hnd* operon that encodes an NADP-dependent hydrogenase in *D. fructosovorans*.[3]

Likewise, it might be possible to use a NAD-dependent [NiFe]-hydrogenase, but until recently there have been few reports of the successful heterologous expression of this class of hydrogenases, apart from the successful expression of a [NiFe]-hydrogenase from *Desulfovibrio gigas* in *D. fructosovorans* (Rousset et al., 1998). Recently, a study on the production and engineering of recombinant [NiFe]-

hydrogenases demonstrated the successful expression in *E. coli* of a recombinant cytoplasmic, NADP-dependent hydrogenase from *Pyrococcus furiosus*, an anaerobic hyperthermophile. This was achieved using novel expression vectors for the co-expression of thirteen *P. furiosus* genes (four structural genes encoding the hydrogenase and nine encoding maturation proteins). Remarkably, the native *E. coli* maturation machinery could also generate a functional hydrogenase when provided with only the genes encoding the hydrogenase subunits and a single protease from *P. furiosus,* the hydrogenase-specific protease required for C-terminal cleavage during insertion of the NiFe active site. Another novel feature is that their expression was induced by anaerobic conditions, whereby *E. coli* was grown aerobically and production of recombinant hydrogenase was achieved by simply changing the gas feed from air to an inert gas (N) (Sun et al., 2010).[3]

Table 2. Examples of heterologus expression of hydrogenases [3]

Organism	Description	References
E. coli HK16	Expression of hydrogenase gene from *C. butyricum* in *E. coli* strain HK16 lacking the native hydrogenase activity.	Karube et al. (1983)
E. coli	Coexpression of maturation genes *hydE*, *hydF*, and *hydG* from the *C. acetobutylicum* supported biosynthesis of active [FeFe]-hydrogenases from different sources.	King et al. (2006)
E. coli BL-21	Expressionof [FeFe]-hydrogenase (*hydA*) from *E. cloacae* IIT-BT 08.	Chittibabu et al. (2006)
E. coli	Developing recombinant *E. coli* with NADH-dependent hydrogenase from *E. cloacae*	Cho et al. (2006)
E. coli TG1/pBS(Kan)Synhox	Expression of bidirectional [NiFe]-hydrogenase from *Synechocystis* sp. PCC 6803 supresses native hydrogen uptake activity.	Maeda et al. (2007c)
E. coli	Coexpression of thirteen *P. furiosus* genes encoding NADP-dependent hydrogenase and maturation genes from *Pyrococcus furiosus.*	Sun et al. (2010)
Enterobacter aerogenes (ATCC13408)	Creation of *E. aerogenes* (ATCC13408) strains with recombinant hydrogenases from *E. cloacae* (IIT-BT 08).	Zhao et al. (2010)
Desulfovibrio fructosovorans	*D. fructosovorans* had been hybridized with the *hydAB* genes from *D. vulgaris* Hildenborough and resulting in developing recombinant NADP-dependent hydrogenase.	Malki et al. (1995)
D. fructosovorans	Expression of [NiFe]-hydrogenase from *D. gigas*.	Rousset et al. (1998)

5. Objectives of the present study

The overall purpose of the research presented in this thesis is to attempt to improve fermentative hydrogen production through physiological manipulation and metabolic engineering.

The main purpose in carrying out physiological manipulation is to use metabolic energy to generate extra hydrogen production. Our hypothesis is that the reducing energy required for driving substrate conversion further towards completion for more hydrogen production can be obtained via microaerobic dark fermentation and/or photofermentation of organic substrates.

Thus, the microaerobic dark fermentation study aimed to verify the principal of using a small amount of respiration with oxygen to furnish the excess reducing energy via oxidative phosphorylation with the induction of the TCA cycle, consequently, extra hydrogen gas might be obtained using dark system instead of using light energy.

The photofermentation studies were focused on improving hydrogen yields from glucose using *Rhodobacter capsulatus* JP91 either in batch and/or continous culture conditions with some technical challenges to establish the proper operational conditions for greater hydrogen yields.

The second focus of this thesis was to use metabolic engineering for improving fermentative hydrogen. The major objective was to engineer efficient hydrogenases using heterologous expression and maturation of [FeFe]-hydrogenases in *E. coli*. The first study aimed to express the orphan *hydA* of *Rhodospirillum rubrum* in *E. coli* with maturation genes from *Desulfovibrio vulgaris* or *Clostridium acetobutylicum*, whereas the second study aimed to express a NADP-dependent [FeFe]-hydrogenase from *Desulfovibrio fructosovorans* with maturation genes from *Clostridium acetobutylicum*.

CHAPTER 1: Microaerobic dark fermentative hydrogen production by the photosynthetic bacterium, *Rhodobacter capsulatus* JP91

Mona Abo-Hashesh[a,b], Patrick C. Hallenbeck[a*]

[a] Département de Microbiologie et Immunologie, Université de Montréal, CP 6128 Succursale Centre-ville, Montréal, Québec, Canada H3C 3J7

[b] Department of Microbiology, Port Said University, Port Said, Egypt

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Microaerobic dark fermentative hydrogen production by the photosynthetic bacterium, *Rhodobacter capsulatus* JP91

Mona Abo-Hashesh[a,b], Patrick C. Hallenbeck[a*]

[a] Département de Microbiologie et Immunologie, Université de Montréal, CP 6128 Succursale Centre-ville, Montréal, Québec, Canada H3C 3J7

[b] Department of Microbiology, Port Said University, Port Said, Egypt

[*]Corresponding author

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ABSTRACT

The photosynthetic bacterium *Rhodobacter capsulatus* produces hydrogen under nitrogen-limited, anaerobic, photosynthetic conditions. The present study examined whether *R. capsulatus* can produce hydrogen under microaerobic conditions in the dark with limiting amounts of O_2 and fixed nitrogen. The relationship between hydrogen production, different O_2 concentrations and carbon sources as well as two different nitrogen sources, glutamate and ammonium, were studied in batch culture using uptake-hydrogenase deficient mutant (*hup*$^-$) *R. capsulatus* JP91. The effect of different O_2 concentrations, ranging from 0.5 to 20%, on hydrogen production was examined in dark batch cultures of *R. capsulatus* grown on RCV medium. Different carbon sources, e.g. glucose, succinate, lactate, acetate and malate, were used at various concentrations (20–40 mM). Similarly, different concentrations of glutamate and ammonium (2–9 mM) were examined for optimum microaerobic dark hydrogen production. Maximum hydrogen production was observed at an O_2 concentration of 4–8%. There was a highly positive correlation between O_2 and growth (r^2 = 0.67), whereas O_2 concentration and hydrogen productivity were negatively correlated (r^2 = -0.3). Succinate (25 mM) together with glutamate (3.5 mM) gave the highest specific hydrogen productivity [5.61 μmols hydrogen/ (mg cell dry weight/ml)]. The maximum average hydrogen yield was 0.6 mol hydrogen/ mol malate followed by 0.41 mol hydrogen/ mol lactate, 0.36 mol hydrogen/ mol succinate, whereas minimum amounts of hydrogen were produced from glucose and acetate (0.16 mol hydrogen/mol and 0.07 mol hydrogen/mol, respectively). The implications for developing a system capable of improved hydrogen production are discussed.

INTRODUCTION

Dark fermentative hydrogen production is one of the promising approaches for biological hydrogen production using anaerobic or facultative anaerobic bacteria. The major advantages in using this process include no light input, possible simultaneous waste treatment and simple reactor technology [1, 2]. Despite these features, there are major obstacles to practical development of this process since fermentative hydrogen yields are limited metabolically to 4 mols of hydrogen per mol of glucose while theoretically 12 mols of hydrogen could be produced from 1 mol of glucose [3, 4].

Low hydrogen yields result from incomplete oxidation of organic compounds, and the need to produce large amounts of reduced compounds to maintain redox poise within the metabolic pathways. Complete oxidation of organic compounds is thermodynamically unfavorable due to various factors, including the oxidation state of the substrate and redox potential equilibrium. Additional energy is required to drive H_2 production from substrates with low negative redox potentials [2, 5]. One process that can be used for this purpose is photofermentation of organic compounds using photosynthetic bacteria where light energy is converted into chemical energy which can then be used for reverse electron transfer, thus releasing more electrons and protons from the feedstock [2, 6]. However, using a photofermentation approach for hydrogen production is not currently cost-effective since it suffers from low light conversion efficiencies, low hydrogen production rates and a requirement for expensive hydrogen impermeable photobioreactors [5, 7]. In the present work, we examine the hypothesis that oxidative phosphorylation might provide the required reducing energy instead of photophosphorylation.

Oxidative phosphorylation is a metabolic pathway that uses the free energy released by the oxidation of nutrients to produce a proton gradient that can then be used to produce adenosine triphosphate (ATP). Maximum energy is generated when the TCA cycle is induced upon exposure to O_2, allowing complete substrate

degradation and production of large amounts of NADH, donor to the aerobic respiratory chain [8, 9]. The enzymes involved in hydrogen production, whether nitrogenase (N_2ase) in photofermentation processes, or hydrogenases in dark fermentation processes, are extremely sensitive to O_2 [10]. Based on the principal of using metabolic energy to drive extra hydrogen production in dark fermentative process, we are testing whether microaerobic fermentation, using a small amount of respiration with oxygen, could furnish energy for hydrogen production while at the same time avoiding the destruction of the enzymes involved in proton reduction; nitrogenase and hydrogenases. A scheme for hydrogen production using photosynthetic bacteria cultured in the dark under microaerobic condition is presented in figure 1. Nitrogenase is the key enzyme functioning in hydrogen production in the photosynthetic bacteria. N_2ase requires a great deal of chemical energy, ATP and low potential electrons, to drive reduction of protons to hydrogen. Although the required energy is commonly provided via photophosphorylation, the present work was undertaken to assess whether microaerobiosis could provide the required energy for nitrogenase activity. This would involve reverse electron transport to release electrons from the feedstock.

During fermentation, bacteria convert substrates such as glucose or other carbon sources to energy for growth and maintenance, producing products such as organic acids, alcohols and hydrogen according to their metabolic pathways. Maintaining a proper C/N ratio is thought to be necessary to optimize anaerobic hydrogen production [11]. Therefore, it was important in the present study to investigate the effect of different concentrations of carbon and nitrogen sources on bacterial growth and hydrogen production under dark microaerobic conditions.

MATERIALS AND METHODS

Bacterial strain and culture conditions

A purple non-sulfur (PNS) photosynthetic bacterium, *Rhodobacter capsulatus*, capable microaerobic nitrogen fixation was used to demonstrate microaerobic hydrogen production using batch cultures under dark conditions. The *R. capsulatus* strain, JP91 [12], a markerless *hup*$^-$ derivative (IS21 insertion in a *hup* (hydrogen uptake) gene) of the wild-type strain, B10 [13], was kindly provided by Dr. John Willison. Precultures were made in screw cap tubes filled with RCV [14] supplemented with sodium lactate and ammonium sulfate as carbon and nitrogen sources. The tubes were incubated at 30°C in a light chamber for optimum growth. Then, cells were washed, collected by centrifugation at 5600 x*g* for 4 min, and resuspended in RCV medium without carbon and nitrogen sources for further experimental investigation.

Screening of optimum parameters for microaerobic dark hydrogen production

Oxygen concentration

JP91 was cultured in RCV medium supplemented with succinate (25 mM) and glutamate (2 mM). The cell density was adjusted to ~ 0.5 mg cell dry weight (CDW)/ml. Different amounts of oxygen were injected into sealed tubes to obtain concentrations ranging from 0.5 to 20%. The tubes were incubated in the dark at 30°C for a maximum of 7 days. Gas sampling to check hydrogen production was done every 24 h.

Carbon source

Examination of the effect of various concentrations of different carbon sources on hydrogen production was done using glucose, succinate, lactate, acetate and malate at concentrations ranging from 20 to 40 mM. The supplemented cultures were incubated under microaerobic dark conditions for 7 days. Oxygen was injected into each tube to provide microaerobic conditions (8% O_2). Glutamate (2 mM) was used as a nitrogen source.

Nitrogen source

Different concentrations of sodium glutamate and ammonium sulfate (2–9 mM) were examined for maximum hydrogen production under microaerobic dark conditions (8% O_2) for 7 days. Twenty-five-millimolar succinate was used as a carbon source.

Analytical procedures

The CDWs of cultures were calculated using a CDW standard curve. *Rhodobacter capsulatus* JP91 cell suspensions of different biomass concentrations [optical density at 600 nm (OD_{600nm})] were placed in pre-weighed plastic microcentrifuges, centrifuged and the pellets were dried at 100°C until the weight remained constant. The dried tubes were weighed, and a CDW versus OD standard curve was developed which gave a linear relationship between OD and CDW. Hydrogen gas accumulated in the head space was detected using a gas chromatograph (Shimadzu GC-8A) equipped with a thermal conductivity detector, a 2 m column packed with molecular sieve 5A and argon as carrier gas. Bacterial growth was determined by measuring the OD_{600nm} using a double-beam spectrophotometer (Shimadzu). High-performance liquid chromatography (HPLC) analysis of the organic acids in the culture media were carried out using a Beckman-Coulter HPLC Gold system, a model 126 solvent module, a model 168 UV spectrophotometric detector. The 32 karat software (Beckman-Coulter) was used to analyze the recorded HPLC spectra. Glucose concentrations in the culture media were measured using a glucose oxidase assay [15].

Detection of nitrogenase activity and N_2ase proteins

Nitrogenase activities of samples were determined by acetylene reduction assay as described previously [16]. The *in vivo* nitrogenase activity of the microaerobic cultures was detected under dark and light conditions in a shaking water

bath maintained at 30°C. The concentration of ethylene present in the gas phase was determined using gas chromatography. Results are presented as the total amounts of ethylene produced per vial. Immunoblotting with chemiluminescence detection was performed to detect N_2ase Fe and Mo–Fe proteins as described previously [17]. Samples were anaerobically withdrawn from the test vials into 3X sample buffer and quickly frozen at -20°C for further analyses [sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting].

Statistical analyses of data

Data are presented in terms of mean, standard deviation and standard error using the data analysis tools of Microsoft Excel and SPSS version 17. K-related samples analysis and correlation analysis tests were used to test the significance between variables at $P < 0.05$. Statistics were also performed using the data tool of SPSS version 17. *P*-value was set at < 0.05 for significant results and < 0.01 for highly significant results. Correlation bivariate analysis was used to find the correlation between continuous quantitative data. The r^2-value was Pearson's correlation coefficient, which measures the strength of the relation between the studied variables.

RESULTS AND DISCUSSION

Growth and O_2 concentration were directly proportional until growth started to decline at high O_2 concentrations (> 20%; data not shown). A highly positive correlation was observed between the growth and O_2 concentration ($r^2 = 0.7$) (Figure 2). On the other hand, there were negative correlations between O_2 concentration and the total amount of H_2 as well as specific H_2 productivity (r^2 = -0.28 and -0.3, respectively). The maximum specific H_2 productivity was obtained at 4, 5 and 6% O_2, 5.32, 5.42 and 5.22 µmols/ (mg CDW/ml), respectively (Figure 2). Previously, it was reported that addition of small amounts of oxygen stimulated hydrogen production under low light conditions by the marine non-sulfur photosynthetic

bacterium, *Rhodovulum* sp. [18]. However, the results reported in the present study represent the first report of dark microaerobic hydrogen production. In addition, the results showed that nitrogenase was active under these conditions, consistent with a previous study that examined the activity and expression of nitrogenase in *R. capsulatus* in the presence of oxygen under dark and light conditions and which concluded that high oxygen concentrations, although influencing enzyme activity, do not inhibit N_2ase expression [19].

The effects of different concentrations of carbon sources on growth and hydrogen production were also examined (Figure 3). The maximum growth was observed with supplementation of glucose (20–40 mM) when compared with other carbon sources tested. There were negative correlations between the growth and the concentration of succinate, lactate and malate (r^2 = -0.68, -0.71, -0.88, respectively). In contrast, there was a high positive correlation between the growth and the concentration of acetate (r^2 = 0.86). Similarly, negative correlations were observed between the specific H_2 productivity and concentrations of glucose, succinate and malate (r^2 = -0.90, -0.77, -0.84, respectively) while it was highly positive in the case of acetate (r^2 = 0.895). This may be due to the chemical structure and oxidation state of acetate ($C_2H_4O_2$) and the relationship between C/N ratio and H_2 production. In a previous study where acetate was used as a carbon source for hydrogen production by photosynthetic bacteria, the acetate concentration was found to strongly affect H_2 production and biomass concentration with a decrease in acetate from 22 to 6 mM resulting in an 87% decrease in the hydrogen evolved [20].

The optimum carbon source and concentration for producing hydrogen in this study was succinate at 25 mM where hydrogen productivity reached 3.7 μmols/ (mg CDW/ml). Lactate gave a broad maximum H_2 productivity within a range of different concentrations. Therefore, variations in both the kind of carbon sources and its concentration considerably influence microaerobic hydrogen production by nitrogenase. Different carbon sources with different oxidation states could cause

variation in electron flux to nitrogenase and therefore influence hydrogen production [21].

Relative hydrogen production from different substrates has been examined to some extent using photosynthetic bacteria under photofermentative conditions. In fact, rates of hydrogen production and relative yields vary with substrate and the variation is different with different species. A previous study has shown that, for *R. capsulatus*, lactate gave higher H_2 production rates than either malate or succinate (acetate was not tested) [22]. However, as this is the first report of microaerobic hydrogen production by a photosynthetic bacterium in the absence of light and photosynthesis, it is not clear to what extent the results of the present study can be compared with previous studies. It is very likely that metabolic pathways, and certainly metabolic fluxes, are different under respiratory conditions. A fuller understanding of the differences observed here will require more detailed analyses, including metabolic flux analysis.

The nature of the nitrogen source and its concentration is one of the major factors influencing total bacterial biomass and hydrogen production. Thus, the influence of the nitrogen source on growth and hydrogen production was examined in some detail. As might be expected, growth increased with increasing concentrations of nitrogen source irrespective of whether the nitrogen source examined was glutamate or ammonium as confirmed statistically with high positive correlations between growth and concentrations of glutamate and ammonium (r^2 = 0.93, 0.94, respectively) (Figure 4). However, even though growth was well supported at different concentrations of fixed nitrogen, hydrogen production was sensitive to the concentration of glutamate and ammonium with maximum H_2 production at 3.5 mM glutamate [5.61 μmols/ (mg CDW/ml)] or 4.0 mM ammonium [4.11 μmols/ (mg CDW/ml)] (Figure 4). Negative correlations were observed between hydrogen production and growth (r^2 = -0.43, -0.54), as well as hydrogen production and ammonium concentrations (r^2 = -0.464). Similar observations were made previously with *R. capsulatus* cultured either as resting cells (no nitrogen source) or growing

cells (3.5 and 7.0 mM glutamate) under light conditions [23]. These results highlight the fact that an increase in glutamate concentration increases the growth rather than H_2 production. In general, a proper C/N ratio is critical in the optimization of anaerobic hydrogen production from organic substrates since under these conditions microorganisms utilize 25–30 times more carbon than nitrogen [24]. For example, one study of limiting factors in hydrogen fermentation by different strains of *E. coli* found that limitation for phosphate or sulfate had little effect whereas strains showed the highest hydrogen yields when cultured at limiting concentrations of either ammonia or glucose [11].

Hydrogen yields were determined for cultures that had been grown at optimal carbon source concentrations for hydrogen production by measuring residual substrate using HPLC and then calculating the hydrogen yield based on substrate utilization (Figure 5). Maximum average hydrogen yields were 0.6 mol hydrogen/ mol malate followed by 0.41 mol hydrogen/ mol lactate and 0.36 mol hydrogen/ mol succinate. Yields with glucose and acetate were low; 0.16 and 0.07 mol/ mol, respectively.

The purple non-sulfur (PNS) bacteria can grow anaerobically and produce hydrogen from reduced substrates such as organic acids but in our study we investigated the growth and hydrogen production under microaerobic conditions on organic acids as well as glucose. It is to be expected that different metabolic pathways might be used under microaerobic conditions when compared with anaerobic conditions. However, the yields of hydrogen obtained in this study are to some extent consistent with most studies carried out under anaerobic phototrophic conditions. Many previous studies have used malate as a model substrate for light-driven hydrogen production by photosynthetic bacteria. For example, malate was found to be a most suitable substrate for the hydrogen production using *Rhodobacter sphaeroides* O.U.001 [25–27]. In our study, the best hydrogen yield was obtained when malate was used at 20 mM together with 2 mM glutamate. Likewise, a previous study showed that the best C/N ratio of malic acid and glutamic acid was 15:2 mM

[27]. Lactate and malate are often used for comparing productivity by different strains as they are the most widely used carbon sources for H_2 production [28, 29].

The lowest hydrogen yields in this study were obtained when glucose and acetate were used as substrates. Likewise, a recent study concluded that the hydrogen production yield from acetate of *R. sphaeroides* RV was lower than that from lactate [30]. Moreover, hydrogen production from glucose by pure and co-cultures of *Clostridium butyricum* and *R. sphaeroides* have been studied in batch cultures where it was shown that pure cultures of *R. sphaeroides* produced hydrogen at lower rates than *C. butyricum* [31]. In fact, in co-culture systems, *R. sphaeroides* could not compete with *C. butyricum* for glucose even at 5.9 times higher cell numbers. In co-culture systems, *R. sphaeroides* uses the acetate and butyrate produced by *C. butyricum* as substrates for hydrogen production [31].

Hydrogen production by photosynthetic bacteria is through the nitrogenase enzyme system and requires large amounts of ATP and high-energy electrons as well as limitation for fixed nitrogen. However, the regulation of nitrogenase under dark microaerobic conditions has been little studied. Therefore, it was worthwhile to examine the levels of nitrogenase enzyme present and its activity under different conditions. It has been reported in previous studies that high concentrations of ammonium inhibit the nitrogenase enzyme by down-regulating expression of the nitrogenase genes and inhibiting enzyme activity. Inhibition is reversible and nitrogenase activity is recovered after depletion of ammonium [32–34]. Nitrogenase expression was investigated by carrying out immunological detection of the N_2ase Fe and Mo–Fe proteins under different culture conditions. The results show that, under microaerobic conditions, high concentrations of fixed nitrogen affect the expression of N_2ase proteins whereas various concentrations of different carbon sources had no effect (Figure 6). These results strongly suggest that the decline in hydrogen production with increasing concentrations of fixed nitrogen noted in the experiments depicted in figure 4 are due to the decreased synthesis of nitrogenase, as shown in figure 6.

It was also of interest to measure nitrogenase activity under controlled conditions by its ability to reduce acetylene to ethylene. N_2ase activities of the microaerobic cultures were measured under two different conditions; in the dark with the same oxygen concentration as used for growth, and anaerobically in the light (Figure 7). Activity was much higher (~10-fold) in the light, suggesting that the activity of nitrogenase in the dark microaerobic cultures was severely limited for supply of ATP and/or low potential electrons. Thus, the hydrogen production observed here under dark microaerobic conditions was not limited by the amount of nitrogenase present and suggesting that, at least in theory, substantially more H_2 could potentially be obtained under microaerobic dark conditions by optimizing operational conditions such that the enzyme present receives substantially more ATP and/or reductant.

A similar conclusion was reached in a previous study that examined the response of *R. capsulatus* to the addition of NH_4^+ under dark microoxic conditions. Different nitrogenase activities were found when the cultures were incubated in the dark than with light incubation, with activity being higher in the light [34]. It was suggested that the lower activity under dark microaerobic conditions was due to a lack of sufficient ATP generation. Future work will be aimed at establishing optimal culture conditions to maximize the use of the TCA cycle for generating greater electron flux and ATP generation required for optimum nitrogenase activity.

CONCLUSIONS

Microaerobic dark fermentation may be one way to improve hydrogen yields. Here we have shown that under the experimental conditions used the optimal O_2 concentrations for microaerobic hydrogen production are between 4 and 8%. The impact of carbon and nitrogen sources on hydrogen production were examined and it was found that maximum H_2 productivity was obtained when succinate (25 mM) and glutamate (3.5 mM) were used as carbon and nitrogen sources. On the other hand, the highest hydrogen yield (0.6) was obtained when malate (20 mM) was used, whereas

acetate gave the lowest value. Moreover, under microaerobic conditions, the optimum conditions for growth are different from that for H_2 production; a similar conclusion had been reported in previous study of photoheterotrophic hydrogen production [35]. Oxygen concentration and C/N ratio limit nitrogenase activity, which in turn influences H_2 production. The technical challenge going forward is to establish operational conditions for maximum H_2 yield, perhaps through the use of continuous cultures.

ACKNOWLEDGMENTS

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FIGURES

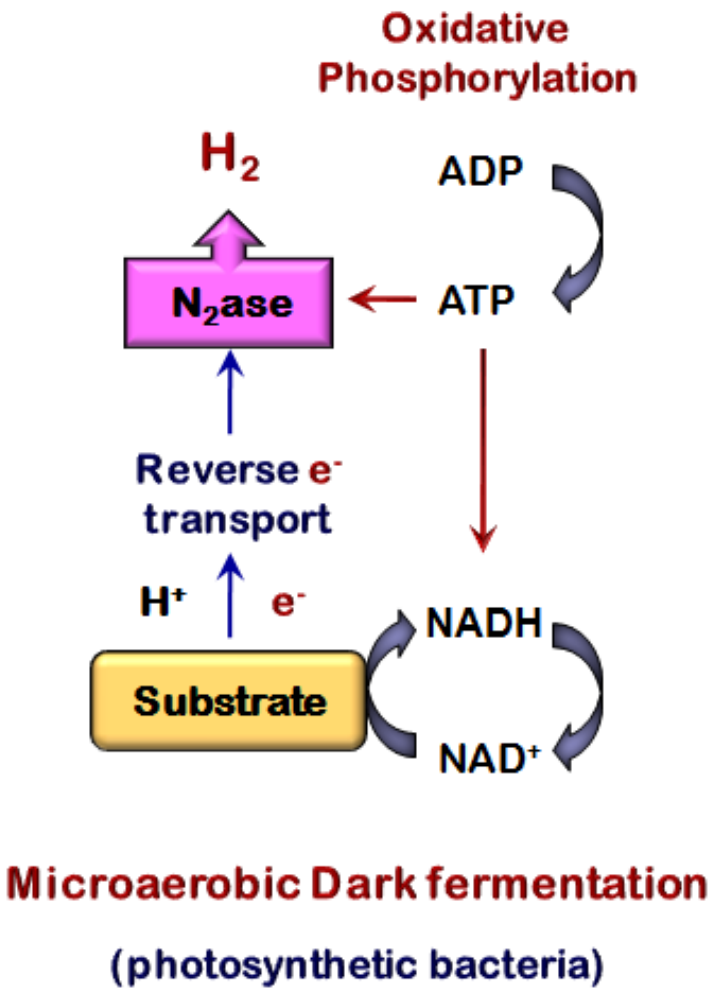

Figure 1. Schematic representation of microaerobic dark fermentative hydrogen production by N_2ase. ATP produced via oxidative phosphorylation could be harnessed to drive the reverse electron transport chain required to release more electrons and protons from the feedstock as well as nitrogenase activity required for reduction of protons to hydrogen.

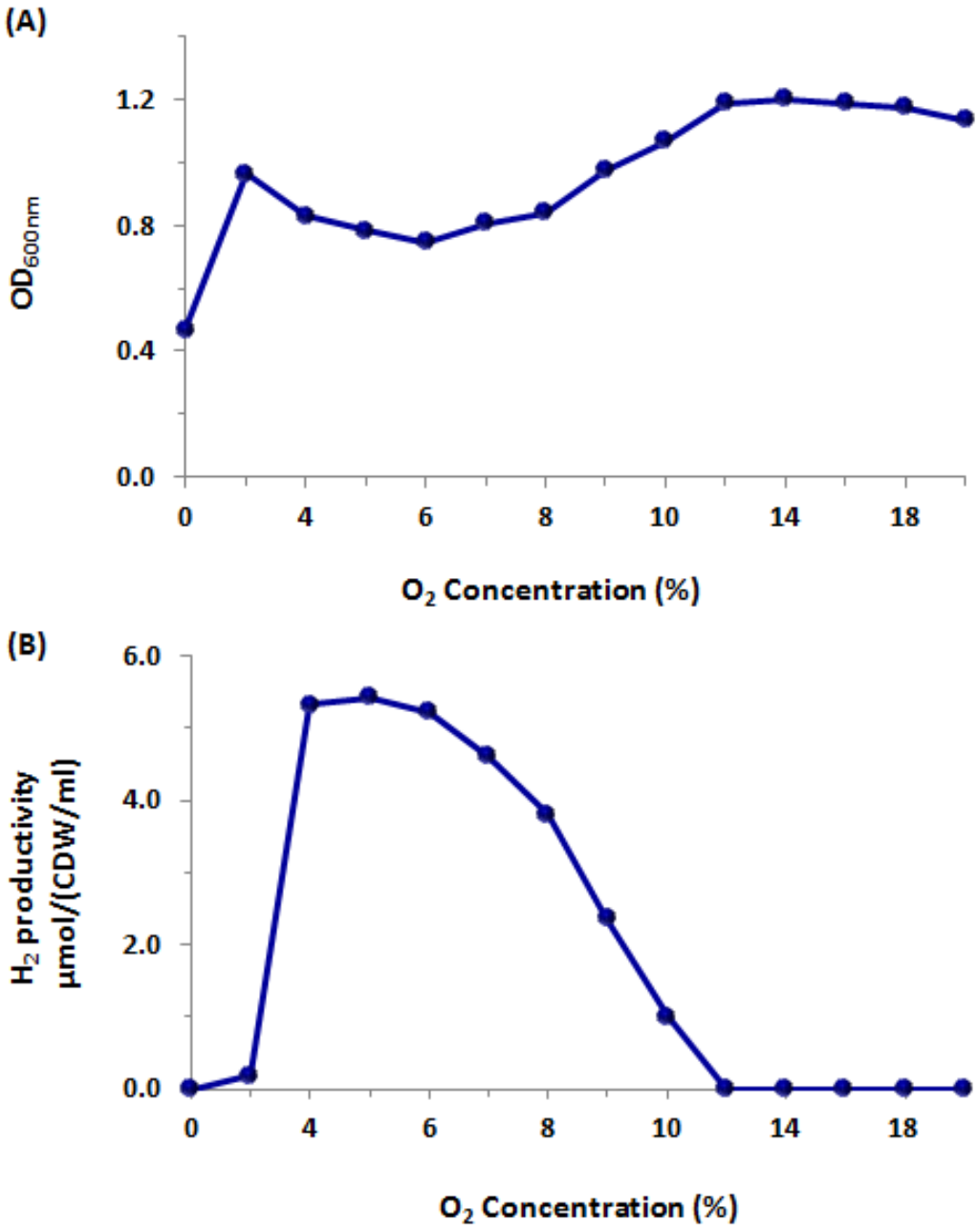

Figure 2. Effect of O_2 concentration on growth (A) and hydrogen productivity (B). (A) OD_{600nm} of *R. capsulatus* JP91 was measured at different O_2 concentrations ranged from 0 up to 20%. (B) Specific hydrogen productivity showed the highest values at O_2 concentrations, 4–8%. Succinate 25 mM and glutamate 2 mM were used as carbon and nitrogen sources, respectively.

