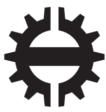

TAMPEREEN TEKNILLINEN YLIOPISTO
TAMPERE UNIVERSITY OF TECHNOLOGY

Rahul Mangayil

Biohydrogen Production:
A Protein to Community Level Perspective Study

Julkaisu 1282 • Publication 1282

Tampere 2015

Tampereen teknillinen yliopisto. Julkaisu 1282
Tampere University of Technology. Publication 1282

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Biohydrogen Production:
A Protein to Community Level Perspective Study

Thesis for the degree of Doctor of Science in Technology to be presented with due permission for public examination and criticism in Festia Building, Auditorium Pieni Sali 1, at Tampere University of Technology, on the 17th of April 2015, at 12 noon.

Tampereen teknillinen yliopisto - Tampere University of Technology
Tampere 2015

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ISBN 978-952-15-3462-1 (printed)
ISBN 978-952-15-3501-7 (PDF)
ISSN 1459-2045

ABSTRACT

Excessive usage of traditional energy reserves leading to increased environmental pollution and global warming have strongly urged for alternative sustainable energy sources. Due to non-polluting nature and high energy yields, hydrogen (H_2) gas is considered as an ideal candidate for alternative fuel. Biohydrogen (bioH_2) production from organic wastes is a sustainable approach, addressing energy production through organic waste disposal. Organic wastes such as lignocellulosic biomass and industrial glycerol, a by-product of biodiesel manufacturing process, have been recently investigated for their bioconversion potential. However, bioconversion of such organic wastes is a challenge due to the presence of impurities, toxic degradation products and complex nature. In comparison to pure bacterial strains, natural microflora could be an ideal inoculum choice offering better adaptability, substrate utilization efficiency and bioconversion rates. Another challenge to ensure efficient fermentation is to optimize various physico-chemical factors such as pH, temperature, substrate selection and concentration, medium compounds, and H_2 removal and collection due to individual and interactive effects on microbial growth, metabolism and hydrogenase enzyme.

Hydrogenases are metalloenzymes that reversibly catalyzes proton reduction to H_2, and are divided into three classes based on the metal cofactor at the active site, [Fe-Fe], [Ni-Fe] and [Fe] hydrogenase. Among the hydrogenase classes, [Fe-Fe] hydrogenases exhibit highest catalytic activity involving mostly in H_2 production. Apart from their pivotal role in fermentative H_2 production, [Fe-Fe] hydrogenases promise an alternative catalyst choice in fuel cells. However, in spite of their preference towards H_2 production, [Fe-Fe] hydrogenases are extremely prone to catalytic inactivation upon oxygen exposure. This is the major challenge, at the protein level, that hinders a cost-effective approach for biotechnological applications and suggests the requirement of targeted tools to investigate the inactivation process at the molecular level.

The purpose of the present study was to investigate bioH_2 production in protein to community level perspective. More specifically the aims were to (1) establish an anaerobic biopanning procedure to enrich antibody binders specific against clostridial [Fe-Fe] hydrogenase protein, (2) develop and standardize a novel enrichment system, (3) implement the enrichment technique to enrich functional inoculum capable of degrading complex substrates, (4) enrich crude glycerol fermenting microbial community and finally, (5) optimize the physico-chemical factors influencing fermentative H_2 production for efficient bioprocess.

In the present study, biopanning with synthetic 'mixed' single chain variable fragment (scFv) libraries against active and inactive clostridial [Fe-Fe] hydrogenases aided the enrichment of anti-hydrogenase antibodies. Out of ninety four (from inactive

hydrogenase) and ninety two (from active hydrogenase) random clones screened, nine potential antibody clones with recognition specificity towards *Clostridium acetobutylicum* [Fe-Fe] hydrogenase were selected. The enriched binders also recognized [Fe-Fe] hydrogenase from *C. butyricum*. Based on the results from this study, it could be reasoned that the binders with generic specificity against closely related clostridial [Fe-Fe] hydrogenases can be used as novel molecular tools for quantitative monitoring [Fe-Fe] hydrogenases at the protein level. Another of-note observation was the specificity of the antibody binders towards active and inactive hydrogenases. Preliminary experiments indicated 7Ac binder (enriched against active hydrogenase) specificity towards the catalytically active [Fe-Fe] hydrogenase rather to the inactive state and 48In (enriched against inactive hydrogenase) recognized both catalytic states. These findings indicate the possibility to apply the isolated antibody clones for functional detection of clostridial [Fe-Fe] hydrogenases.

The study progresses in investigating bioH_2 production in perspective of microbial community. The novel microbial enrichment system was developed and the proof-of-principle experiments conducted using artificial mixed microbial community and varied selection criteria allowed the enrichment of the best H_2 producer. The system was implemented in enriching cellobiose degrading H_2 producer from an environmental sample. The bacterial strain isolated by spread plate technique on agar plates containing CMC was affiliated with *Citrobacter* sp. and named as *Citrobacter* sp. CMC-1. *Citrobacter* sp. CMC-1 utilized glucose, cellobiose and CMC and followed mixed-acid fermentation profile producing H_2 and carbon dioxide (CO_2) as gaseous metabolites and acetate, formate, lactate and ethanol as liquid metabolites. At optimized values of cultivation conditions (pH 6.0 and 34 ˚C) the H_2 yield was 1.82 mol-H_2/mol-glucose. The isolate efficiently fermented monomeric hemi-cellulose sugars to H_2 (mol-H_2/mol-substrate): Galactose, 1.18; Mannose, 1.23; Xylose, 1.22; Arabinose, 0.94 and Rhamnose, 1.01). Except for arabinose, an increase in cultivation period improved the biomass and H_2 yield (mol-H_2/mol-substrate): Galactose, 1.68; Mannose, 1.93 and Xylose, 1.63) followed with observations of reduced formate accumulation in the medium, indicating that *Citrobacter* sp. CMC-1 produced H_2 from formate breakdown via the FHL complex.

Microbial community pre-dominated with *Clostridium* spp. enriched from activated sludge fermented crude glycerol mainly to H_2, CO_2, acetate, butyrate and ethanol. Optimal bioprocess conditions for the enriched inoculum were experimentally observed to be pH 6.5, 40˚C and 1g/L crude glycerol. The H_2 yield from raw glycerol at optimal cultivation conditions was 1.1 mol-H_2/mol-glycerol$_{consumed}$. At elevated crude glycerol concentrations, substrate utilization and H_2 production were limited due to the presence of impurities in the crude glycerol fraction. The bioconversion of crude glycerol to H_2 was further improved by statistical optimization of the growth medium composition.

Initial screening with Plackett – Burman design identified NH_4Cl, K_2HPO_4 and KH_2PO_4 with individual and interactive effects on H_2 yield. Among the three identified media components, NH_4Cl and KH_2PO_4 imparted the maximal significance and were optimized in scrutiny. A series of statistical models identified the optimal media composition for improved H_2 production from crude glycerol fermentations and were successful in improving the H_2 yield by 29% (1.42 mol-H_2/mol-glycerol$_{consumed}$) in comparison to previously reported value (1.1 mol-H_2/mol-glycerol$_{consumed}$).

ACKNOWLEDGMENTS

This thesis is based on the work performed at the Department of Chemistry and Bioengineering, Tampere University of Technology (TUT) during the years 2010 – 2014. I would like to take this opportunity to acknowledge Maj and Tor Nessling Foundation and The Academy of Finland (Project no's. 126974 and 139830) for financial support.

I am extremely grateful to my mentors, Prof. Matti Karp and Adj. Prof. Ville Santala for their valuable guidance, encouragement and support throughout my days at TUT. I would also like to express gratitude towards my co-authors: Dr. Tommi Aho, for introducing me to the world of statistical modelling and Adj. Prof. Urpo Lamminmäki, for patiently educating and providing an expert guidance in phage display and biopanning. Docent Kristiina Takkinen and PhD. Pauli Kallio are kindly acknowledged for reviewing the thesis. Associate Professor Alexander Frey and Docent Kristiina Takkinen are acknowledged for agreeing to act as my opponents.

I would also thank all my co-workers in the lab throughout these years and other personnel at the department. Special thanks to Bobin George Abraham and Rajasekaran Andiappan for the great friendship and their support to maintain my sanity 'in-check' through our occasional get-together. I extend my thankfulness to my colleagues Alessandro Ciranna, Matti Kannisto, Anniina Kivistö, Suvi Santala, Katariina Tolvanen, Nina Virolainen, Joanna Alanko, Noora Männistö and Anna-Liisa Välimaa for their advice, friendship and support at work.

I owe my endless thanks to my family; Dad and Mom for their immense love, encouragement and support throughout my life, my brothers for their love and friendship, my wife Gayathri Menon for her love and mostly for her patience and encouragement in lifting my spirits high during the frustrations along the work, and finally my sweetheart, my daughter Athena Rahul, whose smile gives me the most relief and helps me forget my tensions.

I dedicate this thesis to you all.

Tampere,

Rahul Krishnan Mangayil

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PUBLICATION LISTS

The thesis is based on the following original publications, referred in the thesis by Roman numerals.

I. Mangayil, R., Karp, M., Lamminmäki, U., Santala, V. Isolation of recombinant antibodies for specific detection of clostridial [Fe-Fe] hydrogenases. Submitted for publication.

II. Tolvanen, K.E.S., Mangayil, R.K., Karp, M.T., Santala, V.P. 2011. Simple enrichment system for hydrogen producers. Applied and Environmental Microbiology. 77: 4246-4248.

III. Mangayil, R., Santala, V., Karp, M. 2011. Fermentative hydrogen production from different sugars by *Citrobacter* sp. CMC-1 in batch culture. International Journal of Hydrogen Energy. 36: 15187-15194.

IV. Mangayil, R., Karp, M., Santala, V. 2012. Bioconversion of crude glycerol from biodiesel production to hydrogen. International Journal of Hydrogen Energy. 37: 12198-12204.

V. Mangayil, R.,Aho, T., Karp, M., Santala, V. 2015. Improved bioconversion of crude glycerol to hydrogen by statistical optimization of media components. Renewable Energy. 75: 583–589.

AUTHOR'S CONTRIBUTIONS

I. Rahul Mangayil planned and performed the experimental work, interpreted the results, wrote the paper and is the corresponding author. Matti Karp and Ville Santala assisted in planning experiments, results interpretation and manuscript writing. Urpo Lamminmäki provided synthetic scFv libraries, assisted in implementing the biopanning procedure and results interpretation.

II. Rahul Mangayil planned and performed the experimental work, interpreted the results, drafted the manuscript and is the corresponding author. Katariina Tolvanen and Ville Santala provided the conceptual idea. Ville Santala assisted in planning the experiments, result interpretation and manuscript writing.

III. Rahul Mangayil planned and performed the experimental work, interpreted the results, drafted the manuscript and is the corresponding author.

IV. Rahul Mangayil planned and performed the experimental work, interpreted the results, drafted the manuscript and is the corresponding author.

V. Rahul Mangayil planned and performed the experimental work, interpreted the results, drafted the manuscript and is the corresponding author. Tommi Aho designed the statistical models and assisted in result interpretation.

The experimental work was conducted under the supervision of Prof. Matti Karp and Adjunct Prof. Ville Santala.

ABBREVIATIONS

[Ni-Fe]	Nickel-Iron
[Fe-Fe]	Iron-Iron
[Fe]	Iron
AP	Alkaline phosphatase
BB	Box – Behnken
BCCP	Biotin carboxyl carrier protein
bioH_2	Biohydrogen
CMC	Carboxymethyl cellulose
CCD	Central composite design
CDR	Complementary determining regions
CN	Cyanide
CO	Carbon monoxide
CO_2	Carbon dioxide
CFPS	Cell free protein synthesis
DGGE	Density gradient gel electrophoresis
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
Fab	Fragment antigen-binding
FAME	Fatty acid methyl ester analysis
Fe-S	Iron-sulfur
Fd (ox)	Ferredoxin (Oxidized form)
Fd (red)	Ferredoxin (Reduced form)
FHL	Formate-hydrogen lyase
FISH	Fluorescent *in situ* hybridization
g/L	Gram per liter
H_2	Hydrogen
Hmd	Methylene-tetrahydromethanopterin dehydrogenase
hydA	Hydrogenase
IEA	International energy agency
IPTG	Isopropyl β-D-1-thiogalactopyranoside
O_2	Oxygen
OD_{600}	Optical density (absorbance) at 600 nm
ori	Origin of replication
Mo	Molybdenum
NAD	Nicotinamide adenine dinucleotide
NAD^+	Nicotinamide adenine dinucleotide (Oxidized form)
NADH	Nicotinamide adenine dinucleotide (Reduced form)
NFOR	NADH-ferredoxin oxidoreductase
NGS	Next-generation sequencing
PB	Plackett – Burman

PCR	Polymerase chain reaction
PNSB	Purple non-sulfur bacteria
PFOR	Pyruvate-ferredoxin oxidoreductase
PFL	Pyruvate-formate lysase
PSI	Photosystem I
PSII	Photosystem II
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
RA	Ridge analysis
RFLP	Restriction fragment length polymorphism
RISA	Ribosomal intergenic spacer analysis
RSM	Response surface methodology
SA	Steepest accent
SSCP	Single strand confirmation polymorphism
scFv	Single chain variable fragment
T-RFLP	Terminal-restriction fragment length polymorphism
v/v	Volume by volume

1. INTRODUCTION

1.1. CURRENT HYDROGEN PRODUCTION METHODS

Depletion of petroleum-based energy resources, global warming, increased environmental pollution and strict emission regulations have strongly urged for an alternative energy source (Balat 2008). Currently, as shown in Figure 1.1, the energy derived from fossil fuels covers majority of total global fuel consumption (IEA Key world energy statistics, 2012). H_2 is considered as an alternative fuel due to high specific energy content (Yilmaz et al. 2012), less energy intensive (for a review, Das & Veziroglu 2001) and carbon neutral combustion products (Balat & Kirtay 2010). Though H_2 fulfills all the essential requirements of an ideal fuel, currently it is produced by chemical methods from coal, oil, natural gas (Cheong & Hansen 2006; Valdez-Vazquez & Poggi-Varaldo 2009), naptha (Valdez-Vazquez & Poggi-Varaldo 2009) or physical methods like photolysis, thermolysis (Das & Veziroglu 2001) and electrolysis of water (Oh et al. 2003a). Current major commercial modes of H_2 production are steam-methane reforming (48%), oil reforming (18%), gasification of coal (30%) and water electrolysis (4%) (Mazloomi & Gomes 2012).

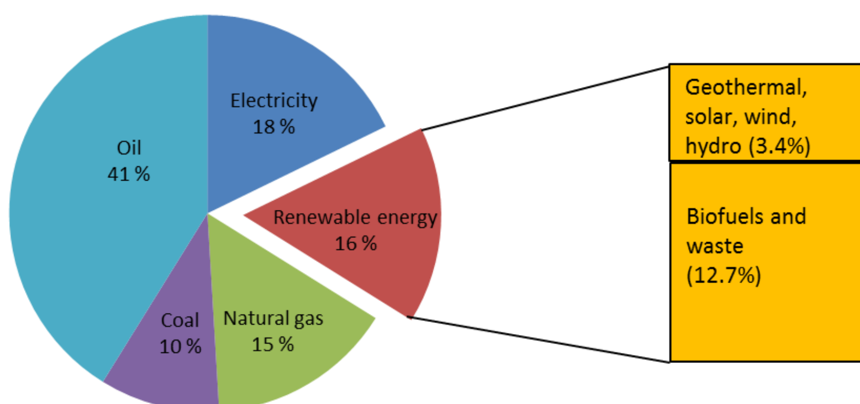
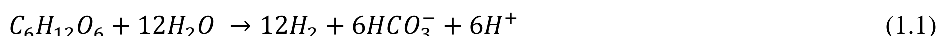


Figure 1.1. Fuel share percentage from global energy consumption in 2010 (Modified from IEA Key world energy statistics, 2012).

1.2. BIOLOGICAL HYDROGEN PRODUCTION

Since the current H_2 production methods are expensive and non-sustainable, biological hydrogen production offers a promising alternative. For example with glucose as substrate, complete oxidation of one molecule of glucose yields 12 molecules of H_2 according to reaction 1.1,



$$C_6H_{12}O_6 + 12H_2O \rightarrow 12H_2 + 6HCO_3^- + 6H^+ \quad (1.1)$$

However, glucose oxidation by fermentative H_2 producing microbes is generally carried out with the production of several reduced fermentation metabolites in turn lowering the H_2 yield values. Thus, intensive research has been concentrated in the field of biological hydrogen production to improve the H_2 yield. Benmann (2008) has reported that a H_2 yield of 10 mol-H_2/mol-glucose is required for commercializing microbe-assisted biohydrogen production. Studies on bioH_2 production have been reported for both pure strains and mixed microbial community. Kleerebezem and Loosdrecht (2007) have reported the importance of 'mixed microbial cultures' in the field of biotechnology and bioprocess technology. Diversity of microbial consortia in natural samples helps in the complete degradation of organic substrates and direct towards the production of H_2 and other high-value compounds. While microbes can ferment sugars to H_2, due to low production yields and use of non-sustainable substrates thus far no commercial biohydrogen production routes are yet available. For a sustainable bioprocess, it is important to use agricultural and industrial wastes containing high organic content as feedstock for bioH_2 production, as eventually these wastes require adequate pretreatment for proper disposal. For this reason, bioconversion of such feedstock for H_2 production can be a way to overcome the economical and bioprocess sustainability issues. Biological production of H_2 can be performed by various processes such as direct and indirect biophotolysis, photo fermentation, dark fermentation and hybrid processes utilizing the efficiency of both fermentative and photosynthetic organisms. However, the current study (Paper **III**, **IV** and **V**) focused on dark fermentation and hence is described in detail, followed with a brief description of other bioH_2 production processes in the later sub-section.

1.2.1. DARK FERMENTATION

Dark fermentation is a light-independent biological H_2 production process performed by strict or facultative anaerobic bacteria wherein H_2 is generated from organic substrates through the catalytic activity of hydrogenases (Figure 1.4). Thus far, the widely studied microbial groups for H_2 production through dark fermentation process are the family of *Clostridiaceae* and *Enterobactericeae*. Fermentative microorganisms lack the ability to use terminal electron acceptors and the reduction of protons to molecular H_2 serves as one of the routes to dispose the excess reducing equivalents generated from oxidation of carbohydrate rich substrates. This biological H_2 production process is suggested as the most promising choice for a large-scale sustainable H_2 production (Hallenbeck et al. 2012). Dark fermentation offers several advantages such as being light-independent process, requires simple bioreactor set-up, yields high H_2 production rate and a wide versatility in the choice of substrate thus addressing both energy production and waste disposal concerns. (Levin et al. 2004; Wu et al. 2006; Chong et al. 2009a). However, the process is not yet optimal for commercialization and major challenges such as removal of inhibitory fermentation metabolites, H_2 separation and collection, process stability and low H_2 yields needs to be addressed for stable and efficient H_2 production (Das and Veziroglu 2008; Show et al. 2012).

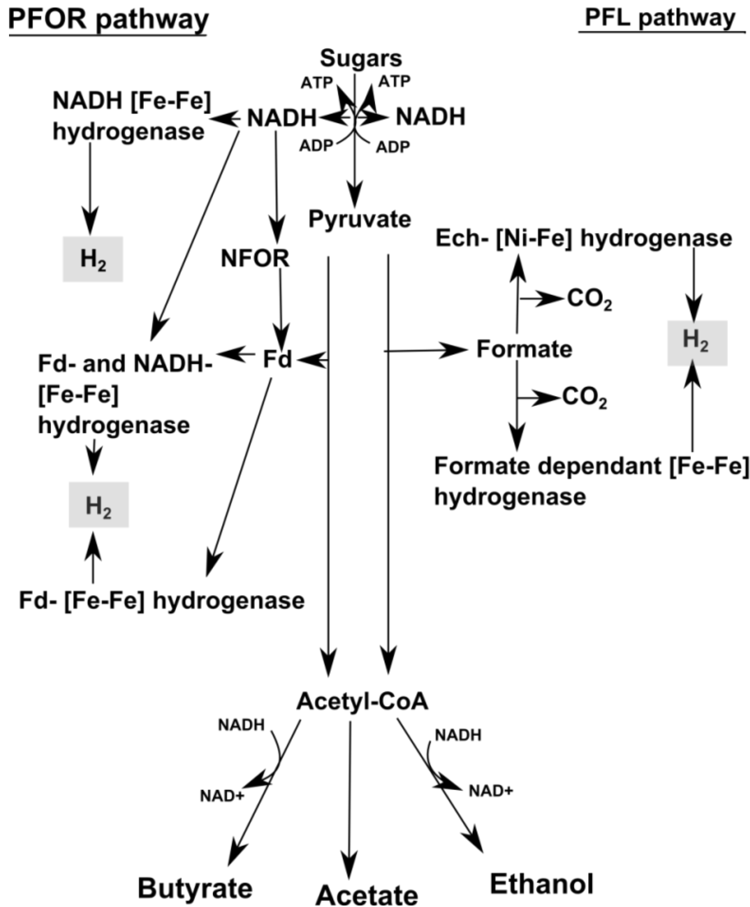


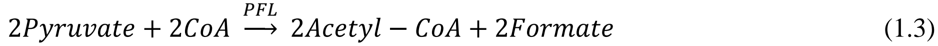
Figure 1.2. Biological H_2 production routes through dark fermentation. In Enterobacteriaceae, pyruvate is converted to acetyl-CoA and formate, which is degraded to H_2 by FHL together with [Ni-Fe] hydrogenase or by formate-dependent hydrogenase. In obligate anaerobes, pyruvate breakdown via PFOR pathway produces acetyl-CoA and reduced ferredoxin. The electron transfer between reduced ferredoxin and [Fe-Fe] hydrogenase results in the evolution of molecular H_2. During low H_2 partial pressure NADH can be used in H2 production, either by reducing ferredoxin (via NFOR) and directing the electron disposal through proton reduction by Fd-[Fe-Fe] hydrogenases or by directly reducing NADH [Fe-Fe] hydrogenases or as a co-substrate with reduced ferredoxin to produce H_2 from NADH-dependent [Fe-Fe] hydrogenase. (Modified from Hallenbeck et al. 2012).

Complete oxidation of one molecule of glucose yield 12 molecules of H_2 according to reaction 1.1. However apart from H_2, the oxidation of carbohydrates is generally accompanied with the production of several reduced end-metabolites (lactate, ethanol, butyrate, succinate, propionate, butanol, acetone and alanine) to maintain the cellular redox balance. In general, during carbohydrate oxidation by fermentative H_2 producers

two molecules of pyruvate and NADH are generated from one molecule of glucose and two molecules of NAD^+ as shown in reaction 1.2,

$$Glucose + 2NAD^+ \rightarrow 2Pyruvate + 2NADH + 2H^+ \quad (1.2)$$

Pyruvate is further metabolized to acetyl coenzyme A (acetyl-CoA) by two metabolic routes (Figure 1.4) (Ntaikou et al. 2010). In facultative anaerobes, the breakdown of pyruvate to acetyl-CoA is catalyzed by pyruvate-formate lysase (PFL; Reaction 1.3) and pyruvate-ferredoxin oxidoreductase (PFOR; Reaction 1.4) (Kim & Kim 2011; Hay et al. 2013).



$$2Pyruvate + 2CoA \xrightarrow{PFL} 2Acetyl - CoA + 2Formate \quad (1.3)$$

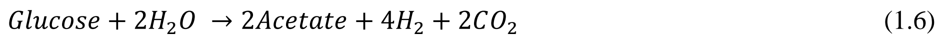
$$2Pyruvate + 2CoA + 4Fd_{ox} \xrightarrow{PFOR} 2Acetyl - CoA + 4Fd_{red} + 4H^+ \quad (1.4)$$

In formate-based H_2 production, the formate generated from pyruvate is subsequently oxidized to H_2 and CO_2 by formate hydrogen lyase (FHL) enzyme complex as shown in reaction 1.5,

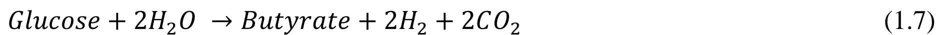
$$2Formate \xrightarrow{FHL} 2H_2 + CO_2 \quad (1.5)$$

The FHL complex comprises of cytoplasmic formate dehydrogenase-H and membrane bound [Ni-Fe] hydrogenase (hydrogenase-3) (Axley et al. 1990; Sawers 1994). The maximal theoretical H_2 yield obtained through this route is limited to 2 moles H_2 per mole of glucose due to incomplete formate utilization, inability to exploit the reducing power of NADH in proton reduction producing H_2 and NADH oxidation routes generating variety of reduced metabolites (Manish & Banerjee 2008; Ntaikou et al. 2010; Hallenbeck et al. 2012; Hay et al. 2013). Table 1.1 lists the H_2 yield obtained in selected publications wherein facultative anaerobes were employed in fermentative H_2 production from glucose.