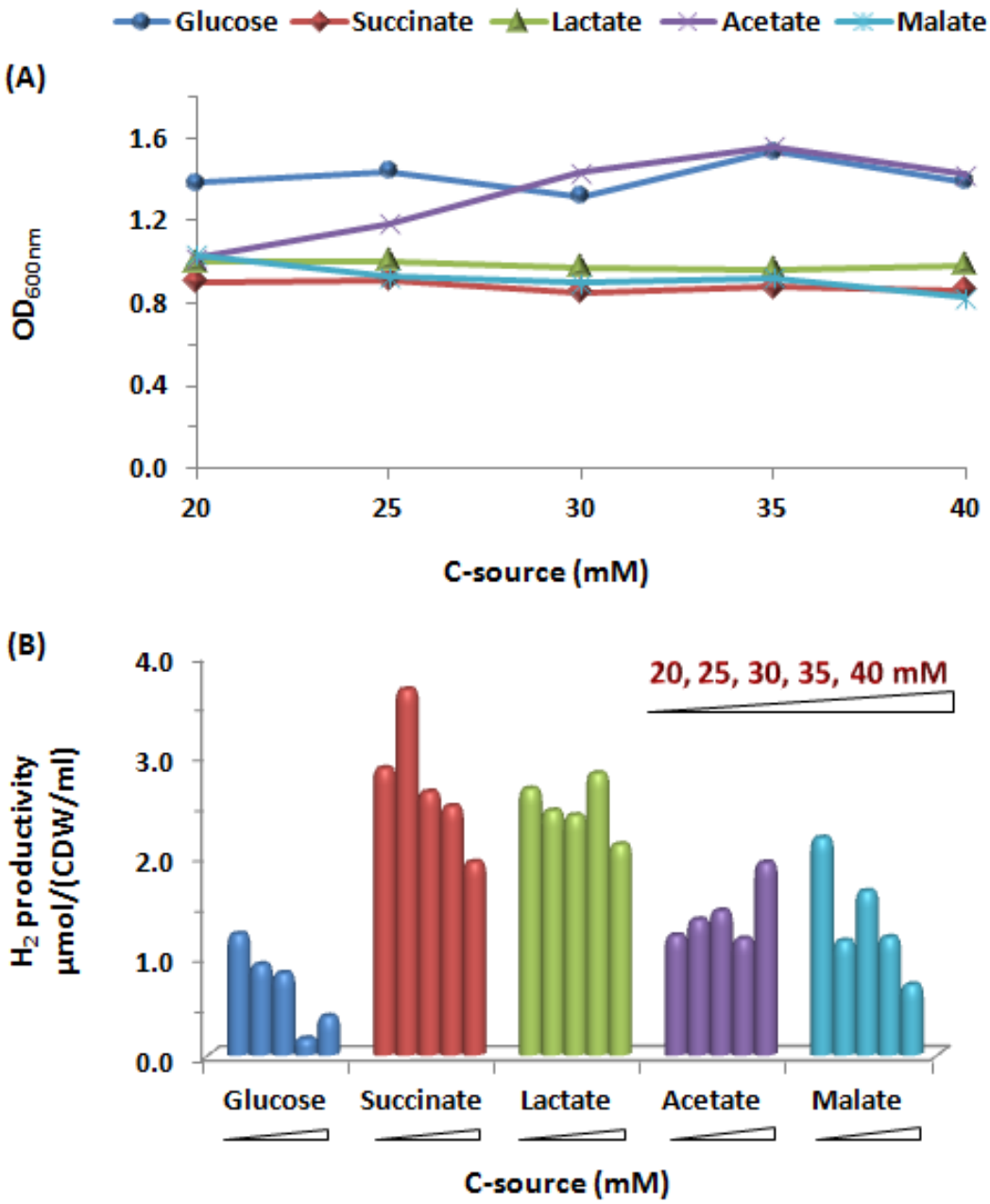

Figure 3. Effect of different concentrations of carbon sources on growth (A) and hydrogen production (B). (A) Average of four sets for values of OD_{600nm} of the cultures grown for a maximum of 7 days on different concentrations of carbon sources, SD varied between ±0.07 to ±0.49. (B) Average of specific hydrogen productivity (µmol/ mgCDW/ml), SD varied between ±0.28 to ±2.33. The carbon concentrations examined were 20, 25, 30, 35 and 40 mM, whereas glutamate 2 mM was used as a nitrogen source.

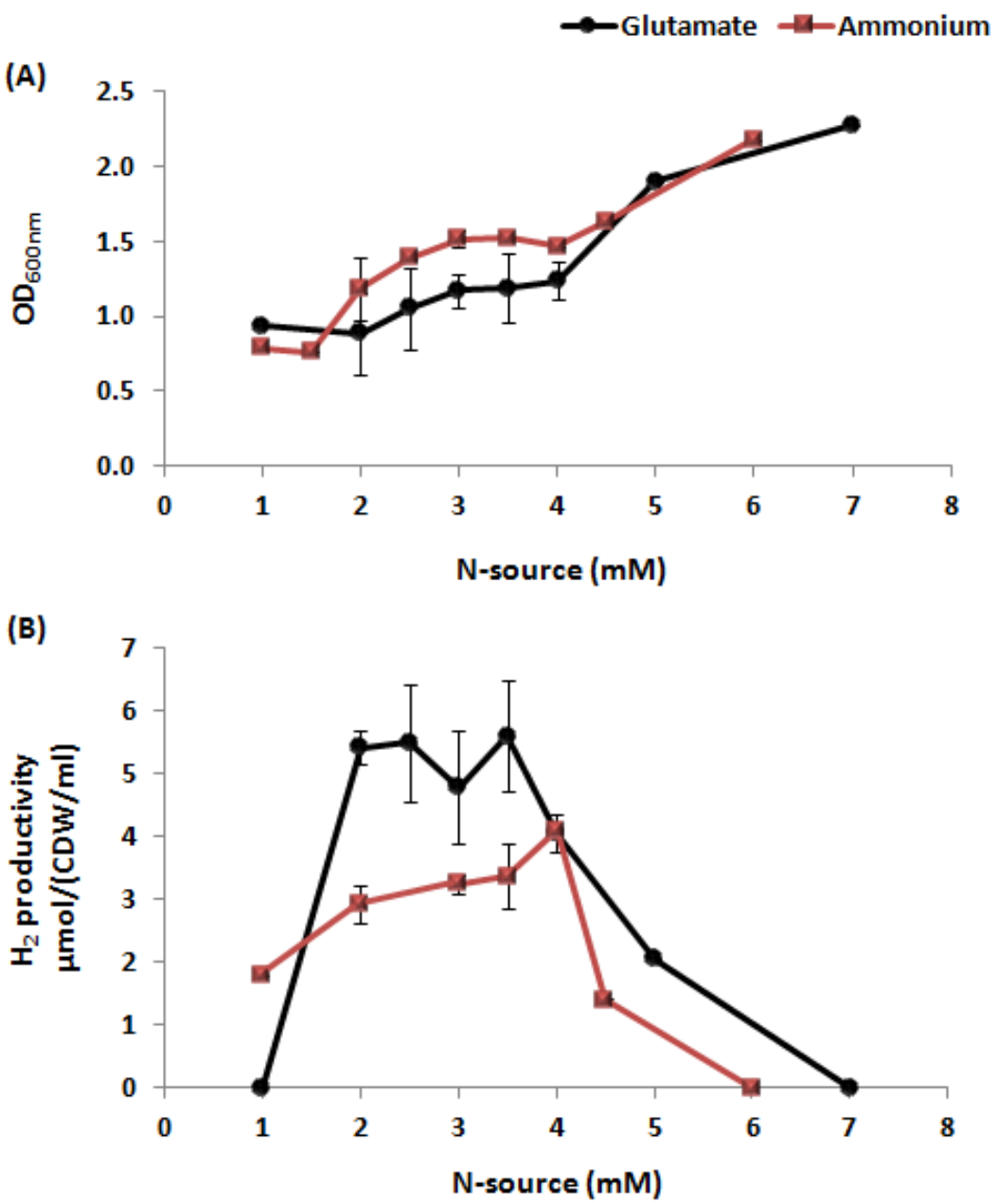

Figure 4. Impact of different concentrations of nitrogen sources on growth (A) and hydrogen production (B). (A) Average of three sets for values of OD_{600nm} of the cultures grown on different concentrations of sodium glutamate and ammonium sulfate, SD varied between ±0.0 to ±0.3. (B) Average of specific hydrogen productivity (mmol/mg CDW/ml), SD varied between ±0.0 and ±0.9. Succinate (25 mM) was used as a carbon source. The nitrogen concentrations examined ranged from 1 to 7 mM.

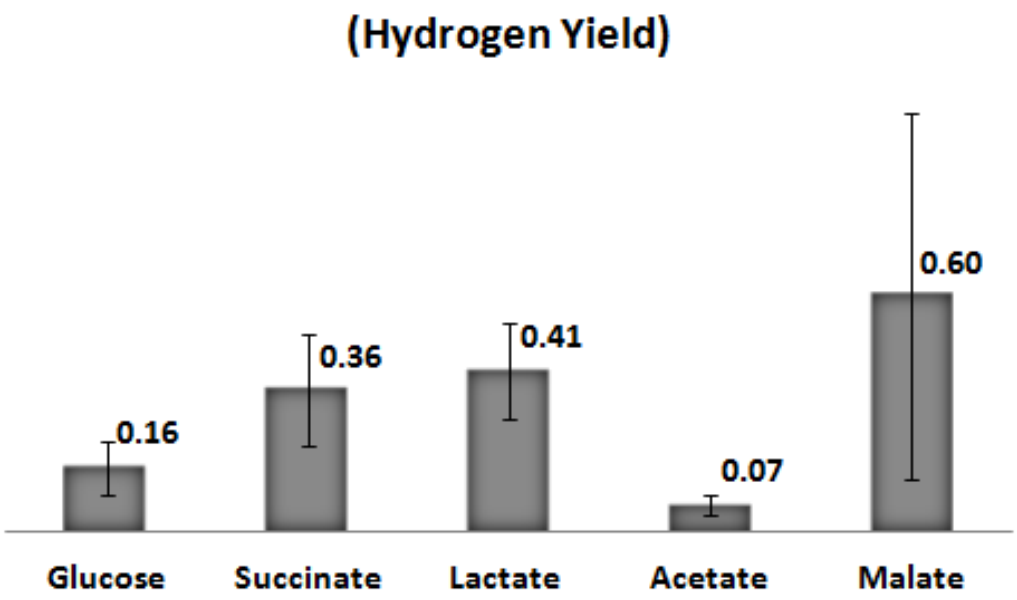

Figure 5. Yield of hydrogen on different carbon sources. Average hydrogen yields of three sets were calculated (mean±SE) for cultures grown on optimum concentrations of different carbon sources for H_2 production; 20 mM glucose, 25 mM succinate, 35 mM lactate, 40 mM acetate and 20 mM malate were used as carbon sources whereas glutamate 2 mM was used as a nitrogen source.

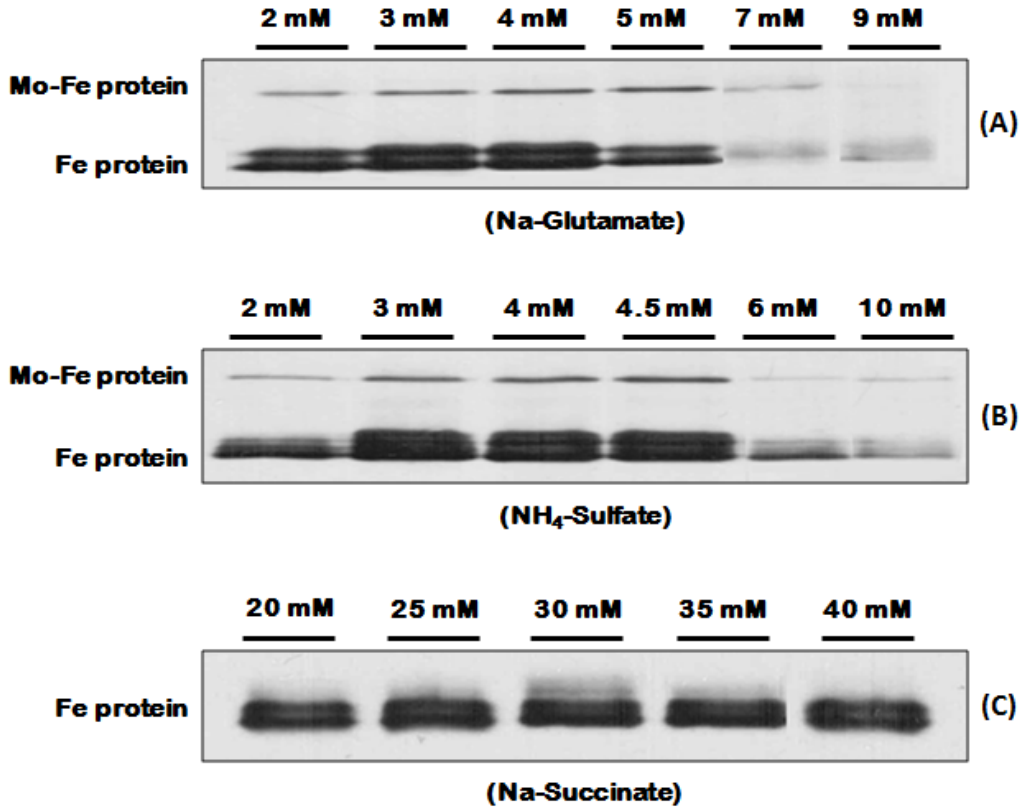

Figure 6. Effect of different concentrations of sodium glutamate (A), ammonium sulfate (B) and succinate (C) on the expression of N_2ase proteins. In (A) and (B), the media contained 25 mM succinate with the indicated nitrogen source and in (C) the media contained 2 mM glutamate with the indicated concentrations of succinate. High concentrations of both sodium glutamate and ammonium as nitrogen sources negatively affect N_2ase protein expression, whereas variation in the concentration of succinate has no effect. Nitrogenase proteins were visualized by immunoblotting. Samples were anaerobically withdrawn from test vials, subjected to SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were reacted with a mixture of polyclonal antibodies to the Mo–Fe and Fe nitrogenase proteins of *R. capsulatus* and visualized with chemiluminescence detection.

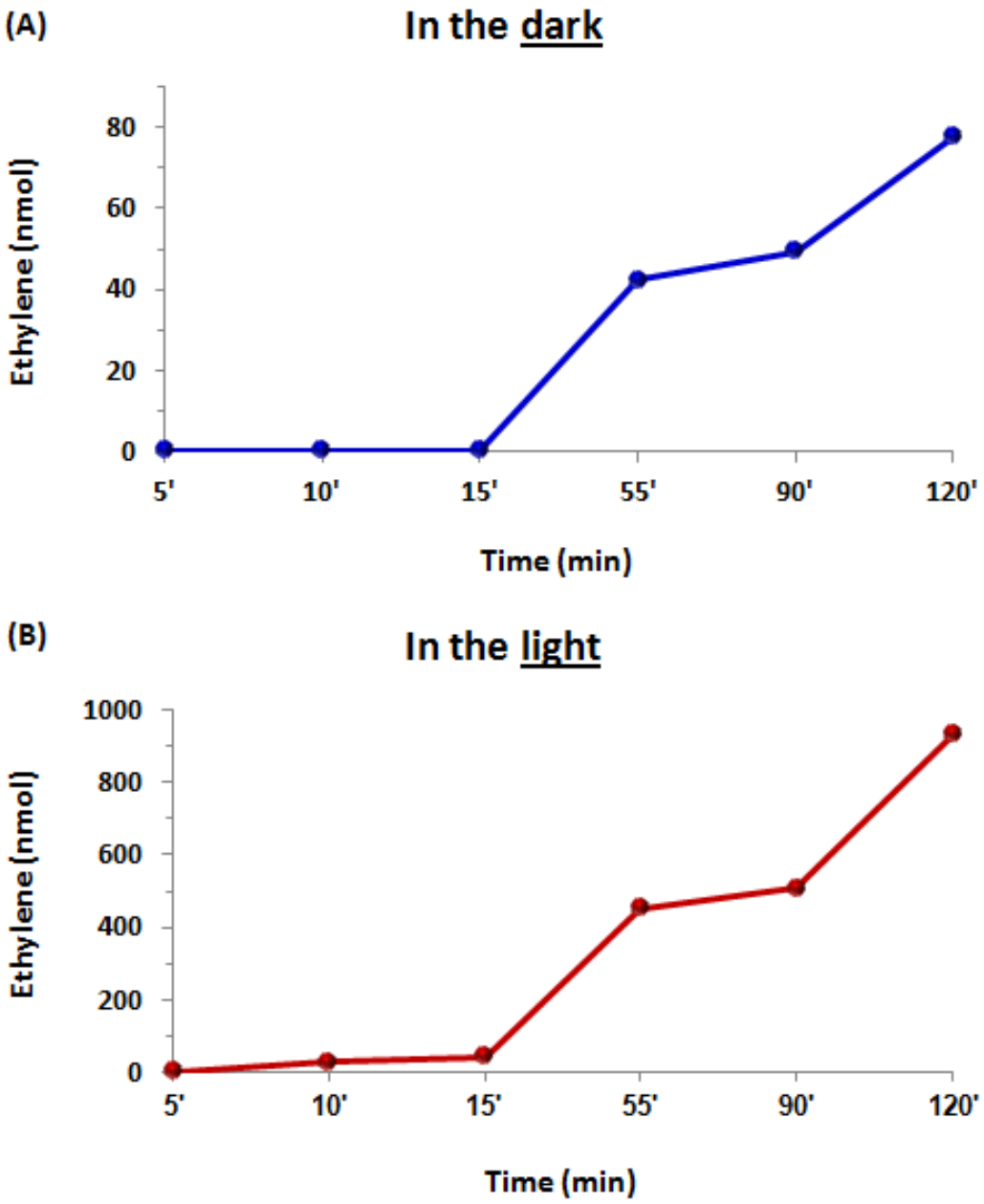

Figure 7. N_2ase activity of microaerobic cultures under the same conditions as used for hydrogen production studies (A) and under optimum assay conditions (B). N_2ase activity under optimum assay conditions (light, anaerobic) was ~10 times higher than that under culture conditions (dark, microaerobic). Results are given as total nanomols of ethylene produced per vial. Each vial contained 5 ml of culture (OD_{600nm}= 1.13).

CHAPTER 2: Single stage photofermentative hydrogen production from glucose: An attractive alternative to two stage photofermentation or co-culture approaches

Mona Abo-Hashesh[a,b], Dipankar Ghosh[a], Alexandre Tourigny[a], Azougui Taous[a], Patrick C. Hallenbeck[a*]

[a] Département de Microbiologie et Immunologie, Université de Montréal, CP 6128 Succursale Centre-ville, Montréal, Québec, Canada H3C 3J7

[b] Department of Microbiology, Port Said University, Port Said, Egypt

Author contribution: A preliminary series of experiments had been carried out by two undergraduate students, A.T. and A.T. partially under my supervision and guidance. All the data in the published study presented was directly collected by me in experiments that I carried out on my own, I perfomed the data analysis as well. D.G. helped in constructing the experimental setup and contributed to writing this article. All the work was under supervision of our professor Dr. Patrick Hallenbeck.

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Single stage photofermentative hydrogen production from glucose: An attractive alternative to two stage photofermentation or co-culture approaches

Mona Abo-Hashesh[a,b], Dipankar Ghosh[a], Alexandre Tourigny[a], Azougui Taous[a], Patrick C. Hallenbeck[a*]

[a] Département de Microbiologie et Immunologie, Université de Montréal, CP 6128 Succursale Centre-ville, Montréal, Québec, Canada H3C 3J7

[b] Department of Microbiology, Port Said University, Port Said, Egypt

[*]Corresponding author

Keywords: Biohydrogen; Photofermentation; Photosynthetic bacteria; Hydrogen yield

ABSTRACT

Photosynthetic bacteria have been extensively investigated for biohydrogen production due to their high intrinsic substrate conversion efficiency. Many studies have examined different aspects of photo-fermentative hydrogen production using various volatile organic fatty acids under nitrogen limited conditions, and in some cases nearly stoichiometric hydrogen yields have been obtained. In addition, there has been great interest in using photosynthetic bacteria to increase the yields of dark fermentation of glucose through either two stages or co-culture approaches. Although these processes can achieve yields of about 7 mols of H_2 per mol of glucose, there have many drawbacks. Thus, we have begun the systematic investigation of a simple one stage system for the conversion of glucose to hydrogen through photofermentation by *Rhodobacter capsulatus*. Yields of about 3 mols of H_2 per mol of glucose have been obtained, which represents a yield of 25%. Thus improvement is needed and can be sought through a variety of means, including process optimization and gene inactivation. These approaches could allow the development of a single stage process for the complete stoichiometric conversion of glucose, or glucose containing wastes, to hydrogen with a minimal lag phase and relative insensitivity to inhibition by fixed nitrogen. This would present an attractive simple alternative to either two stage or co-culture fermentations for the complete conversion of carbohydrate substrates to hydrogen.

INTRODUCTION

Renewable energy sources, including solar energy, wind energy, hydroelectricity, and energy from biomass have received extensive attention by the international community during the last several decades. Hydrogen is an attractive potential alternative energy carrier and possible scenarios for renewable and sustainable biohydrogen production are under active investigation [1]. A large quantity of biomass resources are available from which hydrogen could be extracted including; industrial, municipal and agricultural wastes [2]. Among the possible routes for conversion of biomass-borne carbohydrates, dark fermentation is attractive since it can be carried out using readily available fermentation technology operated at or near ambient temperature and atmospheric pressure [3-5]. A variety of organisms, either in pure culture, or as natural isolates from the environment, can degrade a wide variety of byproducts or wastes to hydrogen. Much recent work has shown that immobilized mixed microbial populations capable of degrading complex materials and giving high volumetric hydrogen production rates can be maintained over extended periods of time [6]. However, the major limiting factor in hydrogen production by dark fermentations appears to be metabolic; no pathway is known that can give more than 4 mols of H_2 per mol of hexose, although in theory 12 mols are available. Not only does this represent a very poor yield (33% at best) when compared with the production of other biofuels from many of the same substrates, for example bioethanol or biomethane can be produced at greater than 70 or 80% yields, this also means that two-thirds of the carbon in the substrate remains, creating a waste disposal problem. Thus, in a scenario where wastes are fermented to hydrogen by dark fermentation, complementary measures must be taken to extract additional energy from the resulting side products. Various avenues are under investigation using different processes in a second stage, including; a methane fermentation, a microbial electrolysis cell, or photofermentation [6].

In photofermentation, purple non-sulfur photosynthetic bacteria use nitrogenase to evolve hydrogen to dispose of excess electrons and recycle redox

cofactors when assimilating reduced carbon compounds under nitrogen limited conditions in the light [7]. Photosynthetic bacteria are well known for their capacity to ferment the various organic acids that are typically produced during hydrogen fermentations; acetate, lactate, butyrate, and some can also produce hydrogen from the ethanol byproduct. Thus, in this respect photofermentation seems ideal as a second stage method to extract additional hydrogen following a dark hydrogen producing fermentation, and a number of recent studies have examined sequential dark and photo-fermentations using a variety of substrates and a number of different organisms, either in pure culture, or, particularly in the first stage, as consortia (Table 1). A large five-year European project, Hyvolution, has been devoted to developing this process [25-27]. These studies do demonstrate that, as predicted, additional hydrogen can be obtained in this way, with combined yields ranging from 5 to 7 mols H_2 per mol of hexose. However, these yields are still lower than desirable and, moreover, a number of problems inherent in directly applying effluents from the first stage have been noted; including inhibition by acidic pH, by excess fixed nitrogen, and by excess substrate concentration. Therefore, the effluent must be extensively treated before being subjected to photofermentation, with dilution, nutrient addition, pH adjustment, and, depending upon the substrate used, even sterilization and centrifugation, being necessary. Thus, although relatively straightforward in principle, sequential dark-photo fermentation will require extensive bioprocess development before it reaches a practical stage and even then potentially cost and energy intensive inputs may be required.

A related process which might in some respects present a possible solution to at least some of these problems is the use of co-cultures where dark and photo fermentations occur simultaneously in the same reactor. This avenue has been investigated in a number of studies (Table 1). The main advantage to this approach would be that the substrates for photofermentation are generated in situ by the fermenting organism and if immediately used for photofermentation would not build up to inhibitory levels. In addition, the acidification due to the dark fermenter could be balanced by the alkalinization brought about by photofermentation. A number of

studies have examined the feasibility of this approach with cultures either freely mixed in the liquid phase or co-immobilized (Table 1). Once again, overall yields of 7 mols of H_2 per mol of hexose were obtained in the best cases. This approach also suffers from a number of potential limiting factors. For one thing, it is not obvious how to provide a growth medium that is appropriate for two different metabolic types, and it is especially difficult to develop a feasible scenario for obtaining the appropriate match in growth rates between the two different organisms. In addition, the highest yields were obtained with immobilized systems and 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 implementation.

Although organic acids are the usual substrates for photofermentation, the purple non-sulfur photosynthetic bacteria are capable of using a number of other substrates [28]. Indeed, *Rhodopseudomonas palustris* is capable of the photofermentative conversion of biodiesel-derived glycerol to hydrogen [29]. Given the number of problems and the inherent complexity of two stage or co-culture systems noted above, a single stage system capable of the photofermentative production of hydrogen from glucose, or other sugars, would be of interest if conversion efficiencies were high enough. Some previous studies, mostly carried out with *Rhodobacter sphaeroides*, have reported the direct production of hydrogen from glucose by photosynthetic bacteria, but with variable yields, with most reporting around 1 mol of H_2 per mol of glucose, although a few were appreciably higher (Table 2). We are initiating a systematic investigation of glucose photofermentation by *Rhodobacter capsulatus* with the aim of finding culture conditions and genetic modifications that would render this process competitive with the more cumbersome two stage and co-culture approaches. Here we report on initial studies of the photofermentation of glucose by *R. capsulatus* under different nitrogen regimes and suggest avenues for improving hydrogen yield performance.

MATERIALS AND METHODS

Bacterial strain and culture conditions

The purple non-sulfur photosynthetic bacterium *R. capsulatus* (JP91), a markerless *hup*$^-$ derivative of B10 lacking uptake hydrogenase activity, 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 [37] glucose amended with thiamine-HCl (1 mg/l) incubated at 30°C in an environmental chamber Biotronette Mark III (Lab-line Instruments) equipped with three 150 W incandescent bulbs.

Photofermentation of glucose

R. capsulatus JP91 was cultured as resting cells along with growing cells in RCV medium supplemented with 22 mM glucose as a carbon source. For growing cells, 3.5 mM or 7mM sodium glutamate was added as nitrogen source, which allows growth and nitrogenase derepression. Resting-cell suspensions were prepared by centrifuging late log phase cultures, resuspending the cell pellets in an anaerobically prepared mineral base solution having the same composition as the growth medium, except that glutamate was omitted, and 30 mM K phosphate buffer (pH 6.9) was added. Cell suspensions were kept under anaerobic conditions until delivery into the reaction serum bottles. 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 in serum bottles which had been sealed with butyryl rubber stoppers and rendered anaerobic by sparging with oxygen free argon for 15 min. Cultures were incubated for 6 days in a glass-sided controlled (Eheim Jager aquarium heaters) water bath (30°C). The cultures were illuminated with a bank of six 50W halogen bulbs placed 25 cm from the culture vessels. The experimental setup is shown in figure 1. Light intensity was adjusted using a potentiometer to give 200 W/m2 on the surface of the bottle. 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 procedures

The concentration of hydrogen in the collected gas was determined using a gas chromatograph (Shimadzu GC-8A) equipped with a thermal conductivity detector, a 2 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 440 nm with a glucose oxidase/peroxidase assay [38]. Light intensities were measured using a Delta OHM photo/radiometer (HD 2102.1).

RESULTS AND DISCUSSION

Effect of nitrogen supply on photofermentative production of hydrogen from glucose

Initial experiements with *R. capsulatus* JP91 which had been acclimatized to RCV-glucose were carried out at varying concentrations of glucose; 5 mM, 20 mM, 40 mM and 60 mM, with 3.5 mM glutamate as nitrogen source. A wide range of concentrations was taken into account to observe the effect of varying concentrations of the glucose on overall performance and on the cumulative production of hydrogen. Furthermore, this might help to establish the probable presence of a threshold concentration above which inhibition of hydrogen production could occur, due to substrate inhibition. Total cumulative hydrogen production was highest at 20 mM glucose concentration with some substrate inhibition being observed at concentrations of 40 mM or above (data not shown).