In the case of strict anaerobes such as *Clostridia* spp., (enriched microbial community in **IV** and **V;** Table 1.1) acetyl-CoA production from pyruvate follows two routes, either towards acetate or butyrate production. Theoretically, acetate production route yields 4 moles of H_2 per mole of glucose (reaction 1.6) and 2 moles of H_2 are produced with butyrate (reaction 1.7) as the main end-metabolite (Levin et al. 2004).



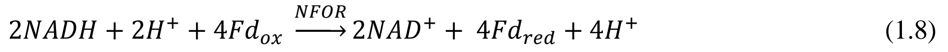
$$Glucose + 2H_2O \rightarrow 2Acetate + 4H_2 + 2CO_2 \quad (1.6)$$



$$Glucose + 2H_2O \rightarrow Butyrate + 2H_2 + 2CO_2 \quad (1.7)$$

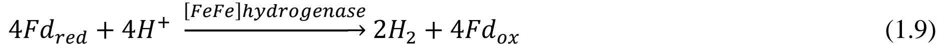
The additional H_2 formed with acetate production is catalyzed by the reduction of ferredoxin and proton transfer to hydrogenase through electrons donated from oxidation of NADH to NAD^+ (Reaction 1.8). Under low H_2 partial pressure, direct oxidation of NADH and Fd_{red} can be catalyzed by variety of hydrogenases using Fd_{red} and/or NADH (Hallenbeck et al. 2012). In addition, the electrons can be transferred between the NADH and Fd_{ox} by NADH:ferredoxin oxidoreductase (NFOR) and molecular H_2 is

produced by hydrogenases using Fd_{red} (Figure 1.4). In the case when butyrate is the final product, the electrons are utilized during the conversion of acetoacetyl-CoA to butyrate (Ntaikou et al. 2010; Kim & Kim 2011; Hay et al. 2013; Levin et al. 2004; Das & Veziroglu 2008).



$$2NADH + 2H^+ + 4Fd_{ox} \xrightarrow{NFOR} 2NAD^+ + 4Fd_{red} + 4H^+ \quad (1.8)$$

The reduced ferredoxin is re-oxidized by donating electrons to [Fe-Fe] hydrogenase generating H_2 (Reaction 1.9)



$$4Fd_{red} + 4H^+ \xrightarrow{[FeFe]hydrogenase} 2H_2 + 4Fd_{ox} \quad (1.9)$$

However at high dissolved H_2 conditions, elevated concentrations of $NADH/NAD^+$ can inhibit the cellular metabolism and reductant disposal through proton reduction. High cellular $NADH/NAD^+$ concentrations can exert a negative impact on glyceraldehyde-3-phosphate dehydrogenase activity resulting in inhibited carbon utilization (Wolfe 2005). Furthermore, in order to maintain the cellular redox balance the excess reducing electrons generated are recycled through a metabolic shift towards the production of reduced metabolites such as ethanol, lactate and butanol which subsequently affects the substrate to H_2 conversion yields (Lay 2000; Mizuno et al. 2000, Van Niel et al. 2003; Kim & Kim 2011; Ciranna et al. 2011).

Table 1.1. Examples of H_2 yields and soluble fermentation metabolites produced from glucose fermentation by *Enterobactericeae* and *Clostridiaceae* family [a]

Inoculum	H_2 yield [b]	Soluble fermentation metabolites	References
Enterobactericeae			
Enterobacter aerogenes HT-34 and HGN-2	~2.2	Ace., But.	Jayasinghearachchi et al 2009
E. cloacae IIT-BT 08	2.2	Ace., EtOH, But.	Khanna et al. 2011
Escherichia coli BW25113 and mutant strains	0.0 - 1.80	ND	Seppälä et al. 2013
E. coli [c]	2.0	ND	Bisallion et al. 2006
Citrobacter sp. Y19	2.5	Ace., EtOH, Pro., Val., But.	Oh et al. 2003
C. freundii CWBI952	0.9	Ace., Lac., Suc., EtOH, For.	Hamilton et al. 2010
Citrobacter sp. *CMC-1*	1.8	Ace., EtOH, For., Lac.	**III**
Clostridiaceae			
Clostridium sp. R1.	2.0	Ace., EtOH, But.	Ho et al. 2010
Clostridium sp. Fanp2	2.5	Ace., But., EtOH, Pro.	Pan et al. 2008
C. beijerinckii L9	2.8	Ace., Lac., But., ButOH.	Lin et al. 2007
C. acetobutylicum	1.8	Ace., But., EtOH., Lac., For.	Lin et al. 2007
C. butyricum	2.3	Ace., But., EtOH., Lac., Pro., For.	Lin et al. 2007
C. tyrobutyricum [d]	2.6	Ace., But.	Liu et al. 2006
C.thermocellum 27405	1.6	Ace. EtOH, Lac., For.	Levin et al. 2006

[a] Batch fermentations, [b] mol- H_2/mol-substrate, [c] E.coli mutant strain with upregulated FHL enzyme complex and inactivation of uptake hydrogenases and lactate dehydrogenase, [d] acetate kinase mutant.

ND = Not determined, Ace. = Acetate, But. = Butyrate, EtOH = Ethanol, Pro. = Propionate, Lac. = Lactate, Val. = Valerate, Suc. = Succinate, For. = Formate

1.2.2. OTHER BIOHYDROGEN PRODUCTION STRATERGIES

Biophotolysis

Green algae and cyanobacteria are able to produce H_2 from water either by direct or indirect biophotolysis. In *direct biophotolysis*, the light energy absorbed by photosystem II (PSII) and photosystem I (PSI) assists in electron transport from water to ferredoxin. The reduced ferredoxin is re-oxidized by transferring the electrons to hydrogenases and/or nitrogenases. Though direct biophotolysis relies on inexpensive substrate and reported to have high energy conversion rates (Melis 2009), one major limitation is the presence of O_2 produced from PSII mediated water oxidation which irreversibly inactivates both hydrogenase and nitrogenase. (For reviews, Eroglu & Melis 2011; Bicakova & Straka 2012).

In *indirect biophotolysis*, the CO_2 is fixed to synthesize sugars via Clavin cycle and the stored carbohydrates (temporal separation) can be fermentatively metabolized to produce H_2 via nitrogenase (spatially separated in heterocycts) or hydrogenase. Under sulfur deprived conditions, H_2 production from nitrogenases and [Fe-Fe] hydrogenases occur through transfer of electrons from NAD(P)H to reduced ferredoxin via PSI (for a review, McKinlay & Harwood 2010). Apart from spatial separation of nitrogenases and [Fe-Fe] hydrogenases in nitrogen fixing organisms, cyanobacteria can also produce H_2 without nitrogen fixing (Bicakova & Straka 2012). In non-nitrogen fixing cyanobacteria such as *Synechocystis* spp., *Synechococcus* spp., *Gloebacter* spp., the NAD(P)H produced via fermentation of stored carbohydrates donates electrons to bidirectional [Ni-Fe] hydrogenases producing H_2 (Baebprasert et al. 2010; for a review Das & Veziroglu 2008).

Photofermentation

Photofermentation is a process in which photofermenting bacteria utilize reduced organic (acetate, butyrate and lactate) and inorganic (H_2S or Fe^{2+}) compounds as electron donors with utilization of solar energy (for reviews, McKinlay & Harwood 2010; Kim & Kim 2011; Hay et al. 2013). Oxidation of organic or inorganic compounds produces CO_2, H^+ and electrons. The electrons are transferred from ubiquinone to PSI by NAD reduction and, undergo electron transfer to nitrogenase enzyme by reduced ferredoxin. One of the best studied species in photofermentation is purple non-sulfur bacteria (PNSB) due to proficient utilization of solar energy, wide substrate utilization range, high substrate conversion efficiencies and lacks PSII therefore eliminating O_2 mediated inhibition (Golomysova et al. 2010; for a review, Eroglu & Melis 2011). In addition, photofermenting bacteria also contribute towards bioconversion of organic wastes (Tao et al. 2008). However, photofermentation is greatly hindered due to low specific activity of nitrogenase enzyme (for reviews, Kim & Kim 2011; Bicakova & Straka 2012).

Biological water-gas shift reaction

Photosynthetic gram-negative bacteria such as *Rhodospirillum rubrum*, *Rubrivax gelatinosus* and *Rhodobacter sphaeroides* and non-photosynthetic gram positive *Carboxydothermus hydrogenoformans* can produce H_2 and CO_2 from carbon monoxide (CO) oxidation. *Rubrivax gelatinosus* and *Rhodospirillum rubrum* produces H_2 in the absence of light through carbon monoxide-mediated water-gas shift reaction. Under anaerobic conditions, carbon monoxide induces the synthesis of carbon monoxide dehydrogenase, iron-sulfur protein and carbon monoxide tolerant hydrogenase. Carbon monoxide dehydrogenase oxidizes CO to CO_2 and the reducing equivalents produced are transferred to hydrogenase via Fe-S cofactor (for reviews, Ni et al. 2006; Saxena et al. 2009). Though the process involves carbon monoxide removal, slow growth kinetics with CO as sole carbon source, low gas-liquid mass transfer rate and

irreversible O_2 mediated hydrogenase inactivation are the major disadvantages of biological water-gas shift process (for a review, Kim & Kim 2011).

Hybrid fermentation

Hybrid fermentation is a promising technique for efficient H_2 production as the method addresses the disadvantages of dark fermentation (accumulation of inhibitory fermentation metabolites) and the merit of photofermentation (utilization of organic acids). Moreover, in theory, 12 moles of H_2 can be produced from 1 mole of glucose through hybrid fermentation (for reviews, Bicakova & Straka 2012; Tekucheva & Tsygankov 2012). One approach involves bioconversion of sugars and organic wastes to H_2 and organic end products. The liquid end products are then converted to H_2 by photosynthetic bacteria. Yokoi et al. (2002) have reported a H_2 yield of 7.2 mol/mol hexose by *C. butyricum*, *Enterobacter aerogenes* HO-39 (dark fermentation) and *Rhodobacter* spp. M-19 (photofermentation) with sweet potato starch residue as the carbon source (Yokoi et al. 2002). Sucrose fermentation by *C. pasteurianum* CH_4 and *R. palustris* WP3-5 resulted in a H_2 yield of 10.1 mol/mol hexose with 72.0% COD removal (Chen et al. 2008). Another approach in hybrid fermentation is the usage of phototrophic accumulation of polysaccharides in microalgal biomass as the substrate for the two-stage fermentation. Kawaguchi et al (2001) investigated the effect of starch in *Dunaliella tertiolecta* biomass as the carbon source for hybrid fermentation involving *Lactobacillus amylovorus* and *Rhodobium marinum* A-501. The authors reported a H_2 yield of 8.3 mol/mol hexose.

2. OPTIMIZATION TOWARDS IMPROVED FERMENTATIVE HYDROGEN PRODUCTION

2.1. THE NEED FOR BIOPROCESS OPTIMIZATION

Fermentative H_2 production is a complex process and in-depth understanding of the microbial physiology is vital to develop an efficient biotechnological process. The microbial cellular metabolism and external physico-chemical parameters are closely related and often the output is directly related to the interaction between these two components. There are several factors that have to be taken into account for maximizing H_2 yield and H_2 production rate. In the context of dark fermentative H_2 production chosen in the current study (**III**, **IV** and **V**), parameters affecting the bioprocess such as inoculum type and strain selection, cultivation conditions (pH and temperature), substrate selection, medium components and H_2 removal and collection are explained in this section. Thus optimization of such parameters enables to understand the dynamics of cellular metabolism and H_2 production. Optimization of key factors for improved bioH_2 production by pure strains and mixed microbial communities from selected studies are shown in Table 2.1.

2.2. INOCULUM TYPE AND STRAIN SELECTION

Pure bacterial strains capable of utilizing wide range of substrates have been investigated for their potential in fermentative H_2 production in closed batch experiments. As observed from Table 2.1, bacteria affiliated to *Clostridiaceae* and *Enterobactericeae* family have been greatly exploited as inoculum for mesophilic bioH_2 production. An attractive substrate utilization range and relatively high H_2 production have resulted in studying biological H_2 production in *Clostridium* spp. (Rajhi et al. 2013). *Clostridium* spp. requires strict anaerobic conditions for H_2 production, thereby requiring the need for reducing agents to maintain anoxic conditions. On the contrary to *Clostridium* spp., *Enterobactericeae* solves this major hurdle by consuming dissolved O_2 and produce H_2 when anaerobic conditions are maintained. Pure cultures (discussed in **III**) as inoculum source eases in metabolic predictions revealing important information regarding the process conditions and metabolic engineering strategies that contribute to high H_2 yield and production rate (Hallenbeck and Ghosh 2012). BioH_2 productions by mixed cultures containing facultative and strict anaerobes have also been studied (Yokoi et al. 1998). (For review, Wang & Wan 2009a; Sinha & Pandey 2011; Show et al. 2012)

Natural H_2-fermenting mixed microbial communities widely exist in environmental samples such as activated sludge (**IV** and **V**), wastewater, open bioreactors, anaerobic digestion sludge, organic wastes, and soil. Kleerebezem and Loosdrecht (2007) introduced the term of ‘Mixed culture biotechnology’ that aims in production of H_2 by natural mixed cultures rather than pure strains. Apart from producing H_2, mixed

microbial consortium also helps in production of other useful metabolites as well, thus increasing the range of products (Valdez-Vazquez & Poggi-Varaldo 2009). The use of environmental samples offers several advantages over pure strains such as utilization of complex substrates, improved bioconversion and adaptable towards bioprocess fluctuations (Kleerebezem & van Loosdrecht 2007; Wang & Wan 2009a). However, in addition to H_2 producers, such microbial communities would contain H_2 consumers that inhibit interspecies electron transfer, reducing the production rate (Kleerebezem & van Loosdrecht 2007; Ren et al. 2008). Pre-treatment of natural samples is highly essential in order to eliminate H_2 consumers, so as to enrich H_2 producing microbial community and increase H_2 production (Ren et al. 2008). Pre-treatment methods such as heat-shock (Wang et al. 2010; Chang et al. 2011), acid (Ren et al. 2008; Chen et al. 2002), base (Lin et al. 2006a; Xiao & Liu 2006) and addition of chloroform (Chidthaisong & Conrad 2000; Hu & Chen 2007), fluoroacetate (Chidthaisong & Conrad 2000), acetylene (Valdez-Vazquez et al. 2006) and 2-bromoethanesulphonic acid (Chidthaisong & Conrad 2000; Venkata Mohan et al. 2008) have been widely used to enrich H_2-producing bacteria from natural samples. The type of inoculum and substrate source play an important role in deciding the type of enrichment technique that should be used for effective removal of H_2 consuming microbes (Ren et al. 2008; Wang et al. 2010; Valdez-Vazquez et al. 2006).

2.3. pH

The culture pH is one of the important physiological factors that influence the redox and energetic state of H_2 producing bacteria. Several researchers have studied the effect of initial pH on H_2 production as fermentative H_2 production by pure strains and mixed microbial communities were largely performed in batch experiments (Table 2.1). It can be inferred from Table 2.1 that in most studies the initial pH required for optimal H_2 production lie in the range of 6.0 – 8.0. But one cannot generalize the applicability of the optimal initial pH range and depends greatly on the inoculum source. An initial pH of 11.0 has been reported optimum for alkali-tolerant H_2 producers (Ren et al. 2008; Cai et al. 2004). Acidic pH ranges of 4.5 – 5.5 have been reported to be optimal for H_2 production (Khanal et al. 2004; Lin et al. 2008; Tang et al. 2008). It is to be noted that the contradictions on optimal culture pH can also be attributed by the different experiment setups employed during the study (pH controlled and non-pH controlled). However, a direct comparison is impossible and the optimal pH value should be individually investigated for each case. At alkaline pH, *Enterobacteriaceae* is affected by cell formate regulation mechanism and subsequent breakdown to H_2 and CO_2 (Seol et al. 2008; **III**). In the case of non-pH controlled fermentations by *Clostridium* spp., at low culture pH a metabolic shift from acidogenesis phase to solventogenesis phase was observed and negatively affected the substrate utilization (Van Ginkel and Logan 2005). At low culture pH the undissociated form of organic acids produced exert stress on the microbial cellular metabolism (Jones and Woods 1986).

2.4. TEMPERATURE

The effect of cultivation temperature on fermentative H_2 production can be assessed experimentally on a broad range of temperatures. The temperature range in which the investigations need to be performed depends on the inoculum type (pure strains) and inoculum source (mixed microbial consortium). Fermentative H_2 production are operated mainly in mesophilic (25-40˚C), thermophilic (40-65˚C) and hyperthermophilic (>80˚C) temperatures. Thermophilic bioprocess contributes in inhibiting pathogens and activity of H_2 consumers, produces less variety of fermentation metabolites and favors higher substrate to H_2 conversion yields. On the contrary, thermophilic bioprocesses require greater energy input and thus increase the operational costs (Noike et al. 2002; Davila-Vazquez et al. 2008). In the present study, the fermentations were conducted at mesophilic temperature ranges (**III**, **IV** and **V**). Report published by Li et al (2007) mentions the dominance of fermentative H_2 production studies at mesophilic conditions. Generally, an increase in cultivation temperature to an optimal value upregulated the carbon metabolism and thus the fermentative capacity of microorganisms. In addition to the optimal metabolic activity of the fermentative microorganisms, cultivation temperature also affects the hydrogenase activity. Adams and Mortenson investigated the effect of temperature on *C. pasteurianum* [Fe-Fe] hydrogenases and reported that high temperatures affected the hydrogenase activity (Adams and Mortenson, 1984). Hence, a declined response in fermentative H_2 production at cultivation temperatures above the optimal value can be explained by inhibited hydrogenase activity and low abundance of cellular proteins involved in maintaining the cellular redox balance and carbon metabolism (Sinha & Pandey 2011; Guo et al. 2010).

2.5. SUBSTRATE SELECTION, CONCENTRATION AND MEDIUM COMPOSITION

Selection of optimal substrate is important for bacterial growth and in obtaining high substrate to H_2 conversion yields. Wide ranges of substrates have been used for the production of H_2 from microbial consortia. Among them, most widely used substrates are glucose, sucrose and starch (Wang & Wan 2009a). However, for sustainable bioH_2 production, the substrate selected should be (i) abundant, (ii) readily available, (iii) cheap and (iv) requires minimum pretreatment. Feedstock such as agricultural residues and industrial effluents/by-products are excellent substrates of choice due to their wide availability, high energy and organic content. In addition to H_2 production, use of such feedstock by fermenting microflora assists in waste disposal issues. In the present study (**III**), H_2 production from monomeric hemi-cellulosic sugars by *Citrobacter* sp. CMC-1

was investigated. Lignocellulosic wastes are excellent choice of feedstock for a cost-effective and sustainable H_2 production. However, such complex feedstock require proper pre-treatment for efficient bioconversion. It is to be noted that the type and concentration of monosaccharides depends on the lignocellulosic feedstock and the pre-treatment process involved. Zhang et al. (2007) investigated bioH_2 production from acid pretreated cornstalk wastes with an inoculum enriched from cow dung compost and reported that proper pretreatment method is essential for optimal availability of soluble sugars for fermentation. The applicability of an optimal substrate concentration also depends on the inoculum and experimental setup. Magnusson et al (2009) reported a total gas production of 56.6 ml/l/h with a substrate concentration of 4 g/L α-cellulose and at higher substrate concentrations the substrate delivery was impaired. Prevalence of monomeric hemicellulosic pentose sugars (xylose and arabinose) also affects the bioconversion yields. Pentose is an unfavorable carbon substrate for H_2 production. Compared with hexose fermentation, a very few pentose fermenting bacteria among *Clostridiaceae* and *Enterobacteriaceae* family have been identified. An optimal concentration of 16.15 g/L of xylose was experimentally observed for bioH_2 production by *Enterobacter* sp. CN1 (Long et al. 2010), whereas Ren et al. (2009) and Jayasinghearachchi et al. (2009) have reported a substrate mediated inhibition affecting the cellular metabolism of *Enterobacter aerogenes* at xylose concentrations over 10 g/L.

Wastes from industrial processes such as crude glycerol (by-product from biodiesel production plants) can also be used as fermentative carbon source. Over the years due to the tremendous growth of biodiesel production, the glycerol surplus has resulted in waste disposal concerns and in turn has affected the crude glycerol prices (for reviews, Chong et al. 2009; Sarma et al. 2012). In addition to the economic advantages of using crude glycerol as feedstock in fermentation processes, crude glycerol requires minimum pretreatment (Sarma et al. 2012) and due to the highly reduced nature of carbon atoms in glycerol reduced metabolites such as fuels (methane, H_2) and chemicals (ethanol, 1,3-propanediol succinic acid and lactic acid) can be produced at higher yields than obtained from model substrates (Muraka et al. 2008; Sarma et al. 2012; **IV** and **V**). However, crude glycerol often contains impurities such as methanol, soaps, organic compounds and ash. Addition of acids cause the dissolved soaps to split to insoluble free fatty acids and salts and at elevated concentrations these impurities can exert high osmotic stress negatively affecting the microbial growth (Ito et al. 2005; Kivistö et al. 2013; Reungsang et al. 2013; **IV**; for a review, Chatzifragkou and Papanikolaou 2012). Thus a careful case by case investigation to identify the optimal substrate concentration is highly necessary to yield efficient bioconversion yields.

In addition to the carbon and energy sources for optimal microbial growth and cellular metabolism during dark fermentation process, the medium needs to be supplemented with essential macronutrients (i.e. nitrogen, sulfur, phosphate, potassium and magnesium) and micronutrients (iron, nickel, vitamins, etc.). The need for macro and micronutrient supplementation greatly depends on the fermentation substrate and inoculum (Wang & Wan 2009a). Though medium components play a great role in

microbial growth and metabolism, an un-optimized medium can adversely affect the overall bioprocess cost. Thus optimization of cultivation medium composition is vital for each fermentation process to determine appropriate concentrations of medium components for improved fermentative H_2 production process.

Nitrogen is an important media component assisting in the synthesis of proteins, nucleic acids and enzymes and an optimal carbon to nitrogen ratio is significant for the microbial growth (Lin & Lay 2004; Lin & Lay 2005; Sittijunda & Reungsang 2012). Different organic and inorganic nitrogen sources such as yeast extract, peptone, ammonium chloride and ammonium bicarbonate have been investigated to study the effect on fermentative H_2 production (Kalil et al. 2008; Sittijunda & Reungsang 2012). Kalil et al (2008) compared the effect of organic and inorganic nitrogen sources on microbial growth, substrate utilization and fermentative H_2 production. They have reported maximum growth and bioH_2 production with organic nitrogen sources. This effect has been widely observed and points to the fact that organic nitrogen sources contains additional carbon and amino acids that can be utilized by the inoculum. However, their use is not optimal at industrial scale. In the case of ammonia nitrogen, discrepancy in optimal concentration for fermentative H_2 production indicates that the appropriate concentration vary with the inoculum type.

Phosphates are indispensable components of nucleic acids, nucleotides and phospholipids. Phosphates are also required for proper maintenance of media buffering capacity. Lin and Lay (2004) investigated the effect of carbonates and phosphates in improving fermentative H_2 production and reported phosphates being significant as buffering agents (Lin & Lay 2004). However, high phosphate ion concentrations are reported to cause increased cytoplasmic osmotic pressure, negatively affecting the microbial growth (Wang & Wan 2009a; Lin & Lay 2004; Lin & Lay 2005; Jitrwung & Yargeau 2011; Jitrwung et al. 2013).

Potassium ions are main cellular inorganic cations and required as cofactor in ATP-dependent ion-pumps. Increased potassium concentrations have a negative effect on cell growth as high cation concentrations may result in cell lysis and thus affecting fermentative H_2 production (Van Niel et al. 2003; Wongtanet & Prapagdee 2008).

Magnesium is an important component for cell wall, ribosome and cell membrane synthesis. Magnesium also functions as a cofactor for glycolytic enzymes such as hexokinase, phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase and enolase. Thus optimization of magnesium in growth media is essential as increased magnesium concentrations directly influence the proportion of glycolytic metabolites thereby resulting in feed-back inhibition and negatively affecting fermentative H_2 production (Lin & Lay 2005; Alshiyab et al 2008; Sinha & Pandey 2011; Jitrwung et al. 2013; **V**).

Apart from the nutrients discussed above, iron and nickel are essential in fermentative H_2 production. Iron and nickel form fundamental components in maturation of [Fe-Fe] and [Ni-Fe] hydrogenases (Valdez-Vazquez & Poggi-Varaldo 2009; Wang & Wan

2009a; Chong et al. 2009b; Alshiyab et al. 2008; Wang & Wan 2008). Although high iron concentrations inhibit bioH_2 production, trace amounts of iron are essential in synthesizing iron-sulfur clusters required for electron transport in hydrogenase (Wang & Wan 2009a). Furthermore, externally supplemented iron affects both microbial growth (Liu & Shen 2004) and metabolism patterns (Dabrock et al. 1992; Lee et al. 2009; Karadag & Puhakka 2010).

2.6. HYDROGEN REMOVAL AND COLLECTION

The concentration of H_2 in the bioprocess system greatly affects the overall thermodynamics of cellular biological reactions. Therefore high dissolved H_2 concentrations should be avoided for continuous and efficient fermentation process. Maintaining low H_2 partial pressure is critical due to its negative effect on hydrogenase. (Valdez-Vazquez & Poggi-Varaldo 2009; Chong et al. 2009b; Levin et al. 2004) Hydrogenases involve in reversible oxidation and reduction of ferredoxin. High dissolved H_2 concentrations inhibit the cellular metabolism and H_2 production (see Section 1.2.1.) Such negative effects of H_2 partial pressure have been reported in investigations by mesophilic (Lay 2000; Mizuno et al. 2000) and thermophilic strains (Van Niel et al. 2003; Ciranna et al. 2011). Sparing with nitrogen gas has been used for H_2 removal (for a review, Nath and Das 2004). However, at commercial scale, such process is not practical as it would hinder the gas separation process (Van Groenestijn et al. 2002; Levin et al. 2004). Though when compared with nitrogen, CO_2 would be a suitable option as it is a by-product from fermentation process and easier to separate (Kim et al. 2006), but CO_2 is also known to negatively affect the microbial metabolism by modifying the media composition (Dixon and Kell 1989). Methane gas has been considered a better choice as it can be easily separated from methane-H_2 gas mixture and does not affect the microbial metabolism (Pawar et al. 2013). Other strategies such as bioreactor design and *in situ* H_2 removal by silicone rubber membrane have been established for H_2 removal (Lay 2000; Mizuno et al. 2000, Van Niel et al. 2003).

2.7. ONE-FACTOR-AT-A-TIME DESIGN VERSUS FACTORIAL DESIGN

One-factor-at-a-time (OFAAT) design is the most commonly used optimization technique wherein the method investigates the effect of varying levels of a single factor towards the response while keeping the other factors constant. Thus after optimization of one factor, the next factor is optimized until all the variables are studied. OFAAT

design is advantageous due to its ease in analysis and has been widely used in optimizing different physico-chemical parameters affecting bioH_2 production (Kyazze et al. 2006; Salerno et al. 2006; Cui et al. 2009; Prasertsan et al. 2009; Kivistö et al. 2010; Kivistö et al. 2011; Ciranna et al. 2011; **III** and **IV**). But when investigating the effects of several parameters on a targeted objective, OFAAT method requires large number of experiments and the technique does not take the effect of interactions between the analyzed factors into account. This method, in certain studies, will fail in obtaining the optimal response from the parameters tested.

The drawbacks in OFAAT design are addressed by factorial design. In factorial design, the factors (variables) investigated are included as coded or actual factor levels and helps in identifying the individual and interactive effect of those factors towards the response studied. The actual factor level, i.e. bioprocess parameter ranges, can be assigned to corresponding coded factor levels (for example, +1, 0, -1). The coded factor levels are irrespective to the dimensional in the actual factor level and thus provide uniformity in analysis. Factorial designs are classified as (i) full factorial design, where every combination of the factor is tested. In full factorial design, with increasing number of factors and their levels, the number of runs increases geometrically. This drawback is addressed in (ii) fractional factorial design where the effects of factors on the response are studied under practical ways. In general, when such design is employed, the first step (Plackett-Burman design) involves identifying the significant factors affecting the response. Next optimization steps (Path of steepest ascent or Ridge analysis) helps to move the experimental region from the design center towards the direction of an optimal response. Central composite or Box-Behnken design together with response surface methodologies (RSM) are then chosen to characterize the response in the optimal region. (For a review, Wang and Wan 2009b) Recently, statistical optimization methods have been widely used for fermentative H_2 production studies (Sittijunda & Reungsang 2012; Jitrwung & Yargeau 2011; Jitrwung et al. 2013; Liu & Fang 2007; Saraphirom & Reungsang 2010; Long et al. 2010; Ghosh & Hallenbeck 2010; Reungsang et al. 2013; **V**). Table 2.1 shows H_2 yield results obtained after optimization of operational parameters and media components or supplements by OFAAT and statistical methods.

Table 2.1. Optimization of key factors for improved bioH_2 production by pure strains and mixed microbial communities

Inoculum; Source	**Microbial community**	**Substrate**	**Design**	**Optimization parameters**		**H_2 yield (mol-H_2 /mol-substrate)**	**Ref.**
				Physico-chemical factors	**Nutrients & media supplements**		
Pure strains							
Halanaerobium saccharolyticum subsp. saccharolyticum		Glu, PG	OFAAT	Initial pH, Salt conc.	YE	2.44 (Glu) 0.62 (PG)	(Kivistö et al. 2010)
H. saccharolyticum subsp. saccharolyticum		PG	OFAAT		Vitamin B_{12}	2.16 (0 µg/ml B_{12})	(Kivistö et al. 2011)
Caloramator celere		Glu	OFAAT	Initial pH, H_2 partial pressure	Phosphate buffer, YE, Tryp, Fe^{2+}	3.36	(Ciranna et al. 2011)
Caloramator celere		Glu	OFAAT	pH control, H_2 partial pressure	Buffering agents	3.07 – 3.95	(Ciranna et al. 2012)
***Thermoanaerobacterium thermosaccharolyticum* PSU-2**		Suc	OFAAT	Initial pH, Temp., Sub. utilization,		2.53	(O-Thong et al. 2008)
***Citrobacter* sp. CMC-1; Activated sludge**		Glu, Xyl, Gal Man, Ara, Rha	OFAAT	Initial pH, Temp., Cultivation time		1.82/NA(Glu) 1.22/1.63(Xyl) 1.18/1.68(Gal) 1.23/1.93(Man) 0.94/0.79(Ara) 1.01/NA (Rha)	**III**
***Escherichia coli* DJT135**		Glu	BB	Initial pH, Temp., Sub. conc.		1.69	(Ghosh & Hallenbeck 2010)
***Enterobacter aerogenes* KKU-S1**		CG	PB, CCD	Initial pH, Temp., Sub. conc.	Vitamin, YE	0.12	(Reungsang et al. 2013)
E. aerogenes		CG	Full factorial design		$MgSO_4$, $CaCl_2$, EDTA, Na_2HPO_4, KH_2PO_4	0.84 (CG)	(Jitrwung et al. 2013)
Mixed cultures enriched from							
Bioreactor treating citrate-producing wastewater	*Clostridium* spp.	Suc	CCD	Initial pH, Temp., Sub. conc.		3.69	(Wang et al. 2005)

Table 2.1. cont.

Municipal sewage treatment plant		Xyl	OFAAT	pH, Sub. uti.		2.25	(Lin & Cheng 2006)
Paper mill wastewater treatment sludge	*Clostridium, Klebsiella, Streptococcus and Pseudomonas* spp.	Sta	OFAAT	Initial pH, Sub. conc., HRT		2.2	(Lin et al. 2008)
Stir tank reactor	*Clostridium* spp.	Glu	OFAAT		Fe^{2+}, Ni^{2+}	1.53	(Karadag & Puhakka 2010)
Ethanol manufacturing and Municipal wastewater treatment plants		Glu	BB	Initial pH, Sub. conc.	Linoleic acid conc.	3.38	(Ray et al. 2010)
Municipal wastewater treatment plant		Xyl	Fractional factorial design	Initial pH, Biomass conc.	Oleic acid conc.	2.64	(Chaganti et al. 2012)
Wastewater treatment plant		CG	PB, SA, BB	Initial pH, Temp., Sub. conc.		0.96	(Varrone et al. 2012)
Activated sludge	*Klebsiella, Escherichia, Shigella* and *Cupriavidus* sp.	PG, CG	OFAAT	Headspace/medium ratio, Sub. conc.	Vitamin and Mineral conc., YE	0.90 (PG) 0.91 (CG)	(Varrone et al. 2013)
Activated sludge	*Clostridium* spp.	CG	OFAAT	Initial pH, Temp., Sub. conc.		1.1	**IV**
Activated sludge	*Clostridium* spp.	CG	PB, SA, BB , RA, CCD		NH_4Cl, K_2HPO_4, KH_2PO_4, $MgCl_2$, KCl	1.41	**V**

*The references to publications from current study are listed in the Reference column (**III** – **V**).*

Glu = Glucose, Gly = Glycerol, PG = Pure glycerol, CG = Crude glycerol, Suc = Sucrose, Sta = Starch, Gal = Galactose, Man = Mannose, Xyl = Xylose, Ara = Arabinose, YE = Yeast extract, Tryp = Tryptone, Temp. = Temperature, Sub. conc. = Substrate concentration, Sub. uti. = Substrate utilization, HRT = Hydraulic retention time, YE = Yeast extract, OFAAT = One-factor-at-a-time, PB = Plackett – Burman, SA = Steepest accent, BB = Box – Behnken, RA = Ridge analysis and CCD = Central composite design, NA = Not applicable.

3. MICROBIAL COMMUNITY PROFILING TECHNIQUES

3.1. IMPORTANCE OF MICROBIAL COMMUNITY PROFILING

Profiling the microbial consortium is important to understand the genetic diversity. There are several methods for characterizing microbial communities and have been widely used in determining the functional consortium of fermentative communities. More importantly in addition to a quantitative analysis of the community, such profiling techniques help to elucidate functional information of the microorganisms involved in fermentative bioH_2 production. Microbial community profiling methods can be divided in two groups i.e. culture-dependent and culture-independent. In this section, strain isolation and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) employed in **III** and **IV** are discussed, followed with brief mention on other community profiling techniques (Figure 3.1).

3.2. STRAIN ISOLATION

Strain isolation is a conventional, cultivation based method for the enumeration of cultivable microorganisms in a mixed culture. This technique is based on the expression of specific phenotypes such as antibiotic resistance, utilization of specific substrates and specific growth conditions (Kirk et al. 2004). Culture-dependent methods remain important tools for targeted isolation of microorganisms. Strain isolation method has been applied in isolating H_2-fermenting microorganisms from environmental samples (Castelló et al. 2009; Jayasinghearachchi et al. 2009; Jayasinghearachchi et al. 2010; Long et al. 2010; **III**). Tam et al. (2005) have compared PCR-DGGE and selective plating techniques to understand the population dynamics in a mixed culture (Tam et al. 2005). They reported selective plating to be advantageous in monitoring the population dynamics of the microbial community. On the contrary, selective plating is labor intensive and applicable only for cultivable microorganisms and cannot be adopted for profiling microbial community. For this reason, in the view of obtaining a comparative analysis of microbial communities and isolated and uncultured strains, this method remains is inefficient and is often used in combination with 16S rRNA gene amplification and sequencing (Ueno et al. 2005; Wang et al. 2007).

3.3. PCR-DGGE AND SEQUENCING

One of the most commonly used profiling techniques to identify individual microbial species from a complex microbial community is the Denaturing Gradient Gel Electrophoresis (DGGE) coupled with PCR amplified 16S rDNA region (in prokaryotes) and 18S rDNA (in eukaryotes) (Fischer & Lerman 1980; Rivas et al. 2004). PCR-DGGE is the most commonly used technique in profiling bioH_2 producing microbial community (Ueno et al. 2001; Cai et al. 2004; Ueno et al. 2006; Lin et al.

2006b; Wang et al. 2007; Lin et al. 2008; Maintinguer et al. 2008; Huang et al. 2010; Karadag and Puhakka 2010; Sittijunda and Reungsang 2012; **IV**). PCR-DGGE technique helps in the separation of DNA fragments of equal lengths but varied sequence. Thus this technique aims in sequence determined DNA separations. DGGE can separate DNA fragments of size up to 500 base pairs. The principle is based on decreased electrophoretic mobility of partially melted double stranded DNA molecules in a linear gradient of DNA denaturant (Urea). When the samples are allowed to run in a polyacrylamide gel containing linear range of denaturing material, the DNA fragments undergo a transition from helical to partially melted stage. The migration, under a constant temperature and a uniform denaturing gradient, is dependent on the basis of sequence differences (Malik et al. 2008). The separation is based on the base composition and melting behavior of the DNA fragment rather than the size. The bands obtained from DGGE are re-amplified and sequenced for further characterization. DGGE allows detection of only 50% of sequence variants in the DNA fragments tested. Inclusion of GC-clamp to PCR primers prevents in complete dissociation of the DNA fragments and improves DGGE sensitivity to nearly 100% (For review, Malik et al. 2008; Muyzer & Smalla 1998; Lerman et al. 1984).

DGGE can be used for identifying the distribution of microorganisms containing the gene of interest from a complex microbial community by sequence dependent separation and hybridization with target gene specific probes. Hybridization of DGGE separated bands can also be used for enrichment, identification and isolation of certain species of interest. For example, Brinkhoff and Muyzer (1997) studied the diversity of sulfur oxidizing bacteria, *Thiomicrospira* spp. and were successful in isolating 7 different strains by using DGGE (Brinkhoff & Muyzer 1997). However, DGGE has several disadvantages as well. The technique is slow, labor intensive and provides limited resolution in complex profiles. PCR is also considered as a main source of error, as heteroduplex formation during the amplification process can lead to misinterpretation of results. This misinterpretation would result in overestimation of the microbial population. Furthermore, DGGE displays only the bands of most predominant species present in the community (1%) (Muyzer & Smalla 1998).

While PCR-DGGE and sequencing methods have been widely used in elucidating H_2 fermenting microbial communities, other techniques such as whole genome and shotgun sequencing of microbial consortia and metagenomics approaches are rapidly gaining importance. In metagenomics, i.e. genomic analysis of microbial communities, the DNA from microbial community is directly cloned into libraries and can be analyzed by sequencing (Handelsman 2004). This approach expands the knowledge of the structure and function of the microorganisms present in the environmental sample in question (Handelsman 2004; Riesenfeld et al. 2004). Development of new sequencing technologies (next generation sequencing, NGS), that are rapid, less-labor intensive and economical, have contributed further in the field of microbial community profiling (Gottel et al., 2011; for reviews, Mardis 2008; Nowrousian 2010; Koboldt et al. 2013).

3.4. OTHER COMMUNITY PROFILING TECHNIQUES

Fatty acid methyl ester analysis (FAME) is a biochemical microbial community profiling method that relies on bacterial phospholipid composition. The method provides information on microbial consortia based on the fatty acid analysis with the help of unique fatty acids able to taxonomically differentiate the community. Though rapid and cost-effective method, FAME analysis requires large samples and is unable to differentiate closely related species (for a review, Zelles 1999).

Restriction fragment length polymorphism (RFLP) is a PCR based profiling technique. RFLP technique is rapid and cost-effect and relies on DNA polymorphism. In RFLP, PCR amplified 16S rDNA fragments are digested with specific restriction enzymes and the digested fragments are resolved using gel electrophoresis (Kirk et al. 2004; Liu et al. 1997). The DNA sequences are restricted at varying locations and result in profile unique to the analyzed community. The cons of RFLP include careful choice on the restriction enzymes, incomplete restriction, nucleic acid extraction and PCR biases and inability to measure and profile the microbial community (for a review, Malik et al. 2008).

Terminal-restriction fragment length polymorphism (T-RFLP) is a modified version of RFLP, in which the fluorescent labeled PCR primers used to amplify 16S rDNA. The amplified products are subjected to digestion by specific 4 base restriction endonucleases and the fragments are separated using capillary electrophoresis. T-RFLP technique is fast, highly reproducible method, helps in analyzing complex microbial communities and can be automated. But similar to RFLP, the biggest drawback is need for restriction and complete digestion (for reviews, Kirk et al. 2004; Malik et al. 2008).

Ribosomal intergenic spacer analysis (RISA) is another PCR-based community profiling technique similar to RFLP and T-RFLP. But instead of targeting 16S rDNA, RISA involves PCR amplification of spacer region between 16S and 23S ribosomal subunits (Kirk et al. 2004; Fisher & Triplett 1999). The intergenic spacer region contains both sequence and length variabilities. This technique is highly reproducible, does not require denaturing conditions or restriction enzymes and facilitates taxonomic identification of organisms. The major hurdle of limited database for ribosomal intergenic spacer sequences limits RISA applications (for a review, Rivas et al. 2004).

Single strand confirmation polymorphism (SSCP) detects sequence variations and DNA point mutations through differences in electrophoretic mobility of single stranded DNA (Kirk et al. 2004). DNA sequences are amplified by (fluorescent labeled) PCR primers and the amplified products are denatured to single stranded DNA. The single DNA strands are separated under non-denaturing conditions by polyacrylamide gel electrophoresis. Altered conformation due to changes in sequences will cause an altered migration pattern. (Kirk et al. 2004; Dong & Zhu 2005) The migration patterns can also be visualized using silver staining. SSCP method is simple, fast and cost-effective

method and has been used to study bacterial and fungal communities (Peters et al. 2000; Stach et al. 2001).

Fluorescent in situ hybridization (FISH) is a taxonomic method to study the microbial community using fluorescent probes. In FISH, the microbial cells are stabilized by fixing to a platform and the cell membrane is permeabilized for hybridization of labelled probes. These probes identify the 16S rRNA sequences and hybridize *in situ* (Malik et al. 2008; Sanz & Köchling 2007). The samples are then analyzed using fluorescence-activated cell sorter, epifluorescence microscopy or confocal laser microscopy. FISH method helps in identifying the presence of a specific taxonomic group (using strain-specific probes) and also helps in quantifying certain microbial groups (using domain or group specific probes) (Malik et al. 2008). FISH techniques are also used in combination with molecular-based methods such as DGGE and T-RFLP (Straub & Buchholz-Cleven 1998; Richardson et al. 2002).

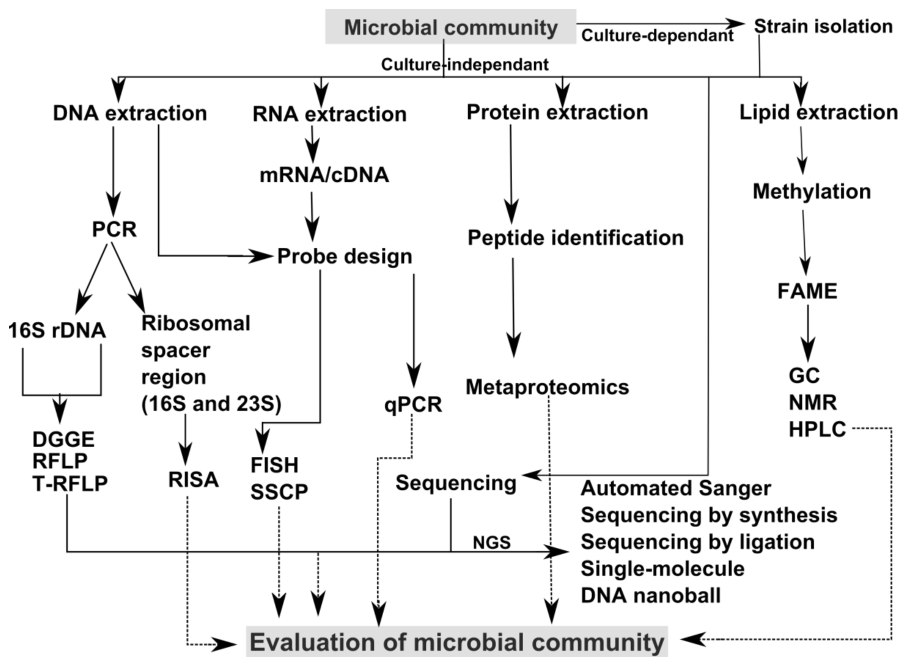


Figure 3.1. Different culture-independent methods used for microbial community profiling. (Modified from Su et al. 2012).

4. HYDROGENASES

4.1. ROLE OF HYDROGENASES IN NATURE

In nature, H_2 is consumed and produced by a variety of microorganisms. For example, fermentative bacteria dispose excess reducing equivalents through proton reduction and generate H_2 as a by-product of their metabolism, whereas methanogenic archea consume H_2 as an energy source for methane production. The key enzyme involved in metabolism of molecular H_2 is hydrogenases. Hydrogenases are a diverse group of metalloenzymes that reversibly catalyze the conversion of dihydrogen to protons and electrons (Stephenson & Stickland 1931),

$$2H^{+} + 2e^{-} \leftrightarrow H_2 \qquad (4.1)$$

4.2. CLASSIFICATION OF HYDROGENASES

Most of the hydrogenases contain iron-sulfur (Fe-S) clusters and Fe and/or Ni metal atoms at the active site (Volbeda et al. 1995). Depending on the metal atom composition at the active site, hydrogenases are grouped to three classes: [Ni-Fe], [Fe-Fe] and [Fe] hydrogenases. The hydrogenase classes are however phylogenetically unrelated i.e. while [Ni-Fe] hydrogenases are found in bacteria and archea, [Fe-Fe] hydrogenases are present in bacteria and eukaryotes and [Fe] hydrogenases exist in methanogeneic archea (Korbas et al. 2006; Shima and Thauer 2007; Vignais & Billoud 2007; Kim & Kim 2011; Winkler et al. 2013). In general, the [Ni-Fe] and [Fe] hydrogenases are associated in energy conservation by H_2 uptake and the [Fe-Fe] hydrogenases mainly involve in H_2 production. The [Ni-Fe] and [Fe-Fe] hydrogenases are further divided into several groups based on the enzyme function and hydrogenase accessory domains (Fe-S clusters), respectively. Figure 4.1 presents a flowchart scheme of hydrogenase classification. The structure of hydrogenase was first elucidated by Volbeda et al. (1995) for [Ni-Fe] hydrogenase in *Desulfovibrio gigas* (Volbeda et al. 1995).