As a result, a more detailed analysis of hydrogen production was performed and the effect of different levels of nitrogen supply at a fixed glucose concentration

of 22 mM was determined (Figure 2). Three different nitrogen regimes were examined; -N (resting cells), 3.5 mM glutamate, and 7 mM glutamate. Total gas production (Figure 2A), glucose concentration in the medium (Figure 2B) and growth (optical density (OD)) (Figure 2C) were assessed on a daily basis. Hydrogen evolution began almost immediately and nearly maximal total hydrogen production was achieved after only 48 h. After this point, cells continued growing and consuming glucose with a reduced production of hydrogen. Not surprisingly, growth was directly influenced by nitrogen supply, with -N (resting cell) cultures, showing very little growth, and 7 mM glutamate supporting a higher level of growth than 3.5 mM glutamate. Non-growing cells, i.e. resting cells, only weakly evolved hydrogen and after 6 days had only consumed ~44% of the original (22 mM) glucose. It is interesting to note that the 3.5 mM and 7 mM glutamate cultures, although demonstrating significantly different growth patterns, had very similar time-dependent profiles of hydrogen evolution and glucose consumption. Both cultures had consumed ~70% of the initial glucose by the end of the six day period. Statistical analyses verified that there was positive correlation between growth and gas production in -N, 3.5mM and 7 mM glutamate cultures (r = 0.47, 0.49 and 0.67, respectively). There was a high correlation between optical density (OD) and glucose consumed in the 7 mM glutamate cultures (r = 0.87) which highlights the fact that an increase in glutamate concentration increases growth rather than H_2 production. As might be expected, there was a high positive correlation between gas produced and glucose consumed (r = 0.92, 0.81 and 0.85) in the -N, 3.5 mM and 7 mM glutamate cultures, respectively.

Variation in hydrogen yields with time

Previous studies on photofermentation of glucose have reported widely different yields (Table 2), so it was of interest to determine the hydrogen yields during photofermentation of glucose by *R. capsulatus* JP91. Most of the reported yields are quite low, ~1 mol of H_2 per mol of glucose, or only 8.3% of the theoretical 12 mols of H_2 available in glucose. One study reported stoichiometric conversion of

glucose to hydrogen by an uncharacterized mutant of *R. sphaeroides* [30], although the actual data only gives 5 mols of H_2 per mol of glucose, and when a "better performing" derivative of the same strain was used in another study, only 0.45 mol of H_2 per mol of glucose was obtained [31]. Therefore, the yields observed in the present study are higher than that previously reported. Worthwhile, much higher H_2 yields were also reported for another *hup*$^-$ strain of *R. capsulatus* [35] or a newly isolated strain of *R. sphaeroides* [36].

To more fully characterize the system, we determined yields on a daily basis using the total amounts of hydrogen accumulated and glucose consumed up to the particular time point (Figure 3), something not done in previous studies. Several interesting observations were made. At every time point yields were in the order 3.5 mM glutamate > 7 mM glutamate > -N ($P < 0.001$). Maximal yields occurred around days two and three (the highest was ~3.3±0.1 mols H_2 per mol of glucose for the 3.5 mM glutamate culture at day 3), declining with time after that. This suggests that there is a shift in metabolism at a certain point that decouples hydrogen evolution from glucose utilization. Future determination of the metabolic changes involved could lead to strategies for maintaining high yields throughout the period of incubation, or possibly even increasing the maximum yields obtained.

Light conversion efficiency

Although we have not optimized this process in this initial study it was of interest to calculate the approximate light conversion efficiency (CE). The calculation was based on total incident radiation as shown below.

$$CE = \frac{\Delta G\ (H_2)}{\Delta G\ (\text{total light input})}$$

After two days, a flask with a surface area of 3×10^{-3} m^2 had received 103.7 kJ. Since the useful energy contained in hydrogen (i.e. ΔG when used in a fuel cell) is -237.1 kJ/mol, a culture that had produced 3 mmols of H_2 had a conversion efficiency

of 0.7%. This is more or less typical of values reported for light conversion efficiencies of photofermentation of a variety of substrates by photosynthetic bacteria. However, an approximate calculation of theoretical light conversion efficiencies (CE) shows that there is certainly room for improvement in this regard. Theoretical CEs can be calculated based on the following equation:

$$CE = \frac{\Delta G\ (H_2)}{\#\ \text{Photons X}\ \Delta G\ h\nu}$$

The useable energy available in a mol of H_2 is 237 kJ (ΔG, $H_2 + ½\ O_2 \rightarrow H_2O$) and the average photon energy in the visible spectrum (550 nm) is 218 kJ/mol. In the photosynthetic bacteria nitrogenase requires both electrons and ATP to reduce protons to hydrogen. Four ATP are needed per H_2 ($2ATP/e^-$) and since the cyclic electron transport induced by each captured photon will pump 2 H^+ through the Q cycle, 8 photons are need to supply the necessary ATP. Additional energy is needed for reverse electron flow to reduce ferredoxin with NADH. Although the exact mechanism is unknown, this additional requirement is likely to be small: $\Delta G^{0'} = -nF\Delta E^{0'} = -(2)(96.48\ kJV^{-1}mol^{-1})(-0.13\ V) = +25.1$ kJ/mol and can easily be supplied by the proton gradient since the movement of 1 mol of protons should give about ~21.5 kJ/mol under cellular conditions, thus $2H^+$, pumped by the capture of one photon, should be more than sufficient. Hence, the minimum photon requirement is 9 photons. This gives a theoretical efficiency of 8.5%, i.e. ten-fold higher than the actual efficiency observed here.

FUTURE PERSPECTIVES

A number of avenues can be pursued to attempt to increase yields, ideally to 8 or more mols of H_2 per mol of glucose. For one thing, optimizing glucose concentrations as well as glutamate concentrations could affect yields. To this end, a multiprocess parameter optimization study is currently underway. Additional improvements could be sought through introducing appropriate mutations. For example, polyhydroxybutyrate (PHB) synthesis uses reducing power that could

otherwise be used for hydrogen production, and high carbon to nitrogen ratios, such as used here, favor PHB synthesis. Several PHB^- mutants have been created previously and shown to increase hydrogen yields during photofermentation of organic acids, but this approach has been little investigated for its effect on photofermentation of glucose. In one study where a Hup^-/PHB^- strain was examined, only a small (13%) increase in yield was noted [34]. An earlier study found that PHB^- mutants of *Rhodobacter sphaeroides* and *Rhodopseudomonas palustris* evolve more hydrogen from organic acids than the wild-type cells [39]. Other, additional metabolic alterations may be desirable and these can be guided by a newly developed metabolic flux analysis model of photosynthetic bacterial photoheterotrophic growth and photofermentation [40]. Finally, light conversion efficiencies might be improved by enabling the more efficient use of high light intensities through reduction in the size of the photosynthetic antennae, as previously shown for photofermentation of lactate [41].

CONCLUSIONS

In this study we have shown that the *hup*$^-$ *R. capsulatus* JP91 strain shows promise for the development of a single stage photofermentation process for converting glucose, and possibly other sugars, to hydrogen. Maximum yields, 3.3 mols of H_2 per mol of glucose with a light conversion efficiency of 0.7%, were found near the end of exponential growth in 3.5 mM RCV-glutamate. Various approaches to increasing yields have been identified.

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TABLES

Table 1. Hydrogen production by combined dark-photo fermentation or co-cultures

Approach (Microorganisms)	Substrate	Biohydrogen yield (mol H_2/mol hexose)	Reference
A. Sequential dark-photo			
Clostridium butyricum/ Enterobacter aerogenes Rhodobacter sp.	Sweet potato starch (glucose)	7.0	[8]
Enterobacter cloacae/ Rhodobacter sphaeroides	Glucose	5.3	[9]
Enterobacter cloacae/ Rhodobacter sphaeroides	Glucose	6.7	[10]
Clostridium pasteurianum/ Rhodopseudomonas palustris	Sucrose	5.0	[11]
Clostridium butyricum/ Rhodopseudomonas palustris	Glucose	5.5	[12]
Caldicellulosiruptor saccharolyticus/ Rhodobacter capsulatus	Beet molasses (sucrose)	6.8	[13]
Microbial consortium *Rhodobacter capsulatus*	Potato starch (glucose)	5.6	[14]
Clostridium butyricum/ Rhodopseudomonas palustris	Sucrose	5.8	[15]
B. Co-culture			
Cellulomonas/ Rhodobacter capsulatus	Cellulose	4 –6	[16]
Clostridium butyricum/ Rhodobacter sphaeroides	Glucose	2.2	[17]
Clostridium butyricum/ Rhodobacter sphaeroides	Glucose	7.0	[18]
Vibrio fluviulis/ Rhodobium marinum	Algal biomass (starch)	6.2	[19]
Lactobacillus amylovorus/ Rhodobium marinum	Algal biomass (starch)	7.2	[20]
Clostridium butyricum/ Rhodobacter sphaeroides	Glucose	0.9	[21]
Lactobacillus delbrueckii/ Rhodobacter sphaeroides	Glucose	7.1	[22]
Ethanoligenens harbinense/ Rhodopseudomonas faecalis	Glucose	3.1	[23]
Microbial consortium *Rhodobacter sphaeroides*	Wheat starch (glucose)	1.45	[24]

Table 2. Direct photofermentation of glucose

Reported yield mol H_2/mol glucose	Organism	Reference
5.0	*Rhodobacter sphaeroides* (Glc^+)	[30]
0.45	*Rhodobacter sphaeroides* VM81 (from Glc^+)	[31]
0.82	*Rhodobacter sphaeroides* ATCC 17026	[32]
0.9	*Rhodobium marinum*	[19]
0.9	*Rubrivivax gelatinosus* L31	[33]
1.0	*Rhodobacter sphaeroides* DSM158	[21]
1.53	*Rhodobacter sphaeroides* KD131	[34]
1.7	*Rhodobacter sphaeroides* KD131(Hup^-, PHB^-)	[34]
4.2	*Rhodobacter capsulatus* B100 (Hup^-)	[35]
6.5	*Rhodobacter sphaeroides* ZX5	[36]

FIGURES

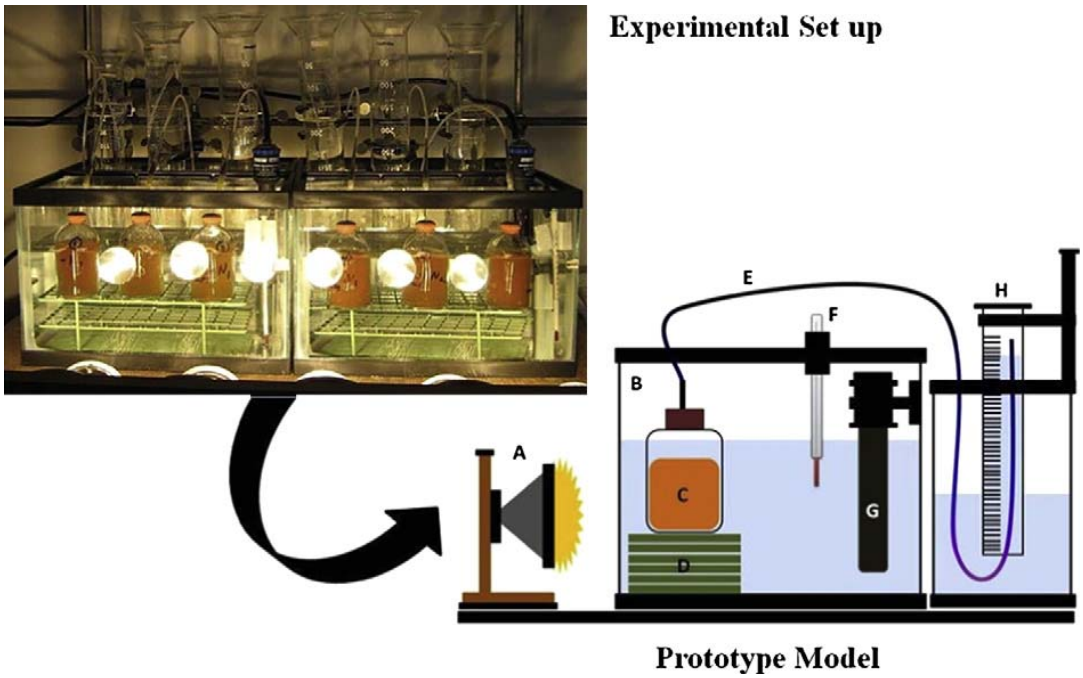

Figure 1. Experimental setup general diagram showing experimental setup with prototype model for generation and quantification of biohydrogen. (A) Panel of halogen bulbs, (B) Transparent water bath, (C) Serum bottle with culture, (D) Support, (E) Tubing (FEP890), (F) Thermometer, (G) Adjustable thermostat, (H) Inverted graduated cylinder.

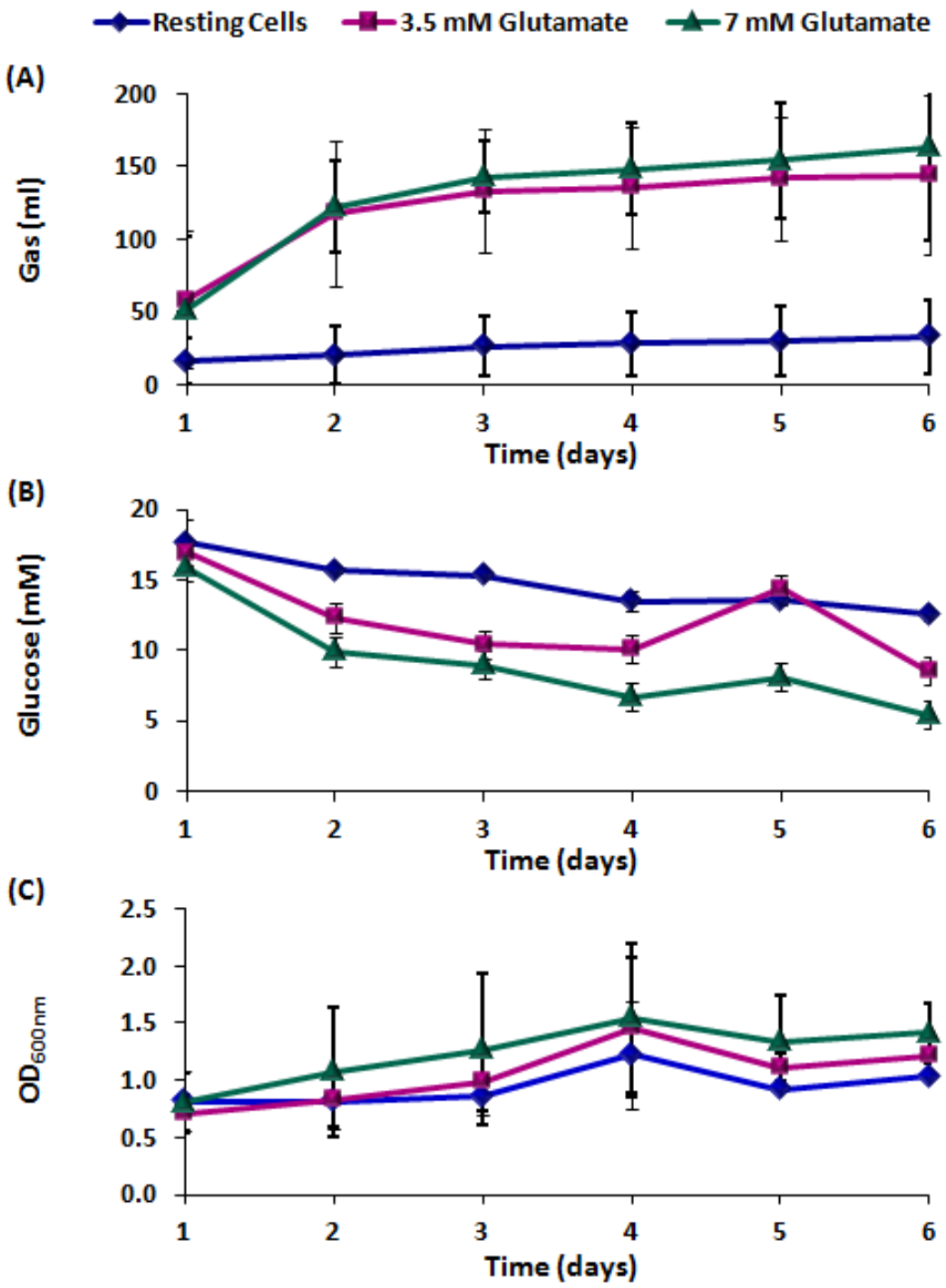
Resting Cells
3.5 mM Glutamate
7 mM Glutamate
(A)
Gas (ml)
200
150
100
50
0
1
2
3
4
5
6
Time (days)
(B)
Glucose (mM)
20
15
10
5
0
1
2
3
4
5
6
Time (days)
(C)
OD600nm
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
4
5
6
Time (days)

Figure 2.

Figure 2. Effect of different nitrogen regimes on the photofermentation of glucose. Experiments were carried out at different glutamate concentrations and 22 mM glucose, using the setup shown in figure 1 illuminated at 200 W/m2, 30°C, pH 7.0 and with a 5% (v/v) inoculum. Cultures were incubated either without added glutamate (-N, resting cells), or with 3.5 or 7 mM glutamate. The results shown are the averages of two independent representative runs with standard deviations shown as error bars. (A) Total gas production. (B) Glucose remaining in the culture. (C) Optical density (OD) at 600 nm.

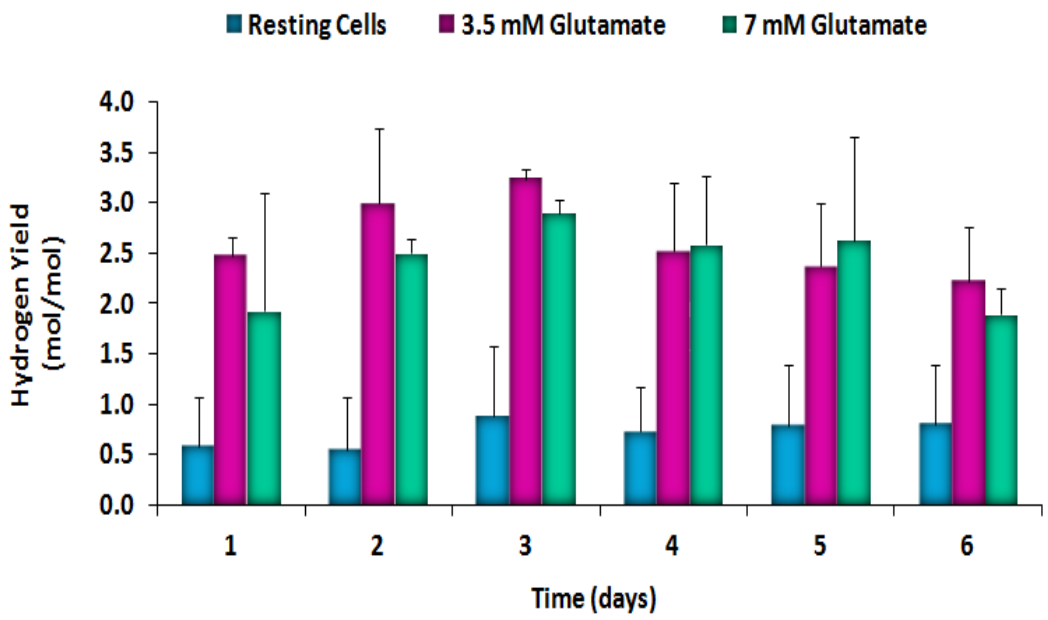

Figure 3. Variation in hydrogen yields with time and nitrogen source. Hydrogen yields were calculated on a daily basis using the amount of glucose consumed (initial concentration minus the amount remaining in the medium at that time) and the total hydrogen produced up to that time based on total gas production and the hydrogen concentration of the gas as determined by gas chromatography.

CHAPTER 3: High yield single stage conversion of glucose to hydrogen by photofermentation with continuous cultures of *Rhodobacter capsulatus* JP91

Mona Abo-Hashesh[a,b], Nicolas Desaunay[a], Patrick C. Hallenbeck[a*]

[a] Département de Microbiologie et Immunologie, Université de Montréal, CP 6128 Succursale Centre-ville, Montréal, Québec, Canada H3C 3J7

[b] Department of Microbiology, Port Said University, Port Said, Egypt

Author contribution: The experimental work was carried out by internship student Nicolas Desaunay under my supervision and guidance. I was directly responsible for the experimental design, data analyses and manuscript preparation. The article was revised by our professor Dr. Patrick Hallenbeck.

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High yield single stage conversion of glucose to hydrogen by photofermentation with continuous cultures of *Rhodobacter capsulatus* JP91

Mona Abo-Hashesh[a,b], Nicolas Desaunay[a], Patrick C. Hallenbeck[a*]

[a] Département de Microbiologie et Immunologie, Université de Montréal, CP 6128 Succursale Centre-ville, Montréal, Québec, Canada H3C 3J7

[b] Department of Microbiology, Port Said University, Port Said, Egypt

[*]Corresponding author

Keywords: Photofermentation; *Rhodobacter capsulatus*; Hydrogen production; One stage chemostat

ABSTRACT

Photofermentative hydrogen (H_2) production from glucose with the photosynthetic bacterium *Rhodobacter capsulatus* JP91 (*hup*$^-$) was examined using a photobioreactor operated in continuous mode. Stable and high hydrogen yields on glucose were obtained at three different retention times (HRTs; 24, 48 and 72 h). The H_2 production rates, varying between 0.57 to 0.81 mmol/h, and optical densities ($OD600_{nm}$) were similar for the different HRTs examined. However, the rate of glucose consumption was influenced by HRT being greater at HRT 24 h than HRTs 48 and 72 h. The highest hydrogen yield, 10.3 mols H_2/ mol glucose, was obtained at 48 h HRT. These results show that single stage photofermentative hydrogen production from glucose using photobioreactors operated in continuous culture mode gives high, nearly stoichiometric yields of hydrogen from glucose, and thus is considerably more promising than either two stage photofermentation or co-culture approaches.

INTRODUCTION

The world is confronting serious concerns about increasing energy demands on the one hand and depletion of fossil fuel reserves, by far the most important source of energy, on the other hand. In addition, environmental hazards caused by utilization of fossil fuels require the development of alternative renewable energy sources which are "eco-friendly". Among these sustainable energy resources, hydrogen is broadly recognized as a clean and effective future energy carrier. It has the highest energy content per unit weight of any known fuel (122 kJ/g) and it is the only common fuel that is not chemically bound to carbon. Thus, hydrogen combustion does not contribute to the greenhouse effect, acid rain, and ozone depletion (Hallenbeck et al., 2009).

Biological hydrogen production has significant advantages over photo, electro or thermo chemical methods because it is safer and more economical. As well, biological hydrogen production processes usually operate at ambient temperatures and pressure, and hence are inherently less energy intensive (Basak and Das, 2007). Biohydrogen production processes can be classified into four major categories; biophotolysis of water using green algae and cyanobacteria, bioelectrohydrogenesis and fermentative methods including photodecomposition (photofermentation) of organic compounds using photosynthetic bacteria and dark fermentative hydrogen production using anaerobic (or facultative anaerobic) bacteria (Kapdan and Kargi, 2006; Hallenbeck, 2011).

Photofermentation is the light-driven conversion of organic substrates to biohydrogen and is carried out by a diverse group of photosynthetic bacteria. Light plays a key role in providing the required energy input needed to drive substrate conversion to hydrogen to completion (Hallenbeck, 2009; Abo-Hashesh and Hallenbeck, 2012a). Much research has been carried out using photofermentation processes for hydrogen production via either one stage systems, where organic acids undergo complete oxidation to carbon dioxide (CO_2) and hydrogen (H_2), or two stage

systems, where organic acids produced during the acidogenic phase of dark fermentation are further converted to hydrogen. In the photofermentation process light energy drives reverse electron transfer required for the complete oxidation of organic acids to H_2 and CO_2. Consequently, it is an attractive method for the complete conversion of feedstocks that are only partially oxidized during dark fermentation and hence increases the yield of hydrogen from those substrates (Hallenbeck, 2005; Hallenbeck, 2012; Hallenbeck et al., 2012).

The purple non-sulphur (PNS) bacteria are the most commonly used for biohydrogen production by photofermentation. They can use a broad spectrum of substrates while growing photoheterotrophically using reduced organic substrates as electron donors and carbon source, while some species can also grow photolithoautotrophically using H_2, S^{-2} or Fe^{+2} as electron donors and CO_2 as the sole carbon source (Larimer et al., 2004; Dasgupta and Das, 2011). Many studies have shown that *Rhodobacter* species in particular have a great capacity for hydrogen production when grown on organic acids as substrate, converting acetic, lactic, propionic, malic and butyric acids to H_2 and CO_2 under anaerobic conditions in the light (Eroğlu et al., 2004; Shi and Yu, 2004; Fang et al., 2005; He et al., 2005). In addition some carbohydrates, including the simple sugars glucose, fructose and sucrose have been reported to be used for hydrogen production by photosynthetic bacteria (Maeda et al., 2003; Eroğlu et al., 2004; Keskin et al., 2011).

A schematic diagram showing the photofermentation of organic substrates by photosynthetic bacteria is given in figure 1. The photosynthetic bacteria produce chemical energy from sunlight (i.e. a proton gradient) and this energy can be used to drive reverse electron to nitrogenase, which reduces protons released through metabolism to hydrogen with the hydrolysis of ATP, produced by photophosphorylation (Hallenbeck, 2011; Abo-Hashesh and Hallenbeck, 2012b). Nitrogenase normally functions to catalyze the biological reduction of dinitrogen to ammonia with the release of H_2 per N_2 reduced (Equation 1), but in the absence of

other reducible substrates nitrogenase continues to turnover reducing protons to hydrogen (Equation 2).

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16\ Pi \quad (1)$$

$$2H^+ + 2e^- + 4ATP \rightarrow H_2 + 4ADP + 4\ Pi \quad (2)$$

A great deal of energy is required, both high energy electrons and ATP, with 2 ATP/e^-, or 4 ATP/H_2. Hydrogen production under these conditions is apparently a response to the metabolic need to maintain redox balance (Figure 1) (Masepohl and Hallenbeck, 2010). In the future it would be interesting to see if replacing the highly energy demanding nitrogenase with a hydrogenase, which would not require ATP, would result in increased efficiency of hydrogen production.

A number of previous studies have examined the efficiency of the direct (one stage) photofermentative hydrogen production from sugars in batch culture and are shown in Table (1).

As can be seen, a variety of studies have shown that hydrogen can be produced from sugars; glucose, sucrose, or complex carbohydrates. Yet, in all cases hydrogen yields are low, 50% or less, and quite often much lower, of the chemically available hydrogen (12 mols H_2/ mol glucose). However, in all cases photofermentation of glucose was carried out using a batch process. Since in batch processes exhibit both varying growth rates and cell densities, which affect light availability. Therefore, in the present study photofermentative hydrogen production from glucose by *Rhodobacter capsulatus* was investigated under continous culture conditions using an illuminated chemostat. The effect of different retention times, 24, 48 and 72 h, on the rates and yields of biohydrogen production were investigated. We were able to define conditions under which more than 80% of the consumed glucose was converted to hydrogen.

MATERIALS AND METHODS

Bacterial strain and pre-culture conditions

The purple non-sulfur photosynthetic bacterium *R. capsulatus* (JP91) *hup*$^-$ strain which lacks hydrogenase activity, kindly provided by Dr. John Willison, was routinely maintained in screw cap tubes (16 mm by 125 mm) completely filled with RCV medium (weaver et al. 1975) which contained 30 mM ammonium sulfate, 35.7 mM lactate, 19 mM phosphate buffer (pH 6.8) and incubated at 30°C in an environmental chamber Biotronette Mark III (Lab-line Instruments) equipped with three 150 W incandescent bulbs. Prior to use in experiments, *R. capsulatus* JP91 was pre-cultured in RCV medium supplemented with 55.5 mM glucose as a carbon source and 7 mM sodium glutamate as nitrogen source, which allows growth and nitrogenase derepression. Cells were cultured under anaerobic conditions with illumination overnight until inoculation into the bioreactor vessel containing RCV with the same composition.

Experimental setup for continuous photofermentation of glucose

Photofermentation experiments were carried out using an automated chemostat, BioFlo® C30 (500 ml vessel capacity), New Brunswick Scientific Co. Inc. The experimental setup is shown in figure 2. A pre-culture of *R. capsulatus* JP91 was used to inoculate (10% v/v) RCV medium that had been sterilized with the reactor vessel. Anaerobic conditions were achieved by flushing with argon at a flow rate of 5 ml/min. The culture was incubated with continuous agitation (250 rpm) under constant illumination using one 50 W incandescent bulb, while the temperature was maintained at 30°C. RCV medium was supplied at the appropriate flow rates to give retention times (HRT) of 24, 48 and 72 h, respectively. The total culture volume in the bioreactor was maintained at 350 ml.

Analytical procedures

Gas and liquid sampling were carried out daily. Hydrogen was measured by injecting 50 µl of the effluent gas into a Shimadzu GC-8A chromatograph equipped with a thermal conductivity detector and a 2 m column packed with molecular sieve 5A with argon as carrier gas. Hydrogen production rates were calculated according to the argon sparging flow rate. Bacterial growth was determined by measuring the OD_{600nm} using a double-beam spectrophotometer (Shimadzu). Correlation of these readings with dry weight measurements (CDW) gave the relationship, CDW (mg/ml) = 0.55 x OD_{600nm}. The concentration of residual glucose was determined by the phenol-sulfuric acid assay (Masuko et al., 2005).

Data statistical analyses

Data are presented in terms of mean, standard deviation using the data analysis tools of Microsoft Excel and SPSS version 17. Statistics were also performed using the data tool of SPSS version 17. One-Way ANOVA analysis was used to test the significance between variables at $P < 0.05$. *P*-value was set at < 0.05 for significant results and < 0.01 for highly significant results.

RESULTS AND DISCUSSION

Growth and operational stability

A late log phase culture of *R. capsulatus* JP91 was used to inoculate (10% v/v) the C30 bioreactor which was incubated with illumination under anaerobic conditions until the growth reached a nearly constant value of 4-5 OD_{600nm} (corresponding to 2.2–2.8 mg CDW/ml). Three different retention times were used (24, 48 and 72 h) over 15 days (315 hrs, continuous operation) starting with 48 h HRT followed by 72 h HRT and then 24 h HRT. At steady state this process is governed by standard chemostat theory (Equation 3).

$$dC_C / dt = (\mu - D)C_C \qquad (3)$$

Where dC_C/dt is the change in cell concentration with time (= 0 at steady state); Cc, Cell cencentration; μ, Specific growth rate; D, Dilution rate.