The [Fe] hydrogenases, also termed as H_2 forming methenyl-tetrahydromethanopterin dehydrogenase (Hmd), are devoid of Fe-S cluster and contain one iron at the active site. The [Fe] hydrogenase was first observed in *Methanobacterium thermoautotrophicum* (Zirngibl et al. 1990). The [Fe] hydrogenase catalyzes an intermediary step in the CO_2 reduction to methane, i.e. the reduction of methenyl tertrahydromenthanopterin ($methenyl = H_4MPT^{+}$) with molecular H_2 to methylene tertrahydromethanopterin ($methylene \equiv H_4MPT^{+}$) and H^{+} according to the reaction 4.2 (Zirngibl et al. 1990; Zirngibl et al. 1992; Korbas et al. 2006; Shima and Thauer 2007).

$$methenyl = H_4MPT^{+} + H_2 \leftrightarrow methylene \equiv H_4MPT + H^{+} \qquad (4.2)$$

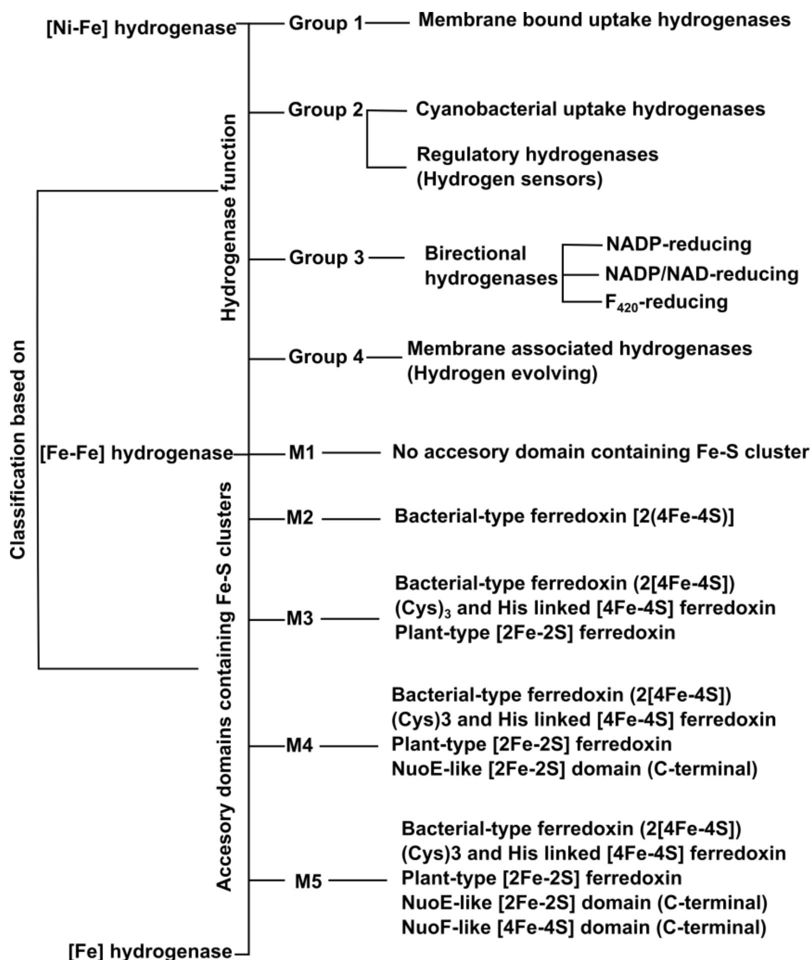


Figure 4.1. Hydrogenase classification. (Modified from Kim & Kim 2011).

Although phylogenetically different, these hydrogenases share some common structural features. The metal atoms are buried deep inside the active site and, except in [Fe] hydrogenase, the electrons are transferred to and from through Fe-S clusters. The metal atoms at the active site are stabilized by CO, CN and cysteine ligands. The active site arrangement in [Ni-Fe], [Fe-Fe] and [Fe] hydrogenases are presented in Figure 4.2.

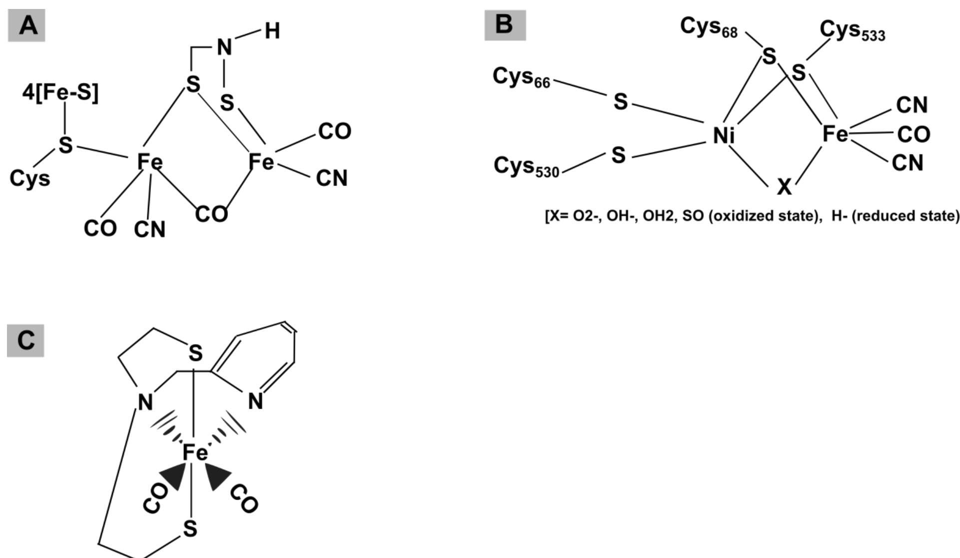


Figure 4.2. Active site of [Fe-Fe] hydrogenase (A), [Ni-Fe] hydrogenase (B) and [Fe] hydrogenase (C). (Adapted from Kim & Kim 2011).

4.3. [Ni-Fe] HYDROGENASES

The [Ni-Fe] hydrogenases are the best studied hydrogenase classes and are associated with a number of distinct cellular functions in bacteria and archea (Vignais & Colbeaua 2004; Kim & Kim 2011). The [Ni-Fe] hydrogenases exist mainly as heterodimers (Vignais & Colbeaua 2004). The large subunit (60 kDa) contains Ni-Fe cluster buried deep inside the active site and the small subunit (30 kDa) hosts up to three Fe-S clusters involving in electron transport to the catalytic site (Peters 1999; Vignais & Colbeaua 2004). Based on enzyme function, [Ni-Fe] hydrogenases are divided into four groups - uptake hydrogenases (Group 1), cytoplasmic H_2 sensors and the cyanobacterial uptake hydrogenases (Group 2), bidirectional cytoplasmic hydrogenases (Group 3) and membrane-associated hydrogenases (Group 4) (For reviews, Vignais & Colbeaua 2004; Vignais & Billoud 2007).

The [Ni-Fe] uptake hydrogenases are membrane bound or periplasmic enzymes important in oxidation of H_2 for the reduction of electron acceptors intended for aerobic (O_2) or anaerobic (fumarate, CO_2, sulfate or nitrate) respiration (Vignais & Colbeaua 2004). In the [Ni-Fe] uptake hydrogenases, the signal peptide at the N-terminal position of the small subunit assisting in transport of functional hydrogenase enzyme from cytoplasm to membrane and periplasm. The hydrophobic C-terminus of the small subunit together with a third subunit consisting of cytochrome-b aids in anchoring to the membrane. For example, *E. coli* uptake hydrogenases (hydrogenase-1 and hydrogenase-2) fall in Group 1 [Ni-Fe] hydrogenase category. (for reviews, Vignais & Colbeaua 2004; Pandelia et al. 2012)

The Group 2 [Ni-Fe] hydrogenase class comprises of cyanobacterial uptake hydrogenases and cytoplasmic regulatory (H_2 sensor) hydrogenase. The cyanobacterial uptake hydrogenases present in *Nostoc* and *Anabaena variabilis* are localized in the cytoplasm and are induced under N_2 fixing conditions. *Ralstonia eutropha* and *Rhodobacter capsulatus* contains cytoplasmic H_2 sensing hydrogenases (Kleihues et al. 2000; Vignais et al. 2000). The main distinguishing feature of Group 2 from Group 1 [Ni-Fe] hydrogenases is the absence of signal peptide in the small subunit. Group 2 hydrogenases are heterodimers with a large subunit involving in hydrogen detection and the small subunit takes part in non-reversible reduction process (Volbeda et al. 1995).

The [Ni-Fe] bidirectional hydrogenases (Group 3) are found in achaea and bacteria and are multimeric proteins that contain three to five species dependent subunits (Vignais & Billoud 2007; Dutta et al. 2005). These hydrogenases take part in cofactor binding. They are termed bidirectional as these [Ni-Fe] hydrogenases can thus re-oxidize the cofactors under anaerobic conditions by using the protons of water as electron acceptors (Vignais & Billoud 2007). Soluble cofactors such as NAD^+, $NADP^+$ or cofactor 420 are required for carrying in oxidation/reduction reactions under anaerobic conditions (Vignais & Colbeaua 2004; Vignais & Billoud 2007).

The Group 4 [Ni-Fe] membrane hydrogenases, found in *E. coli* (hydrogenase-3) and *Enterobacter* spp. takes part in H_2 evolution and energy conservation by the breakdown of formate under anoxic conditions (Sawers 2005). Carbon monoxide induced group 4 [Ni-Fe] hydrogenases, structurally resembling to the *E. coli* FHL enzyme, are found in *Rhodospirillum rubrum*, (Vignais & Colbeaua 2004). Certain archaea such as *Methanosarcina barkeri*, *Methanothermobacter marburgensis* and *Pyrococcus furiosus* contains *Ech* hydrogenases involving in H_2 evolution (Vignais & Billoud 2007).

4.4. [Fe-Fe] HYDROGENASES

The [Fe-Fe] hydrogenases are found mostly in strict anaerobic microorganisms like *Clostridia* spp., sulfate reducing bacteria and eukaryotes (Horner et al. 2002). In eukaryotes, hydrogenases are localized in organelles devoid of membrane structure (Vignais & Colbeaua 2004). The [Fe-Fe] hydrogenases catalyze the redox reaction presented in reaction 4.1 and generally favor in proton reduction to molecular H_2 (Kim & Kim 2011). However, the [Fe-Fe] hydrogenases are also reported to function in H_2 uptake in some sulfate-reducing bacteria (Pohorelic et al. 2002). Di-Iron containing hydrogenase exist mainly monomeric form, even though few di, tri and tetrameric structures have been elucidated. The smallest [Fe-Fe] hydrogenases (44.5 kDa) are found in green algae *Scenedesmus obliquus* (Florin et al. 2000).

Biochemical characterization and protein structural studies have reported that the hydrogenases have conserved domains of 350 amino acid residues at the active site or H-cluster (Vignais & Billoud 2007). The H-cluster comprises of an iron-sulfur [4Fe-4S] domain and binuclear iron unit ($2Fe_H$) linked by CO, CN and dithiolate bridging ligands (Stripp et al. 2009). The structural differences in [Fe-Fe] hydrogenases are related to the accessory domains containing Fe-S moieties (F-cluster) involved in electron transport to and from the active site (Figure 4.3). The F-clusters are absent in [Fe-Fe] hydrogenases found in *Chlamydomonas reinhardtii*, *Chlorella fusca*, and *Scenedesmus obliquus* (Happe & Naber 1993; Florin et al. 2001). Such [Fe-Fe] hydrogenases correspond to structure type M1. Structure type M2 hydrogenase consists of two cubane bacterial-ferredoxin type [4Fe-4S] clusters linked to N-terminal of the active site. The [Fe-Fe] hydrogenases displaying M2 structural type is studied extensively in dimeric *Desulfovibrio desulfuricans* [Fe-Fe] hydrogenase. Additional two N terminal ferredoxin units (Histidine ligated and plat type ferredoxin types) are observed in M3 structural type and usually found in *Clostridium* spp. Structural types M4 and M5 are found in trimeric (*Thermotoga maritima*) or tetrameric (Proteobacteria) forms comprising of additional NADP/NADH-reducing hydrogenase subunits (Akhmanova et al. 1998; Schut & Adams 2009). (For review, Mulder et al. 2011; Winkler et al. 2013)

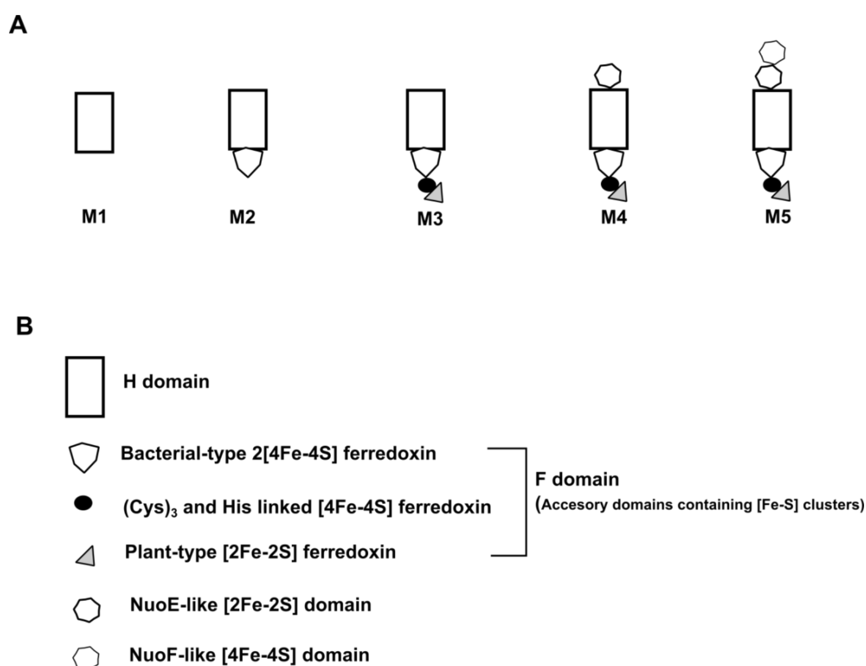


Figure 4.3. Representation of different types of [Fe-Fe] hydrogenases classified based on accessory domains containing [Fe-S] clusters (A) and presented along with the names corresponding to the accessory domains. (Modified from Winkler et al. 2013).

Assistance of maturation proteins, HydE, HydF and HydG are required for proper assembly of di-iron moieties and maturation of [Fe-Fe] hydrogenases. Although not fully understood, HydF maturation protein acts as a GTPase and HydG and HydE accessory proteins are S-adenosyl methionine enzymes, involved in [Fe-Fe] hydrogenases maturation process (Kuchenreuther et al. 2012; Posewitz et al. 2004).

4.5. [Fe] HYDROGENASE

The third group of hydrogenase, devoid of iron – sulfur core, found in methanogenic archea is structurally and functionally different from the other two counterparts. Under nickel starvation conditions, methanogenic archea reduces CO_2 with H_2 to produce methane for energy conservation. The [Fe] hydrogenase catalyzes an intermediary step in the CO_2 reduction process, according to the reaction 4.2 (Zirngibl et al. 1992; Korbas et al. 2006)

Considering the structural basics of Hmd, it consists of two identical subunits of 38 kDa (Korbas et al. 2006). Studies indicate that apoenzyme Hmd is converted to its active state with the binding of a cofactor, where both the cofactor and hydrogenase are light, oxygen and heat sensitive (Korbas et al. 2006). Structural investigations of Hmd hydrogenase revealed the presence of one iron per monomer of the hydrogenase (Lyon et al. 2004). Zirngibl et al. (1992) reported the presence of Hmd hydrogenase in *Methanobacterium thermoautotrophicum*, which had a molecular mass of 43kDa,

sensitive to O_2 and stable in the presence of CO and CN. Apart from the structural and functional dissimilarities between the other two hydrogenase counterparts, the iron present in the Hmd hydrogenase is not redox active (Vignais & Colbeaua 2004; Kim & Kim, 2011).

4.6. OXYGEN SENSITIVITY

In addition to the physico-chemical factors that affect efficient H_2 production, a major biochemical issue i.e. requirement of strict anoxic conditions for hydrogenase maturation due to oxygen sensitivity of hydrogenases is considered as a major challenge for practical applications. In general, all hydrogenases are sensitive towards O_2, but some of them are more tolerant than others. In case of [Ni-Fe] hydrogenase, the O_2 inactivation is reversible in nature i.e. the catalytic activity can be regained after treating with reducing agent (Liebgott et al. 2011). However, O_2 tolerant [Ni-Fe] hydrogenases have been reported. Pandelia et al (2010) have reported the presence of thermostable and O_2 tolerant membrane-bound [Ni-Fe] hydrogenase in *Aquifex aeolicus* (Pandelia et al. 2010). Membrane-bound hydrogenase from *Ralstonia eutropha* H16 have also observed to undergo H_2 oxidation in presence of O_2. It has been reported that the reduced nature of [4Fe-4S] clusters play a pivotal role in maintaining the O_2 tolerance in [Ni-Fe] hydrogenase from *Ralstonia eutropha* H16 (Goris et al. 2011; Fritsch et al. 2011). However, the [Fe-Fe] hydrogenases are irreversibly damaged by O_2. The irreversible inactivation of [Fe-Fe] hydrogenase by O_2 is schematically presented in Figure 4.4. Movement of O_2 through the gas migration pathway, and finally to the catalytic center, is one of the parameters affecting O_2 tolerance (Cohen et al. 2005; Liebgott et al. 2010).

'Rational design' approach by site-directed mutagenesis to introduce mutations around the gas migration pathways (Buhrke et al. 2005; Lautier et al. 2011) and random mutagenesis of hydrogenase protein (Stapleton & Swartz 2010a; Bingham et al. 2012) have been reported. This also substantiates the finding that O_2 does not diffuse specifically through the gas migration pathways, rather accesses the active site through transient cavities formed by the dynamic nature of the protein (Cohen et al. 2005). In the work by Nagy et al (2006), gene shuffling technique was applied to generate novel [Fe-Fe] hydrogenase libraries (Nagy et al. 2007). Another approach in engineering hydrogenase protein was by directed evolution techniques. Stapleton and Swartz (2010a) reported success in creating *C. reinhardtii* [Fe-Fe] hydrogenase mutant libraries by random mutagenesis and screened mutants with improved specific activity using cell free protein synthesis (CFPS) approach. Stapleton and Swartz (2010b) further adopted microbead *in vitro* compartmentalization display methodology, wherein biotinylated mutant hydrogenase gene libraries coated on streptavidin microbeads were used as the platform for emulsified CFPS and hydrogenases with decreased O_2 sensitivity were screened by fluorescence activated cell sorting method (Stapleton & Swartz 2010b).

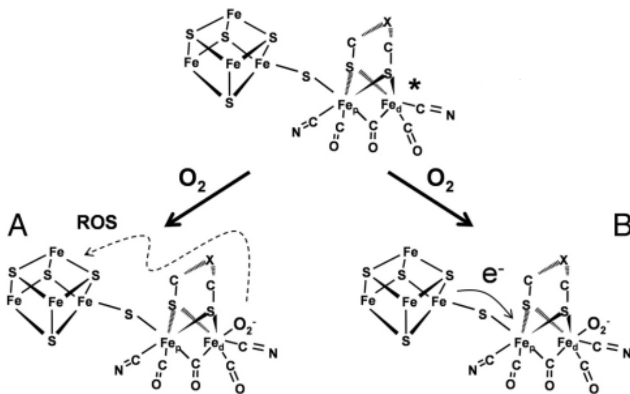


Figure 4.4. Two proposed mechanisms on oxygen attack towards the catalytic site. Upon binding of oxygen to the vacant binding site at the distal Fe, reactive O_2 species are formed. Either the reactive O_2 species can migrate and oxidize the [4Fe-4S] subunit (A) or remain bound at the distal Fe atom and oxidizes the [4Fe-4S] subunit by bound-electron transfer (B). In either two mechanisms, the O_2 first hinders electron transfer by binding to di-iron domain before destroying the catalytic site. (Modified from Stripp et al. 2009).

5. PHAGE DISPLAY AND BIOPANNING TECHNIQUES

5.1. PHAGE DISPLAY TECHNOLOGY

Phage display is an *in vitro* selection technique in which peptide, protein or antibody gene is genetically fused to a phage coat protein gene resulting in its display at the exterior of the phage virion while the genetic information encoding the displayed protein remain within the phage DNA, establishing a physical link between the phenotype and genotype. Since its invention by Smith in 1985, phage display has become a widely applied and efficient tool in the field of drug discovery (Hetian et al. 2002; Su et al. 2005), clinical diagnostics (Lamminmäki et al. 1999; Du et al. 2010; Chan et al. 2013) and protein engineering (Speck et al. 2011; Vithayathil et al. 2011; Brockmann et al. 2011). The display of peptide, protein or antibody libraries as fusions with the phage coat proteins pIII (Brockmann et al. 2005; Marks et al. 1991; Korpimäki et al. 2004; Paschke & Höhne 2005), pVI (Jespers et al. 1995; Hufton et al. 1999), pVII (Gao et al. 1999; Løset et al. 2011a; Løset et al. 2011b), pVIII (Weiss et al. 2000; Held & Sidhu 2004) or pIX (Brockmann et al. 2011; Løset et al. 2011b) have been extensively studied. In the current study (**I**), phage displayed antibody libraries were used for the screening of anti-[Fe-Fe] hydrogenase antibodies with the aim of employing such molecular tools towards monitoring hydrogenases in bioprocess system.

Alternative to hybridoma technology, phage display enables isolation of diverse antibodies through combinatorial libraries of heavy-chain and light-chain variable antibody domains (McCafferty et al. 1990; Barbas III et al. 1991; Hoogenboom et al. 1991). Antibody combinatorial libraries are filamentous phages displaying antigen binding domains on a phage coat protein. Antibodies are composed of a light-chain, comprising of single variable and constant domain, and a heavy-chain containing single variable and three constant domains. The antigen-binding sites in each light and heavy chain consist of three hyper-variable domains. Among the recombinant antibody formats, Fab (fragment antigen-binding) and scFv (single-chain variable fragment) are most commonly used in phage display libraries. Single variable and constant domains from light and heavy-chain connected by a disulfide bond create a Fab fragment. The light-chain and heavy-chain variable fragments connected by a flexible linker produce a scFv fragment (Huston et al. 1988; Bird et al. 1988; Marks et al. 1991). Figure 5.1 schematically illustrates the general immunoglobulin, Fab and scFv structures.

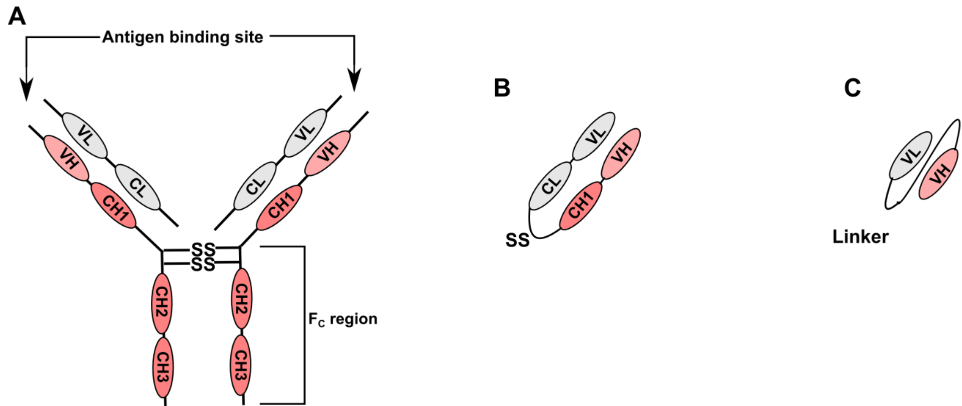


Figure 5.1. Schematic structural representation of immunoglobulin (A), Fab (B) and scFv (C). (Modified from Tikunova & Morozova 2009).

The choice of appropriate bacteriophage, phage library, phage display system and phagemid vectors are crucial for efficient phage display.

Filamentous phages

Phage display is usually performed using filamentous phages such as M13, f1 and fd phages, of which M13 filamentous phages are most commonly used due to detailed knowledge on phage structure and life cycle (Smith & Petrenko 1997; Willats 2002). Filamentous bacteriophages contain a 6400 nucleotide long ssDNA encoding 11 genes: I, IV, and XI for virion assembly; II, V and X for phage DNA replication and III, VI, VII, VIII and IX for phage capsid proteins. In addition to the above mentioned genes, phage DNA carries a 'packaging site' and origin of replication for both (+) and (-) strand synthesis required for rolling circle type replication (Schaller et al. 1979; Zinder & Boeke 1982). The M13 filamentous phages has several advantages such as ease of manipulation, ability to incorporate large amounts of foreign DNA, high stability under wide range of temperature and pH and non-lytic propagation in the bacterial cell (Sidhu 2001; Pande et al. 2010).

Antibody libraries – Diversity and design

Mammalian immunoglobulins provide the antibody variable domains required for phage antibody library construction (McCafferty et al. 1990). Such phage antibody libraries are classified as natural, semi-synthetic and synthetic libraries.

Natural antibody libraries are constructed by *in vivo* rearrangement immunoglobulin variable domain regions obtained from mammalian spleen B cells. Natural libraries can be further classified into immune or naïve libraries. Upon immunization, the donor generates antibodies that are highly specific towards the introduced antigen (Willats 2002). Rearrangement of variable domains of such specific antibodies gives rise to immune libraries (Tikunova & Morozova 2009; Willats 2002).

The first cloned immune libraries were reported by Ward et al. (1989) and Huse et al. (1989). Immune libraries were also constructed for antibodies against HIV (Burton et al. 1991), hepatitis B virus (Zebedee et al. 1992) and herpes simplex virus (Williamson et al. 1993). Though the libraries are highly specific towards a certain antigen, individual libraries have to be built for each antigen (Hoogenboom & Chames 2000). *Naïve antibody libraries* (dominated by IgM) are constructed from non-immunized (non-intentionally immunized) donors and were first demonstrated by Marks et al (1991) (Willats 2002; Pande et al. 2010). The antibodies present in such libraries would have a broader specificity with moderate antigen affinity. In both immune and naïve libraries, the genetic diversity is an essential determinate for the construction of a proper antibody library. The library diversity can be improved by optimized cloning procedure, use of several donors and by use of several B cell sources.

Semi-synthetic libraries further improved the library diversity from natural libraries. Such libraries are constructed by either PCR assembly of immunoglobulin genes or by introducing synthetic diversity into a naïve library through recombination of *in vivo* formed complementary determining regions (CDR) (Hoogenboom 2005). In constructing semi-synthetic library, the CDR in the immunoglobulin structure is the prime target (Braunagel 2003). Among the six CDR domains, CDR3 domain of heavy and light-chain is the most variable domain contributing majority of antigen binding. The diversity in first semi-synthetic antibody library was controlled by the oligonucleotide synthesis (Barbas III et al. 1992). Using PCR technologies, strategies such as random mutagenesis of CDR3 domains may lead to an improvement in library diversity. Apart from improving the diversity, such strategy is adopted in order to improve the binding characteristics of suitable antibodies, screened from natural antibody libraries, towards the target antigen (Braunagel 2003).

Synthetic antibody libraries contain *in vitro* constructed un-rearranged antibodies using oligonucleotides wherein the diversity is introduced in CDR domains of one or more variable genes. Diversity in antibody library in the first synthetic library was by introducing randomization in the heavy chain CDR3 domain (Barbas III et al. 1992; Hoogenboom & Winter 1992). The library diversity was further improved with completely randomized CDR domains in light and heavy chain (Garrard & Henner 1993). Synthetic antibody libraries can be constructed aiming for specific applications (Hoogenboom & Chames 2000). Kirkham et al. (1999) developed the first synthetic antibody library featuring specific antibody fragments with *de novo* binding-site design recognizing an antigen epitope (Kirkham et al. 1999). Such synthetic libraries help in fine tuning the antibodies with desirable properties that would ease further manipulation and/or to improve binding specificity to the target antigen.

Phage display systems

Though pIII and pVIII display systems have been widely studied in phage display, the proteins or peptides of interest have been reported to display on all coat proteins

(Tikunova & Morozova 2009; Løset & Sandlie 2012). The crucial step in efficient phage display is to place the polypeptide of interest between the signal sequence and the coat protein (Løset & Sandlie 2012). As explained earlier, choice of appropriate vector system is also pivotal for successful phage display and can be classified as natural vectors or phagemid.

Natural vectors are derived from filamentous phage genome (Smith & Petrenko 1997; Paschke 2006). The simplest mode of displaying the foreign protein on phage surface is to create a fusion with the coat proteins, for example pIII or pVIII (Willats 2002). Though theoretically possible, the insert size determines the phage viability since no wild type coat proteins are retained (Willats 2002; Paschke 2006; Kehoe & Kay 2005). This limitation could be overcome using hybrid phage-based expression system. In hybrid phage-based expression, in addition to the foreign protein fusion to phage coat protein genes, a wild type coat protein gene is retained in the phage genome thus displaying both recombinant and wild type coat proteins (Smith & Petrenko 1997).

Another hybrid phage display is the *phagemid*-based system. Phagemids are filamentous phage derived vectors generally containing (i) promoter, (ii) signal peptide, (iii) multiple cloning site, (iv) amber stop codon, (v) coat protein gene, (vi) intergenic sequences harboring sequences for phage packaging and phage *ori*, (vii) plasmid *ori*, (viii) selective marker and (ix) terminator sequence (Figure 5.2; Krebber et al. 1997; Qi et al. 2012). Upon induction, the phagemids infect *E. coli* strains and the fusion proteins formed are transferred to the host inner cell membrane with the assistance of signal peptide. The phage lysogenic cycle is complete upon co-infection of phagemid with helper phages, containing phage assembly sequences and defective origin of replication. The phage assembly signal sequences from helper phages allow the interaction with packing sequences located in phagemids, assisting in the formation of filamentous phages (Smith & Petrenko 1997; Qi et al. 2012). This differs from phage-based hybrid systems as the two coat protein genes are on separate genomes (phagemid and helper phage genomes). Compared to natural vectors, phagemids are genetically more stable, efficient and easily controllable expression vectors; providing a better mode of incorporating large amounts of foreign DNA (Qi et al. 2012). Phagemid vectors display systems can also ensure a polyvalent antibody display by deleting the coat protein gene from helper phage (Rondot et al. 2001).

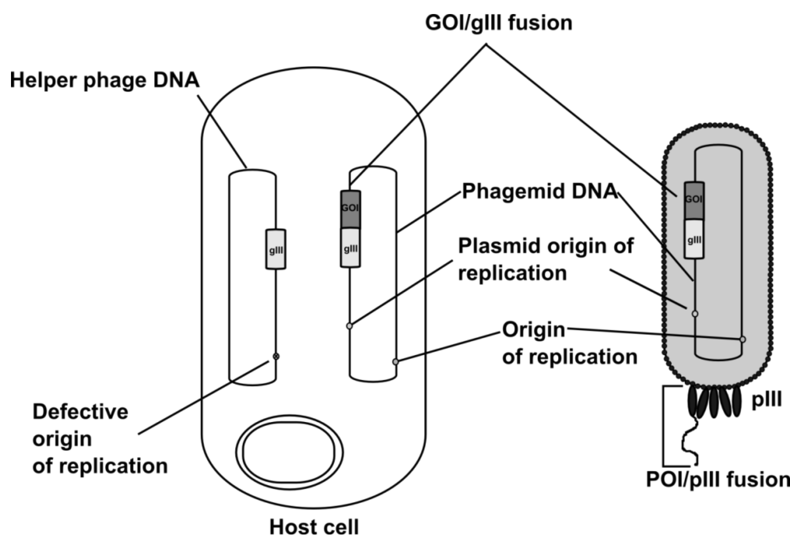


Figure 5.2. General scheme of phagemid-based pIII fusion display system. (Modified from Willats 2002).

5.2. BIOPANNING

In phage display, selection of antibodies against target antigens from phage libraries is equally important as determining the right antibody library or phage display system. Panning and screening are the two important steps in antibody selection and the most common technique is the biopanning procedure (Kretzschmar & von Rüden 2002). Biopanning technique is based on affinity selection of antibodies specific to the desired antigen (Tikunova & Morozova 2009). The panning can be performed in solution or solid phase and process is repeated three to six times (Pande et al. 2010; Kretzschmar & von Rüden 2002; Griffiths & Duncan 1998). The biopanning steps are schematically presented in Figure 5.3 and are briefly explained in following text.

a. ***Immobilization of target antigen*** – Several supports has been used to immobilize desired antigens for the biopanning procedure. Extreme care should be taken in choice of panning support as this initial step in biopanning process though helps in enriching specific antibody binders, also allows the enrichment of target unrelated or background binders. A negative screening or subtractive panning steps should be included in the procedure, aiming to reduce the enrichment of target unrelated binder moieties. (Vodnik et al. 2011)

    i. *Direct non-specific passive adsorption* on polystyrene surfaces has been widely used for antigen immobilization due to merits such as ease of immobilization and absence of requiring any antigen pre-treatment prior to introducing phage libraries (Willats 2002; Zebedee et al. 1992; Vodnik et al. 2011; Menendez & Scott 2005). However, due to weak bonds formation with the plastic surface, this method may result in loss of target antigens or partial denaturation of adsorbed antigens. Similar to the antigens, the

antibodies displayed on phage surface also shows high affinity towards the plastic surface thus can lead to enrichment of unspecific antibodies. (Vodnik et al. 2011; Menendez & Scott 2005) Elimination of background antigens can be ensured by either incubating the phage library with the panning platform prior to the target antigen or by immobilizing the solid surface with antigens at high densities (Hawkins et al. 1992; Kretzschmar et al. 1995; Katakura et al. 2004).

ii. *In-direct immobilization* of target antigen by affinity tags or biotinylation solves the disadvantages from passive adsorption technique and maintains the antigen structural integrity (Pande et al. 2010; Vodnik et al. 2011). Affinity tag such as Histidine tag enables immobilization of antigens in highly specific proper orientation onto Ni^{2+}, Cu^{2+}, or Co^{2+} coated surfaces (Pande et al. 2010). Another approach is capturing biotinylated antigens on avidin or streptavidin coated microtitre plates or paramagnetic beads. This approach is based on rapid kinetics and strong covalent bond formation between biotin and avidin (Pande et al. 2010; Menendez & Scott 2005). Antigens can be biotinylated either by chemical or enzymatic methods. Though advantageous, in-direct immobilization approach also allows enrichment of background binders specific towards the exposed capture surfaces (Menendez & Scott 2005). A subtractive panning step involving incubation of input phage library with the panning surface prior to antigen panning reduces the chance of unspecific antigen enrichment (Vodnik et al. 2011).

b. ***Phage binding*** – The phage library from negative screening round are incubated with target antigens coated on the selection platforms. Highly diverse phage library and high concentrations of target antigen and phage library displaying polyvalent scFv's are crucial in the first biopanning round (Katakura et al. 2004; Ravn et al. 2004). The diversity of phage library is better represented in polyvalent libraries ensuring highly diverse phage antibodies (Rondot et al. 2001; Ravn et al. 2004). In subsequent panning rounds, the concentration of both input phages and antigen are decreased to improve the panning stringency (Schier et al. 1996). This approach allows the enrichment of antibodies with improved specificity and avidity towards the target antigen. Biopanning in solution guarantees reduced non-specific phage binding. Addition of glycerol has also been reported to improve in reducing non-specific binding, but it also lowers the specific binding kinetics with a biased enrichment (Kjær et al. 1998). In the case of biopanning with solid support, the prevalence of non-specific antibody binders can be controlled by altering the selection platforms, for example between streptavidin and neutravidin coated surfaces.

c. ***Washing*** – Washing step is important to remove unbound and non-specific phages. Phage libraries may contain antibodies specific towards a target antigen with

varying degrees of affinity. Apart from the removal of non-specific phages, extensive washing may also result in loss of specific antibodies with low affinity. The length and number of washing steps in a biopanning round are crucial and thus optimization of the same is required (Willats 2002).

d. ***Phage elution*** – Phage elution step is required to select the antibodies recognizing the target antigen for additional panning rounds or to evaluate the specificity and affinity of the binders towards the antigen. The elution can be categorized into non-specific and specific approaches.

   Non-specific approach involves the elution of all antibodies that recognizes the antigen of interest. Due to high stability of filamentous phages, extreme conditions such as base or acid based elution are commonly preferred. More subtle elution techniques are opted when specially designed phage display vectors, that introduce a protease cleavage site, DNA linker or a disulfide bond between the displayed antibody and the coat protein, are used in phage display. Protease or enzymatic cleavage site for example trypsin cleavage site is chosen as a less harsh elution technique (Rondot et al. 2001). Santala and Saviranta (2004) have engineered a DNA linker between paramagnetic bead and streptavidin and thus the elution was carried out by DNAase action (Santala & Saviranta 2004). Introducing disulfide bond between antibody and phage allows the elution by cleaving the disulfide bond (Rothe et al. 2008).

   Specific approach involves the elution of phages that are bound to the target epitope in the antibody. In competitive elution method, the phages are eluted by introducing a known soluble antigen for the epitope, thus causing a competition for the binding site. In the case of non-competitive elution, a known compound that weakens the phage-epitope binding but does not actively involve in binding to the epitope is used (Griffiths & Duncan 1998). Another approach in specific elution is the use of selectively infective phage technique. In this technique, the antibody is cloned to C-domain of N-terminal truncated pIII protein. The antigen contains the pIII N-terminal domain, pivotal for infection, as fusion with the target epitope. Thus only the phages that recognize the epitope are infective and are propagated by infection. This technique is devoid of any washing or elution steps and the binders are highly specific (Duenas & Borrebaeck 1994; Dueñas et al. 1996).

After phage elution, the eluate is infected with freshly grown *E. coli* cells. The biopanning process is followed by phage production, wherein the phage infected bacterial cells are co-infected with helper phages and the phage stock prepared is used as the input phage stock in the following panning round (Brockmann et al. 2005).

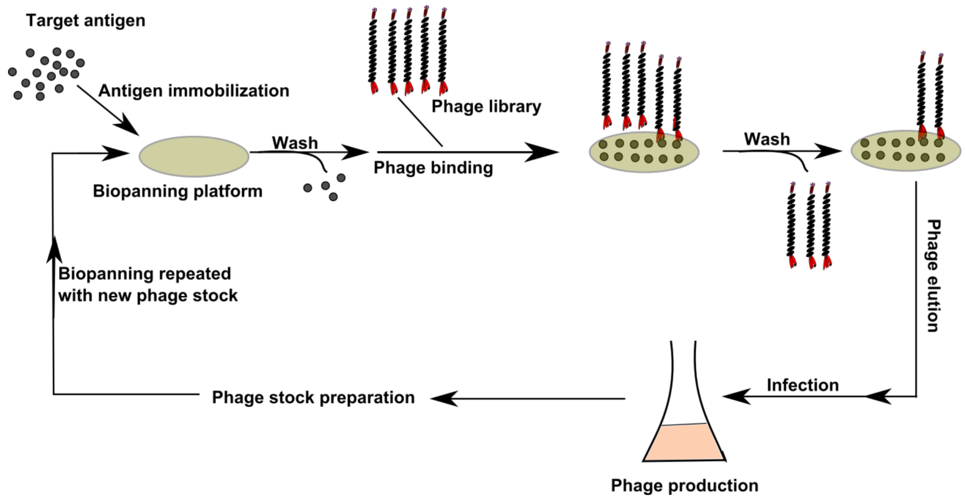


Figure 5.3. Schematic representation of biopanning process. (Modified from Tikunova & Morozova 2009).

6. AIMS OF THE PRESENT STUDY

The present study investigates bioH_2 production in protein to community level perspective. One of the goals of this study was to standardize anaerobic biopanning with synthetic 'mixed' antibody libraries and purified *Clostridium* [Fe-Fe] hydrogenases as target antigen. The study aimed to progress by enriching anti-hydrogenase antibody binders, using aerobic and anaerobic biopanning, specific against inactive and active [Fe-Fe] hydrogenases and their implementation to use as molecular tools for protein level quantitative monitoring of [Fe-Fe] hydrogenases in closely related *Clostridia* spp. Another objective of this study was to establish a novel enrichment technique to select H_2 producers from natural samples and its employment to isolate a bacterial strain that utilizes cellobiose, carboxymethyl cellulose (CMC) and monomeric hemi-cellulose sugars. Furthermore, in the quest for an optimized conversion of substrate to H_2 by the isolated strain and glycerol-enriched functional consortia, 'One-Factor-At-A-Time' (OFAAT) and statistical optimization strategies were used in this study.

More specifically the aims were to;

- Establish an anaerobic biopanning procedure (**I**).
- Enrich and select antibody binders' specific against *Clostridium* [Fe-Fe] hydrogenases (**I**).
- Develop a novel technique to enrich H_2 producers from microbial consortium (**II**).
- Employ the enrichment technique for the isolation of cellobiose degrading H_2 producer from activated sludge (**III**).
- Optimize the bioconversion of monomeric hemi-cellulosic sugars to H_2 (**III**).
- Implement 'One-factor-at-a-time' and statistical optimization strategies for improved bioconversion of crude glycerol to H_2 by an enriched microbial community (**IV**, **V**).

7. SUMMARY OF MATERIALS AND METHODS

All the microbial cultivation and optimization studies were conducted as small scale batch fermentation experiments in closed serum bottles (10 – 50 ml working solution) under sterile working conditions. The summary of experiments performed, substrate used, inoculum, microbial screening, and enrichment methods used in the original publications of the thisis **I –V** is listed in Table 7.1.

7.1. BACTERIAL STRAINS AND MIXED MICROBIAL INOCULUM

In Paper **I**, *E.coli* BL21 (DE3) ΔiscR harbouring pFEGA vector was chosen as the host for expression and purification of *C. acetobutylicum* [Fe-Fe] hydrogenase as deletion of iscR gene has been reported to be advantageous in improving the total hydrogenase activity (Nakamura et al. 1999; Akhtar & Jones 2008). *E. coli* XL1-Blue (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [[F´ proAB lacI^q ZΔM15 Tn10 (Tet^r)]]) (Stratagene, USA) strain containing the F' plasmid was chosen as the host for phage infection and phage production in biopanning procedure. The *E. coli* XL1-Blue strain containing phoA gene were also used in periplasmic expression and screening of anti-hydrogenase antibody binders. A redox-modified *E. coli* strain, Origami B ({F$^-$ ompT hsdS$_B$ (r$_B$$^-m_B$$^-$) gal dcm lacY1 ahpC gor522::Tn10 trxB (Kan^r Tet^r)}) (Novagen, EMD Millipore, USA), was used for immunoassay experiments as the strain contains oxidizing cytoplasmic environment aiding in proper folding of proteins with di-sulfide bonds (Santala & Lamminmäki 2004).

E. coli strains BL21 (DE3), a non-H_2 producing strain naturally deficient in *lon* and *ompT* proteases; *E. coli* JW2409-1 ({F$^-$, *Δ (araD-araB) 567*, *Δ lacZ4787* (::rrnB-3), *λ*$^-$, *ΔldhA744::kan*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*}), H_2 producing Keio collection strain; and *E. coli* JW1375-1 ({F$^-$, *Δ (araD-araB) 567*, *Δ lacZ4787* (:: rrnB-3), *λ*$^-$, *ΔptsI745::kan* , *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*}), Keio strain deficient in glucose utilization were used as model organisms to create an artificial mixed culture in Paper **II** due to their different growth and gas production capabilities (Baba et al. 2006; Luo et al. 2008; Vardar-Schara et al. 2008).

Activated sludge collected from waste water treatment plant (Viinikanlahti, Finland) was used as the seed inoculum source in Paper **III – V**. Cellobiose degrading functional community was enriched from activated sludge in cellobiose amended minimal media and *Citrobacter* sp. CMC-1 strain was isolated from growth media containing CMC by spread plate technique (**III**). Enriched microbial consortia capable of degrading crude glycerol were used as inoculum in Paper **IV** and **V**.

Table 7.1. Summary of experiments performed, substrate used, inoculum, screening and optimization methods employed for protein to community level perspective studies in original publication(s) of this thesis.

	Summary of experiments	Substrate(s)	Bacterial strains	Screening and Optimization method(s)
Protein level	**Isolation of recombinant antibodies for specific detection of clostridial [Fe-Fe] hydrogenases (I)**			
	Recombinant expression and purification of hydrogenase	Glu	BL21 (DE3) ΔiscR pFEGA *C. acetobutylicum* [Fe-Fe] hydrogenase	
	Biopanning	NA	XL1-Blue; *E. coli* BL21 (DE3) lysate *C. acetobutylicum* [Fe-Fe] hydrogenase	
	Identification of anti-hydrogenase binders	Glu	XL1-Blue-pAK600; Origami B-pAK400c	One-step ELISA, Sandwich ELISA
Strain level	**Fermentative hydrogen production from different sugars by *Citrobacter* sp. CMC-1 in batch culture (III)**			
	Enrichment and strain isolation (as described in **II**)	Cel CMC	Activated sludge from waste water treatment plant	
	Optimization of initial pH and temp.	Glu	*Citrobacter* sp. CMC-1	OFAAT
	Characterization of growth and H_2 production kinetics	Glu	*Citrobacter* sp. CMC-1	
	Characterization of growth, sub. uti. and bioconversion of monomeric hemi-cellulosic sugars	Gal, Man, Xyl, Ara, Rha	*Citrobacter* sp. CMC-1	
Community level	**Simple enrichment system for hydrogen producers (II)**			
	Selection pressure - gas production		BL21&JW1375-1	
	Selection pressure - gas production and glu. uti.	Glu	BL21, JW1375-1& JW2409-1	
	Selection pressure - gas production and sta. uti.	Sta	BL21, JW2409-1 & JW1375-1-pCSS4	
	Specific growth rate and H_2 production	Glu or Sta	BL21, JW2409-1, JW1375-1&JW1375-1-pCSS4	

	Table 7.1. cont.			
Community level	**Bioconversion of crude glycerol from biodiesel production to hydrogen (IV)**			
	Enrichment of microbial community (Liquid sub-cultivation)	PG, CG	Activated sludge from waste water treatment plant	
	Optimization of initial pH, substrate concentration and temperature	CG	Enriched community	OFAAT
	Kinetic analysis of cumulative H_2 and end metabolite production at optimized conditions	PG, CG	Enriched community	
	Characterization of enriched microbial community (PCR-DGGE)		Enriched community	
	Improved bioconversion of crude glycerol to hydrogen by statistical optimization of media components (V)			
	Identifying the effect of NH_4Cl, K_2HPO_4, KH_2PO_4, $MgCl_2.6H_2O$ and KCl on H_2 production	CG	Microbial community predominated with *Clostridium* species	PB
	Optimization of significant variables	CG	Microbial community predominated with *Clostridium* species	SA, RA
	Response surface methodology assisted identification of optimized concentration of the significant variables	CG	Microbial community predominated with *Clostridium* species	BB, CCD, RSM
	Bioconversion of crude glycerol to H_2 with the optimized media	CG	Microbial community predominated with *Clostridium* species	

Glu = Glucose, PG = Pure glycerol, CG = Crude glycerol, Suc = Sucrose, Sta = Starch, Gal = Galactose, Man = Mannose, Xyl = Xylose, Ara = Arabinose, Temp. = Temperature, Sub. uti. = Substrate utilization, OFAAT = One-factor-at-a-time, PB = Plackett – Burman, SA = Steepest accent, BB = Box – Behnken, RA = Ridge analysis, CCD = Central Composite Design, RSM = Response Surface Methodologies and NA = Not applicable.

7.2. VECTORS, PHAGE LIBRARIES AND SELECTION PLATFORMS

The vectors used in Paper **I** and **II** are summarized along with relevant vector properties in Table 7.2. Phagemid vector pEB32X, a modified version of pAK200 vector, was chosen for monovalent phage display of multi-purpose (1.54 × 10e13 cfu) and anti-protein (7× 10e12 cfu) libraries with VCS M13 helper phage (Kan^r; Stratagene, USA) (**I**). Vector pAK600 was used to screen the affinity of enriched anti-hydrogenase antibodies towards the target antigen by periplasmic expression of the binders as a fusion with bacterial alkaline phosphatase (phoA) (**I**). Vector pAK400c, a pelB signal sequence deleted version of pAK400 vector, was employed to introduce site-specific biotinylation and express the recombinant antibodies as fusions with bacterial biotin carboxyl carrier protein (BCCP) domain (**I**). In Paper **II**, enrichment of starch utilizing *E. coli* strain was demonstrated with JW1375-1 containing pCSS4 plasmid harboring α-amylase gene.