Cell density (OD_{600nm}) did not show significant variations throughout the entire process at each HRT while showed low significant variation (P = 0.043) among different dilution rates (14.6, 7.3 and 4.9 ml/h) (Figure 3A), showing that the cultures were at steady state and that therefore their growth rate was being controlled by the dilution rate as expected. Moreover, the reactor was in the range of stable operation; i.e. above the threshold for washout which occurs when the dilution rate exceeds the specific growth rate ($D > \mu$) where the dC_C/dt term becomes negative. The advantage of continuous culture over batch culture is that with continuous cultures fresh nutrients are continually supplied and accumulated cells and waste products are removed usually resulting in significantly increased yields of the desired product. Therefore, we examined hydrogen production and yields with these cultures.

Influence of HRT on the photohydrogen production efficiency

HRT could obviously affect photo-bioreactor process performance and has been suggested to be a limiting factor in photofermentative hydrogen production using continuous cultures (Show et al., 2011). Here we carried out continuous cultures at different HRTs to determine the applicability of a continuous process to hydrogen production from glucose in a single stage system. As shown in figure 3B, the continuous culture gave a H_2 production rate varying significantly in the range 0.57 to 0.81 mmol/h (P = 0.013) with H_2 production rate patterns being quite similar for the various HRTs. The average H_2 production rate was the same (0.72 mmol/h) for both 48 and 24 h HRT, and was only slightly decreased to 0.63±0.04 mmol/h in case of 72 h HRT. This is in contrast to a recent study of continuous hydrogen production from acetate where HRT had a much greater influence, but that study was

carried out with immobilized cells, which effectively decouples dilution rate and bacterial growth rate (Ren et al., 2012).

The influence of different HRTs on the glucose consumption rate was highly significant ($P < 0.01$), as shown in figure 3C. More glucose per unit time was consumed at the lowest HRT, 24 h (0.29±0.05) mmole/h, than at HRTs of 48 h (0.08±0.01) and 72 h (0.15±0.01). This is likely due to the increased growth rate at this HRT and therefore the increased demand for carbon assimilation to maintain the same level of biomass. On the other hand, the slow growth rate at the longest HRT (72 h) might mean that these cells require excess glucose consumption for cell maintenance and survival. Thus under the conditions examined here, an HRT of 48 h would appear optimal in that the rate of glucose consumption is minimal. Thus, if the percentages of total glucose utilized are calculated, an HRT of 48 h represents the optimal retention time studied with a quite low percentage (20%) of total glucose utilized whereas it was higher at 24 h and 72 h HRT (Figure 4A), ($P < 0.01$).

Of course, the significant influence of different HRTs on glucose consumption also affected the hydrogen yields ($P < 0.01$) with an HRT of 48 h giving the highest H_2 yield (10.3 mols H_2/ mol glucose), as shown in figure 5. Yields at 24 h and 72 h HRTs were much lower. This is similar to a previous study on the production of hydrogen from acetate by immobilized cells of *Rhodopseudomonas faecalis* RLD-53 where the highest hydrogen yield (2.26 mols H_2/mol acetate) was obtained at an HRT of 48 h while 36 and 60 h HRTs giving lower yields (Ren et al., 2012).

These results clearly indicate that HRT is an important factor for the photohydrogen production by continuous cultures by *R. capsulatus* JP91. Our previous study using batch cultures to examine photofermentation of glucose by the same bacterium reached to maximum 3.3 mols H_2/ mol glucose (Abo-Hashesh et al., 2011). This could be somewhat improved by Response surface methodology (RSM) optimization of culture conditions (Ghosh et al., 2012) but even then the maximum

value observed, 5.3 mols H_2/ mol glucose, was much lower than observed here with continuous cultures under optimal conditions. This shows that continuous cultures are capable of achieving higher H_2 yields. Indeed, many previous studies employing batch, single stage photofermentation of sugars reported very low yields of hydrogen (Table 1). Although this is the first study on single stage photofermentation of glucose with continuous cultures, a previous study examined the efficiency of continuous photofermentation of effluents from dark fermentation (Chen et al., 2008). This study showed that this process was stable, maintaining an overall average H_2 yield of 10.21 mols H_2/mol sucrose (5.1 mols H_2/mol hexose) for over 10 days, at 96 h HRT and under the optimal conditions determined from a previous batch study. Thus, the yield obtained in the present study, 10.3 mols hydrogen/ mols glucose, is the highest yield yet obtained, and is 86% of the maximum theoretical value, 12 mols hydrogen/ mols glucose.

Heat value and energy conversion efficiency

High heating values and total energy conversion efficiencies were calculated based on equations (4 and 5). The average heating value conversion efficiencies (HVCE) for 24, 48 and 72 h HRTs were calculated according to heating value of glucose (2816 J/mmol) and hydrogen (286 J/mmol) as shown in figure 6A. The highest HVCE was to 91% at 48 h HRT whereas it was 43% at 72 h HRT and even lower at 24 h HRT ($P < 0.01$), thus confirming that an HRT of 48 h is optimum.

$$\text{HVCE} = \frac{\text{Heating value of } H_2 \text{ produced (KJ)}}{\text{Heating value of substrate (glucose) (KJ)}} \text{ X } 100 \qquad (4)$$

Although the present study is the first to examine conversion efficiencies of single stage glucose photofermentation, other groups have reported on the conversion efficiencies of either two-stage systems, or single stage photofermentation of organic acids. The values obtained in the present study are high in comparison with previous

reports. For example, a study of the two-stage (dark fermentation followed by photofermentation) reported that the maximum heating value of hydrogen produced from glucose 0.11 KJ/h (Su et al., 2009) while in the present study 0.231 KJ/h was obtained at 48 h HRT. As well, the HVCE obtained in that study ranged from 5.5 to 34.9% whereas in the present study it ranged from 26.24 to 91.14%.

As well, the average total energy conversion efficiencies (TECE) for 24, 48 and 72 h HRTs were calculated according to the heating values of glucose and hydrogen (KJ) as well as input light energy (KJ) as shown in figure 6B.

$$\text{TECE} = \frac{\text{Heat value of } H_2 \text{ produced (KJ)}}{\text{Heat value of substrate (glucose) (KJ)} + \text{Input light energy (KJ)}} \text{ X } 100 \qquad (5)$$

Using this metric there were not very significant differences between the different HRTs (P = 0.017), average TECEs were 1.31, 1.27 and 1.13% for 48, 24 and 72 h HRTs, respectively. Of course, the reason for this is that, as previously observed in many other studies, light conversion efficiencies (LCE) during photofermentation are quite low. In the present study they were 1.3, 1.3 and 1.2% for 24, 48 and 72 h HRTs (P = 0.016), respectively. Other studies have reported slightly higher TECEs. For example, for a two-stage photofermentation of glucose by *Rhodopseudomonas palustris* a TECE of 2.96 to 3.55% was reported (Su et al., 2009), whereas a single stage photofermentation of acetate by *R. palustris* WP3–5 was reported to give a TECE of 1.93% (Chen et al., 2006). In both cases this was because the LCEs were higher than what we determined here. In fact, comparing TECE with HVCE in this, and other studies, shows that light conversion efficiencies need to be increased. Improvements in LCEs can be sought by development of optimized photobioreactors as well as using genetically modified PNS bacteria capable of producing higher rates of hydrogen at high light intensities.

CONCLUSIONS

Single stage photofermentative hydrogen production from glucose by continuous cultures of a photosynthetic bacterium was examined for the first time. Bacterial growth was stable and H_2 production rates varied between 0.57 and 0.81 mmol/h. The highest H_2 production rate was obtained at an HRT of 48 h which also gave the highest hydrogen yield, 10.3 mols H_2/ mol glucose, much higher than reported previously for batch cultures and 86% of the theoretical value. Thus, this could be a promising system for the direct stoichiometric conversion of sugars to hydrogen, but light conversion efficiencies would need to be improved.

ACKNOWLEDGEMENTS

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TABLES

Table 1. Hydrogen yields obtained from sugars in various photofermentation studies

Organism	Carbon source	H_2 Yield	Reference
Rhodobacter sphaeroides	Glucose	5.0	Macler et al. (1979)
R. sphaeroides VM81	Glucose	0.45	Margaritis and Vogrinetz (1983)
R. sphaeroides DSM158	Glucose	0.56	Fang et al. (2006)
R. sphaeroides KD131	Glucose	1.53	Kim et al. (2006)
R.sphaeroides KD131 (*hup*$^-$, *phb*$^-$)	Glucose	1.70	Kim et al. (2006)
R. sphaeroides ATCC 17026	Glucose	0.82	Jeong et al. (2008)
R. sphaeroides ZX5	Glucose	6.5	Tao et al. (2008)
R. sphaeroides RV	Wheat starch	1.23	Kapdan et al. (2009)
R. capsulatus JP91 (*hup*$^-$)	Glucose	3.3	Abo-Hashesh et al. (2011)
R. capsulatus JP91 (*hup*$^-$)	Beet molasses (sucrose)	10.5	Keskin and Hallenbeck (2012)
	Black strap (sucrose)	8	
	Pure sucrose	14	
R. capsulatus JP91 (*hup*$^-$)	Glucose	5.5	Ghosh et al. (2012)
Rhodopseudomonas palustris	Glucose	4.16	Su et al. (2009)
Rubrivivax gelatinosus L31	Starch	1.1	Li et al. (2008)
	Sucrose	1	
	Glucose	0.9	

FIGURES

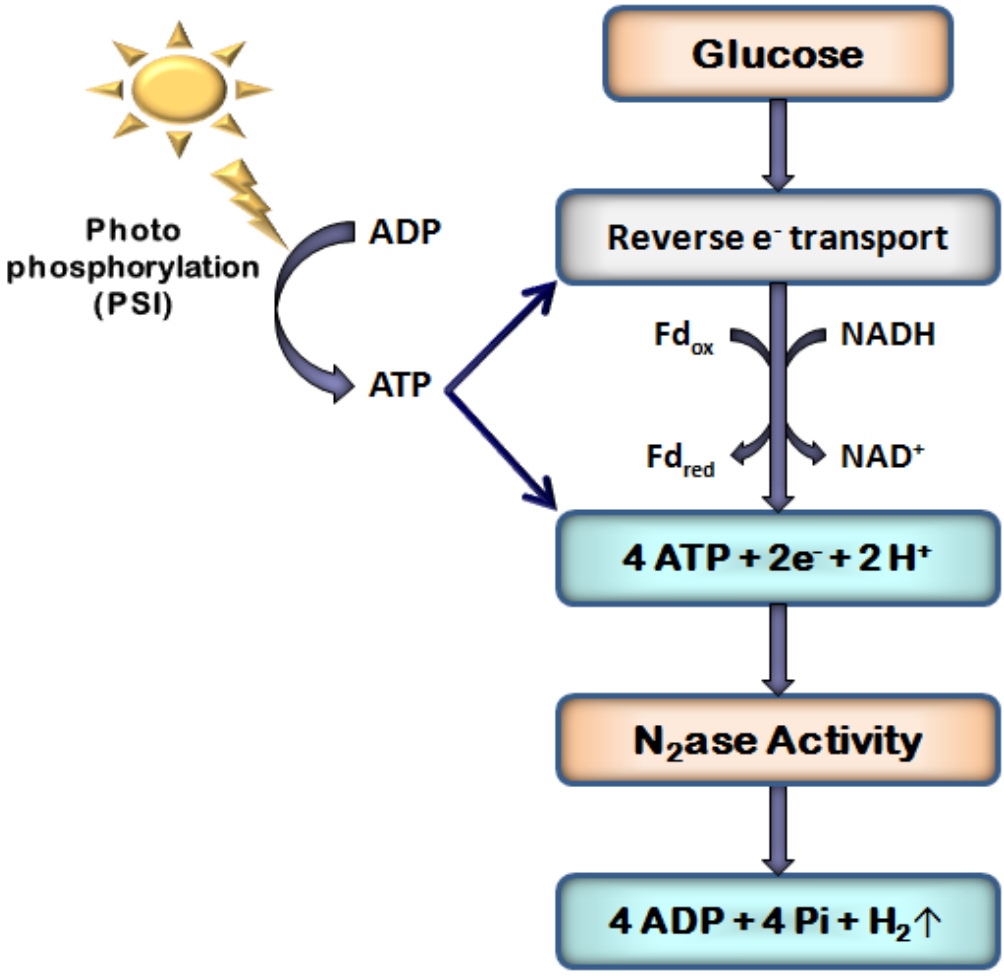

Figure 1. Schematic representation of photo-fermentative hydrogen production by N_2ase. ATP produced via photo-phosphorylation is harnessed to drive the reverse electron transport chain required to release more electrons and protons from the feedstock as well as nitrogenase activity required for reduction of protons to hydrogen. Nitrogenase catalyzes the biological reduction of protons to hydrogen and dinitrogen to ammonia. Light energy converts ADP to ATP which supplies the reducing power. Also, it converts oxidized ferredoxin to strong reducing agent of reduced ferredoxin that mediates electron transfer required for reduction of protons to hydrogen. The final products for this process are ADP, inorganic phosphate, ammonia and hydrogen.

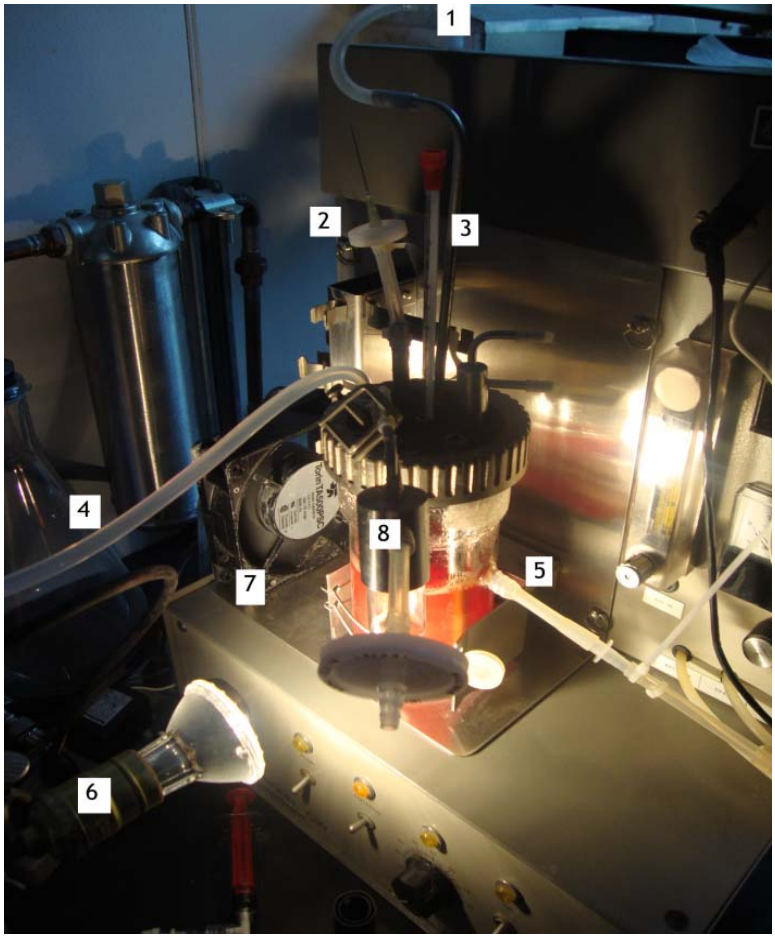

Figure 2. Experimental setup of the BioFlo® C30 chemostat. (1) Argon gas flushing at 5 ml/min. (2) Outlet gas. (3) Thermometer. (4) Inlet medium. (5) Outlet medium that maintain the total culture volume at 350 ml. (6) Illumination and heating by using 50 W incandescent bulb. (7) Fan for cooling and adjust the temperature at 30°C. (8) Liquid sampling.

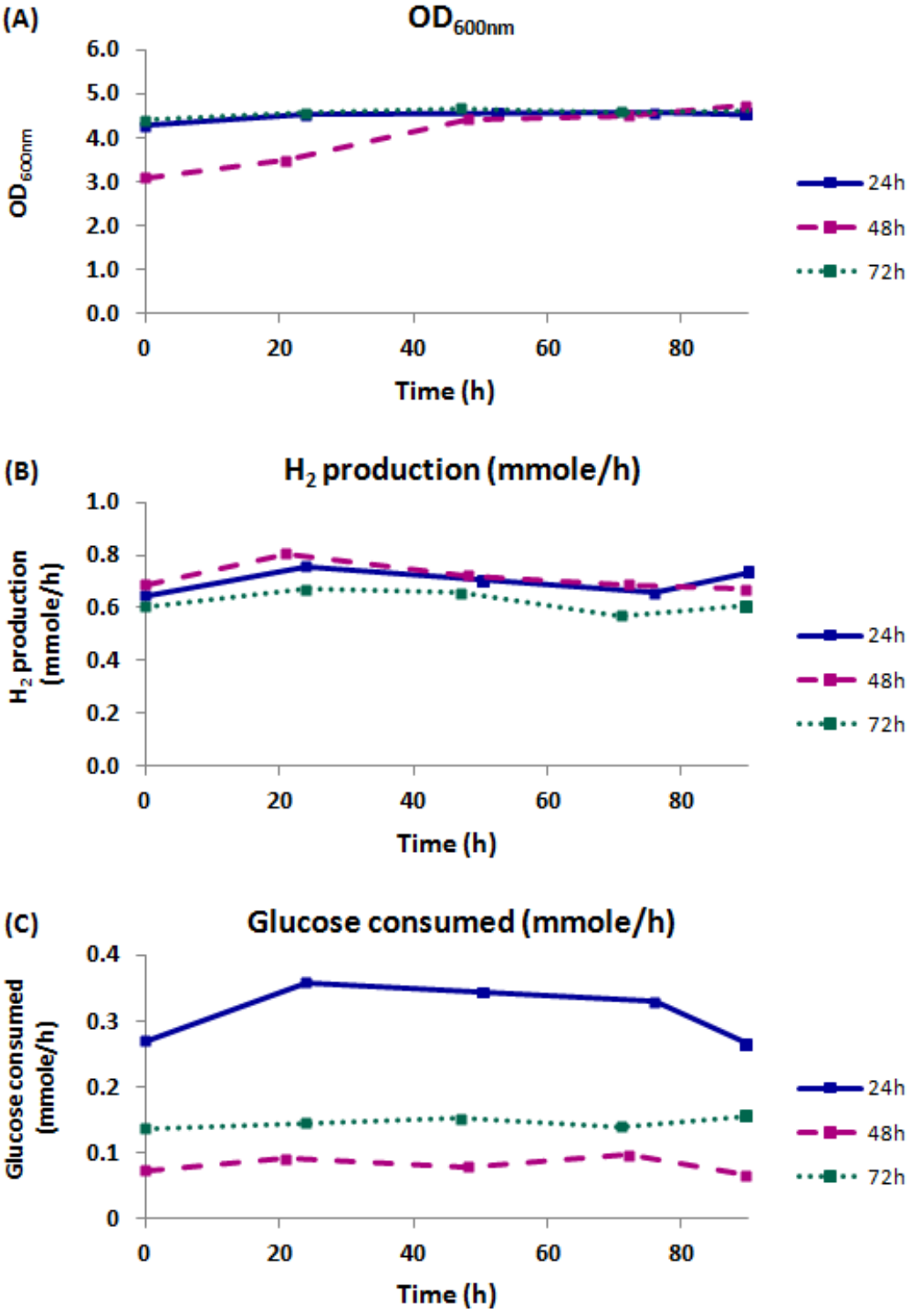
(A)
OD600nm
OD600nm
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
20
40
60
80
Time (h)
24h
48h
72h
(B)
H2 production (mmole/h)
H2 production (mmole/h)
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
Time (h)
24h
48h
72h
(C)
Glucose consumed (mmole/h)
Glucose consumed (mmole/h)
0.4
0.3
0.2
0.1
0
0
20
40
60
80
Time (h)
24h
48h
72h

Figure 3.

Figure 3. Effect of different HRTs on photo-biohydrogen fermentation process. Experiments were carried out using BioFlo® C30 for achieving continous culture condition. Culture was stirring and incubated anaerobically with light supply. Three different HRTs (24, 48 and 72 h) were used to optimize photofermentation process for more hydrogen production. (A) Values of OD_{600nm} of the cultures grown on RCV medium supplemented with 55.5 mM glucose as carbon source and 7mM of glutamate as nitrogen source. (B) Biohydrogen production rate mmol/h during the examined HRTs. (C) Utilization rate of glucose (mmol/h).

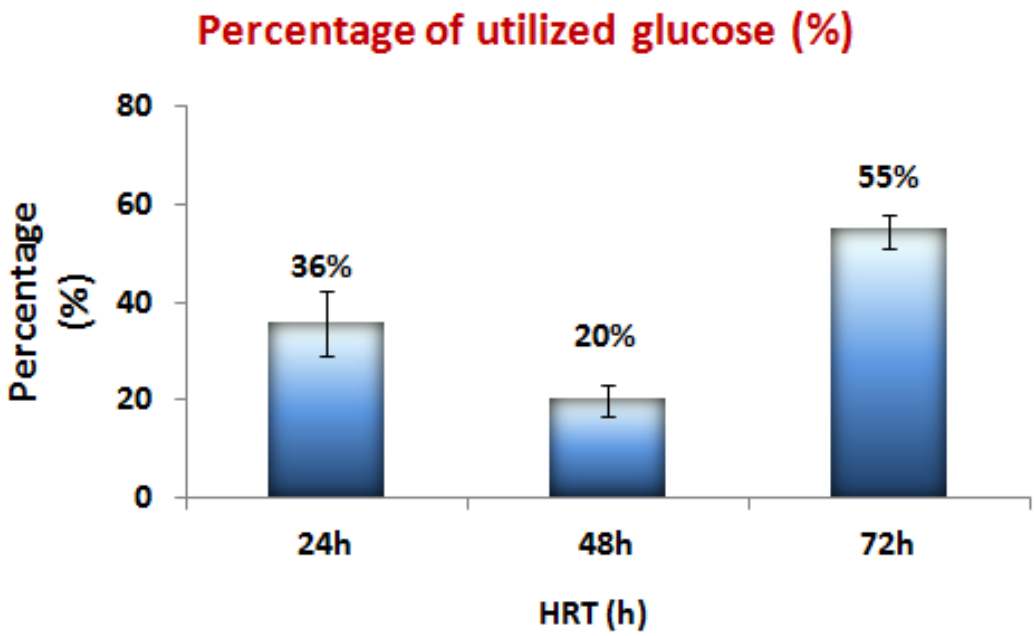

Figure 4. Percentage of glucose utilized at the different HRTs (%). Mean±SD for values of total glucose consumed percentage over the period of the run (15 days) for each HRT.

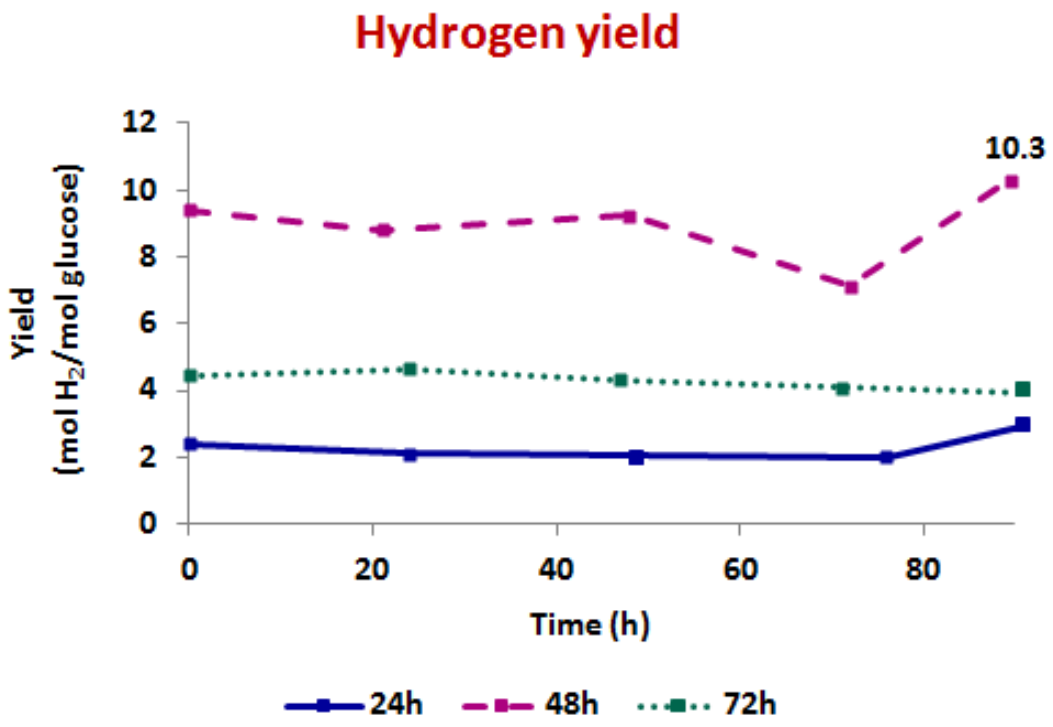

Figure 5. Yields of hydrogen at different HRTs. Hydrogen yields were calculated (mol H_2/ mol glucose) according to the hydrogen production rate (mmol/h) and consumption rate of glucose (mmol/h).

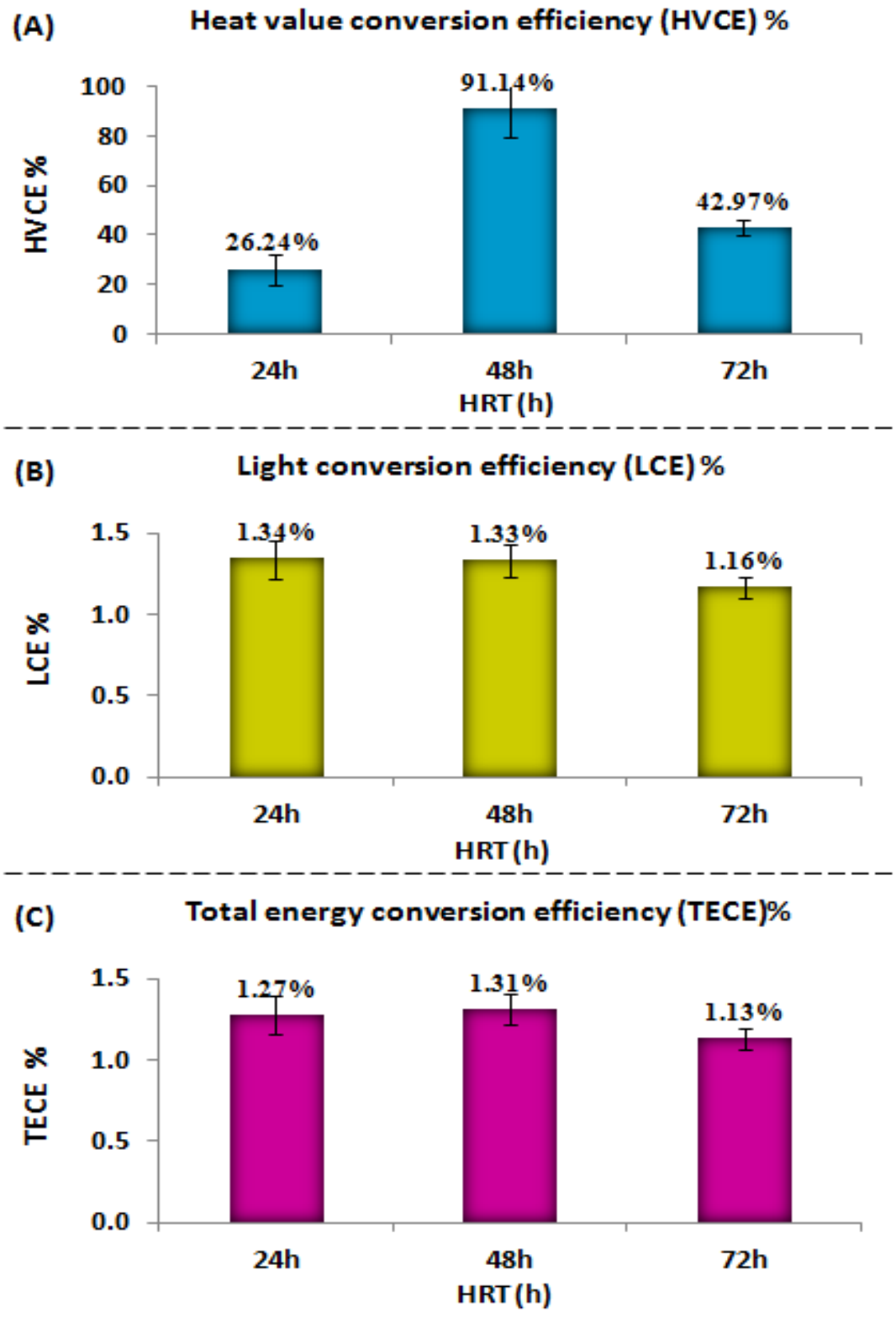
(A)
Heat value conversion efficiency (HVCE) %
HVCE %
100
80
60
40
20
0
26.24%
91.14%
42.97%
24h
48h
72h
HRT (h)
(B)
Light conversion efficiency (LCE) %
LCE %
1.5
1.0
0.5
0.0
1.34%
1.33%
1.16%
24h
48h
72h
HRT (h)
(C)
Total energy conversion efficiency (TECE)%
TECE %
1.5
1.0
0.5
0.0
1.27%
1.31%
1.13%
24h
48h
72h
HRT (h)

Figure 6.

Figure 6. Energy conversion efficiency for hydrogen production from glucose (%). (A) Means±SDs of heat value conversion efficiencies (HVCE) for 24, 48 and 72 h HRTs were calculated according to heat value of glucose (2816 J/mmol) and hydrogen (286 J/mmol). (B) Means±SDs of light conversion efficiencies (LCE) for 24, 48 and 72 h HRTs were calculated according to heat values of hydrogen (KJ) as well as the effective input light energy 15.37 (KJ). (C) Means±SDs of total energy value conversion efficiencies (TVCE) for 24, 48 and 72 h HRTs were calculated according to heat values of glucose and hydrogen (KJ) as well as input light energy (KJ).

CHAPTER 4: Expression and maturation of the nonfunctional *hydA* gene of *Rhodospirillum rubrum* S1^T in *Escherichia coli*

Mona Abo-Hashesh[a,b], Guillaume Sabourin[a], Patrick C. Hallenbeck[a*]

[a] Département de Microbiologie et Immunologie, Université de Montréal, CP 6128 Succursale Centre-ville, Montréal, Québec, Canada H3C 3J7

[b] Department of Microbiology, Port Said University, Port Said, Egypt

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Expression and maturation of the nonfunctional *hydA* gene of *Rhodospirillum rubrum* S1^T in *Escherichia coli*

Mona Abo-Hashesh[a,b], Guillaume Sabourin[a], Patrick C. Hallenbeck[a*]

[a] Département de Microbiologie et Immunologie, Université de Montréal, CP 6128 Succursale Centre-ville, Montréal, Québec, Canada H3C 3J7

[b] Department of Microbiology, Port Said University, Port Said, Egypt

[*]Corresponding author

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ABSTRACT

[FeFe]-Hydrogenases, encoded by *hydA* are thought to require the products of three accessory genes, *hydE*, *hydF* and *hydG*, for the biosynthesis of a functional H-cluster and maturation into a functional protein capable of reducing protons to hydrogen. Genome sequencing has shown that some organisms, including several strains of purple non-sulfur photosynthetic bacteria, possess orphan *hydAs*. It is unknown if these orphan HydAs can be matured into functional proteins. The orphan *hydA* of *Rhodospirillum rubrum* was cloned and overexpressed in *Escherichia coli* BL21(DE3) along with *hydE*, *F* and *G* maturation genes from *Desulfovibrio vulgaris* Hildenborough or *Clostridium acetobutylicum* ATCC 824. *In vitro* hydrogenase activity was measured using a methyl viologen assay (MV). Coexpression of *R. rubrum hydA* with the maturation genes of *C. acetobutylicum* gave the highest hydrogenase activity, 251 nmol/min, whereas it was 19.6 nmol/min when coexpressed with the maturation genes from *D. vulgaris*. Interestingly, *R. rubrum* HydA was twice as active as the HydA of *C. acetobutylicum* when both were matured with the genes from the latter organism. Variation in the activity of recombinant [FeFe]-hydrogenases between these *E. coli* strains might be related to variation in the expression conditions.

INTRODUCTION

Hydrogen is being touted as a fuel of the future and much recent work has been devoted to investigating and improving the biological production of hydrogen. A fundamental understanding of the capability of different hydrogen producing organisms is essential for improving their performance either by physiological manipulation or through metabolic engineering, including the expression or overexpression of hydrogenase. Many photosynthetic bacteria are well known for the capacity to use light energy to produce hydrogen from a variety of substrates. One of the well studied photosynthetic bacteria is *Rhodospirillum rubrum,* a Gram-negative, purple proteobacterium, and the subject of a considerable amount of physiological and genetic research, including studies on nitrogen fixation, carbon monoxide oxidation, CO_2 fixation, H_2 production, photosynthesis, and energy conservation (ATP synthase). In fact, *R. rubrum* was the first photosynthetic bacterium shown to produce hydrogen from organic acids (Gest and Kamen, 1949). At least four enzymes are involved in hydrogen metabolism, either hydrogen production or consumption, in *R. rubrum* (Najafpour et al., 2004). Hydrogen can be produced, as first observed, under some conditions by nitrogenase. Nitrogenase reduces protons to hydrogen during the reduction of nitrogen to ammonia, resulting in a low rate of hydrogen production (Kim et al., 2008a; Wang et al., 2010). However, in the absence of N_2, nitrogenase turnover continues unabated, producing hydrogen, as shown in equation (1).

$$2H^+ + 2e^- + 4\ ATP \rightarrow H_2 + 4ADP + 4\ Pi \qquad (1)$$

In the presence of CO, *R. rubrum* can carry out the water gas shift reaction, producing hydrogen with a [NiFe]-hydrogenase while oxidizing CO using a carbon monoxide dehydrogenase as shown in equation (2) (Najafpour et al., 2004).

$$CO + H_2O \rightarrow H_2 + CO_2 \qquad (2)$$

Finally, fermentative hydrogen production has also reported when *R. rubrum* is cultured in the dark with pyruvate as carbon source (Gorrell and Uffen, 1977; Kim et al., 2008b). The hydrogen is produced via pyruvate-formate lyase (PFL) where pyruvate is metabolized to formate and acetyl-CoA. Formate is then oxidized to carbon dioxide by formate-hydrogen lyase (FHL) complex, releasing H_2 (Gorrell and Uffen, 1977; Sawers and Bock, 1988). The FHL complex includes formate dehydrogenase-H (FDH-H) and a [NiFe]-hydrogenase (Yoshida et al., 2005). In addition to these three enzymes shown to catalyze hydrogen production under different conditions, *R. rubrum* also possesses additional [NiFe]-hydrogenase which is poised to act in hydrogen oxidation. The necessary genes for maturation of [NiFe]-hydrogenases are present in the genome and apparently actively transcribed.

In addition, genome sequencing has revealed that *R. rubrum* contains a sequence with considerable homology to *hydA*, encoding [FeFe]-hydrogenase, of other organisms. This *hydA* may have been acquired by *R. rubrum* through lateral gene transfer (Meyer, 2007). In fact, very little information is known on the regulation of the synthesis of this hydrogenase and the fermentation pathways to which it belongs. It would be interesting to try to overexpress this enzyme in various conditions to potentially increase the production of hydrogen and learn more about this enzyme. [FeFe]-hydrogenases are thought to act primarily in the direction of proton reduction to hydrogen (Vignais et al., 2001; Hallenbeck, 2012). X-ray crystallographic and infrared spectroscopic studies have shown that the active site of [FeFe]-hydrogenases, the so-called H-cluster, consists of a di-iron center that shares four cysteine ligands. Three of the 4 cysteines are non protein ligands; CO, CN, and a small dithiolate [Fe]-bridging molecule, and one protein ligand with a regular [4Fe-4S] subcluster. Each Fe atom in the di-iron center has one CO and one CN ligand and connects to the other Fe through an additional bridging CO ligand. In addition, many [FeFe]-hydrogenases contain additional [4Fe–4S] and [2Fe–2S] ferredoxin clusters that work as electron transfer centers and connect the buried active site electrically to the protein surface (Pierik et al., 1998; Nicolet et al., 2001; Nicolet et al., 2010). Maturation of [FeFe]-hydrogenase is thought to require a series of three other

metalloproteins, HydE, HydF and HydG which carry out the synthesis and assembly of the H-cluster (McGlynn et al., 2007; Nicolet et al., 2010; Nicolet and Fontecilla-Camps, 2012).

However, even though *R. rubrum* has what appears to be a bona fide *hydA* sequence, the known maturation genes, *hydE*, *hydF* and *hydG*, appear to be absent. This raises several questions. Can this putative hydrogenase be matured and functional in *R. rubrum*? Can it be matured and functional in other organisms? Not only is this of fundamental interest, but if nitrogenase could be replaced by a [FeFe]-hydrogenase in the photofermentative production of hydrogen, hydrogen yields and/or rates might be improved since this hydrogenase would not have the tremendous energy requirement necessary for nitrogenase activity (Equation 1). Accordingly, in the present study, we have examined some aspects of the *R. rubrum hydA*, including its heterologous maturation in *E. coli* BL21(DE3). Since *E. coli* does not contain either *hydA* or any of the required maturation genes, we also coexpressed the three other gene products required, HydE, HydF and HydG from either *Desulfovibrio vulgaris* or *Clostridium acetobutylicum*.

MATERIALS AND METHODS

Bacterial strains and plasmids and growth condition

Strains of *R. rubrum* were grown in screw cap tubes filled with RCV medium (Weaver et al. 1975) under anaerobic conditions. The tubes were incubated at 30°C in a light chamber for optimum growth. *E. coli* strains were grown in glass test tubes supplemented with LB medium (Luria et al., 1960) at 37°C overnight. The bacterial strains and plasmids that were used are shown in Tables 1 and 2.

Cloning strategies

DNA (plasmid or genomic) purification and gel extraction were performed using QIAGEN QIAprep Spin Miniprep, QIAquick Gel Extraction and QIAamp DNA Mini Kits.

Cloning *RrPnifH2hydA*Fu in pCRBlunt

To examine the expression of *R. rubrum hydA* (*RrhydA)* under regulation of *nifH2* promoter (*nifH2* is the marker gene which encodes nitrogenase reductase in *R. rubrum*), *RrhydA* has been fused with *nifH2* promoter region. Polymerase chain reactions (PCR) were performed using the GeneAmp PCR system 2400 (Perkin Elmer) and the primers used are indicated in Table 3. Both the *hydA* gene and *nifH2* promoter region were amplified from genomic DNA of *R. rubrum* by PCR, generating the DNA fragments *RrhydA*_HindIIIXhoI and *RrPnifH2*, respectively. The DNA fragment *RrhydA*_HindIIIXhoI was subsequently used with the primer Olap-*PnifHhydA*_F and the primer *RrhydA*XhoI_R to generate a chimeric DNA fragment by PCR, sharing a region of homology with the 3' end of the fragment *RrPnifH2*. Similarly, the DNA fragment *RrPnifH2* was modified by PCR using the primer *RrPnifH2*_F and primer Olap-*PnifHhydA*_R to generate a DNA fragment with homology to the 5' end of the fragment previously generated. Both DNA fragments were then combined in a single PCR reaction with *PnifH2*_F primer and *RrhydA*XhoI_R to produce a fusion between the fragment *RrPnifH2* and *RrhydA*_HindIIIXhoI, giving the fragment *RrPnifH2hydA*Fu. This was then cloned into pCRBlunt to generate plasmid pGSP02.

Cloning *RrPnifH2hydA*Fu in pJB3Tc20

The fragment *RrPnifH2hydA*Fu was extracted from the constructed plasmid pGSP02 by HindIII and XbaI and cloned in pJB3Tc20 to generate plasmid pGSP03.

After transformation into *E. coli* S17-1, it was transferred by conjugation to *R. rubrum* strain UR206 to generate UR206-*hydA*.

Cloning *RrhydA*_HindIIIAflII in pRSFDuet-1 and coexpression with the other maturation genes

The DNA fragment *RrhydA*_HindIIIAflII was amplified from the genomic DNA of *R. rubrum* and cloned in pRSFDuet-1 to generate pRSF-$hydA_{Rr}$. A series of transformations were done to examine the maturation of *R. rubrum* HydA. Transformant 1 (T1): pRSF-$hydA_{Rr}$ was transformed into *E. coli* BL21 (DE3) cells along with pET-$hydEF_{Dv}$ and pCDF-$hydG_{Dv}$ containing the hydrogenase maturation genes of *D. vulgaris* in a three way transformation generating BL21-$hydA_{Rr}EFG_{Dv}$. Transformant 2 (T2): pRSF-$hydA_{Rr}$ was transformed along with pET-$hydE_{Ca}$ and pCDF-$hydFG_{Ca}$ containing the hydrogenase maturation genes of *C. acetobutylicum* into *E. coli* BL21 (DE3) generating BL21-$hydA_{Rr}EFG_{Ca1}$. Transformant 3 (T3); a positive control was obtained by transforming pET-$hydAE_{Ca}$ and pCDF-$hydFG_{Ca}$ of *C. acetobutylicum* into *E. coli* BL21 (DE3) in a two way transformation generating BL21-$hydAEFG_{Ca}$. Transformant 4 (T4); pRSF-$hydA_{Rr}$ was transformed into *E. coli* BL21 (DE3) along with pCDF including the artificial operon of *hydEFG* engineered from *C. acetobutylicum* generating BL21-$hydA_{Rr}EFG_{Ca2}$. Transformant 5 (T5); a negative control was obtained by transforming pRSF-$hydA_{Rr}$ only into *E. coli* BL21 (DE3) generating BL21-$hydA_{Rr}$. All transformants were selected on LB agar plates supplemented with the appropriate antibiotics. Gene expression was induced by adding Isopropyl-D-thiogalactopyranoside (IPTG; Novagen) to the culture medium to a final concentration 1.5 mM.

Overexpression of [FeFe]-hydrogenase genes in *E. coli*

To analyze expression of the *hyd* genes, transformants of *E. coli* strain BL21(DE3) that harbored all the plasmids including a complete set of structural and maturation genes were grown in LB medium plus required antibiotics overnight. The

overnight cultures were subcultured (1:50 dilution) into 115 ml of fresh LB medium supplemented with antibiotics and 100 μM Fe-EDTA (1:1). Cultures were grown aerobically at 37°C with agitation at 250 rpm until an optical density (OD) at 600 nm of 0.5 to 0.7 was reached. For induction of *hyd* gene expression, 1.5 mM IPTG was added to the cultures which were then incubated at room temperature for 1 hr with agitation (100 rpm). The cultures were then transferred to 120-ml serum bottles, sealed with rubber stoppers, sparged with argon for fifteen minutes to establish anaerobic conditions and incubated overnight at room temperature for the induction of [FeFe]-hydrogenase biosynthesis (King et al., 2006).

Samples were taken from the serum bottles before and after IPTG induction, treated with 3X sample buffer and they were kept at -20°C prior to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses. SDS-PAGE for protein samples was performed as previously described (Wilder et al., 1979). Gels were stained using coomassie blue R-250 and scanned using a Typhoon™ 9410 scanner. ImageQuant TL gel analysis software was used for determining the concentration of specific proteins. The total protein concentration of whole cell lysates was determined using Pierce BCA Protein Assay Kit from Thermo Scientific.

Samples were transferred under anaerobic condition into centrifuge bottles and centrifuged at 13000 rpm for 15 min. The cell pellets were resuspended in 1 ml BugBuster™ protein extraction reagent. Cell lysates were kept at -20°C for further protein analyses.

Measurement of gene expression

Extraction of total RNA from bacterial cultures was performed with TRIzol® reagent from Invitrogen. RNAs were then converted to cDNA using a QuantiTect® Reverse Transcription kit from QIAGEN. Levels of gene expression were measured using qRT-PCR with a QuantiTect® SYBR® Green PCR QIAGEN kit and a Rotor-Gene™ 6000 (Corbett Life Science). For each reaction, a pair of primers specific for

the target gene were chosen and optimized. The constitutive gene *rplC*, the gene encoding for large subunit ribosomal protein L3, was used as a reference gene for *R. rubrum* while 16S rRNA was used in the case of *E. coli*. The primers were designed to measure the expression levels of *hydA* in UR206-*hydA* strain under conditions of *nifH2* promoter repression by growth in the presence of 10 mM ammonium sulfate, and induction by growth in the presence of 10 mM glutamate. In addition, expression levels of *R. rubrum hydA* and *D. vulgaris hydE* and *hydG* were measured in *E. coli* BL21 (DE3) prior to and after induction by adding IPTG to a final concentration 1.5 mM. The primers used in qRT-PCR reactions are indicated in Table 3.

Detection of [FeFe]-hydrogenase activity *in vitro*

The [FeFe]-hydrogenase activity of whole cell extracts was determined using a reduced methyl viologen assay (MV) as previously described (King et al., 2006). All procedures were carried out anaerobically. A total amount of 200 µl of each cell extract was added to 9 ml serum vial containing 55 mM Tris-HCl, (pH 7), 20 mM sodium dithionite and 1 mM methyl viologen. Reaction mixtures were incubated at room temperature and every twenty minutes 50 µl of the headspace gas was drawn with a gas-tight syringe and the produced H_2 gas was measured using a gas chromatograph (Shimadzu GC-8A) equipped with a thermal conductivity detector, a 2 m column packed with molecular sieve 5A, and argon as carrier gas.

RESULTS AND DISCUSSION

Gene expression of *R. rubrum hydA* in UR206 strain

Creating a chimeric DNA fragment from *hydA* and *PnifH2* of *R. rubrum*

The gene *hydA* (1.6 kb) and the promoter region upstream of the gene *nifH2* (400 bp) were amplified from genomic DNA of *R. rubrum* (Figure 1a and b). A chimeric DNA fragment (1.8 kb) of the *RrhydA* gene fused to the promoter of *nifH2*

gene was constructed stepwise and cloned into pCRBlunt to give pGSP02 (Figure 1c).

Overexpression of the gene *hydA* in UR206 under the control of *nifH2* promoter

To test the expression of *R. rubrum hydA*, *PnifH2hydA* was transferred to the mobilizable plasmid pJB3Tc20 and conjugated into *R. rubrum* UR206 (*nifH2*$^-$), generating strain UR206-*hydA*. Since in this construction *RrhydA* is under the control of the *nifH2* promoter, and since UR206 does not have a functional nitrogenase (Lehman et al., 1990), any hydrogen that is evolved should be due to the activity of *Rr*HydA. The levels of *hydA* gene expression in UR206-*hydA* strain was measured prior to and after *nifH2* promoter induction conditions. The results show that the level of *hydA* expression is about 50 times higher under conditions where the *nifH2* promoter is induced, confirming that *PnifH2hydA* in UR206-*hydA* is functional and that *hydA* can be regulated by conditions that repress or induce *nifH2* promoter.

Hydrogen production of UR206-*hydA* strain

The cumulative gas production of three strains of *R. rubrum*; wild type, UR206 and UR206-*hydA* is shown in figure 2 under conditions of *nif* repression (N^+; 10 mM ammonium sulfate) and *nif* derepression (N^-; 10 mM glutamate). Although the qRT-PCR results showed that *hydA* is overexpressed under *nif* deprepressing conditions, no hydrogen was produced under this condition. Thus, unlike the wild type strain, where there was abundant hydrogen production under conditions where nitrogenase is active, neither UR206, which does not possess a functional nitrogenase, nor UR206-*hydA* produces hydrogen under any condition. Thus it would appear that either *Rr*HydA is not active under these conditions, perhaps due to the apparent lack of a maturation system, shown in other organisms to be required for the synthesis of active [FeFe]-hydrogenases (Nicolet et al., 2010; Nicolet and Fontecilla-Camps, 2012), or *Rr*HydA cannot be matured into an active hydrogenase.

Coexpression of *RrhydA* in *E. coli* with hydrogenase maturation genes

Expression level of *hydA* and other maturation genes in *E. coli*

A DNA fragment of 1.6 kb corresponding to *RrhydA* was amplified from genomic DNA of *R. rubrum* introducing a HindIII and AflII restriction sites at the 5' end and 3' end, and cloned into pRSFDuet-1 to generate pRSF-$hydA_{Rr}$. Five transformants were constructed (T1, T2, T3, T4 and T5) to study the maturation of *Rr*HydA in *E. coli* BL21 (DE3). *RrhydA* was coexpressed with the *hydE*, *hydF* and *hydG* maturation genes of *D. vulgaris* (T1) and *C. acetobutylicum* (T2 and T4) while the *hydA* of *C. acetobutylicum* (*CahydA*) was overexpressed with its maturation genes as a positive control (T3). *RrhydA* was overexpressed without any maturation genes as a negative control (T5).

The expression levels of genes *RrhydA* together with *hydE* and *hydG* of *D. vulgaris* were evaluated prior to and after induction by IPTG in BL21(DE3). The results show that after induction these genes are expressed in 2 to 6 times more than the uninduced control (Figure 3). The proteins extracted from transformants T1, T2, T3, T4 and T5 were resolved in SDS-PAGE prior to and after induction of their encoding genes are shown in figure 4.

Gene product identification on the gel was by the molecular weight as deduced using the Kyoto Encyclopedia of Genes and Genomes (KEGG) website and Protein BLAST (Basic Local Alignment Search Tool) databases via NCBI (National Center for Biotechnology Information) web site. Details are given in Table 4.

Hydrogen production by *E. coli* BL21 (DE3) strains harboring [FeFe]-hydrogenase structural and maturation genes

Hydrogen evolution by the *E. coli* BL21 (DE3) strains T1, T2, T3, T4 and T5 was examined using an assay where sodium dithionite reduced methyl viologen is

oxidized by the action of the matured HydA. The maximum hydrogen production rate of all the strains tested was obtained when *RrhydA* was matured with the maturation genes of *C. acetobutylicum* (strain T2) (251±12 nmol/min). The positive control T3 strain that harbored *hydA* of *C. acetobutylicum* with its maturation genes evolved hydrogen at a rate of 124±51 (nmol/min). Less hydrogen was obtained with the T4 strain where *RrhydA* was coexpressed with the maturation genes of *C. acetobutylicum* contained in an artificial operon (65±17 nmol/min). The T1 strain, which contains *RrhydA* with the maturation genes of *D. vulgaris*, showed a very low production rate (20±12 nmol/min) close to the value of the negative control T5 strain (5.3±0.3 nmol/min), Figure (5A).

Specific hydrogen productivities were calculated by taking into account the protein concentration in the cell lysate used in the MV hydrogenase assay. Strain T2 gave the highest specific hydrogen production, 107 nmol/min/mg, which is higher than the positive control T3, 49 nmol/min/mg, and T4, 21 nmol/min/mg, whereas the lowest values were found for T1, 6.6 nmol/min/mg, and the negative control T5, 2.2 nmol/min/mg (Figure 5B). Variations seen in specific activity might be due to differences in the expression conditions for example that may play a role in the maturation process.

The use of Gel analyzer software made it possible to nearly calculate the specific amount of hydrogenase protein that was expressed which allowed the calculation of specific activities on HydA protein basis. The T2 strain gave the highest value, 31 nmol/min/µg HydA, and both T3 and T4 gave comparable values, (13 and 12 nmol/min/µg HydA, respectively). No HydA protein could be reliably detected for both strains T1 and the negative control T5 although trace amounts of hydrogen were detected. It is possible that incomplete maturation of *Rr*HydA due to the poor functioning of the *D. vulgaris* maturation system may have caused misfolding of HydA structural genes, exposing them to degradation by cellular proteases.

Several studies have demonstrated the need for the expression of maturation system gens in *E. coli* to achieve synthesis of active hydrogenase. Heterologous expression in *E. coli* of *hydA* alone from either the green alga *Chlamydomonas reinhardtii* or *C. acetobutylicum* failed to produce any active enzyme unless the maturation genes *hydE*, *hydF* and *hydG* from *C. acetobutylicum* were also co-expressed. However, the resulting recombinant [FeFe]-hydrogenase showed less specific hydrogen productivity compared to the native [FeFe] hydrogenase of *C. acetobutylicum* **(**King et al., 2006).

Although hydrogenase maturation gene sequences are highly conserved across species (King et al., 2006), the extent to which different maturation systems carry out the biosynthesis of a given HydA is variable. Co-expression of HydE, HydF and HydG maturases with the active hydrogenase structural gene *hydA* of *D. vulgaris* Hildenborough in *E. coli* only gave very modest hydrogenase activity, about 4000-fold lower for native hydrogenase as isolated from *D. vulgaris* (Laffly et al., 2010). Interestingly, this activity was increased when these proteins were co-expressed with the iron-sulfur cluster biosynthetic operon (*isc*). However, the resulting activity was still very low compared to the native hydrogenase and the enzyme proved unstable suggesting that other factors besides HydE, F, and G are required for full activity (Laffly et al., 2010). Similarly, another study demonstrated that deletion of the gene encoding the IscR transcriptional inhibitor*, iscR,* stimulates the specific activity of the recombinant clostridial [FeFe]-hydrogenase and H_2 accumulation in *E. coli* (Akhtar and Jones, 2008b). Taken together these results suggest that incomplete maturation of HydA is one of the limiting factors for the overproduction of [FeFe]-hydrogenase in *E. coli*. This is corroborated by a study where a host that normally produces [FeFe]-hydrogenase, *C. acetobutylicum*, was used for homologous and heterologous hydrogenase maturation (Abendroth et al., 2008). Hydrogenase specific activity was 28 fold higher in *C. acetobutylicum* when *hydA* and *hydEFG* were supplied on plasmids than when similar constructs were used in *E. coli*.

In the present study we attempted to optimize overexpression conditions by using different Fe-EDTA concentrations as well as adding iron and cysteine to the culture medium (data not shown). Fe-EDTA (1:1) gave the optimum results whereas the addition of iron and cysteine decreased subsequent hydrogenase activity. These results support the idea that use of a *E. coli* Δ*iscR* strain may enhance insertion of Fe-S clusters and hence the maturation of [FeFe]-hydrogenase proteins. In line with this, a recent study found that hydrogenase maturation and protein yields in an *E. coli*-based expression system were improved when glucose was added to the growth medium along with iron and cysteine, thought to aid in increased iron-sulfur cluster synthesis (Kuchenreuther et al., 2010).

CONCLUSIONS

Overexpression of the orphan *R. rubrum hydA* in a *nif* strain failed to produce evidence of active hydrogenase. To determine if *Rr*HydA could be matured, we used an *E. coli* system and demonstrated that the orphan *hydA* of *R. rubrum* is functional and active when expressed in *E. coli* if HydE, F and G are provided from a suitable heterologous source. In the future higher activities might be obtained if *RrhydA* was expressed in its native background together with the three maturases, HydA, HydE and HydG.

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TABLES

Table 1. Bacterial strains used

Strain	Characteristic	Reference
E. coli		
DH5α	*ϕ80dlacZΔM15Δ(lacZYA-argF) recA endA hsdR supE*	Bethesda Research Laboratories
BL21(DE3)	F^- *dcm ompT hsdS($r_B^- m_B^-$) gal λ(DE3)*	Studier et al. (1986)
S17-1	*rp4 2-(Tc::Mu) (Km::Tn7)* integrated into the chromosome	Simon and coll (1983)
BL21-*hydA$_{Rr}$EFG $_{Dv}$* (T1)	BL21 with pRSF-*hydA$_{Rr}$*, pET-*hydEF* and pCDF-*hydG$_{Dv}$*	This study
BL21-*hydA$_{Rr}$EFG$_{Ca1}$* (T2)	BL21 with pRSF-*hydA$_{Rr}$*, pET-*hydEF$_{Ca}$* and pCDF-*hydG$_{Ca}$*	This study
BL21-*hydAEFG$_{Ca}$* (T3)	BL21 with pET-*hydAE$_{Ca}$* and pCDF-*hydFG$_{Ca}$*	This study
BL21-*hydA$_{Rr}$EFG$_{Ca2}$* (T4)	BL21 with pRSF-*hydA$_{Rr}$*, and pCDF-*hydEFG$_{Ca}$*	This study
BL21-*hydA$_{Rr}$* (T5)	BL21 with pRSF-*hydA$_{Rr}$*	This study
R. rubrum		
ATCC® 11170™	Wild type	Pfennig and Trüper (1971)
UR206	Sm^R *nifH*::Km^R	Lehman and coll (1990)
UR206_*hydA*	UR206 + pJB3Tc20 with *nifH2hydA*	This study

Table 2. Characteristics of plasmids used in this study

Plasmid	Characteristic	Reference
pCRBlunt	Km^R, pCR-series cloning vector (4.6)	Invitrogen
pETDuet-1	Amp^R, co-expression vector (5.4 kb)	Novagen
pCDFDuet-1	Sm^R, co-expression vector (3.8 kb)	Novagen
pRSFDuet-1	Km^R, co-expression vector (3.8 kb)	Novagen
pBluescript ǁ KS-	Amp^R, *lacPOZ*, SCM, (2960 pb)	Stratagene
pJB3Tc20	Amp^R, Tc^R, conjugation,vector, (7069 pb)	Blatny (1997)
pET-$hydEF_{Dv}$	Amp^R, *D. vulgaris hydE* and *hydF* in pETDuet-1 (5.4 kb)	Mansure and Hallenebeck (2008)
pCDF-$hydG_{Dv}$	Sm^R, *D. vulgaris hydG* in pCDFDuet-1 (3.8 kb)	Mansure and Hallenebeck (2008)
pET-$hydE_{Ca}$	Amp^R, *C. acetobutylicum hydE* in pETDuet-1	King et al. (2006)
pET-$hydAE_{Ca}$	Amp^R, *C. acetobutylicum hydA* and *hydE* in pETDuet-1	King et al. (2006)
pCDF-$hydFG_{Ca}$	Sm^R, *C. acetobutylicum hydF* and *hydG* in pCDFDuet-1	King et al. (2006)
pCDF-$hydEFG_{Ca}$	Sm^R, *C. acetobutylicum hydEFG* in pCDFDuet-1	Akhtar and Jones (2008a)
pRSF-$hydA_{Rr}$	Km^R, *R. rubrum hydA* in pRSFDuet-1	This study
pGSP02	Km^R, *RrPnifH2hydA*Fu (2.2 kb) in pCRBlunt	This study
pGSP03	Amp^R, Tc^R, *RrPnifH2hydA*Fu (2.2 kb) in pJB3Tc20 (7 kb)	This study

Table 3. Primers used in different PCR and qRT-PCR reactions

Product	Primer	Sequence (5´-3´)
PCR		
*RrhydA*_HindIIIXhoI	*RrhydA*HindIII_F	CCAAGCTTAGAACGTCCTGTCGAT
	*RrhydA*XhoI_R	CCCTCGAGTCATCGTTCGCCTCAA
RrPnifH2	*RrPnifH2*_F	GAAGGGCGTTCTGGGCAGTAG
	*RrPnifH2*_R	TCGCAGCCGACAATCAGGA
*RrPnifH2hydA*Fu	Olap-*PnifHhydA*_F	AAAGGATTGATTCCATGTCGATCCAAC
	Olap-*PnifHhydA*_R	GTTGGATCGACATGGAATCAATCCTTT
*RrhydA*_HindIIIAflII	*RrhydA*HindIII_F	CCAAGCTTAGAACGTCCTGTCGAT
	*RrhydA*AflII_R	CCCTTAAGTCATCGTTCGCCTCAA
qRT-PCR		
*RrhydA*_RT	*RrhydA*_RT_F	TTCGACAAGGCCGTTCCCTATCTT
	*RrhydA*_RT_R	TTTCTCGGTTTCCAGCAGCACCTT
*RrrplC*_RT	*RrrplC*_F	GGTCTTATCGCCCAGAAGGT
	*RrrplC*_R	TTAGAGGTACGCTTGACCTTGG
*DvhydE*_RT	*DvhydE*_RT_F	GACTCGGCTACGAGATAGGC
	*DvhydE*_RT_R	CATCTCCACACCGAGTTCAC
*DvhydG*_RT	*DvhydG*_RT_F	CTCAAGAAGATGCGGTGAAG
	*DvhydG*_RT_R	CGGTCTGGAACATCTCCTCT
*Ec*16S_RT	*Ec*16S_RT_F	CGGAGGGTGCAAGCGTTAATC
	*Ec*16S_RT_R	TACGCATTTCACCGCTACAC

Table 4. Genes and gene products used

Gene accession #	Protein Molecular Weight (KDa)
***R. rubrum* S1**	
hydA (RruA0310)	HydA (48.94)
***D. vulgaris* Hildenborough**	
hydE (DVU0182)	HydE (35.55)
hydF (DVU0929)	HydF (40.97)
hydG (DVU1765)	HydG (51.61)
***C. acetobutylicum* ATCC 824**	
hydA (CAC3230)	HydA (50)
hydE (CAC1631)	HydE (39.95)
hydF (CAC1651)	HydF (45.79)
hydG (CAC1356)	HydG (53.59)

FIGURES

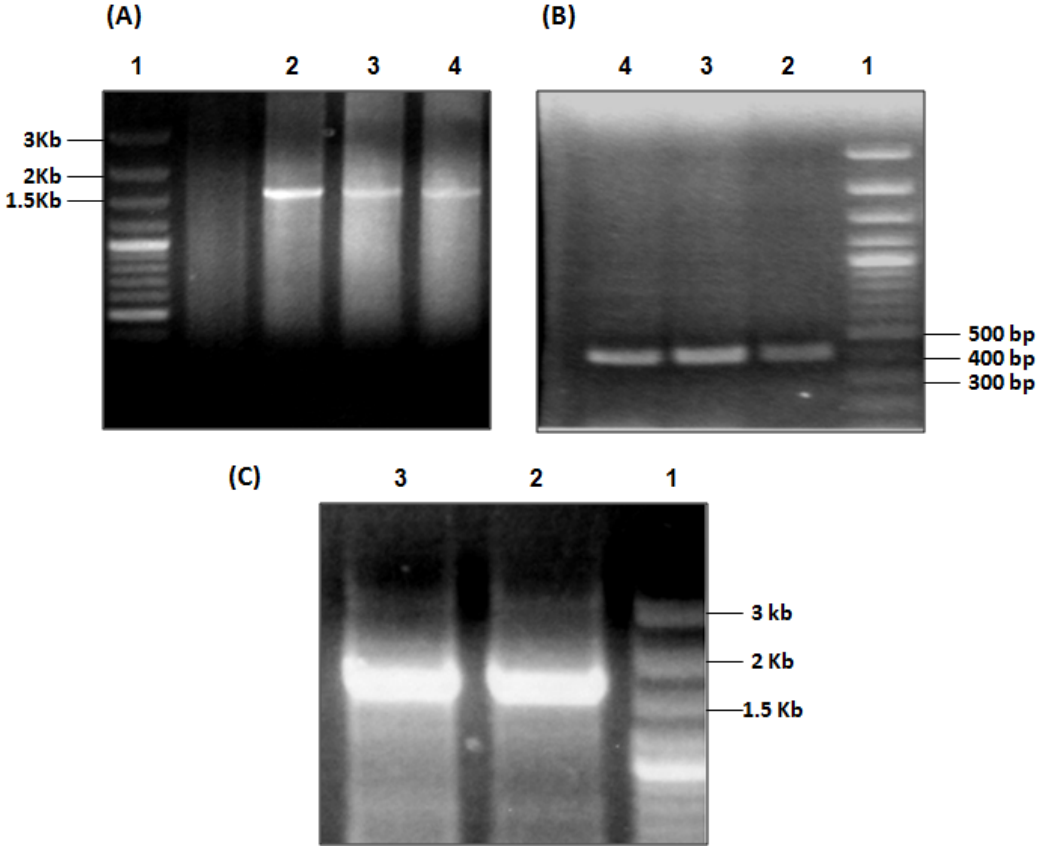

Figure 1. Analysis of PCR products by agarose gel electrophoresis. (A) Rr*hydA* amplification by PCR. Lanes 2, 3 and 4 show a fragment of 1.6 kb corresponding to *hydA*. (B) The promoter region of Rr*nifH2* amplified by PCR. Lanes 2 , 3 and 4 show a fragment of 400 bp corresponding to p*nifH2*. (C) Completed merger between *hydA* and *nifH2* promoter of *R. rubrum* obtained by PCR. Lanes 2 and 3 show p*nifH2hydA* (1.8 kb). GeneRuler ™ 100 bp DNA Ladder was loaded in lane 1 as molecular weight marker.