Table 7.2. Summary of vectors used in original publication(s) of the thesis (I and II).

Vector	Relevant vector properties	Ref.
pEB32X	Modified from pAK200 vector, fusion with truncated pIII CT domain, monovalent phage display, trypsin and factor Xa sites and chloramphenicol resistance.	(Krebber et al. 1997; Huovinen et al. 2013)
pAK600	Periplasmic expression by pelB signal sequence, expressed as fusion with phoA gene, tetracycline resistance and IPTG inducible.	(Krebber et al. 1997)
pAK400c	Modified from pAK400 vector (Krebber et al. 1997), no signal sequence, fusion with BCCP domain, tetracycline resistance and IPTG inducible.	(Santala & Lamminmäki 2004)
pCSS4	*Bacillus stearothermophilus* 3 kb DNA containing pamy (constitutive promoter in *E. coli*) and α-amylase gene, β-lactamase gene and amplicillin resistance.	(Suominen et al. 1987)

7.3. EXPERIMENTAL PROCEDURES

7.3.1 SELECTION OF ANTIBODY BINDERS RECOGNIZING CLOSTRIDIAL [Fe-Fe] HYDROGENASES

Chemically biotinylated (EZ-Link Sulfo-NHS-Biotin, Thermo Scientific, USA) His-tagged *C. acetobutylicum* hydrogenase expressed and purified from *E. coli* BL21 (DE3) ΔiscR pFEGA strain was chosen as the target antigen in the biopanning process. Biopanning was performed with synthetic ‘mixed’ scFv libraries (scFv-M, diversity 1.54 × 10e13 c.f.u) and scFv-P, diversity 7× 10e12 c.f.u) for both catalytically active and inactive hydrogenases under anaerobic and aerobic conditions, respectively. The

antibody panning with inactive hydrogenase was performed with O_2 saturated buffers in aerobic conditions at room temperature (RT). Whereas in the case with active [Fe-Fe] hydrogenase, the biopanning buffers were purged with N_2 for 30 minutes and the panning rounds were carried out under strict anaerobic conditions in an anaerobic glove box. Streptavidin or neutravidin coated microtitre plates (for active hydrogenase) and streptavidin or avidin coated paramagnetic beads (for inactive hydrogenase) were chosen as the biopanning platforms. Subtractive panning was performed by incubating the phage stock with selection platforms and biotinylated BL21 (DE3) lysate. In anaerobic panning conducted in an anaerobic glove box, the input phage library was prepared by incubating the phage stock with empty streptavidin wells and BL21 (DE3) lysed cells for 15 minutes followed by phage precipitation using PEG_{8000}-NaCl solution. In aerobic panning, the subtractive panning step was performed by incubating phage stock with biotinylated BL21 (DE3) lysate coated on streptavidin beads for 5 minutes and thereafter collected. Phages unspecific to background proteins and selection platforms were then incubated with biotinylated target antigens (70 mg L^{-1}) for 1 hour at RT. Unspecific phages were removed by washing the panning platforms twice with TBT-0.5 and once with TSAT-0.05 buffers. Bound phages were eluted with 50 mg L^{-1} trypsin in TSAT-0.05 buffer at 37°C for 30 minutes. New phage stocks for subsequent panning rounds were prepared by infecting fresh XL1-Blue cells (OD_{600} 0.5 – 0.6) with the eluate and VCS M13 helper phage. The number of eluted phages from the panning round was estimated by plating the infected cells in LB-agar amended with tetracycline (10 mg L^{-1}) and chloramphenicol (25 mg L^{-1}). The subtractive and biopanning rounds were iterated twice for both target antigens. In the second round, neutravidin coated microtitre plate wells and avidin coated magnetic beads were used as selection platforms with reduced phage diversity (5 × 10e11 c.f.u.) and target antigen concentrations (10 mg L^{-1}). In the final round, streptavidin coated microtitre wells and paramagnetic beads were used under stringent conditions (input phages diversity, 5 × 10e10 c.f.u.; antigen concentration, 5 mg L^{-1}).

After the biopanning process, the antibody genes were restricted from the phagemid vector, using *Sfi*I restriction sites, and sub-cloned to pAK600 vector expressed as fusion with phoA gene. Antibody binders' specific against the target antigen was measured in one-step ELISA experiments by alkaline phosphatase (AP) activity under strict anoxic conditions. The scFv-phoA fusion proteins were incubated with empty streptavidin paramagnetic beads/wells and blank BL21 (DE3) lysate to determine any unspecific binding. *E. coli* XL1-Blue-pAK600 lysates lacking an antibody gene was also included as control to determine any phoA background activity. The genes of potential anti-hydrogenase antibody binders selected from one-step ELISA were sequenced. Further, the scFv genes from the selected binders were amplified by PCR using rm13_1 and rm13_2 primers (see Table S1/**I**). The amplified product was sub-cloned into pAK400c expression vector using NdeI and EcoRI restriction sites. The construct was transformed into Origami B cells and the transformed colonies were confirmed by plasmid restriction analysis and PCR. The binding specificity of the antibody binders towards [Fe-Fe] hydrogenase from *C. acetobutylicum, C. butyricum* and purified

active and inactive hydrogenases were investigated by sandwich immunoassay with scFv-BCCP (capture) and scFv-phoA (tracer) antibody fusions. Finally, the scFv-BCCP and scFv-phoA binder pairs that exhibited maximal AP signal for respective target antigens (*C. acetobutylicum* or *C. butyricum* hydrogenases in cell lysate or purified active hydrogenase) were selected for dose response assay performed under anaerobic conditions. Figure 7.1 represents the biopanning procedure of phage displayed antibody libraries and immunoassay experiments conducted in sequential order in Paper **I**, aiming to select the antibody binders specific towards clostridial [Fe-Fe] hydrogenases.

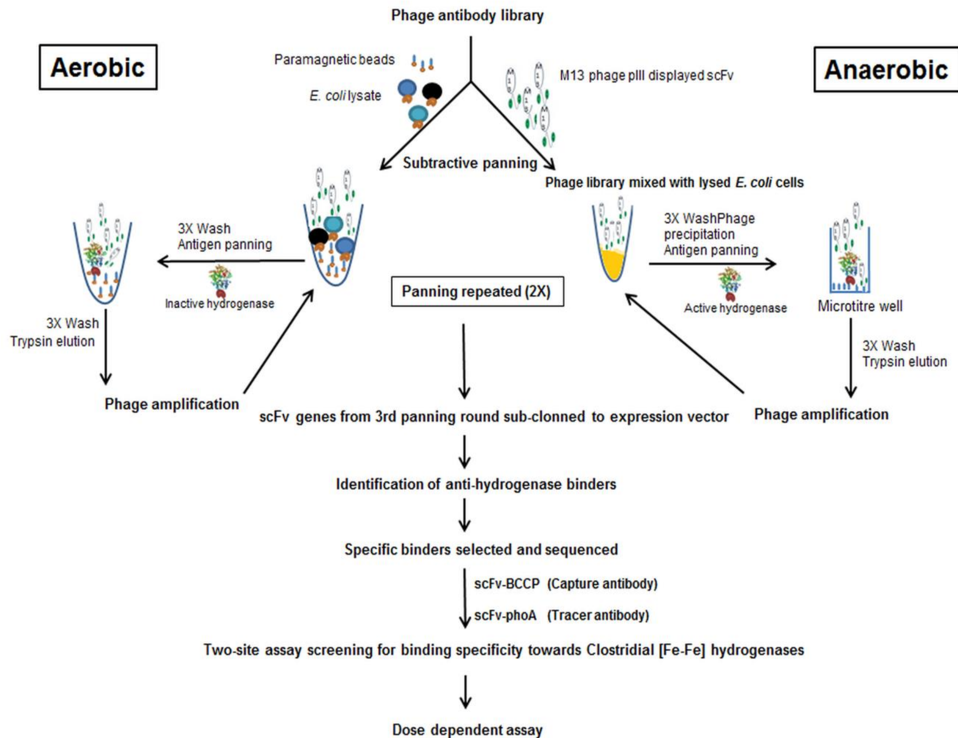


*Figure 7.1. Flowchart of phage display, cloning and immunoassay studies in the order performed, aiming in selection of clostridial [Fe-Fe] hydrogenase specific binders. The flowchart presents the phage library, biopanning platforms and biopanning procedure used for aerobic (left) and anaerobic (right) phage display. (Fig. S1./**I**)*

7.3.2. DEVELOPMENT OF A NOVEL ENRICHMENT SYSTEM – PROOF-OF-PRINCIPLE

The general structure of the enrichment system consists of eight 25 ml serum bottles connected using gas tight tubes with needles fixed at either ends. Figure 7.2 shows the pictorial and schematic representation of the basic experimental set-up. A 1.2 mm * 50 mm dimensioned needle intended for transferring bacterial population was connected to one end of the gas tubing. In the first bottle, inoculated with initial bacterial population, the needle tip was positioned below the liquid surface while the same was kept just above the liquid surface in following vessels. The other end of the gas tubing was connected with a 0.6 mm * 25 mm dimensioned needle for culture transfer to the nearest vessel and was positioned in the vessel headspace. A 'water-lock' with gas outlet was placed at the end of the system to inhibit gas over pressure and maintain the anoxic state. The connections in the enrichment system ensure a unidirectional flow.

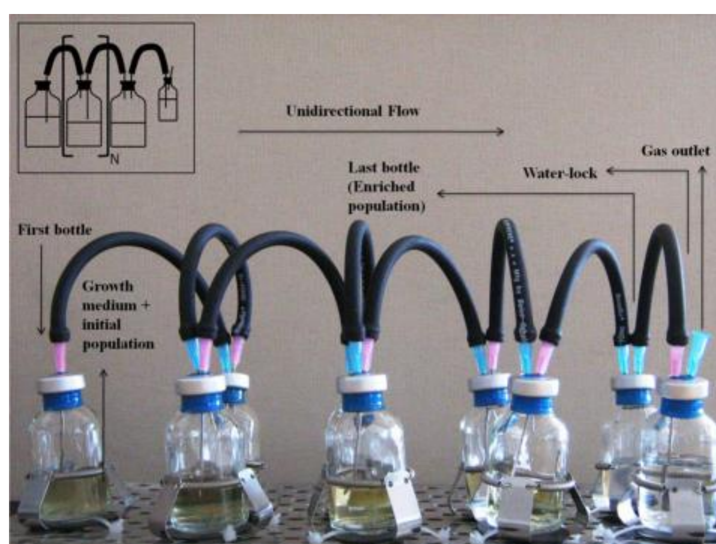


*Figure 7.2. Experimental set-up indicating the serum bottles, gas tight tubes connected with needles (Pink: culture uptake and Blue: culture transfer), water-lock, gas outlet and the population enrichment direction. Inset: Schematic representation of the system. (Fig.1./**II**)*

The proof-of-principle experiments, i.e. to enrich the best H_2 producer(s) from a mixed culture, were performed by creating an artificial microbial population using *E. coli* BL21, JW1375-1 and JW2409-1 strains, differing in growth, substrate utilization and H_2 production characteristics. Three experiments were conducted to prove the proof-of-principle. Table 7.1 lists the bacterial strains employed in each proof-of-principle experiments conducted. Spread plate and colony counting techniques were used to identify the enriched microbial population. In the first experiment, the enrichment of microbial population was studied with respect to gas production. Glucose utilization along with gas production was chosen as the selection pressure in the second experiment. In the third experiment, more stringent selection pressure i.e. starch

utilization was implemented. Parallel experiments on bacterial growth and H_2 production with pure *E. coli* strains were also conducted.

7.3.3. ENRICHMENT OF FERMENTATIVE HYDROGEN PRODUCTING STRAIN AND FUNCTIONAL COMMUNITY

Activated sludge from wastewater treatment plant (Viinikanlahti, Finland) was chosen as the seed inoculum source to enrich fermentative H_2 producing strain or functional microbial community.

In Paper **III**, the mixed microbial community was initially cultivated in glucose supplemented anoxic LB medium. The enrichment process was performed by inoculating the pre-cultivated inoculum in M9 medium supplemented with cellobiose using the enrichment system described in **II**. Gas production and stepwise increment in cellobiose concentration (1 – 60 g/L) were chosen as the selection pressures. After the run, cultures from the last bottle of enrichment system were spread plated on M9 minimal agar plates containing CMC (3 g/L). The plates were incubated under anaerobic conditions. Single colonies were further sub-cultivated on M9-CMC agar plates for five additional rounds. Initial strain identification was performed by colony morphology, microscopy and Gram staining tests. The genomic DNA was isolated and 16S rRNA gene was amplified and sequenced. The nucleotide sequence was compared with existing related GenBank sequences using Nucleotide BLAST program. Following strain identification, the specific growth rate and doubling time of the isolated strain grown on glucose, cellobiose and CMC were investigated.

Enrichment of fermentative H_2 producing microbial community capable of utilizing crude glycerol is explained in detail in Paper **IV** and used in Paper **IV** and **V**. The crude glycerol, a by-product from biodiesel manufacturing process, was kindly provided by Savon Siemen Oy (Iisalmi, Finland). The crude glycerol had an alkaline pH (pH ~ 12) and contained 45% (v/v) glycerol, 30% (v/v) methanol. Enrichment process was conducted by inoculating pre-cultivated activated sludge (pure glycerol; 100 g/L) in sterile anoxic modified HM100 medium (NH_4Cl 1.0 g/L, K_2HPO_4 0.3 g/L, KH_2PO_4 0.3 g/L, $MgCl_2$.$6H_2O$ 2.0 g/L, KCl 4.0 g/L, Na-acetate $3H_2O$ 1.0 g/L, tryptone 1.0 g/L, cystein-HCl 0.5 g/L and resazurin 0.002 g/L) containing crude glycerol (2.5 g/L). By the onset of H_2 production, the cultures (10%) were sub-cultured twice in the same medium and the enriched inoculum was used for later studies. Prior to bioprocess optimization, the functional community was tested for methanol utilization present in the crude glycerol.

7.4. OPTIMIZATION STRATERGIES FOR IMPROVED HYDROGEN PRODUCTION

7.4.1. ONE-FACTOR-AT-A-TIME OPTIMIZATION

One-factor-at-a-time optimization method was opted for the process optimization studies in Papers **III** and **IV**. Table 7.1 provides the summary of experiments

performed, substrate and inoculum used in the original publications. The parameters studied along with their range and targeted response aimed for bioprocess optimization is shown in Table 7.3.

Table 7.3. OFAAT approach of parameters and their ranges investigated towards the targeted response employed in original publication(s) of the thesis (III and IV).

Parameters	Range	Targeted response	Ref.
Temperature	25 – 40˚C	Cell growth, H_2 yield	III
	25 – 46˚C	Substrate utilization, H_2 yield	IV
Initial pH	4 – 7	Cell growth, H_2 yield	III
	5 – 8	Substrate utilization, H_2 yield	IV
Substrate (CG)	0.5 – 5 g/L	Substrate utilization, H_2 yield	IV
Cultivation period (Substrates – Gal, Man, Xyl, Ara and Rha)	24 and 48 hours	Cell growth, Substrate utilization, H_2 yield	III

CG = Crude glycerol, Suc = Sucrose, Sta = Starch, Gal = Galactose, Man = Mannose, Xyl = Xylose, Ara = Arabinose, Rha = Rhamnose.

7.4.2. STATISTICAL OPTIMIZATION

Designs of experiments together with RSM were chosen in optimizing the concentrations of modified HM100 media components in Paper **IV**. Variables that had significant influence on H_2 yield were identified using Plackett – Burman (PB) design. The identified variables were subjected to path of steepest accent (SA) design in order to move from the design center towards the optimal response. The selected variable concentrations were then optimized using Box – Behnken (BB) design. In BB optimization experiments the optimal region was beyond the design range. Further optimization was carried out using ridge analysis (RA). Finally, the central composite design (CCD) enabled in identifying and predicting the optimal variable concentration and H_2 yield, respectively. The statistical designs, media components chosen for optimization, along with their studied range are summarized in Table 7.4.

Table 7.4. Statistical designs and media components along with their studied range and factor levels (in parenthesis) chosen for optimization in original publication of the thesis (V)

Statistical design	**NH_4Cl (g/L)**	**K_2HPO_4 (g/L)**	**KH_2PO_4 (g/L)**	**$MgCl_2.6H_2O$ (g/L)**	**KCl (g/L)**
PB[a]	0.5 (-1), 2.0 (+1)	0.1 (-1), 1.0 (+1)	0.1 (-1), 1.0 (+1)	1.0 (-1), 4.0 (+1)	1.0 (-1), 8.0 (+1)
SA[a]	1.25 – 5.2	0.6 – 5.6	0.6 – 5.9	1[b]	1[b]
BB[a]	0.5 (-1), 2.0 (0), 3.5 (+1)	1.1 (-1), 1.6 (0), 2.1 (+1)	1.1 (-1), 1.6 (0), 2.1 (+1)	1[b]	1[b]
RA[a]	2 – 5.7	1.5 – 1.6	1.6 – 4.2	1[b]	1[b]
CCD[a]	2.95 (-1), 4.05 (+), 5.15 (+1)	1.6[c]	1.52 (-1), 2.52 (0), 3.52 (+1)	1[b]	1[b]

[a] *PB = Plackett – Burman, SA = Steepest accent, BB = Box – Behnken, RA = Ridge analysis and CCD = Central composite design.*

[b] *After PB, $MgCl_2.6H_2O$ and KCl concentrations in later optimization experiments were kept at 1 g/L.*

[c] *Concentration of K_2HPO_4 was optimized to be at 1.60 g/L.*

7.5. ANALYTICAL TECHNIQUES

The analytical methods employed in the original publication(s) of this thesis (**I** – **V**) are listed in Table 7.5.

Table 7.5. Methods employed for protein to community level analysis in the original publication(s) of the thesis (I-V).

	Analysis	Method(s)	Ref.
Protein level	Hydrogenase activity	Methyl viologen oxidation assay + Gas chromatography	**I**
	One-step and sandwich ELISA	Alkaline phosphatase assay + Spectrophotometer (A_{405})	**I**
	Protein concentration	Bradford assay + Spectrophotometer (A_{595})	**I**
Metabolite level	Gaseous compounds: *H_2 and CO_2*	Gas chromatography	**II – V**
	Liquid metabolites: *lactate, acetate, ethanol, butyrate, formate and propionate*	High-Performance Liquid Chromatography	**III – V**
	Substrate utilization: *glucose, galactose, mannose, arabinose, xylose, rhamnose, pure and crude glycerol*	High-Performance Liquid Chromatography	**III – V**
Strain level	Cell morphology	Phase-contrast microscope	**III**
	Cell staining	Gram staining	**III**
	Optical density (OD)	Spectrophotometer (A_{600})	**II – III**
	Dry cell weight (DCW)	OD converted to DCW using equation; $\text{DCW} = 0.294 \times OD_{600} + 0.0717$	**II**
Community level	Enriched microbial community profiling	Selective plating PCR – DGGE	**II** **IV**

8. SUMMARY OF RESULTS AND DISCUSSION

The following sections describe the summary of results and discussions of the experiments conducted in the original publications of thesis (**I** –**V**).

8.1. SELECTION OF ANTIBODY BINDERS RECOGNIZING CLOSTRIDIAL [Fe-Fe] HYDROGENASES

In Paper **I**, in order to target the enrichment of antibody binders recognizing clostridial [Fe-Fe] hydrogenases, synthetic 'mixed' antibody libraries were screened against purified inactive and active hydrogenases. Following three biopanning rounds, the enriched antibody genes were cloned from the pEB32X phagemid vector into pAK600 vector allowing the binder expression as scFv-phoA fusion proteins (Brockmann et al. 2005; Huovinen et al. 2013). Ninety four (for inactive hydrogenase) and ninety two (for active hydrogenase) random single clones were selected for identifying specific antibody binders using one-step ELISA by alkaline phosphatase assay (Figure S2 & S3/**I**). In one-step ELISA, blank *E. coli* XL1-Blue lysate (carrying empty plasmid), biotinylated BL21 (DE3) lysate (devoid of hydrogenase expression plasmid) and empty selection platforms were included as controls. Based on the binding signal, twelve (from inactive hydrogenase) and eight (from active hydrogenase) potential binders were selected that specifically recognized the target antigens and were named with the clone number and suffixes namely 'In' (for inactive binders) and 'Ac' (for active binders). The amino acid sequence analysis revealed that all the eight active hydrogenase binders (7Ac, 23Ac, 31Ac, 43Ac, 49Ac, 59Ac, 82Ac and 88Ac) were different and among the twelve inactive hydrogenase binders only two unique clones (7In and 48In) were found (Table 8.1).

Identification of antibody binder pairs with strong binding characteristics towards [Fe-Fe] hydrogenase from *C. acetobutylicum* was conducted by a two-site immunoassay. The scFv genes from the selected anti-hydrogenase antibodies were amplified by PCR using rm13_1 and rm13_2 primers to generate NdeI and EcoRI restriction sites (see Table S1/**I**). The PCR products were cloned into pAK400c expression vector and transformed to redox modified *E. coli* Origami B strain. The scFv gene fused to N-terminal of the BCCP domain aided in *in vivo* site specific biotinylation for the production of biotinylated antibody binders (Santala & Lamminmäki 2004). The binder 7In was excluded from sandwich immunoassay experiments owing to poor expression. Two-site immunoassay performed with scFv-BCCP (capture binder) and scFv-phoA (tracer binder) fusions and [Fe-Fe] hydrogenase from *C. acetobutylicum* lysate indicated 82Ac and 48In as the best tracer binders. Capture antibodies in accordance to the signal obtained with 82Ac as tracer antibody is as follows; 48In > 7Ac > 59AcP > 49Ac- > 88Ac > 82Ac > 31Ac > 43Ac > 23Ac (Figure 8.1A). The assay configuration based on the 7Ac & 82Ac as capture and tracer binder pair respectively, tested against escalated concentrations of purified active *C. acetobutylicum* [Fe-Fe] hydrogenase and *C. acetobutylicum* lysate indicated a linear fit (Figure 8.1 C).

To study the recognition profile of the anti-hydrogenase binders towards [Fe-Fe] hydrogenases from closely related *Clostridium* spp., sandwich immunoassay with binder combinations that gave alkaline phosphatase signal against *C. acetobutylicum* [Fe-Fe] hydrogenase as target antigen were investigated for their binding specificity against *C. butyricum* [Fe-Fe] hydrogenase (see alignment in Figure S4/**I**). Interestingly, the anti-hydrogenase binders selected against [Fe-Fe] hydrogenase from *C. acetobutylicum* also recognized *C. butyricum* [Fe-Fe] hydrogenase (Figure 8.1B) and the dose-dependent immunoassay with 23Ac-BCCP and 49Ac-phoA as capture and tracer binders respectively, provided a linear fit with an R-square value of 0.98 (Figure 8.1 C). This recognition characteristic could probably be due to the amino acid sequence similarity in the area containing the active site at the C terminal of [Fe-Fe] hydrogenases in *Clostridium* spp. (Schmidt et al. 2010; Calusinska et al. 2011). These results strongly suggest that such recombinant antibodies with generic specificity towards clostridial hydrogenases can be attributed as novel molecular tools for bioprocess monitoring at protein level. However, one binder pair (7Ac-BCCP and 82Ac-phoA) showed stronger binding characteristics towards *C. acetobutylicum* [Fe-Fe] hydrogenase compared with [Fe-Fe] hydrogenase from *C. butyricum*. In the view of monitoring [Fe-Fe] hydrogenases in bioH_2 fermentation systems at protein level, the results demonstrate that the antibody binders can be employed for quantitative monitoring of [Fe-Fe] hydrogenases from cultivations. For that purpose, such binder pairs can offer better resolution in hydrogenase based monitoring and provide more insight on the bioprocess performance.