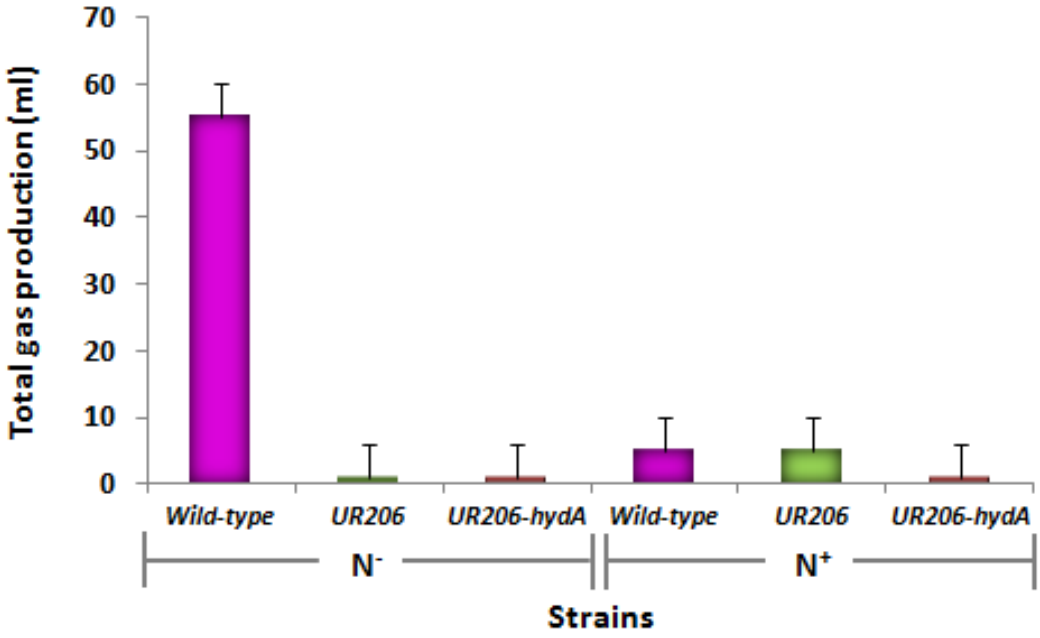

Figure 2. Mean±SE of total cumulative gas production for three strains of *R. rubrum* under conditions of *nif* repression (N^+) or *nif* derepression (N^-). *R. rubrum* (wt), UR206, and UR206-hydA were cultured under anaerobic light conditions with 10 mM succinate as carbon source for maximum of 7 days. The total volume of gas evolved was measured by water displacement.

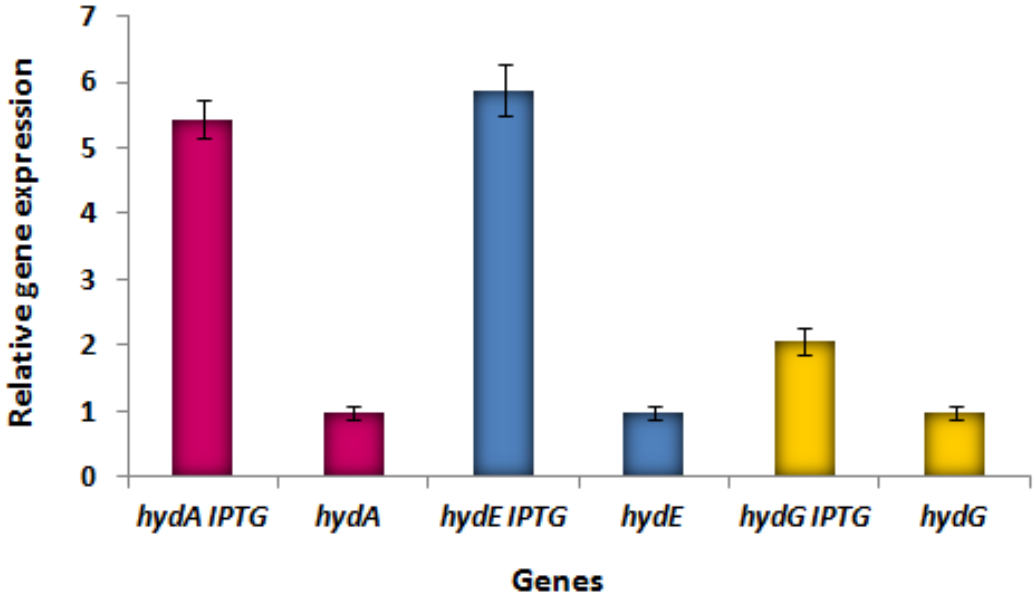

Figure 3. Relative gene expression of *hydA*, *hydE* and *hydG*. The levels of gene expression of *hydA* of *R. rubrum* and *hydE* and *hydG* of *D. vulgaris* in *E. coli* BL21 (DE3) prior to and after induction by adding IPTG to a final concentration 1.5 mM as measured by qRT-PCR.

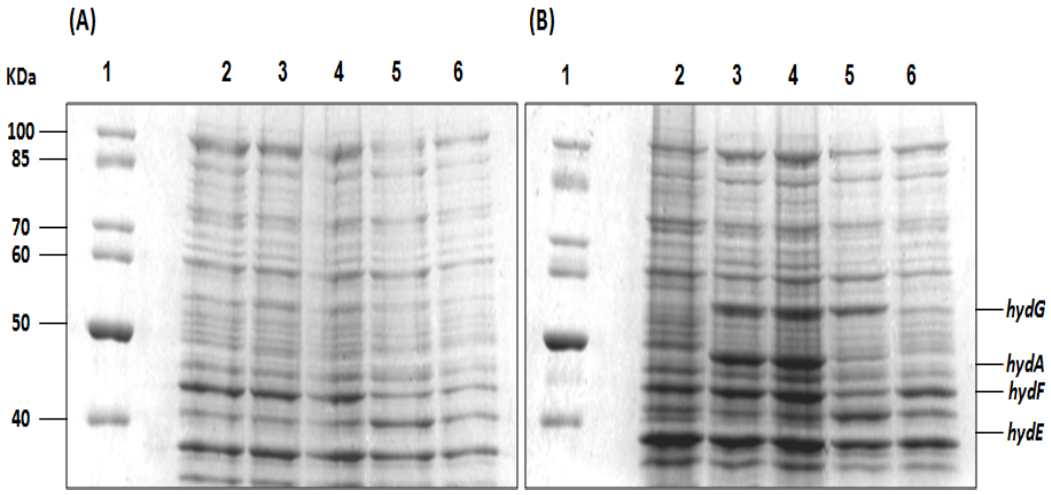

Figure 4. **SDS-PAGE of cell extracts of transformed strains T1, T2, T3, T4 and T5.** (A) Cell proteins prior to induction of structural and maturation encoding genes *hydA*, *hydE*, *hydF* and *hydG*. (B) Cell proteins after induction by IPTG. Lanes 2, 3, 4, 5, 6 show cell proteins of T1, T2, T3, T4 and T5 strains, respectively while lane 1 shows PageRuler^{TM} Unstained Protein Ladder.

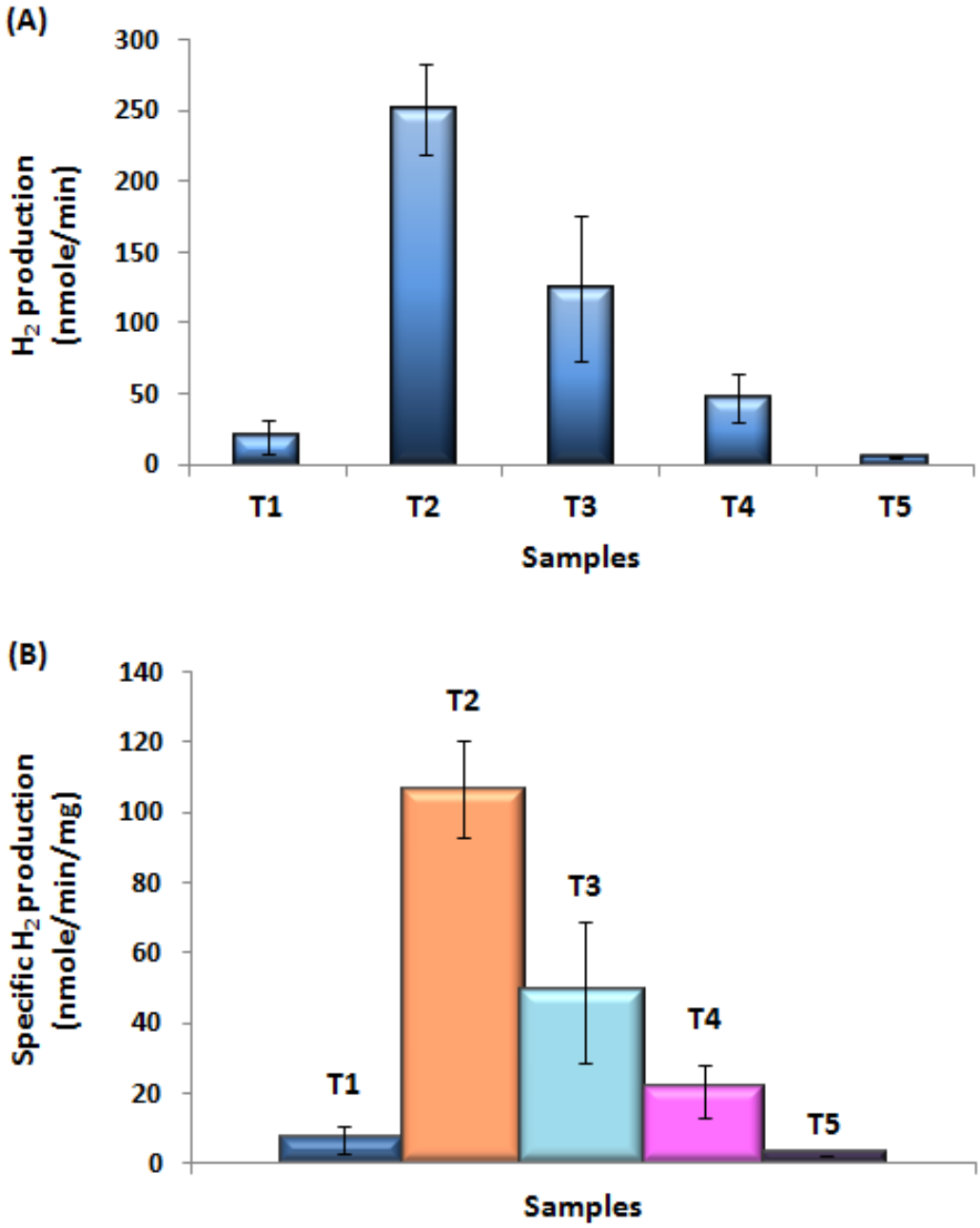

Figure 5. **[FeFe]-hydrogenase activity of tansformed strains T1, T2, T3, T4 and T5.** (A) Means±SDs of three sets for values of hydrogen production rate (nmol/min). (B) Means±SDs of three sets for values of specific hydrogen productivity (nmol/min/mg). T1, T2, T3, T4 and T5 represent transformants of BL21-*hydA*$_{Rr}$*EFG*$_{Dv}$, BL21-*hydA*$_{Rr}$*EFG*$_{Ca1}$, BL21-*hydAEFG*$_{Ca}$, BL21-*hydA*$_{Rr}$*EFG*$_{Ca2}$ and BL21-*hydA*$_{Rr}$, respectively.

CHAPTER 5: Expression and maturation of a NADP-dependent [FeFe]-hydrogenase in *Escherichia coli*

Mona Abo-Hashesh[a,b], Patrick C. Hallenbeck[a*]

[a] Département de Microbiologie et Immunologie, Université de Montréal, CP 6128 Succursale Centre-ville, Montréal, Québec, Canada H3C 3J7

[b] Department of Microbiology, Port Said University, Port Said, Egypt

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Expression and maturation of a NADP-dependent [FeFe]-hydrogenase in *Escherichia coli*

Mona Abo-Hashesh[a,b], Patrick C. Hallenbeck[a*]

[a] Département de Microbiologie et Immunologie, Université de Montréal, CP 6128 Succursale Centre-ville, Montréal, Québec, Canada H3C 3J7

[b] Department of Microbiology, Port Said University, Port Said, Egypt

[*]Corresponding author

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ABSTRACT

Various strategies are being developed to attempt to increase hydrogen yields in biohydrogen fermentative processes. One strategy is to overexpress one of the highly active [FeFe]-hydrogenases in a suitable *Escherichia coli* host strain. Expression of a hydrogenase that can directly interact with NADPH is desirable as this, rather than reduced ferredoxin, is naturally produced by its metabolism. However, the successful maturation of this type of hydrogenase in *E. coli* has not yet been reported. The *Desulfovibrio fructosovorans hnd* operon (*hndA*, *B*, *C*, and *D*), encoding a NADP-dependent [FeFe]-hydrogenase, was cloned into an overexpression vector and transformed into *E. coli* BL21(DE3) and *E. coli* BL21(DE3)Δ*iscR* strains carrying compatible expression vectors harboring the maturation genes *hydE*, *hydF* and *hydG* of *Clostridium acetobutylicum*. Hydrogenase activity was measured *in vitro* using a reduced methyl viologen assay. *E. coli* BL21(DE3)Δ*iscR* (iron sulfur cluster regulator deficient strain) gave a higher hydrogen production rate (20.4 nmol/min) compared to the isogenic strain *E. coli* BL21(DE3), 7.1 nmol/min. The corresponding specific hydrogen productivities, based on total protein, were 6.2 and 2.4 nmol/min/mg. In order to test the hypothesis that increased yields of hydrogen might be obtained with a NADP-dependent [FeFe]-hydrogenase, Δ*aceF* was introduced into these *E. coli* strains by P1 transduction, to give BL21(DE3)Δ*iscR*Δ*aceF* and BL21(DE3)Δ*aceF*. Δ*aceF* has been shown to enhance gluconeogenesis as well as to increase metabolic flux through the pentose phosphate pathway. This would allow increased glucose oxidation if the excess NADPH produced is reoxidized through hydrogen production by the NADP-dependent [FeFe]-hydrogenase. Thus a multi-subunit NADP-dependent [FeFe]-hydrogenase was successfully matured in *E. coli* with the HydE, F and G maturases of *C. acetobutylicum* for the first time. This sets the stage for increased hydrogen yields via the pentose phosphate pathway.

INTRODUCTION

Hydrogenases are metalloproteins that catalyze either hydrogen uptake or evolution according to the requirements of the host organism and the redox potential of the hydrogenase redox partner. Thus, the hydrogenase redox partners such as NAD^+, cytochromes, coenzyme F420 or ferredoxins play a crucial role in catalyzis depending upon whether they are poised to act as electron donors or acceptors. There are two main classes of hydrogenases: [NiFe] and [FeFe]. Both hydrogenases have been shown to be involved in hydrogen evolution in different organisms where electrons coming from a carrier and hydrogen ions react together at the hydrogenase's buried H-cluster active site to produce H_2 (Vignais et al., 2001; Abo-Hashesh et al., 2011). The [FeFe]-hydrogenases can have an activity about 10-100 times higher than the [NiFe]-hydrogenases (Frey, 2002; Horner et al., 2002). All hydrogenases are sensitive to oxygen especially [FeFe]-hydrogenases which are irreversibly inactivated after oxygen exposure while [NiFe]-hydrogenases are less oxygen sensitive and can be partially reactivated (Lenz et al., 2010; Saggu et al., 2010).

Current fermentative biohydrogen processes suffer from low H_2 yields, 2-4 mols of H_2 per mol glucose, leading to the accumulation of large amounts of fermentative byproducts such as ethanol and organic acids (Hallenbeck, 2009). Metabolically engineering a synthetic enzymatic pathway to increase flux of electrons to hydrogenase for H_2 synthesis is one possible way to improve yields. One strategy is to overexpress one of the highly active [FeFe]-hydrogenases in a suitable *Escherichia coli* host strain as it is known to be an ideal microorganism commonly used in genetic engineering due to its well-characterized genome and metabolism as well as its ability to utilize a wide range of carbon sources (Hallenbeck et al., 2011; Abo-Hashesh et al., 2011).

The NAD(P)H- and ferredoxin-dependent hydrogen production pathways are thought to have great potential for improving production yields compared to the formate hydrogen lyase (FHL) pathway (Cho et al., 2011). Since *E. coli* lacks [FeFe]

hydrogenase structural and maturation genes, they need to be introduced in order to attempt to increase yields. One target would be to use glucose oxidation by the pentose phosphate pathway to drive hydrogen production, but this requires connecting NADPH oxidation to proton reduction. Previously, Malki et al. (1995) cloned a genomic DNA fragment from *Desulfovibrio fructosovorans* which hybridized with the *hydAB* genes from *D. vulgaris* Hildenborough. This fragment was sequenced and found to consist of a *hnd* operon containing four genes, *hndA*, *hndB*, *hndC*, and *hndD*, reported to encode an NADP-dependent hydrogenase (Malki et al., 1995). Here, the *D. fructosovorans hnd* operon was cloned and overexpressed in *E. coli* along with the maturation genes from *Clostridium acetobutylicum* that are required for the enzyme to be functional. This sets the stage for driving hydrogen production through oxidation of NADPH.

The cellular NADPH pool can be increased by directing carbon flux through the pentose phosphate (PP) pathway. The pentose phosphate (PP) pathway starts with the oxidation of glucose-6-phosphate to 6-phosphogluconate which is further oxidized to pentose ribulose-5-phosphate and CO_2. A great deal of NADPH is produced during these oxidations as well as CO_2 and the two intermediates of fructose-6-phosphate and glyceraldehyde-3-phosphate. These intermediates are returned to the PP pathway resulting in the complete oxidation of glucose-6-phosphate to CO_2 and NADPH. The NADPH that is formed could be reoxidized by the NADP-dependent hydrogenase to produce hydrogen (Smith et al., 2012). Thus, the NADPH-dependent pentose phosphate pathway together with NADP-dependent hydrogenase activity could be a very efficient path for dark fermentative biological hydrogen production, resulting in the maximum yield of H_2 as shown in figure 1 (Dennis and Blakeley, 2000).

Gluconeogenic activity is necessary for activation of the PP pathway, and hence for enhancement of NADPH production (Kim et al., 2011). The *E. coli* mutant Δ*aceF*, which lacks dihydrolipoamide acetyltransferase (E2p) activity, has been shown to have increased gluconeogenesis as well as increased flux through the

pentose phosphate pathway (Langley and Guest, 1977). Dihydrolipoamide acetyltransferase is one of the proteins of the pyruvate dehydrogenase complex which directs carbon flux from pyruvate to acetyl CoA. Thus, knocking out the dihydrolipoyl transacetylase component of this complex by using an *aceF* mutant results in enhancement of gluconeogenic activity which in turn activates the PP pathway (Lee et al., 2004; Kim et al., 2011). This might allow increased glucose oxidation if the excess NADPH produced could be reoxidized through hydrogen production by a NADP-dependent [FeFe]-hydrogenase. Consequently, activation of the PP pathway and enhancing gluconeogenic flux by using an *aceF* mutant could play a critical role in increasing NADPH-dependent hydrogen production in *E. coli*.

The overall aim of the current study is to clone and overexpress structural and maturation genes of NADP-dependent hydrogenase from different organisms in mutated *E. coli* that can overproduce NADPH via PP pathway.

MATERIALS AND METHODS

Bacterial strains and plasmids

Escherichia coli strains were grown in glass test tubes supplemented with LB medium (Luria et al., 1960) at incubated 37°C overnight. The bacterial strains and plasmids that were used in this study are shown in Table 1.

Cloning of *hnd* operon in pACYCDuet-1 and coexpression with *hyd* maturation genes

Plasmids and genomic DNA were purified and gel extracted using QIAGEN QIAprep Spin Miniprep, QIAquick Gel Extraction and QIAamp DNA Mini Kits. Polymerase Chain Reactions (PCR) were carried out using the GeneAmp PCR system 2400 (Perkin Elmer).

NADP-dependent FeFe-hydrogenase consisting of four genes, *hndABCD* organized in a *hnd* operon. This operon was already available cloned in pBMG6 by (De Luca et al., 1998) and recloned in the present study to place the entire operon in a duet expression vector pACYCDuet-1 that is suitable for coexpression with the *hyd* maturation genes from *C. acetobutylicum*. As the entire *hnd* operon is large (~5000 bp) and to avoid the probability of amplification induced mutations, the operon was cloned stepwise in pBluescript II SK-. The 3´-end region of *hnd* operon (NotI/HindIII) was first cloned in pBluescript (Notl/Hindlll) generating pBluescript-*hnd*3´ (Figure 2A and 2B). Then, the 5´-end region was amplified from pBMG6 with the introduction of upstream Sacl and Pcil restriction sites and a downstream Notl restriction site (Figure 2B). Introduction of an upstream Pcil restriction site gives compatible ends with NcoI, required for the correct insertion of the *hnd* operon directly after the T7 promoter of pACYCDuet-1. The modified 5´-end region was then cloned (SacI/NotI) into pBluescript- *hnd*3´ generating pBluescript-*hnd* (Figure 2B). Finally, the reconstructed *hnd* operon was cloned from pBluescript-*hnd* (Pci1/HindIII) into pACYCDuet-1 (NcoI/HindIII) generating plasmid pACYCDuet-1-*hnd* (Figure 2C).

Plasmid pACYCDuet-1-*hnd* was transformed into *E. coli* strains BL21(DE3) and BL21(DE3) Δ*iscR* along with plasmids pET-*hydEF* and pCDF-*hydG* that harbor the *hyd* maturation genes of *C. acetobutylicum* in a three way transformation, producing BL21(DE3)-$hnd_{Df}hydEFG_{Ca}$ (T1) and BL21(DE3)Δ*iscR*–$hnd_{Df}hydEFG_{Ca}$ (T2), respectively. The structural and maturation genes, *hydAEFG*, of the [FeFe]-hydrogenase of *C. acetobutylicum* were transformed into the same strains, producing BL21(DE3)-$hydAEFG_{Ca}$ (T3) and BL21(DE3)Δ*iscR*-$hydAEFG_{Ca}$ (T4) as positive controls.

Overexpression of NADP-dependent [FeFe]-hydrogenase in *E. coli*

Overnight cultures of transformed *E. coli* strains were subcultured (1:50 dilution) into 115 ml of fresh LB medium supplemented with the required antibiotics

and 100 µM Fe-EDTA (1:1). Then, cultures were grown aerobically at 37°C with 250 rpm of agitation until optical densities (OD_{600nm}, measured using a double beam spectrophotometer (Shimadzu)) of 0.5 up to 0.7 were reached. Isopropyl-D-thiogalactopyranoside (IPTG) 1.5 mM was added to the culture media for pre-induction of *hnd* and *hyd* genes expression and the cultures were incubated aerobically at room temperature for 1 hr and 100 rpm. To achieve induction of NADP-dependent [FeFe]-hydrogenase biosynthesis under anaerobic conditions, the cultures were transferred into 120 ml serum bottles, sealed with rubber stoppers, flushed with argon and incubated overnight at room temperature (King et al., 2006).

Samples were transferred under anaerobic condition into centrifuge bottles and centrifuged at 13000 rpm for 15 min. Cell pellets were resuspended in 1 ml BugBuster protein extraction reagent and the cell lysates were kept at -20°C for further protein analyses. Total protein concentrations of the whole cell lysates were determined using a Pierce BCA Protein Assay Kit from Thermo Scientific.

Samples were taken from the serum bottles before and after IPTG induction, treated with 3X sample buffer, and kept at -20°C for subsequent sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses. SDS-PAGE for protein samples was carried out as described in Wilder et al. (1979). Polyacrylamide gels were stained using coomassie blue R-250 and scanned using a Typhoon™ 9410 scanner. Gel analyzer software ImageQuant TL was used for calculating specific protein concentrations.

Detection of NADP-dependent [FeFe]-hydrogenase activity *in vitro*

A reduced methyl viologen assay (MV) was used to determine [FeFe]-hydrogenase activity of the whole cell extracts *in vitro* as previously described (King et al., 2006). A total amount of 200 µl of cell extract was added to a 9 ml vial serum vial and Tris-HCl (pH 7), sodium dithionite and methyl viologen were added to a final concentrations of 55mM, 20mM and 1 mM, respectively. The reaction mixtures

were incubated at room temperature for 20 min. To calculate the H_2 production rate (nmol/min) 50 µl of headspace gas were withdrawn every twenty minutes with a gas-tight syringe and the H_2 gas produced was measured using a gas chromatograph (Shimadzu GC-8A) equipped with a thermal conductivity detector, a 2 m column packed with molecular sieve 5A, and argon as carrier gas. All the above assay procedures were carried out under anaerobic conditions.

Developing *E. coli* strains for overproduction of NADPH

In order to test the NADP-dependent [FeFe]-hydrogenase activity *in vivo*, *E. coli* strains that overproduce NADPH needed to be developed. As mentioned above, *E. coli* Δ*aceF* is required to enhance gluconeogenesis as well as to increase flux through the pentose phosphate pathway which in turn will increase glucose oxidation and produce large amounts of NADPH possibly resulting in maximum yields of H_2 via NADP-dependent hydrogenase activity.

Δ*aceF* was introduced into recipient cells of *E. coli* strains BL21(DE3) and BL21(DE3) Δ*iscR* from *E. coli* Δ*aceF* JW0111 donor cells by P1 transduction as described in Thomason et al. (2007), giving BL21(DE3)Δ*iscR*Δ*aceF* and BL21(DE3)Δ*aceF*.

RESULTS AND DISCUSSION

The *hnd* operon including the encoding genes of NADP-dependent [FeFe]-hydrogenase (*hndABCD*) was successfully cloned stepwise into an expression vector pACYCDuet-1 generating pACYCDuet-1-*hnd*. Four *E. coli* strains harboring both the structural genes of the NADP-dependent [FeFe]-hydrogenase as well as the maturation genes of the Fd-dependent [FeFe]-hydrogenase were constructed. Thus, *C. acetobutylicum hydEFG* were used to mature the NADP-dependent [FeFe]-hydrogenase, HndABCD of *D. fructosvorans* in the *E. coli* strains, BL21(DE3)-$hnd_{Df}hydEFG_{Ca}$ and BL21(DE3)Δ*iscR*-$hnd_{Df}hydEFG_{Ca}$. As well, the same maturases

from *C. acetobutylicum* were used with the Fd-dependent [FeFe]-hydrogenase structural gene *hydA* of the same organism as a positive control for maturation activity.

Details of the genes and their protein products were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) website and Protein BLAST (Basic Local Alignment Search Tool) databases via NCBI (National Center for Biotechnology Information) web site. Some details of the genes and their products are given in Table 2.

The maximum hydrogen production rate observed *in vitro* was 73 nmol/min for *E. coli* strain BL21(DE3)Δ*iscR* harboring the structural and maturation genes, *hydAEFG* of the Fd-dependent [FeFe]-hydrogenase from *C. acetobutylicum*, while the specific activity was 20.7 nmol/min/mg. In contrast, BL21(DE3) harboring the same genes gave a lower production rate, 33 nmol/min, and the specific activity was 11.81 nmol/min/mg (Figure 3 and 4).

E. coli strains BL21(DE3)Δ*iscR* and BL21(DE3) harboring *hndABCD*, the structural genes of the NADP-dependent [FeFe]-hydrogenase, together with the maturases of *C. acetobutylicum* gave much less activity as the H_2 production rates were 20 and 7.1 nmol/min, respectively while specific activities were 6.12 and 2.4 nmol/min/mg, respectively (Figure 3 and 4).

Thus, the activity of the NADP-dependent [FeFe]-hydrogenase was much less than that of the Fd-dependent [FeFe]-hydrogenase. One plausible explanation for this observation might be that the homologous expression system is more efficient than the heterologous one since the structural genes of the former (*hndABCD*) were matured by genes (*hydEFG*) from another organism (*C. acetobutylicum*) while the structural gene (*hydA*) of the latter was matured by those genes (*hydEFG*) from the same organism. This idea is supported by the results of a previous study by Girbal et al. (2005) demonstrated that homologous expression of Fe-only hydrogenase of

Clostridium acetobutylicum showed higher activity than heterologous expressions of *Chlamydomonas reinhardtii* and *Scenedesmus obliquus* Fe-only hydrogenases. However, Sybirna et al. (2008) showed that heterologous production system of *Chlamydomonas reinhardtii* [Fe-Fe] in *Shewanella oneidensis* appeared to be more efficient than that of *C. acetobutylicum* in *E. coli*. Moreover, King et al. (2006) demonstrated that heterologous expression of *C. acetobutylicum* [FeFe]-hydrogenase in *E. coli* resulted in a specific hydrogen activity that was 7.5-fold higher than the value reported previously for the same enzyme in the native recombinant system. As well, different algal and bacterial [FeFe] hydrogenases that were heterologously expressed in *E. coli* provided enzymes with specific activities that were comparable to those of the enzymes extracted from native sources.

Interestingly, using a *E. coli* Δ*iscR* strain as the expression host enhances hydrogen production, an effect that is probably related to the increased availability of Fe-S clusters which are required for proper biosynthesis and assembly of [FeFe]-hydrogenase proteins. Previous studies have described the impact of deletion of *iscR* on the up-regulation of expression of the *isc* operon (Schwartz et al., 2001; Giel et al., 2006). Therefore, using a Δ*iscR* strain has a positive influence on total hydrogenase activity via enhancing Fe–S cluster assembly and/or repair (Nakamura et al., 1999; Kriek et al., 2003).

Future work should focus on the expression of the NADP-dependent [FeFe]-hydrogenase and maturation genes in Δ*acef E. coli* strains BL21(DE3)Δ*iscR*Δ*aceF* and BL21(DE3)Δ*aceF* that overproduce NADPH. This would allow an analysis of *in vivo* hydrogenase activity and perhaps demonstrate increased hydrogen yields. In addition, it would be interesting to determine *in vitro* the efficiency with which the enzyme matured in *E. coli* is able to use NADPH

CONCLUSIONS

Metabolic engineering is currently being used by a number of researchers in attempts to improve hydrogen production. The development of efficient hydrogenases through heterologus expression in *E. coli* is one promising strategy to increase hydrogen yields in biohydrogen fermentative processes. A NADP-dependent [FeFe]-hydrogenase, encoded by *hndABCD* of *D. fructosovorans* was overexpressed in *E. coli* BL21(DE3) and *E. coli* BL21(DE3)Δ*iscR* along with compatible expression vectors harboring the maturation genes *hydEFG* of *C. acetobutylicum*. A reduced methyl viologen assay was used to determine hydrogenase activity *in vitro*. *E. coli* strains BL21(DE3)Δ*iscR* and BL21(DE3) harboring the structural and maturation genes of *C. acetobutylicum* gave higher hydrogen production rates than those harboring structural genes *hndABCD* of *D. fructosovorans* with the maturases of *C. acetobutylicum*. The ratios of specific hydrogen production, based on the total protein, were similar to those of hydrogen production rates. A *aceF* knockout was introduced by P1 transduction to boost pentose phosphate pathway which in turn could lead to production of a great amount NADPH. Finally, those NADPH could be harnessed for hydrogen production through activity of NADP-dependent FeFe]-hydrogenase that already was successfully matured in *E. coli* with the HydEFG maturases of *C. acetobutylicum* for the first time. This may present a new strategy for increasing hydrogen yield from glucose via the pentose phosphate pathway.

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TABLES

Table 1. Characteristics of bacterial strains and plasmids used

Strain / plasmid	Characteristic	Reference
E. coli		
DH5α	*ϕ80dlacZΔM15Δ(lacZYA-argF) recA endA hsdR supE*	Bethesda Research Laboratories
BL21(DE3)	F⁻ *dcm ompT hsdS($r_B^-m_B^-$) gal λ(DE3)*	Studier et al. (1986)
BL21(DE3)Δ*iscR*	F⁻ *ompT hsdSB($r_B^-m_B^-$) gal dcm iscR::kan (DE3)*	Akhtar and Jones (2008)
aceF(del) (Keio:JW0111)	F⁻ Δ*(araD-araB)567*, *ΔaceF733::kan*, *ΔlacZ4787*(::*rrnB*-3), λ^-, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	Baba et al. (2006)
BL21(DE3)-$hnd_{Df}hydEFG_{Ca}$	BL21(DE3) with pACYCDuet-*hnd,* pET-*hydEF* and pCDF-*hydG* of *C. acetobutylicum*	This study
BL21(DE3)Δ*iscR* -$hnd_{Df}hydEFG_{Ca}$	BL21(DE3)Δ*iscR* with pACYCDuet-*hnd,* pET-*hydEF* and pCDF-*hydG* of *C. acetobutylicum*	This study
BL21(DE3)-$hydAEFG_{Ca}$	BL21(DE3) with pET-*hydAE* and pCDF-*hydFG* of *C. acetobutylicum*	This study
BL21(DE3)Δ*iscR*-$hydAEFG_{Ca}$	BL21(DE3)Δ*iscR* with pET-*hydAE* and pCDF-*hydFG* of *C. acetobutylicum*	This study
Plasmids		
pACYDuet-1	Cm^R, co-expression vector (4008 bp)	Novagen
pBluescript ‖ SK-	Amp^R, *lacPOZ*, SCM, (2961 bp)	Stratagene
pBMG6-*hnd*	Gm^R *D. fructosovorans hnd* in pBMG6 (10 Kb)	De Luca et al. (1998)
pET-*hydE*	Amp^R, *C. acetobutylicum hydE* in pETDuet-1	King et al. (2006)
pET-*hydAE*	Amp^R, *C. acetobutylicum hydA* and *hydE* in pETDuet-1	King et al. (2006)
pCDF-*hydFG*	Sm^R, *C. acetobutylicum hydF* and *hydG* in pCDFDuet-1	King et al. (2006)
pBluescript-*hnd*3´	Amp^R, 3´-end region of *hnd* operon in pBluescript ‖ KS- (6601 bp)	This study
pBluescript-*hnd*	Amp^R, *hnd* operon (5 kb) in pBluescript ‖ KS- (7764 bp)	This study
pACYCDuet-1-*hnd*	Cm^R, *hnd* operon (5 kb) in pACYDuet-1(8860 bp)	This study

Table 2. Genes and their protein products used in the current study

Gene accession #	Protein Molecular Weight (KDa)
D. fructosovorans **(U07229)**	
hndA (AAA87054)	HndA (18.81)
hndB (AAA87055)	HndB (13.79)
hndC (AAA87056)	HndC (52.59)
hndD (AAA87057)	HndD (63.44)
C. acetobutylicum **ATCC 824**	
hydA (CAC3230)	HydA (50)
hydE (CAC1631)	HydE (39.95)
hydF (CAC1651)	HydF (45.79)
hydG (CAC1356)	HydG (53.59)

FIGURES

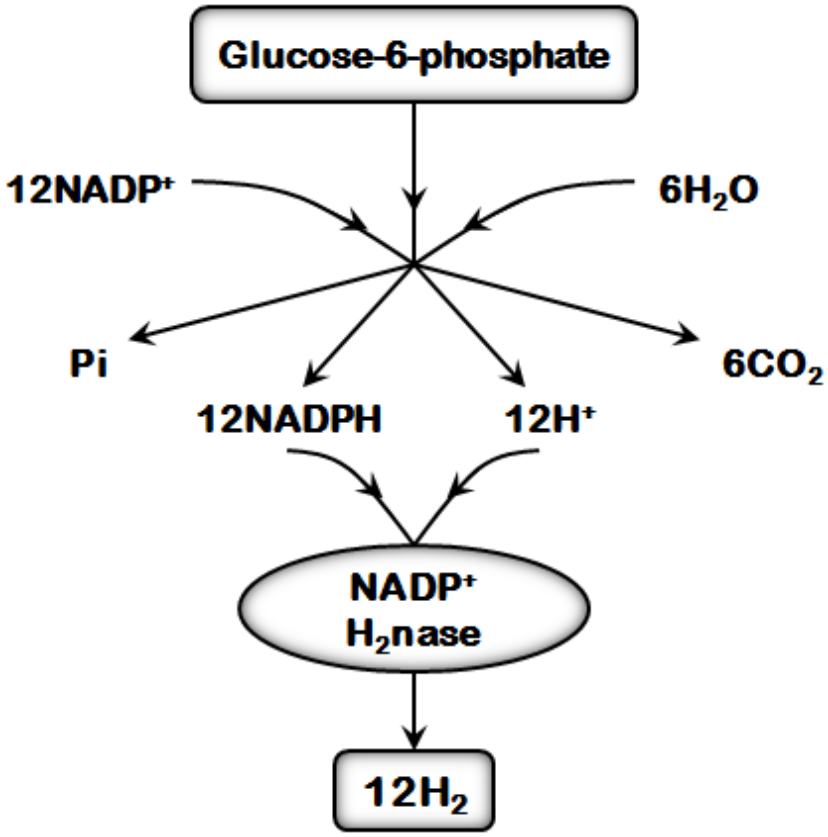

Figure. 1 Complete oxidation of glucose-6-phosphate through the oxidative pentose phosphate (PP) pathway. The production of large amounts of NADPH through PP pathway results in maximum yields of H_2 via NADP-dependent hydrogenase activity.

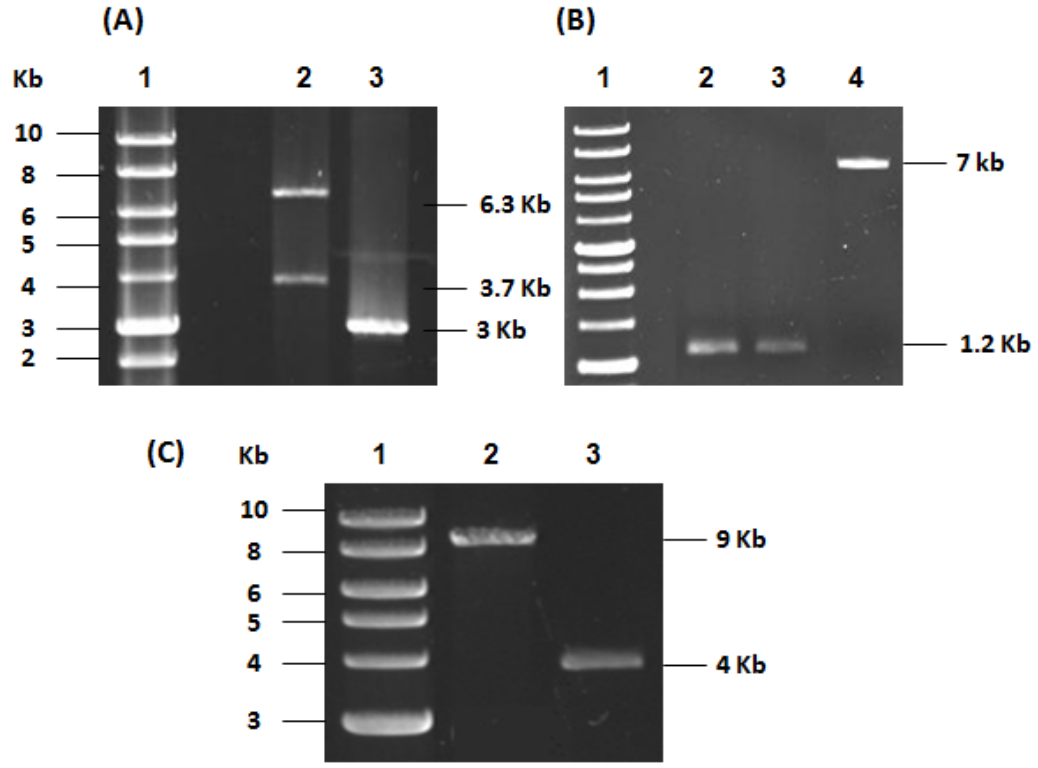

Figure. 2 Analysis of DNA fragments by agarose gel electrophoresis. (A) 3′-end region of *hnd*. Lane 2 shows 3′-end region of *hnd* (3.7 kb) and the rest of digested pBMG6 (NotI/HindIII). Lane 3 shows digested pBluescript with HindIII (3 kb). (B) 5′-end region of *hnd*. Lane 2 and 3 show the amplified fragments of 1.2 kb corresponding to 5′-end region of *hnd*. Lane 4 shows the constructed pBluescript-*hnd3′* (7 kb). (C) Cloning of *hnd* operon in pACYCDuet-1. Lane 2 shows the constructed pACYCDuet-1-*hnd* (9 kb). Lane 3 shows intact pACYCDuet-1 (4 kb). Lane 1 shows a 1 kb DNA Ladder.

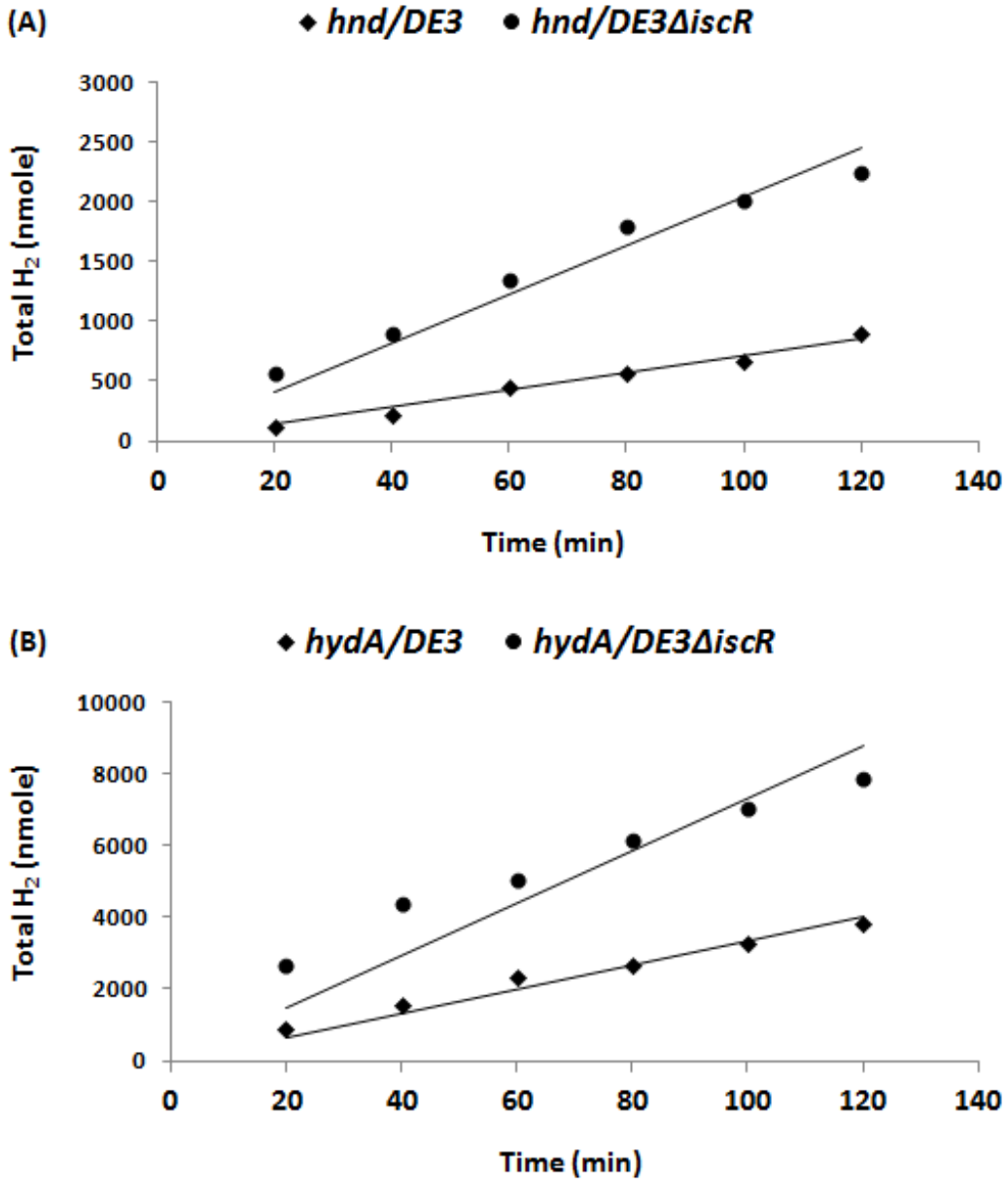

Figure 3. *In vitro* hydrogenase activity of the engineered [FeFe]-hydrogenase in *E. coli* strains. (A) Hydrogen production rates (nmol/min) for constructed *E. coli* strains BL21(DE3)-$hnd_{Df}hydEFG_{Ca}$ (*hnd*/DE3) and BL21(DE3)Δ*iscR*-$hnd_{Df}hydEFG_{Ca}$ (*hnd*/DE3Δ*iscR*) which display NADP-dependent [FeFe]-hydrogenase activity. (B) Hydrogen production rates (nmol/min) for constructed *E. coli* strains BL21(DE3)-$hydAEFG_{Ca}$ (*hdA*/DE3)and BL21(DE3)Δ*iscR*-$hydAEFG_{Ca}$ (*hydA*/DE3Δ*iscR*) which reveal Fd-dependent [FeFe]-hydrogenase activity.

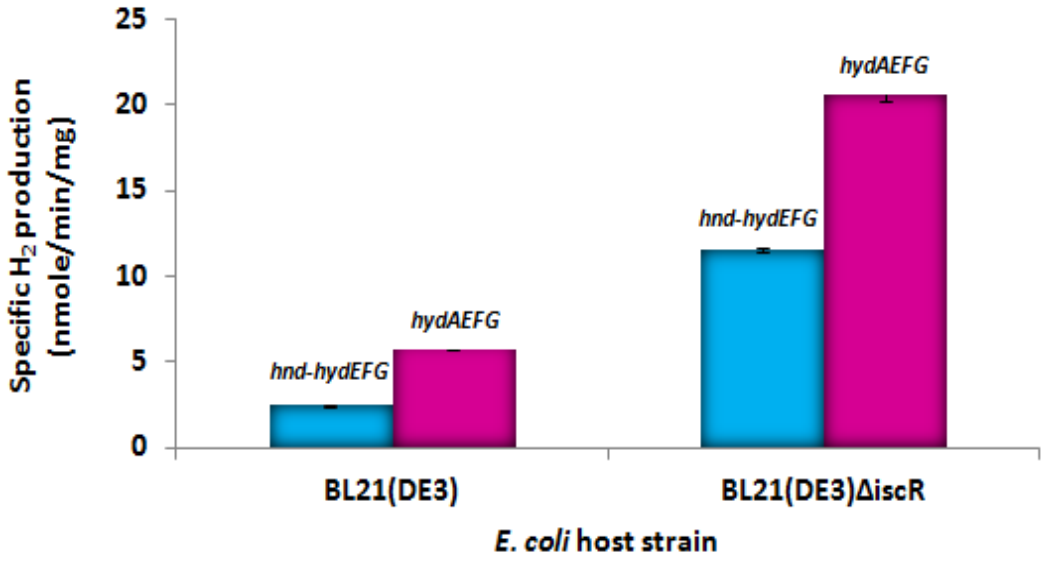

Figure 4. Specific hydrogen productivity of the engineered [FeFe]-hydrogenase in *E. coli* strains. BL21(DE3) and BL21(DE3)Δ*iscR* are holding structural and maturation genes of either NADP-dependent [FeFe]-hydrogenase (*hnd-hydEFG*) or Fd-dependent [FeFe]-hydrogenase (*hydAEFG*).

DISCUSSION

This thesis examined various means to improve hydrogen production whether via physiological manipulation or metabolic engineering.

1. Physiological manipulation

The first part of the thesis concerned using physiological manipulation to attempt to improve hydrogen production. Three studies were performed to meet this goal including microaerobic dark fermentative hydrogen production, photofermentive hydrogen production from glucose in batch and continuous culture.

1.1 Microaerobic dark fermentative hydrogen production

One major avenue for potentially improving hydrogen production was an examination of microaerobic dark fermentation for hydrogen production using the photosynthetic bacterium *Rhodobacter capsulatus* which typically produces hydrogen under nitrogen-limited, anaerobic photosynthetic conditions. However, if the use of light could be avoided, the fermentative process might be more economical. Microaerobic dark fermentation depends on using a low concentration of oxygen to induce the TCA cycle and replace photo-phosphorylation with oxidative-phosphorylation to provide the energy required for driving substrate oxidation to completion. In this way more hydrogen gas could potentially be obtained. In addition, the use of limiting amounts of oxygen is necessary to prevent the oxidative destruction of the nitrogenase enzyme that catalyzes the hydrogen production process. The optimal O_2 concentration for microaerobic hydrogen production was determined. As well, different concentrations of carbon sources and two different nitrogen sources, glutamate and ammonium, were evaluated for optimal microaerobic hydrogen production conditions in batch culture using a *hup*$^-$ strain of *R. capsulatus*.

As *R. capsulatus* is a facultative anaerobic bacterium, its growth increases with an increase in the concentration of O_2 within limits, since above 20% O_2 the growth declined. On the other hand, there was a negative correlation between the O_2 concentration and the total amount of H_2 as well as specific H_2 production and this can be related to the negative impact of high O_2 concentrations on the nitrogenase enzyme and hence on H_2 production.

Maximal specific H_2 productivities were obtained at 4, 5 and 6% O_2. The present study represents the first report of microaerobic hydrogen production under dark conditions. A previous study by Matsunaga et al. (2000) reported that addition of small amounts of oxygen stimulated hydrogen production under low light conditions by the marine non-sulfur photosynthetic bacterium, *Rhodovulum* sp.

The effects of different concentrations of five carbon sources (glucose, succinate, lactate, acetate and malate) on growth and hydrogen production were also examined at the previously determined optimal O_2 concentration for microaerobic hydrogen production. Maximal growth was observed with glucose supplementation compared to the other carbon sources tested. There were negative correlations between growth and the concentration of succinate, lactate and malate. In contrast, there was a high positive correlation between growth and the concentration of acetate. Likewise, there were negative correlations between specific H_2 productivity and the concentrations of glucose, succinate and malate while it was highly positive in the case of acetate. The differences observed between acetate and the other carbon sources might be due to the oxidation state of acetate ($C_2H_4O_2$) which is less than the other examined carbon sources and has a direct relation to carbon/nitrogen (C/N) ratio in the culure medium.The C/N ratio is important in any biological process and it effects on fermentative hydrogen production (Lin and Lay, 2004). In a previous study (Barbosa et al., 2001), the influence of different acetate concentrations on hydrogen production using photosynthetic bacteria had been investigated with the concentration of acetate being found to positively affect H_2 production and biomass accumulation.

Succinate (25 mM) was found to be the optimal carbon source and the best concentration for producing hydrogen under microaerobic condition whereas lactate gave a broad maximum H_2 productivity within a range of different concentrations. Thus, variance in both the kind of carbon source and its concentration significantly has an impact on microaerobic hydrogen production by nitrogenase. Different carbon sources with different oxidation states could lead to variations in electron flux to nitrogenase and therefore influence hydrogen production (Dutta et al., 2005).

Previous studies have examined H_2 production from different substrates using photosynthetic bacteria under photofermentative conditions. The hydrogen production rates as well as relative yields vary with substrate and the variation is different with various species. A previous study by Hillmer and Gest (1977) investigated the metabolism of *R. capsulatus* under illumination with lactate giving higher H_2 production rates than either malate or succinate. However, it is indistinct to what extent the results of the present study could be compared with previous studies, as this study is the first report of microaerobic hydrogen production by a photosynthetic bacterium in the absence of light and photosynthesis. It is probably that metabolic pathways, and certainly metabolic fluxes, are different under respiratory conditions. Therefore, more detailed analyses, including metabolic flux analysis will be required for understanding the differences observed here.

Likewise, nature of the nitrogen source and its concentration are considering major factors influencing total bacterial biomass and hydrogen production. In the present study, growth was well supported at different concentrations of fixed nitrogen irrespective of whether the nitrogen source examined was glutamate or ammonium. On the other hand, hydrogen production was sensitive to the concentration of glutamate and ammonium with maximum H_2 production at 3.5 mM glutamate or 4.0 mM ammonium. This observation is in agreement with previous studies that have shown that nitrogenase activity is reversibly inhibited at high ammonia or N/C ratios (Koku et al., 2003). Similar observations were made in our previous study with *R. capsulatus* cultured either as resting cells (no nitrogen source) or growing cells (3.5

and 7.0 mM glutamate) under light conditions (Abo-Hashesh et al., 2011b). These results highlight the fact that an increase in nitrogen concentration increases growth rather than H_2 production.

In general, a proper C/N ratio is essential for optimization of fermentative hydrogen production processes especially for those depend on nitrogenase activity in producing hydrogen. Bisaillon et al. (2006) studied limiting factors in hydrogen fermentation by different strains of *E. coli* and they observed that limitation for phosphate or sulfate had little effect whereas strains showed the highest hydrogen yields when cultured at limiting concentrations of either ammonia or glucose. Also, Kalil et al. (2008) studied the influence of C/N ratio in anaerobic hydrogen production from organic substrates and they observed that under these conditions microorganisms utilize 25–30 times more carbon than nitrogen.

Maximum average hydrogen yields obtained in this study were 0.6 mol hydrogen/ mol malate followed by 0.41 mol hydrogen/ mol lactate and 0.36 mol hydrogen/ mol succinate whereas the yields with glucose and acetate were low; 0.16 and 0.07 mol /mol, respectively. Different metabolic pathways might be involved under microaerobic conditions when compared with anaerobic conditions due to the induction of the TCA cycle. However, the yields of hydrogen obtained in this study are to some extent consistent with most studies carried out under anaerobic phototrophic conditions. For example, in our study, the best hydrogen yield was obtained when malate was used at 20 mM together with 2 mM glutamate. This result is nearly consistent with a previous study showed that the best C/N ratio of malic acid and glutamic acid was 15:2 (Eroğlu et al., 1999). In addition, malate was found to be the most suitable substrate for hydrogen production using *Rhodobacter sphaeroides* O.U.001 (Eroğlu et al., 1998; Koku et al., 2003). Lactate and malate are often used for comparing productivity by different strains as they are the most widely used carbon sources for H_2 production (Eroğlu et al., 1999, Sasikala et al. 1997).

Otherwise, the lowest hydrogen yields in this study were obtained when glucose and acetate were used as substrates. This result coincides with a recent study in which the hydrogen production yield from acetate of *R. sphaeroides* RV was lower than that from lactate (Kobayashi et al., 2011). In another study of hydrogen production from glucose in batch culture by pure and co-cultures of *Rhodobacter sphaeroides* and *Clostridium butyricum*, pure cultures of *R. sphaeroides* produced hydrogen at lower rates than *C. butyricum*. In co-cultures, *R. sphaeroides* could not compete with *C. butyricum* for glucose even at 5.9 times higher cell numbers and it utilized the acetate and butyrate produced by the latter as substrates for hydrogen production (Fang et al., 2006).

Nitrogenase enzyme plays a key role in hydrogen production by photosynthetic bacteria and it requires large amounts of ATP, high-energy electrons and limitation for fixed nitrogen. The regulation of nitrogenase has been well studied under light anaerobic conditions while little information is available for microaerobic conditions. Therefore, it was worthwhile to examine the levels of nitrogenase expression and its activity under different conditions. The results showed that, under microaerobic conditions, high concentrations of fixed nitrogen affect the expression level of N_2ase proteins whereas various concentrations of different carbon sources had no effect. These results strongly suggest that the decline in hydrogen production with increasing concentrations of fixed nitrogen noted in the experiments is due to a decreased synthesis of nitrogenase. In previous studies, it has been reported that high concentrations of ammonium inhibit the nitrogenase enzyme by down-regulating expression of the nitrogenase genes and inhibiting enzyme activity. This inhibition is reversible and nitrogenase activity can be recovered after depletion of ammonium (Yakunin and Hallenbeck, 1998, 2000, 2002).