Table 8.1 – Single clone sequence alignment of CDR regions from the selected anti-hydrogenase antibodies [a, b] (I/ Table 2)

Antibody clones	Light chain		Heavy chain		
	CDR-L1	CDR-L3	CDR-H1	CDR-H2	CDR-H3
Clone selected against inactive hydrogenase					
48In	YLN	LQDYYTPF	SYSMD	GITPSGGSTY	ASYKDWGFDY
Clone selected against active hydrogenase					
7Ac	PLN	LQNTSDPF	SYLMS	RISPSGGSTD	ARGQYNGPDY
23Ac	NLA	QQSYSTPW	SDVMH	SINPNTGYTT	ASWTRDSLDI
31Ac	YLN	QQAYYIPH	SYLMQ	WIASSGGSTD	ARHDDPAD
43Ac	PLG	LQGYYIPY	SYSMD	EINPSGGSTN	ARWYDWDGVFDY
49Ac	NLA	QQSSSTPW	DYWMH	EIRPSTGSTN	ARWDFDY
59Ac	NLA	QQSSSLPW	NYGMH	EIWPSSGYTY	ARSWNTGEGWLDY
82Ac	NLA	QQSYSTPW	NYGMH	EINTVSGETY	ARKSDQFDY
88Ac	SLN	LQWNSSPY	SYLMH	SIAPSGGSTD	ARGGDFDI

[a] Identical amino acid sequences of anti-hydrogenase antibodies are excluded.

[b] Amino acid sequences were aligned using ClustalW.

Another very interesting finding was the identification of anti-hydrogenase binders (enriched from recombinant active and inactive *C. acetobutylicum* [Fe-Fe] hydrogenases) capable to recognize the hydrogenase functional states. In the initial trial, three antibody binders (7AC, 59Ac and 48In as capture antibodies) that gave the highest alkaline phosphatase response with *C. acetobutylicum* lysate were investigated for functional detection of [Fe-Fe] hydrogenase (data not shown). The validation experiment was conducted with antibody binders enriched against active and inactive hydrogenases (7Ac and 48In, respectively) as capture binders along with 82Ac as tracer binder for the functional detection of purified hydrogenases (active and inactive). The results highlight the specificity of 7Ac binder towards the catalytically active state of [Fe-Fe] hydrogenase rather than to the inactive state. On the other hand, 48In binder showed specificity towards both active and inactive target antigens. Hence, these anti-hydrogenase antibodies indicate as promising tools for functional detection of clostridial [Fe-Fe] hydrogenases. (Preliminary results; Figure 8.1D)

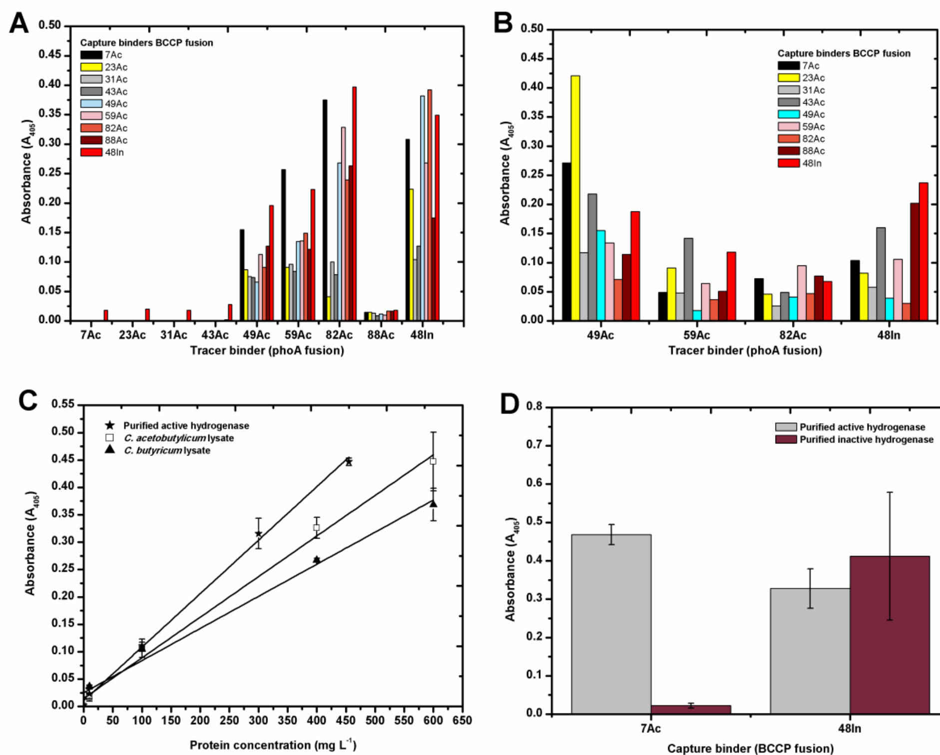


*Figure 8.1. Graph plotting results from (A) two-site immunoassay with the isolated antibody binders and C. acetobutylicum and (B) C. butyricum crude lysates as target antigens, (C) linearly fitted data-point from dose-dependent immunoassay with the immunoassay pairs, 7Ac-BCCP-82Ac-phoA (C. acetobutylicum crude lysate and active hydrogenase) and 23Ac-BCCP-49Ac-phoA (C. butyricum crude lysate) and (D) recognition profile of 7Ac and 48In with active and inactive hydrogenases as target antigens. The background absorbance values determined from sandwich immunoassay with blank XL1-Blue-pAK600 and Origami B-pAK400c lysates were deducted from the sample readings obtained from the target antigens and graphically plotted. The error bars indicate standard deviation of averaged data from triplicate experiments. (Fig.1 – 4/**I**)*

8.2. DEVELOPMENT OF A NOVEL ENRICHMENT SYSTEM – PROOF-OF-PRINCIPLE

In Paper **II** the poof-of-principle experiments for the enrichment system, i.e. enriching H_2 producers from a mixed consortium, were conducted using *E. coli* BL21, JW1375-1 and JW2409-1 strains. Lactate dehydrogenase and phosphotransferase enzyme system were replaced with kanamycin resistance gene in JW1375-1 and JW2409-1 strains, respectively. In the preliminary experiment with gas production as the selection factor, JW1375-1 strain was observed to be enriched. Figure 8.2 graphically represents the colony forming units (CFU) calculated from the first and last bottles out of eight bottle enrichment system. *E. coli* undergoes mixed-acid type fermentation and H_2 is produced from formate breakdown via FHL (See section 1.4). Lactate, a primary end-metabolite in mixed-acid fermentation is formed by direct pyruvate oxidation via lactate dehydrogenase. Thus *E. coli* mutants with deleted lactate dehydrogenase enable efficient anaerobic growth and H_2 production (Mat-Jan et al. 1989). Along with the enriched JW1375-1 strain, un-enriched BL21 were also present in the last bottle. This was due to less stringent selection process and subsequent co-transfer with JW1375-1 strain.

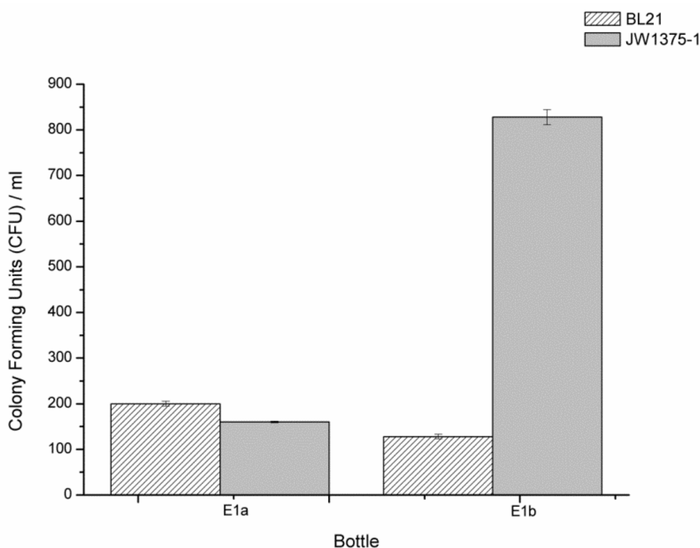


Figure 8.2. Graph plotting the colony forming units of individual E. coli strains in the artificial mixed culture. E1a and E1b indicate the first and last bottle out of eight bottle enrichment system. The error bars indicate standard deviation of averaged data from duplicate experiments.(Mangayil 2009/ plotted from the data presented in ***II****)*

The enrichment process was further investigated with substrate utilization as an additional selection pressure. The *E. coli* JW2409-1 strain deficient in phosphotransferase enzyme system thus deficient in glucose uptake was introduced to the artificial mixed culture. The enrichment was based on the different substrate utilization, growth and H_2 production properties of the *E. coli* strains. After the run, a

1.9-fold enrichment for JW1375-1 and 5-fold drop in prevalence of strains BL21 and JW2409-1 were observed. Dominance of JW1375-1 strain in the last enrichment bottle was also indicated in the parallel growth and H_2 production experiments. Pure *E. coli* strains BL21 and JW2409-1 accumulated little or no H_2 and the enrichment drive was contributed by JW2409-1. Similar to the preliminary experiment, co-transfer of non-H_2 producing strains was observed. The enrichment and parallel batch experiments with bottle samples and pure *E. coli* strains are shown in Figure 8.3.

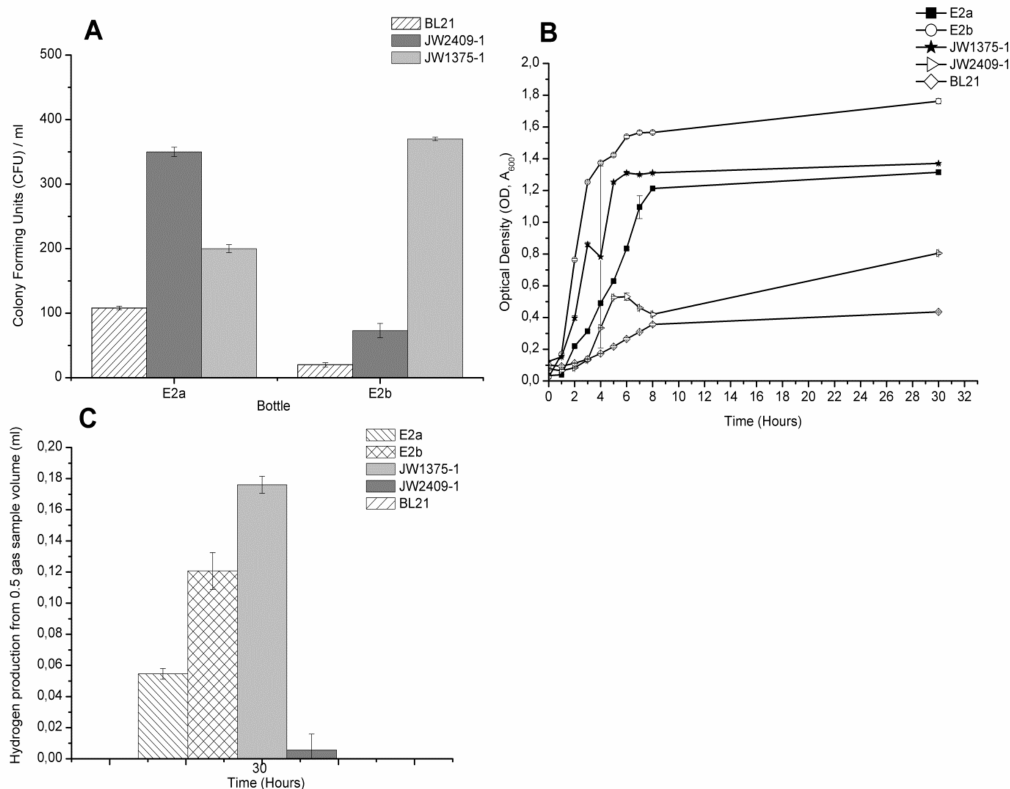


*Figure 8.3. Graph plotting (A) CFU of individual E. coli strains in the artificial mixed culture, (B) Growth curve and (C) end-point H_2 production plots for bottle and pure strain samples. E2a and E2b indicate the first and last bottle from the enrichment system. The error bars indicate standard deviation of averaged data from duplicate experiments.(Mangayil 2009/ plotted from the data presented in **II**)*

To further improve the enrichment process, starch was introduced as the sole carbon source. The H_2 producing JW1375-1 strain was replaced with JW1375-1 harboring pCSS4 vector, containing *Bacillus stearothermophilus* α-amylase gene under the control of *pamy* promoter. Promoter *pamy* is constitutively expressed in *E. coli* (Suominen et al. 1987). Compared to the previous experiment, under stringent selection pressures the process resulted in 9-fold enrichment of JW1375-1-pCSS4 strain and a 6-fold reduction and absence of JW2409-1 and BL21 strain, respectively. The growth curve experiments indicated a similar specific growth rate between samples from last bottle of the enrichment system (0.8 h^{-1}) and pure JW1375-1-pCSS4 (0.6 h^{-1}) strain. Similar to

previous H_2 production batch experiment, BL21 and JW2409-1 strains did not produce substantial amount of H_2 required for the enrichment process. Thus the gas pressure required for the enrichment was contributed by JW1375-1-pCSS4. The enrichment and parallel batch experiments with bottle samples and pure *E. coli* strains are shown in Figure 8.4. The results from Paper **II** provide evidence that the developed system was able to enrich the best H_2 producer from a mixed consortium.

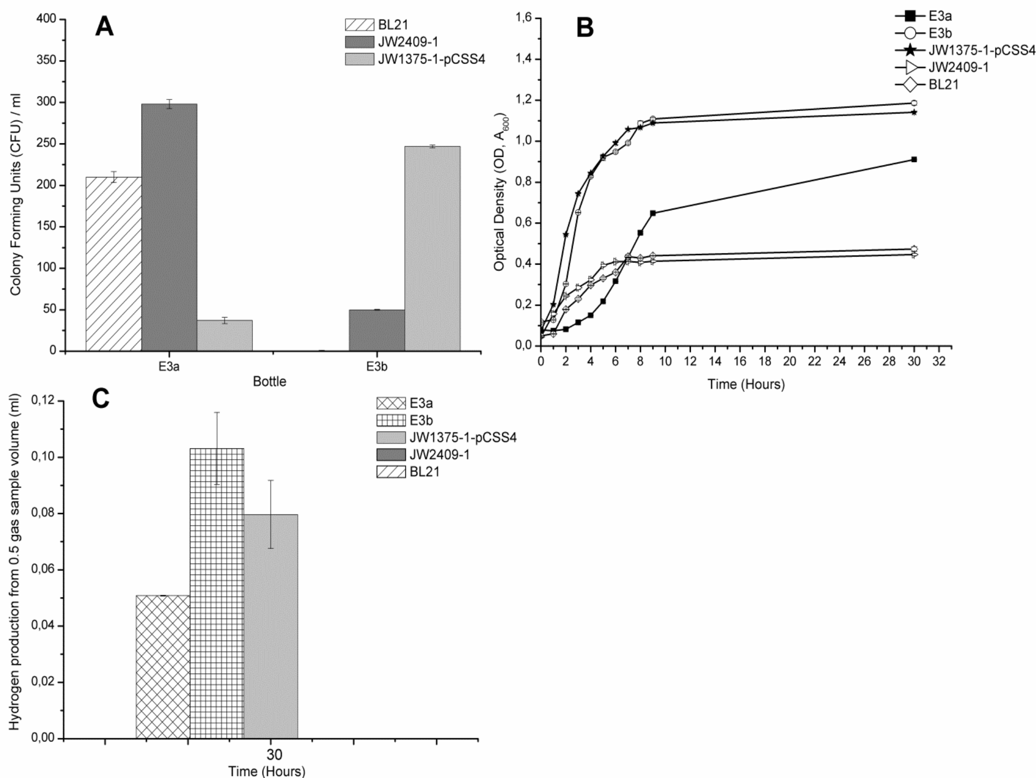


*Figure 8.4. Graph plotting (A) CFU of individual E. coli strains in the artificial mixed culture, (B) Growth curve and (C) end-point H_2 production plots for bottle and pure strain samples. E3a and E3b indicate the first and last bottle from the enrichment system. The error bars indicate standard deviation of averaged data from duplicate experiments. (Mangayil 2009/plotted from the data presented in **II**)*

8.3. ENRICHMENT OF FERMENTATIVE HYDROGEN PRODUCING STRAIN AND FUNCTIONAL COMMUNITY

Paper **III** reports the enrichment and strain isolation of a cellobiose degrading microorganism. Activated sludge was used as the inoculum source and the enrichment was performed by the system developed in Paper **II**. Depending on the selection pressure, the enrichment system enables to enrich the best H_2 producer(s) from microbial consortia. The enrichment system employed in Paper **III** contained a step-wise increment of cellobiose concentration as the selection pressure. Strain isolation with M9-CMC agar plates was successful in isolating a single bacterial strain. The

morphology of the isolate when grown on M9-CMC agar plates appeared to be yellow round shaped colonies with irregular edges. The strain was identified as Gram-negative and microscopic observations revealed a small rod-shaped bacterium. Amplification and sequencing of partial 16S rRNA gene and nucleotide BLAST searches against GeneBank database indicated the isolate's affiliation to *Enterobacteriaceae* family; more specifically with *Citrobacter amalonaticus* SA01 strain (99%). Thus the isolate was termed as *Citrobacter* sp. CMC-1 and the 16S rRNA gene sequence was deposited in GenBank (Accession number: GU324417). Following strain identification, the growth experiments identified glucose as the best carbon source. The specific growth rates and generation time for *Citrobacter* sp. CMC-1 was calculated to be 0.32 h^{-1} and 2.1 h (glucose), 0.13 h^{-1} and 5.5 h (cellobiose) and 0.08 h^{-1} and 8.3 h (CMC), respectively.

In Paper **IV**, the functional community was enriched from repeated liquid sub-cultivation of seed inoculum source (activated sludge) in modified HM100 media supplemented with crude glycerol (2.5 g/L). The growth curve and end-metabolite profile of the enriched microbial community when grown in a media (devoid of substrate) supplemented with/without 25 mM methanol indicated that the microbial consortium did not utilize methanol as a carbon source (Figure 8.5).

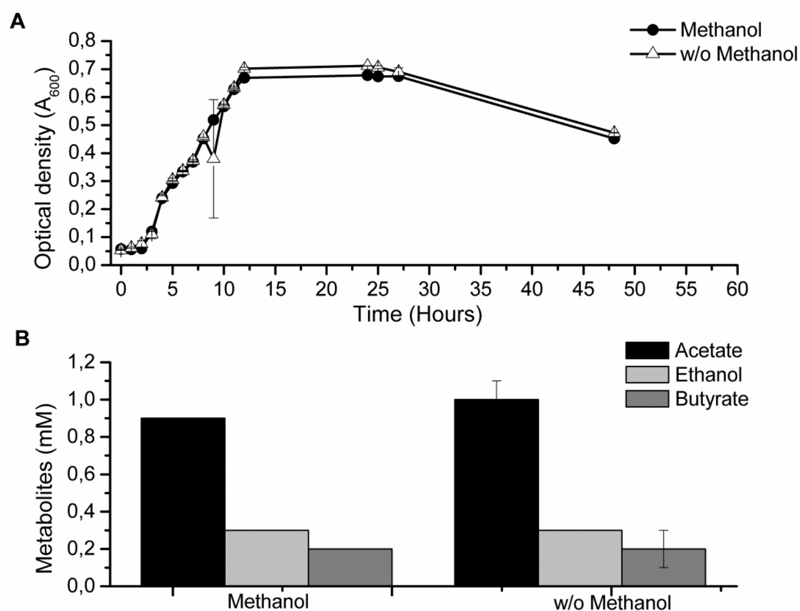


*Figure 8.5. Graphical presentation for (A) growth-curve of enriched microbial consortium in media amended with (●) and without (Δ) 25 mM methanol and (B) metabolites produced in presence and absence of methanol. The error bars indicate standard deviation of averaged data from duplicate experiments. (Data send to the reviewers, unpublished results/**III**)*

The dominant bacteria in the enriched microbial community were analyzed using PCR-DGGE technique. Following seriel dilution, reduction in microbial diversity led to three

prominent bands in the DGGE profile (Figure 8.6). Sequencing and nucleotide BLAST search against GenBank database affiliated the functional consortia towards uncultured rumen bacterium clone GRC39 ([1], 97%), uncultured firmicutes bacterium clone M0042_014 ([2], 93%), uncultured bacterium clone ([3], 98%), *Clostridium sporogenes* strain CL3 ([4], 98%) and *Clostridium subterminale* isolate DSM 758 ([5], 99%). Figure 8.6 shows the DGGE profile of the enriched microbial culture.

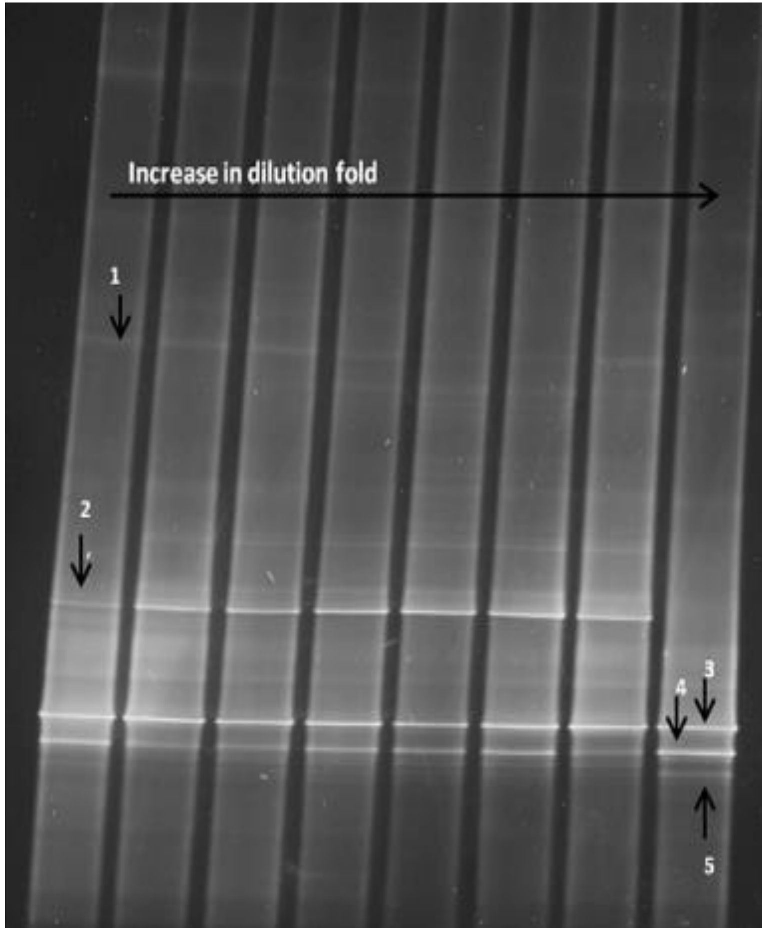


Figure 8.6. DGGE profile of the enriched microbial community. Bands 1, 2, 3, 4 and 5 showed affiliation towards uncultured rumen bacterium clone GRC39 (Identity, 97%), uncultured firmicutes bacterium clone M0042_014 (Identity, 93%), uncultured bacterium clone (Identity, 98%), Clostridium sporogenes strain CL3 (Identity, 98%) and Clostridium subterminale isolate DSM 758 (Identity, 99%), respectively. (Fig.4/ ***IV****)*

8.4. ONE-FACTOR-AT-A-TIME OPTIMIZATION

The effects of several physico-chemical factors namely initial culture pH, cultivation temperature and carbon sources on fermentative H_2 production were studied by OFAAT optimization method in Paper **III** and **IV**. The effects of these individual bioprocess parameters have been discussed (Chapter 2) in relation to cell growth, H_2 yield, fermentation end-metabolites or substrate utilization. The values for targeted response under optimal culture conditions and parameters optimized in the original publications are listed in Table 8.2.

Table 8.2. Bioprocess parameters and their values optimized by OFAAT approach accounting for the highest average values for the targeted responses studied in original publications of the thesis (III-IV) [a]

Parameter studied (cultivation conditions)	Optimized parameter value (range tested)	Targeted response: optimal value
Fermentative hydrogen production from different sugars by *Citrobacter* sp. CMC-1 in batch culture (III)		
Initial pH (5mM, 30˚C & 24 hrs) [b]	6.0 (4 – 6)	Biomass:0.16 g/L H_2 yield: 1.75 (mol-H_2/mol-glycerol$_{consumed}$)
Temperature (˚C) (5mM, pH 6.0 & 24 hrs) [b]	34 (25 – 40)	Biomass:0.30 g/L H_2 yield: 1.75 (mol-H_2/mol-glycerol$_{consumed}$)
Cultivation period (5mM, pH 6.0, 34 ˚C) [b]	48 (24 and 48)	Biomass:0.32; 0.36; 0.27 and 0.22 g/L [c] H_2 yield: 1.68; 1.93; 1.63 and 0.79 (mol-H_2/mol-glycerol$_{consumed}$) [c]
Bioconversion of crude glycerol from biodiesel production to hydrogen (IV)		
Initial pH (2.5 g/L, 37˚C & 72 hrs) [d]	6.5 (5 – 8)	Substrate utilization: 36.7% H_2 yield: 0.7 (mol-H_2/mol-glycerol$_{consumed}$)
Temperature (˚C) (2.5 g/L, pH 6.5 & 72 hrs) [d]	40 (25 – 46)	Substrate utilization: 35.7% H_2 yield: 1.0 (mol-H_2/mol-glycerol$_{consumed}$)
Substrate concentration (g/L) (40˚C, pH 6.5 & 72 hrs) [d]	1.0 (0 – 5)	Substrate utilization: 56.5% H_2 yield: 1.1 (mol-H_2/mol-glycerol$_{consumed}$)

[a] *Optimization studies were conducted as triplicate experiments in closed batch experimental set-up and the averaged values for targeted response are reported.*
[b] *Glucose concentration , temperature, pH and cultivation period chosen for optimization studies.*
[c] *Biomass and H_2 yield obtained from fermentation of monomeric hemi-cellulosic sugars in the order: Galactose, Mannose, Xylose and Arabinose.*
[d] *Crude glycerol concentration, temperature, pH and cultivation period chosen for optimization studies.*

Microbial growth, substrate consumption and fermentation end product synthesis are profoundly affected by the initial medium pH. In Paper **III**, *Citrobacter* sp. CMC-1 strain was observed to grow and produce H_2 at all tested pH ranges (4 – 7). The optimal culture pH value of 6.0 reported in Table 8.2 is in line with the reports published for other *Citrobacter* strains (Oh et al. 2003b; Hamilton et al. 2010). In Paper **IV**, The optimal initial medium pH was observed to be 6.5 (Table 8.2). The trend involving an

increase in H_2 production up to the optimal pH was also observed in crude glycerol fermentation with enriched microbial community as the inoculum. Furthermore, the initial pH also showed noteworthy effects on the production of soluble fermentation metabolites (**III** and **IV**). The *Citrobacter* sp. CMC-1 strain isolated in Paper **III** followed mixed-acid type fermentation profile. Comparing with the other pH points, at pH 6.0 a slight increase in acetate production and decrease in lactate and ethanol accumulation was observed. Low H_2 yield at pH 7.0 can be correlated to extra-cellular formate accumulation at alkaline conditions (Seol et al. 2008; Rossmann et al. 1991). In Paper **IV**, the mixed microbial inoculum underwent acetate-butyrate type fermentation. Reduced substrate utilization (4.6%) and an absence in H_2 production at low pH (pH 5.0) can be hypothesized due to hindrance in formation of intracellular ATP (Ciranna et al., 2011). As observed in Papers **III** and **IV**, acetate was the main by-product of cell growth and an increase in acetate concentration generally indicate the improvement in cell growth and H_2 production (Ueno et al. 2001; Zhang et al. 2009).

Determining optimal cultivation temperature for cell growth and H_2 production is pivotal in fermentation experiments. In the case of glucose fermentations by *Citrobacter sp*. CMC-1 (**III**), the strain grew well at temperature ranges 25˚C-34˚C producing the highest biomass yields at 30.5˚C (0.322 g/L). However, the optimal temperature for H_2 production was observed to be at 34˚C (1.82 mol-H_2/mol-glucose). Fermentation at higher temperatures (>37˚C) resulted in decreased biomass and H_2 yields indicating an inhibition on proteins involved in reductant disposal and H_2 production, i.e. [Ni-Fe] hydrogenase (Guo et al., 2010; Sinha & Pandey 2011). Analyzing the end-metabolites produced from glucose fermentation; at higher temperatures a sharp decline in acetate production and increased lactate and formate accumulation was observed. In the case of crude glycerol fermentation by the enriched microbial community (**IV**), substrate bioconversion to H_2 was noted to be highest at 40˚C. A decrease and subsequent cessation of growth and metabolite production was observed for the enriched inoculum when grown at high temperatures (>40˚C). These results signify the requirement to optimize fermentation temperature.

Crude glycerol was observed to be a suitable substrate of choice for the enriched functional consortium in fermentative H_2 production (**IV**). An optimal substrate concentration of 1 g/L of crude glycerol was identified from closed batch fermentations. Comparing the substrate utilization (1 g/L of substrate) and H_2 production profile with pure glycerol (74% and 0.95 mol-H_2/mol-$\text{glycerol}_{\text{consumed}}$) in batch experiments, after a cultivation period of 72 hours the enriched inoculum utilized only 56.5% of crude glycerol yielding 1.1 mol-H_2/mol-crude $\text{glycerol}_{\text{concumed}}$. These results suggest that the microorganisms may utilize the organic compounds present in the unpurified glycerol fraction contributing positively towards H_2 production and negatively in efficient substrate utilization. Similar observations of such positive effect of organic compounds in crude glycerol on bacterial growth and fermentation have been reported (Ngo et al. 2011; Srinophakun et al. 2012). Conversely, fermentations at higher crude glycerol concentration further reduced the substrate utilization efficiency. Venkataramanan et al. (2012) studied the impact of crude glycerol impurities on 1,3-propanediol and butanol

production by *C. pasteurianum* and reported strong inhibitory effects of stearic and oleic acids present in crude glycerol towards glycerol utilization (Venkataramanan et al. 2012). However, it is worth to note that a direct comparison of fermentation data is impossible due to inconsistency of crude glycerol compositions produced from biodiesel production plants. In Paper **IV**, the enriched microbial community fermented crude glycerol mainly to acetate, butyrate, ethanol and an unidentified compound, yielding 1.1 mol-H_2/mol-$glycerol_{consumed}$.

8.5. STATISTICAL OPTIMIZATION

Paper **V** investigated the effect of medium component optimization by statistical methods on bioconversion of crude glycerol to H_2 by the enriched microbial community (used in Paper **IV**). Nutrients and buffering components in modified HM100 medium (NH_4Cl, K_2HPO_4, KH_2PO_4, $MgCl_2.6H_2O$ and KCl) were selected to screen and optimize the components that contributed significantly towards H_2 production. The details of optimum independent variable concentrations from each experimental step and the final media composition yielding the highest H_2 yield are provided in Table 8.3.

Table 8.3. Details of optimum independent variable concentrations from each experimental step and final optimized media composition yielding the highest response.

Statistical design[a]	NH_4Cl (g/L)	K_2HPO_4 (g/L)	KH_2PO_4 (g/L)	$MgCl_2.6H_2O$ (g/L)[b]	KCl (g/L)[b]	H_2 yield (mol-H_2/mol-$glycerol_{consumed}$)
PB	2.0	1.0	1.0	1.0	1.0	0.72
SA	2.0[c]	1.6	1.6[c]	1.0	1.0	1.04
RA	4.05[d]	1.62	2.53[d]	1.0	1.0	1.39
CCD	4.05	1.6 [e]	2.52	1.0	1.0	1.41
Confirmation	4.4[f]	1.6	2.27[f]	1.0	1.0	1.42

Results from Box – Behnken design are not presented in the table as the optimal region inferred was beyond the design range.

[a] *PB = Plackett – Burman, SA = Steepest accent, RA = Ridge analysis and CCD = Central composite face-centered design.*

[b] *PB indicated negative effect of $MgCl_2.6H_2O$ and KCl on hydrogen yield. Thus their concentrations were kept at 1 g/L (low levels) in later optimization experiments.*

[c] *Concentrations were chosen as center point for BB design.*

[d] *Concentrations were chosen as center point in CCD.*

[e] *Concentration of K_2HPO_4 was optimized to be at 1.60 g/L.*

[f] *Calculated by partial differentiation of second order polynomial model obtained from CCD.*

Screening for significant media components on H_2 yield as the targeted response was performed by Plackett – Burman design. The analysis of variance (ANOVA) data indicated that NH_4Cl, K_2HPO_4 and KH_2PO_4 displayed a positive effect, whereas $MgCl_2.6H_2O$ and KCl had a negative effect towards the response. It could be noted that the degree of significance towards the response followed the order $NH_4Cl > KH_2PO_4 > K_2HPO_4$. In the following optimization step (Steepest accent), the variables that positively influenced H_2 production were selected to identify the optimal concentration

by moving towards the optimal response in the design area. The variables were subjected to Box – Behnken optimization experiments and the response surface graph produced from the model indicated the optimal response beyond the design area. The results suggests an incorrect choice of concentration limits opted for the experimental runs in steepest accent experiments, indicating the requirement for further optimization experiments. The optimization experiments were carried out with ridge analysis, a non-linear analog of steepest ascent design, by moving the variables in specified step size until the predicted response was beyond the theoretical maximum H_2 yield obtained from glycerol fermentations, thus ensuring the presence of the optimal response within the design area. From the data obtained from the ridge analysis experiments, it was decided that the concentration of K_2HPO_4 can be kept at 1.60 g/L as the compound showed the least significance among the selected variables and in the ridge analysis experimental design the tested K_2HPO_4 concentrations remained around 1.5 - 1.6 g/L. The NH_4Cl and KH_2PO_4 were subjected to final optimization by CCD and RSM, keeping the other media component concentrations as listed in Table 8.3. The response surface and contour graphs plotted from the experimental data indicated an interdependent significance of the selected variables towards the response. The second order polynomial equation generated from the CCD model predicted a H_2 yield of 1.41 mol-H_2/mol-glycerol$_{consumed}$ with 4.40 g/ and 2.27 g/L NH_4Cl and KH_2PO_4, respectively.

8.6. OPTIMIZED BIOPROCESS

The optimized bioprocess parameters were applied in closed batch or kinetic experiments (wherein the gaseous content in the headspace of the cultivation bottles were quantified at specific time points) to study the bacterial growth, substrate utilization and H_2 production profiles in Paper **III** – **V**.

In Paper **III**, the kinetic analysis on growth and H_2 production in glucose amended minimal media by *Citrobacter* sp. CMC-1 strain was conducted under optimal conditions (5mM glucose, pH 6.0 and 34˚C) (Figure 8.7A). The strain yielded the highest H_2 production rate (0.2 mmol-H_2 h^{-1}) and cumulative H_2 volume (20.1 ml) by the 4th and 12th hour, respectively, after which a decline in both H_2 production and biomass was observed (Figure 8.7B). This decline in H_2 production can be due to the activation of H_2 evolving FHL complex and membrane bound uptake hydrogenases at exponential and stationary phases, respectively (Kim et al. 2008).

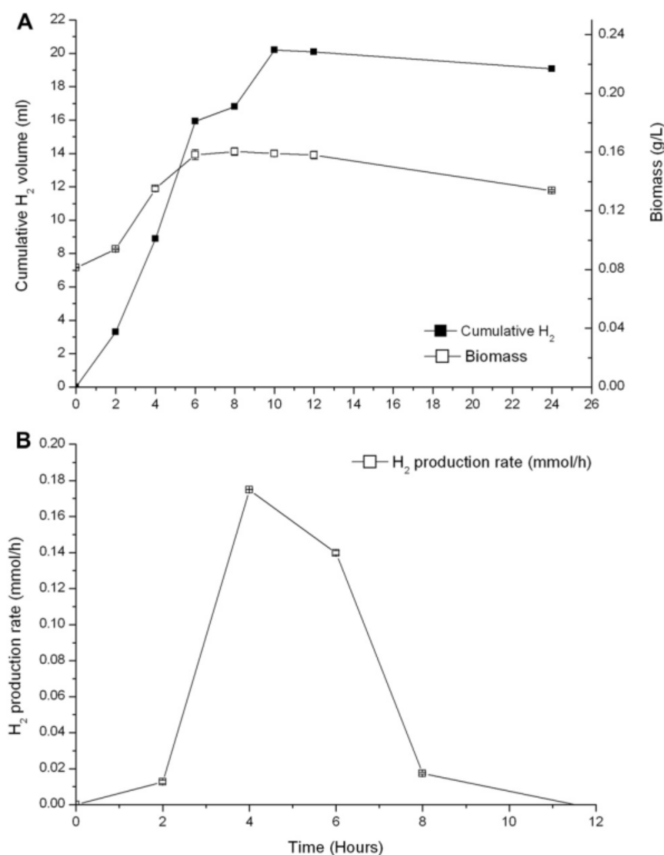


Figure 8.7. Kinetic study on (A) growth (□), cumulative H_2 volume (■) and (B) H_2 production rate by Citrobacter sp. CMC-1 when fermenting glucose under optimal bioprocess conditions. (Fig. 3/ ***III****)*

Monomeric hemi-cellulose sugars, i.e. galactose, mannose, xylose, arabinose and rhamnose were observed to be excellent carbon sources for fermentative H_2 production by *Citrobacter* sp. CMC-1 under optimal process conditions. Better growth, substrate utilization and H_2 production was observed when the cultivation time was increased from 24 to 48 hours. The biomass and H_2 yield data from 48 hour fermentation experiments are listed in Table 8.2. By analyzing the HPLC data it could be inferred that when compared with 24 hour fermentation, reduced formate accumulation was observed in longer cultivations. This observation along with the increased H_2 yields substantiates that the strain produced H_2 from formate breakdown via the FHL complex. Due to energy intensive arabinose utilization route, similar results were not observed in arabinose fermentation (Li et al. 2008; Ntaikou et al. 2008).

In Paper **IV**, kinetic experiments by the enriched inoculum indicated crude glycerol as a suitable carbon source for fermentative H_2 production. The results from substrate utilization, cumulative H_2 volume and end-metabolites produced during the kinetic study are shown in Figure 8.8.

A lag phase of 6 hours for H_2 production was observed in crude glycerol fermentation. The pre-cultivation of inoculum in pure glycerol can be the reason for long lag phase due to the acclimatization time required when introduced to media supplemented with crude glycerol. In addition, the impurities present in crude glycerol could also contribute towards extending the lag phase (Chatzifragkou & Papanikolaou 2012; Jensen et al. 2012). Comparing the substrate utilization efficiencies by the enriched inoculum in closed batch cultivations, kinetic experiments on pure (91%) and crude glycerol (93%) fermentations showed similar substrate degradation pattern (Figure 8.8B) with different metabolite production profiles (Figure 8.8C). This may be due to the growth of other bacterial species in the mixed culture, prevalent under low dissolved H_2 concentrations, contributing towards the production of fermentation metabolites, for example ethanol. Cumulative H_2 volume produced from pure and crude glycerol fermentation were 20.9 ml and 14.8 ml, respectively. Increased H_2 production observed in crude glycerol fermentation further explains the utilization of non-glycerol organic matter in the crude glycerol. Kinetic studies on pure and crude glycerol fermentations indicated decreased acetate and increased butyrate production from 120th hour (Figure 8.8C), signifying recycling of acetate to butyrate at late-exponential phase in *Clostridium* spp. (Zhang et al. 2009). Comparing the produced end-metabolites from the glycerol substrates, a prominent decline in acetate production and an exponential increase in ethanol production was observed with pure glycerol as the substrate.

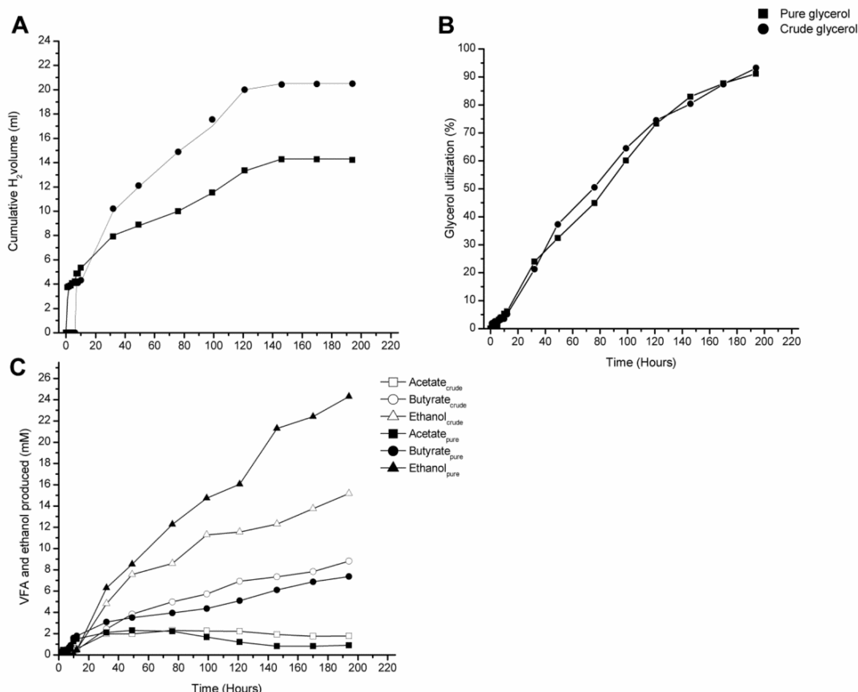


Figure 8.8. Kinetic analysis data on (A) cumulative hydrogen production [pure (■) and crude (●) glycerol], (B) glycerol utilization [pure (■) and crude (●) glycerol] and (C) end-metabolites produced from pure (open symbols) and crude (closed symbols) glycerol fermentations by the enriched inoculum. (A, Fig. 2/ ***IV****; B, plotted from the results obtained and C, Fig. 3/* ***IV****)*

After optimizing the bioprocess parameters in Paper **IV**, statistical amendment of growth media composition (Paper **V**) further improved the H_2 yield. The model validation experiments performed in optimized HM100 media supplement with crude glycerol (1 g/L) resulted in a H_2 yield of 1.42 mol-H_2/mol-glycerol$_{consumed}$. Comparing with the predicted H_2 yield from CCD and RSM models the results showed only 0.6% variation. The results signify successful implementation of design of experiments and RSM techniques in optimizing media composition for fermenting crude glycerol by the enriched functional microbial community and a 29% improvement in H_2 yield in comparison to the value reported in Paper **IV**.

9. CONCLUSIONS

The work presented in this thesis focused in studying bioH_2 production in protein to community level perspective and consisted of four published papers (**II** –**V**) and one manuscript (**I**).

Paper **I** reported successful anaerobic biopanning on purified *C. acetobutylicum* [Fe-Fe] hydrogenase and enrichment of anti-hydrogenase antibody binders. The antibodies enriched against purified *C. acetobutylicum* hydrogenase were successful in recognizing [Fe-Fe] hydrogenases from *C. acetobutylicum* lysate. In addition, scFv's with binding specificities towards *C. acetobutylicum* hydrogenase also recognized closely related *C. butyricum* [Fe-Fe] hydrogenase suggesting the employment of such antibodies with generic specificities as tools for quantitative monitoring of clostridial hydrogenases in bioprocess systems at protein level. Preliminary experiments on functional detection of clostridial [Fe-Fe] hydrogenases propose as an excellent tool for hydrogenase quantification. Detailed studies such as epitope mapping and binding kinetics are required to further understand and improve the binding kinetics of anti-hydrogenase antibodies. So far there are no reports on anti-hydrogenase antibodies and their applicability towards hydrogenase monitoring at protein level. The present study also points to the direction of using the recombinant antibodies to monitor structural changes during hydrogenase inactivation process. Moreover, the study implies to investigate the effect of intra-molecular interactions on hydrogenase protein and leeway a possibility to close the O_2 migration 'door' by finding a specific binder from the panned library. Furthermore, future studies with the isolated antibodies also offer chance in developing a direct cost-effective method for hydrogenase purification from bacterial lysates.

Paper **II** serves as the proof-of-principle that the developed system could enrich H_2 producers from a microbial population. Gas production along with stringent selection conditions, such as utilization of complex substrates, allowed in lowering the background and isolating the best H_2 producer. In future, apart from linear connection; the enrichment system could be used as branched or series format, allowing isolation of microbes capable of degrading various complex sugars in a single run (**II**).

In Paper **III** successful implementation of the enrichment system in isolating a cellobiose degrading H_2 producer from activated sludge is reported. Though the strain fermented cellobiose and CMC producing H_2, glucose was observed to be the optimal carbon source. Nucleotide BLAST search affiliated the strain to *Citrobacter* sp. Optimizing initial medium pH and cultivation temperature were identified as crucial parameters in fermentative H_2 production. Glucose fermentation at pH 6.0 and 34˚C by *Citrobacter* sp. CMC-1 strain yielded 1.82 mol-H_2/mol-glucose and followed mixed-acid type fermentation. In kinetic studies, the strain registered maximum biomass yield and H_2 production rate of 0.16 g/L and 0.2 mmol-H_2/h, respectively. The isolate was capable in utilizing monomeric hemi-cellulose sugars and yielded biomass and H_2, with

respect to the fermented sugars, in the order; mannose > galactose > xylose > rhamnose > arabinose.

Glycerol utilizing functional community was enriched from activated sludge in Paper **IV**. DGGE profile identified *Clostridium* sp. predominant in the enriched inoculum. Similar in Paper **III**, initial pH, cultivation temperature and substrate concentration were to be optimized for efficient fermentation. Crude glycerol was proven as a suitable fermentation substrate for the enriched inoculum producing H_2 and CO_2 as gaseous metabolites and acetate, butyrate and ethanol as liquid metabolites. At optimal cultivations conditions (pH 6.5, 40˚C and 1g/L of substrate) the inoculum yielded 1.1 mol-H_2/mol-glycerol$_{consumed}$.

In addition to bioprocess optimization, statistical optimization of medium components improved the H_2 yield from crude glycerol fermentation was conducted in Paper **V**. PB design identified NH_4Cl, K_2HPO_4 and KH_2PO_4 as components that positively influenced H_2 yield. RA and CCD aided in optimizing the concentrations of significant media components. The CCD and RSM models predicted H_2 yield of 1.41 mol-H_2/mol-glycerol$_{consumed}$ for crude glycerol fermentation with an optimized media comprising, NH_4Cl, 4.40 g/L; K_2HPO_4,1.6 g/L; KH_2PO_4, 2.27 g/L; $MgCl_2.6H_2O$, 1.0 g/L; KCl, 1.0 g/L; Na-acetate.3H_2O, 1.0 g/L and tryptone 2.0 g/L. Optimization of media components was effective in improving the H_2 yield from 1.1 to 1.42 mol-H_2/mol-glycerol$_{consumed}$; emphasizing its importance in improving fermentative H_2 production.

Taken together, this thesis emphasizes the robustness of the novel enrichment system in isolating the best H_2 producer capable of degrading complex substrates from environmental samples and highlights the importance of bioprocess optimization for enhanced bioH_2 production. Furthermore, for the first time, anaerobic biopanning of purified O_2 labile [Fe-Fe] hydrogenase and enrichment of generic antibody binders recognizing clostridial [Fe-Fe] hydrogenases are reported in this thesis.

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Mangayil, R., Karp, M., Lamminmäki, U., Santala, V. Isolation of recombinant antibodies for specific detection of clostridial [Fe-Fe] hydrogenases.

Submitted for publication.

II

Tolvanen, K.E.S., Mangayil, R.K., Karp, M.T., Santala, V.P. 2011. Simple enrichment system for hydrogen producers. Applied and Environmental Microbiology. 77: 4246-4248.

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V

Mangayil, R.,Aho, T., Karp, M., Santala, V. 2015. Improved bioconversion of crude glycerol to hydrogen by statistical optimization of media components. Renewable Energy. 75: 583–589.

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PL 527
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P.O.B. 527
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ISBN 978-952-15-3462-1
ISSN 1459-2045