On the other hand, nitrogenase activity was much higher (~10-fold) in the anaerobic light cultures than that in the microaerobic dark cultures, suggesting that nitrogenase activity in the latter was severely limited for supply of ATP and/or low potential electrons. Thus, the hydrogen production observed under microaerobic dark

conditions was not limited by the amount of nitrogenase present and proposing that, at least in theory, substantially more H_2 could potentially be obtained by optimizing microaerobic operational conditions for supplying more ATP and/or reductants to nitrogenase enzyme. A similar conclusion was reported by Yakunin and Hallenbeck (2000). They examined the response of *R. capsulatus* to the addition of NH_4^+ under dark microoxic conditions. Different nitrogenase activities were found when the cultures were incubated in the dark than with light incubation, with activity being higher in the light. It was suggested that the lower activity under dark microaerobic conditions was due to a lack of sufficient ATP generation. Therefore, future work should be aimed at establishing optimal culture conditions to maximize the use of the TCA cycle for generating greater electron flux and ATP generation required for optimal nitrogenase activity.

Several nitrogen-fixing bacteria have been reported to regulate nitrogenase in the short term by post-translational covalent modification via reversible ADP-ribosylation of the Fe protein in response to various environmental stimuli as ammonium addition, darkness, and the absence of oxygen (Yakunin et al., 1999). ADP-ribosylation of N_2ase Fe protein was detected in the experiments carried out here which suggests that the dark conditions negatively affected nitrogenase activity resulting in lower hydrogen production. Thus, it is recommended to develop a mutant that lacks the *draT* gene (encoding dinitrogenase reductase ATP-ribosyltransferase). Mutants lacking a functional *draT* have no dinitrogenase reductase ADP-ribosyltransferase (DRAT protein) activity and thus are incapable of modifying dinitrogenase reductase with ADP-ribose (Liang et al., 1991; Zhang et al., 1992). Consequently, using such a mutant under the microaerobic conditions used here might result in increased hydrogen production making this approach a more promising substitute for photofermentative approaches.

In conclusion, the research reported in this thesis proved the capability of *R. capsulatus* JP91 (*hup*$^-$) to produce H_2 under microaerobic dark conditions with limiting amounts of O_2 and fixed nitrogen. The oxygen concentration and C/N ratio

limit nitrogenase activity, which in turn influences H_2 production. Further work should be undertaken to increase H_2 yields using this technology, perhaps through the use of continuous cultures, the development of *R. capsulatus* mutants that are not sensitive to O_2 and/or a *draT* knockout mutant.

1.2 Photofermentative hydrogen production in batch cultures

The improvement in hydrogen production via physiological manipulation was also attempted using single stage photofermentative hydrogen production from glucose. The effect of nitrogen supply on growth of *R. capsulatus* JP91 (*hup*$^-$) as well as hydrogen production was investigated in batch culture under illumination. Three different nitrogen concentrations were examined; -N (resting cells), 3.5 mM glutamate, and 7 mM glutamate. Growth was directly influenced by nitrogen supply as very little growth was observed with -N (resting cells) cultures whereas 7 mM glutamate gave a higher level of growth than 3.5 mM glutamate.

Resting cells gave the lowest accumulated hydrogen values and after 6 days had only consumed ~44% of the original (22 mM) glucose. Interestingly, the 3.5 mM and 7 mM glutamate cultures, although demonstrating significantly different growth patterns, had very similar patterns of hydrogen evolution and glucose consumption. Both cultures had consumed ~70% of the initial glucose by the end of the day six. Generally, there was a positive correlation between growth and gas production in -N, 3.5 mM and 7 mM glutamate cultures. Similarly, there was a high correlation between growth and glucose consumed in the 7 mM glutamate cultures which highlights the fact that an increase in glutamate concentration increases growth rather than H_2 production. As might be expected, there was a high positive correlation between gas produced and glucose consumed in the -N, 3.5 mM and 7 mM glutamate cultures.

The main purpose of the study was to obtain the maximal yield of hydrogen per glucose by examining different cultures. Maximal total hydrogen evolution was

achieved after 48 h. Yields were determined on a daily basis during the operation process which had never been done in previous studies. Maximal yields occurred after two and three days and the highest value was 3.3 mols H_2 per mol of glucose for the 3.5 mM glutamate culture, followed by the 7 mM glutamate culture while resting cells (-N) showed the minimum yields during the study. After three days of the operation the yields declined with time which suggests that there is a shift in metabolism at a certain point that decouples hydrogen evolution from glucose utilization. Future determination of the metabolic changes involved could lead to strategies for maintaining high yields throughout the period of incubation, or possibly even increasing the maximum yields obtained.

Previous studies on the photofermentation of glucose have reported widely different yields with most of the reported yields being quite low to the theoretical value. In the present study ~27% of the theoretical 12 mols of H_2 available in glucose was obtained. Therefore, compared to most previously reported yields, the yields observed in the present study are higher, nearly as high as reported for another *hup*$^-$ strain of *R. capsulatus* (Ooshima et al., 1998) or a newly isolated strain of *R. sphaeroides* (Tao et al., 2008).

The maximal light conversion efficiency observed in this study was 0.7%. This is more or less typical of values reported for light conversion efficiencies of photofermentation of a variety of substrates by photosynthetic bacteria. However, an approximate calculation of theoretical light conversion efficiency, 8.5% is ten-fold higher than the actual efficiency observed in this study which in turn shows that there is certainly room for improvement in this regard.

A number of strategies can be used to increase H_2 yields from glucose in a photofermentation process. Optimizing glucose concentrations as well as glutamate concentrations was one way attempted in this study to affect yields. Another study was undertaken to increase H_2 yields from glucose using continuous cultures with various retention times and is discussed in detail below.

Developing appropriate mutations might introduce additional improvements in photofermentation process. For example, polyhydroxybutyrate (PHB) synthase deficient mutants 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; 2011; Hustede et al., 1993). This approach has been little studied on photofermentation of glucose and needs to be well examined. Further metabolic alterations certainly required for photosynthetic bacteria to increase hydrogen yields in photofermentation processes. These can be guided by a newly developed metabolic flux analysis model for photoheterotrophic growth of photosynthetic bacteria pertinent to biohydrogen production (Golomysova et al., 2010).

Moreover, a mutant lacking ribulose 1, 5-bisphosphate carboxylase (Rubisco), the key CO_2-fixing enzyme of the Calvin-Benson-Bassham (CBB) cycle (Calvin cycle), appears to be a possible strategy for optimizing hydrogen production. In a Rubisco mutant the reduction of metabolically produced CO_2 is blocked and the reductants generated during photo-heterotrophic growth can be fully utilized for cellular redox balance through the reduction of protons to H_2 instead (Wang et al., 2011). Finally, light conversion efficiencies might be improved as previously shown by Kondo et al. (2002), since they developed a *Rhodobacter sphaeroides* mutant capable of efficiently using high light intensities through reduction in the size of the photosynthetic antennae.

1.3 Photofermentative hydrogen production in continuous culture

Another study for achieving more hydrogen yields from glucose using the photofermentative approach was undertaken using continuous cultures; three different retention times (HRTs; 24, 48 and 72 h) were examined for optimizing hydrogen production, again using the photosynthetic bacterium *Rhodobacter capsulatus* JP91 (*hup*$^-$).

Growth was stable during the duration of the operation for all HRTs examined as the bacterial cells increase their growth rate to maintain optimal growth in relation to the dilution rate used. Therefore, optimizing cell production rates in continuous culture can be achieved by controlling the dilution rate. The observed growth demonstrates the advantage of using continuous cultures over batch cultures since the latter keeps a culture growing indefinitely. On the other hand, continuous removal of accumulated cells and waste products with a supply of fresh nutrients into the culture medium results in the accumulation of significant amounts of metabolic products.

Retention time is considered a critical limiting factor in continuous cultures and certainly affects the performance of photofermentation process for hydrogen production. Similarly to the growth patterns, the H_2 production rate was quite stable during the operation at the examined HRTs. However, a 48 h HRT gave better H_2 production than 24 and 72 h HRTs while the latter showed the lowest rates of H_2 production. Ren et al. (2012) have recently examined three other HRTs (36, 48 and 60 h) and observed that the lowest H_2 production rate was obtained at the highest HRT (60 h) since the hydrogen production rate increased with decreasing retention time.

It is apparent that using different HRTs has a direct influence on glucose consumption rates. The bacterial cells consumed more glucose at the lowest HRT (24 h) as well as at the highest HRT (72 h) whereas the consumption rate was the lowest at a HRT of 48 h. This observation might be explained as follows; at low HRT the bacterial cells increase their growth rate to maintain the stability of cell biomass as mentioned above. On the other hand at high HRT, the aged cells in the decline phase may consume more substrate to maintain their survival.

The percentage of utilized glucose at each HRT examined reinforces the idea that the 48 h HRT was the optimal retention time for limiting glucose consumption

rather than HRTs of 24 and 72 h since it was 20% at 48 h HRT whereas as it reached 36% and 55% at 24 and 72 h HRTs, respectively.

As different HRTs have a significant influence on glucose consumption, the hydrogen yield was also influenced and 48 h HRT gave the highest H_2 yield, 10.3 mols H_2/ mol glucose, much higher than ever before reported and ~ 86% close to the theoretical maximal value (12 mols hydrogen/ mol glucose). A study using batch cultures to examine photofermentation of glucose by the same bacterium reached a maximum of 3.3 mols H_2/ mol glucose (Abo-Hashesh et al., 2011b). A previously published study Fang and his colleagues (2006) reported a H_2 yield of 0.56 from glucose using photofermentation with *R. sphaeroides* DSM158 in batch culture. Likewise, Li et al. (2008) reported a yield of 0.9 using *Rubrivivax gelatinosus* L31 under the same conditions whereas Su et al. (2009) reported a yield of 4.16 using *R. palustris*.

Similar to the observations in this thesis, a study by Ren and his colleagues (2012) used continuous cultures in a photofermentation process for hydrogen production. The highest hydrogen yield of 2.26 mols H_2/ mol acetate was obtained at a HRT of 48 h rather than 36 and 60 h HRTs using *Rhodopseudomonas faecalis* RLD-53. The yield obtained represents 56.5% of the theoretical value (4 mols hydrogen/ mol acetate).

The highest conversion efficiency of the heating value of glucose was 91% when 48 h HRT was used whereas it was 43% and 26% for 72 and 24 h HRTs, respectively. These results confirm the previous observations in this study where the optimal HRT was 48 h. Compared to a previous photofermentation study by Su and his colleagues (2009), the maximum heating value of hydrogen produced from glucose 0.231 KJ/h obtained in this thesis was twice the value they obtained 0.11 (KJ/h). As well, the heating value conversion efficiency (HVCE) range they obtained (5.5-34.9%) was less than that obtained in the current study (26.24 - 91.14%).

Total energy conversion efficiencies (TECE) did not show high significant differences between the examined HRTs, with values ranging from 1.13-1.31% which is pretty low in comparison to the HVCE values. This decrease is due to the low light conversion efficiency (LCE) obtained which ranged from 1.16-1.34%. Comparing to previous studies, the TECE values obtained in this study were less than those reported by Su et al. (2009) who obtained a TECE of 2.96 to 3.55% in the two-phase process in which glucose was used for photo-hydrogen production by *Rhodopseudomonas palustris*. Likewise, Chen et al. (2006) obtained 1.93% TECE using *R. palustris* WP3–5 for photo-hydrogen production from acetate.

In general, the results of his study indicate that continuous cultures appear to be a better technique capable of attaining greater H_2 production. Also, Chen et al. (2008) confirmed the efficiency of using a continuous mode for a photo-hydrogen process as continuous photofermentation operated at 96 h HRT using effluent from dark fermentation was quite stable, maintaining an overall average H_2 yield of 10.21 mol H_2/ mol sucrose for over 10 days.

The results of total energy conversion efficiency in comparison to heating value conversion efficiency give an indication that light conversion efficiencies need to be improved, possibly by developing optimized photobioreactors to increase the LCEs as well as using genetically modified PNS bacteria capable of producing higher rates of hydrogen at high light intensity.

2. Metabolic engineering

The second part of this thesis concerned using metabolic engineering to improve hydrogen production. Two studies were undertaken to accomplish this purpose via heterologous expression of [FeFe]-hydrogenase.

2.1 Expression and maturation of the nonfunctional *hydA* gene of *Rhodospirillum rubrum* S1^T in *Escherichia coli*

Genome sequencing has revealed that *R. rubrum* contains an orphan *hydA* encoding [FeFe]-hydrogenase (HydA) while it lacks the other known maturation genes *hydE*, *hydF* and *hydG* (Nicolet et al., 2010; Munk et al., 2011). In addition, Meyer (2007) has demonstrated that *R. rubrum hydA* gene is nonfunctional as *R. rubrum* has acquired the gene *hydA* by lateral gene transfer. Therefore, the main aim of the this study was to express the non functional HydA from *R. rubrum* in *E. coli* BL21(DE3), which does not contain *hydA* or any of the maturation genes required for enzyme activity, and maturing it by coexpressing three other bacterial gene products (HydE, HydF and HydG) from *D. vulgaris* and *C. acetobutylicum*.

hydA of *R. rubrum* (*RrhydA*) was hybridized with the promoter region of the gene *nifH2* (encodes nitrogenase reductase) and transformed into *R. rubrum* UR206 which does not have a functional nitrogenase (Lehman et al., 1990). Since *hydA* was under the control of the *nifH2* promoter in UR206-*hydA*, the level of *hydA* gene expression in UR206-*hydA* strain was measured under conditions of *nifH2* promoter repression and induction. The level of gene expression of *hydA* was about 50 times higher when the *nifH2* promoter was induced. Consequently, the constructed *PnifH2hydA* incorporated into the UR206-*hydA* strain of *R. rubrum* is functional and it is possible to regulate *hydA* gene expression by repressing or inducing the *nifH2* promoter. However, overexpression of *hydA* in UR206-*hydA* strain does not generate hydrogen production and unlike *R. rubrum* wild type, neither UR206 nor UR206-*hydA* produces hydrogen under inducing or repressing conditions. This result strongly suggests that the *hydA* structural gene alone is not enough for generating an active hydrogenase and other maturation genes are required for synthesis and assembly of [FeFe]-hydrogenase that drives hydrogen production, as previously shown in other organisms (Nicolet et al., 2010; Nicolet and Fontecilla-Camps, 2012).

R. rubrum hydA was cloned into pRSFDuet-1 generating pRSF-*hydA*. To assess if *Rr*HydA could be matured into a functional hydrogenase, it was cloned and overexpressed in *E. coli* BL21(DE3) along with the *hydE*, *F* and *G* maturation genes from *D. vulgaris* or *C. acetobutylicum*. The expression levels of *R. rubrum hydA* together with *hydE* and *hydG* of *D. vulgaris* were evaluated prior to and after induction in *E. coli* BL21(DE3). The results revealed that induced genes are expressed 2 to 6 times more compared to the uninduced control.

Hydrogenase activity of the *E. coli* BL21(DE3) harboring the structural and maturation genes of [FeFe]-hydrogenase was examined *in vitro*. Compared to the positive control (*E. coli* BL21 (DE3) transformed with plasmids harboring *hydA* and the maturation genes of *C. acetobutylicum*), the maximum hydrogen production rate was obtained when *RrhydA* was matured with the maturation genes of *C. acetobutylicum* while less hydrogen was produced when it was matured with the maturation genes of *C. acetobutylicum* constructed as an artificial operon. In addition, *RrhydA* with the maturation genes of *D. vulgaris* showed a very low production rate close to the value of the negative control (*E. coli* BL21(DE3) transformed with the plasmid harboring *RrhydA* alone without maturation genes).

The specific hydrogen productivities that based on the total protein mass of each constructed strain gave results similar to the observations mentioned above. The variations in the specific activity ranges between those recombinant [FeFe]-hydrogenases might possibly be due to differences in the expression conditions for example; culture components or any other unknown factors that play a role in the maturation process.

Moreover, the amount of the HydA protein for each constructed strain was calculated using gel analyzer software allowing the evaluation of specific hydrogen productivities based on the target encoded protein (HydA). As expected, the maximum specific hydrogen productivity was for the strain harboring *RrhydA* with the maturation genes of *C. acetobutylicum*. Lower values were found for the positive

control and the HydA matured with the maturation genes of *C. acetobutylicum* constructed as an artificial operon. No HydA protein could be detected in the SDS gel for the negative control and the constructed strain harboring maturation genes of *D. vulgaris*. As well, only traces of hydrogen were detected for both strains. These results may be due to the incomplete maturation of *R. rubrum* HydA by the *D. vulgaris* maturation genes causing misfolding or incomplete folding of HydA. Misfolded proteins are known to be unstable as they are prone to degradation by cellular proteases. Even though hydrogenase maturation gene sequences are highly conserved across species (King et al., 2006), these results demonstrate that maturation of *R. rubrum hydA* by the maturation genes of *C. acetobutylicum* is much more effective rather than by those of *D. vulgaris*. Similarly, Laffly and his co-workers demonstrated that co-expression of the HydE, HydF and HydG maturases with the hydrogenase structural gene of *D. vulgaris* Hildenborough in *E. coli* gave only very low hydrogenase activities which were about 4000-fold lower than for the native hydrogenase isolated from *D. vulgaris* (Laffly et al., 2010).

From the results presented here and previous observations, it can be concluded that incomplete maturation by HydE, HydF and HydG is one of the limiting factors for the overproduction of [FeFe]-hydrogenase in *E. coli*. Similar observations have been reported by Nagy et al. (2007), who cloned six indigenous [FeFe]-hydrogenase genes from six species of the genus *Clostridium* separately expressed them in *E. coli* with the assembly proteins HydE, HydF and HydG required for [FeFe]-hydrogenase maturation. Five of the six strains exhibited significant activity when expressed. As well, King and his co-workers (2006) have observed that heterologous expression of *hydA* alone from green alga *Chlamydomonas reinhardtii* or *C. acetobutylicum* in *E. coli* failed to produce active enzyme unless the maturation genes *hydE*, *hydF* and *hydG* from *C. acetobutylicum* were co-expressed. However, the resulting recombinant [FeFe]-hydrogenase showed lower specific hydrogen productivity compared to the native [FeFe] hydrogenase of *C. acetobutylicum*. Abendroth and his colleagues (2008) studied heterologous and even homologous [FeFe]-hydrogenase in *C. acetobutylicum* and observed unexpectedly high protein

yield and specific activity of the recombinant hydrogenase, suggesting that *E. coli* might lack factors besides *hydE, F* and *G* required for full maturation of HydA.

In the present study, attempts were made to optimize overexpression conditions by using different Fe-EDTA concentrations as well as by adding iron and cysteine to the culture medium. Fe-EDTA (1:1) gave the best results whereas adding iron and cysteine decreased hydrogenase activity. This observation supported the idea of using *E. coli* Δ*iscR* strain as an expression system since the correct insertion of Fe-S clusters and hence the maturation of [FeFe]-hydrogenase proteins may be enhanced in this strain.

Ineffective hydrogenase maturation and low protein yields in an *E. coli*-based expression system have been improved upon by Kuchenreuther et al. (2010). They added glucose to the growth medium for enhanced anaerobic metabolism and growth during hydrogenase expression. Also, they added iron and cysteine to increase iron-sulfur cluster protein accumulation (Kuchenreuther et al., 2010). In a similar manner, Akhtar and Jones (2008) described that the deletion of the gene encoding the transcriptional negative regulator of the *isc* operon*, iscR,* stimulates the specific activity of the recombinant clostridial [FeFe]-hydrogenase and H_2 accumulation in *E. coli*.

Therefore, it has been shown in this thesis that the orphan *hydA* of *R. rubrum* is functional and active when coexpressed in *E. coli* with *hydE, F* and *G* from different organisms and that coexpression of [FeFe]-hydrogenase structural and maturation genes in microorganisms that lack a native [FeFe]-hydrogenase can successfully result in the assembly and biosynthesis of active hydrogenases. However, other factors may be required for significantly increased protein yield and hence specific activity of the recombinant hydrogenase.

2.2 Expression and maturation of a NADP-dependent [FeFe]-hydrogenase in *Escherichia coli*

In another part of this thesis, a study for engineering a highly efficient hydrogenase was undertaken which consisted of cloning and expressing a NADP-dependent [FeFe]-hydrogenase (encoded by *hndABCD*) in *E. coli* strains BL21(DE3) and BL21(DE3)Δ*iscR*. The *D. fructosovorans hnd* operon (*hndABCD*) encoding a NADP-dependent [FeFe]-hydrogenase, has been successfully cloned in the expression vector pACYCDuet-1 generating pACYCDuet-1-*hnd*.

Four *E. coli* strains were constructed carrying compatible expression vectors harboring the structural and maturation genes of [FeFe]-hydrogenase. Two *E. coli* strains were made, BL21(DE3)-hnd_{Df}/$hydEFG_{Ca}$ and BL21(DE3)Δ*iscR*–hnd_{Df}/$hydEFG_{Ca}$, carrying plasmids that harboring NADP-dependent [FeFe]-hydrogenase structural genes $hndADCD_{Df}$ of *D. fructosvorans* as well as the maturation genes $hydEFG_{Ca}$ of *C. acetobutylicum*. The other two constructed *E. coli* strains BL21(DE3)-$hydAEFG_{Ca}$ and BL21(DE3)Δ*iscR*-$hydAEFG_{Ca}$, carried plasmids harboring the Fd-dependent [FeFe]-hydrogenase structural gene $hydA_{Ca}$ of *C. acetobutylicum* as well as its maturation genes $hydEFG_{Ca}$. Hydrogenase activity was detected *in vitro* using reduced methyl viologen assay (MV) to investigate the efficiency of NADP-dependent [FeFe]-hydrogenase versus Fd-dependent [FeFe]-hydrogenase as well as to examine the performance of *E. coli* strain BL21(DE3)Δ*iscR* versus BL21(DE3).

The results obtained indicate that *E. coli* strains BL21(DE3)Δ*iscR* and BL21(DE3) harboring the structural and maturation genes, $hydAEFG_{Ca}$, of *C. acetobutylicum* gave higher hydrogen production rates and specific hydrogen productivities than those harboring the structural genes $hndABCD_{Df}$ of *D. fructosovorans* with the maturases of *C. acetobutylicum*. As revealed in the previous studies of Schwartz et al. (2001) and Giel et al. (2006), it was observed in the current study that using *E. coli* Δ*iscR* strain as the expression host has a positive effect on

hydrogenase activity that may related to the enrichment of Fe-S clusters required for biosynthesis and assembly of [FeFe]-hydrogenase proteins.

Moreover, *E. coli* strains expressing NADP-dependent [FeFe]-hydrogenase gave less activity than those expressing Fd-dependent [FeFe]-hydrogenase. This may be due to the fact that the structural genes of the former (*hndABCD*) were matured by genes (*hydEFG*) from another organism (*C. acetobutylicum*) while the structural gene (*hydA*) of latter was matured by those genes (*hydEFG*) from the same organism. The homologous expression system may be more effective than the heterologous one as previously reported (Laffly et al., 2010). Likewise, Girbal et al. (2005) reported that homologous expression of the Fe-only hydrogenase of *C. acetobutylicum* gave higher activity than heterologous expression of *Chlamydomonas reinhardtii* and *Scenedesmus obliquus* Fe-only hydrogenases.

On the other hand, a different observation was reported by Sybirna et al. (2008), who found that the heterologous production system of *C. reinhardtii* [Fe-Fe] in *Shewanella oneidensis* appeared to be more efficient than that of *C. acetobutylicum* in *E. coli*. As well, heterologous expression of *C. acetobutylicum* [FeFe]-hydrogenase in *E. coli* displayed specific hydrogen productivity 7.5-fold higher than the value reported for the same enzyme in the native recombinant system (King et al., 2006). Various algal and bacterial [FeFe]-hydrogenases that were heterologously expressed in *E. coli* gave enzymes with specific activities that were comparable to those of the enzymes extracted from native sources (King et al., 2006).

Mutated *E. coli* strains BL21(DE3)Δ*aceF* and BL21(DE3)Δ*iscR*Δ*aceF* that overproduce NADPH have being constructed by P1 transduction to enhance metabolic flux through the pentose phosphate pathway which would lead to production of a great deal of NADPH. The NADPH produced could then be utilized for hydrogen production via activity of NADP-dependent [FeFe]-hydrogenase that was already successfully matured in *E. coli* with the HydEFG maturases of *C. acetobutylicum* for the first time. Consequently, a higher hydrogen yield from

glucose in *E. coli* could be obtained as a result of enhancing pentose phosphate pathway and the activity of the engineered NADP-dependent [FeFe]-hydrogenase .

Further work is required to introduce engineered NADP-dependent [FeFe]-hydrogenase into mutated *E. coli* strains Δ*acef* that overproduce NADPH and examine hydrogenase activity *in vivo* for the recombinant *E. coli* strains, BL21(DE3)Δ*iscR*Δ*aceF* and BL21(DE3)Δ*aceF*.

CONCLUSIONS AND PERSPECTIVES

Microaerobic dark fermentation was one attempt to improve hydrogen production using *R. capsulatus* JP91 (*hup*$^-$). The optimal O_2 concentration range for achieving microaerobic hydrogen production was 4-6%. Maximum H_2 production was obtained when succinate (25 mM) and glutamate (3.5 mM) were used as carbon and nitrogen sources whereas the highest hydrogen yield (0.6) was found when 20 mM malate was used. The results show that in general, under microaerobic conditions, the optimal conditions for growth are different from those for H_2 production. The concentration of oxygen and the C/N ratio limit nitrogenase activity, and hence influence H_2 production. The technical challenge in going forward is to establish the optimal operational conditions for maximum H_2 yields, perhaps through the use of continuous cultures, develop *R. capsulatus* mutants that are not as sensitive to O_2 as well as a *draT* knockout mutant which has no DRAT activity and that are therefore incapable of inhibiting nitrogenase activity. Construction of *draT* knockout mutant is currently in progress in the laboratory of Dr. Hallenbeck.

Another study demonstrated the promise of using *R. capsulatus* JP91 (*hup*$^-$) in the development of a single stage photofermentation process for converting glucose, and possibly other sugars, to hydrogen. Glucose and glutamate concentrations were optimized for maximal hydrogen production. The maximal yield obtained, 3.3 mols of H_2 per mol of glucose with a light conversion efficiency of 0.7%, was found near the end of exponential growth in 3.5 mM RCV-glutamate. A number of strategies can be suggested for increasing H_2 yields from glucose in photofermentation process. The use of continuous cultures to increase H_2 yields from glucose was investigated. As well, the development of appropriate mutants that might introduce additional improvements in the photofermentative process such as, a PHB mutant which would increase the reducing power available for hydrogen production, and a Rubisco mutant which would block the reduction of metabolically produced CO_2 allowing reducing equivalents to be used for the reduction of protons to H_2 are currently in progress in the laboratory of Dr. Hallenbeck. Furthermore, light conversion efficiencies might be

improved by enabling the more efficient use of high light intensities through reducing the size of the photosynthetic antennae.

Photofermentative hydrogen production from glucose in continuous cultures as carried out in this thesis was very successful since the highest hydrogen yield ever reported, 10.3 mols per mol glucose, was obtained. This represents more than 85% of the theoretical maximal value. Three retention times were examined (HRTs; 24, 48 and 72 h) to optimize operation performance. A retention time of 48 h was the optimal retention time as it gave the highest H_2 production rate and the lowest glucose consumption, resulting in maximal H_2 yields. As well, it gave the highest conversion efficiency of the heating value of glucose to hydrogen (91.14%). However, these cultures were also characterized by low light conversion efficiencies; hence total energy conversion efficiencies were quite low. These results demonstrate the great advantage of using a single stage photofermentative hydrogen production from glucose process in continuous culture rather than batch culture. Such a process appears to be more promising than two stage photofermentation or co-culture approaches. Nevertheless, light conversion efficiency needs to be optimized for a practical and cost-effective photofermentative hydrogen production process. This could be attained by constructing effective impermeable photo-bioreactors as well as using genetically modified PNS bacteria capable of producing higher rates of hydrogen at high light intensities.

In this thesis two studies were carried out to improve hydrogen production using heterologous expression of [FeFe]-hydrogenase. The capacity to express a hydrogenase from one species with the maturation genes from another was examined. One study demonstrated that the orphan *hydA* of *R. rubrum* is functional and active when co-expressed in *E. coli* with *hydE, hydF* and *hydG* from different organisms. Therefore, co-expression of [FeFe]-hydrogenase structural and maturation genes in microorganisms that lack a native [FeFe]-hydrogenase can successfully result in the assembly and biosynthesis of active hydrogenases. However, other factors may be required for significantly higher protein yields and hence specific activities of

recombinant hydrogenase. Some efforts were performed to optimize overexpression conditions such as using different Fe-EDTA concentrations as well as adding iron and cysteine to the culture medium. In addition, using an *E. coli* Δ*iscR* strain as the expression host may enhance the correct insertion of Fe-S clusters and hence the maturation of [FeFe]-hydrogenase proteins. On the other hand, it is recommended to mature HydA of *R. rubrum* in the native *R. rubrum* strain together with the three maturases HydA, HydE and HydG from another bacterium to attempt to create strains which may show higher hydrogen production since the requirement for ATP for this process will be eliminated.

Finally, expression in *E. coli* of a hydrogenase that can directly interact with NADPH is desirable as this, rather than reduced ferredoxin, is naturally produced by its metabolism. However, the successful maturation of this type of hydrogenase in *E. coli* has not yet been reported. The *D. fructosovorans hnd* operon (*hndA*, *B*, *C*, and *D* genes), encoding a NADP-dependent [FeFe]-hydrogenase, was expressed in various *E. coli* strains with the maturation genes *hydE*, *hydF* and *hydG* from *Clostridium acetobutylicum*. *In vitro* hydrogenase activity was detected, thus demonstrating that an active multi-subunit NADP-dependent [FeFe]-hydrogenase had been successfully expressed and matured in *E. coli* for the first time. Further studies should be aimed at expressing this hydrogenase in *E. coli* mutant strains that overproduce NADPH, setting the stage for increased hydrogen yields via the pentose phosphate pathway.

In conclusion, several strategies have been investigated for improving biological hydrogen production through physiological manipulation of the culture conditions and/or metabolic engineering of the hydrogen producing bacteria. Nevertheless, there are a number of technical challenges in each strategy that must be overcome for obtaining maximum hydrogen yield with less energy consumption in the dark- and photo-fermentation processes. Further studies are being conducted to meet this goal and make such processes cost-effective and applicable on a practical large scale.

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