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Anniina Kivistö

Halophilic Biohydrogen and 1,3-Propanediol Production from Raw Glycerol:

A Genomic Perspective

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A Genomic Perspective

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ABSTRACT

Glycerol is produced in large amounts as a by-product in biodiesel industry (10 kg per 100 kg biodiesel). By-products and waste materials are typically economical substrates for bioprocesses. Furthermore, microorganisms are able to combine the degradation of organic material with production of a wide range of metabolites and other cellular products. The current biotechnological interest of industrial glycerol lies on bioprocesses yielding environmentally friendly energy carrier molecules (hydrogen, methane, ethanol, butanol) and reduced chemicals (1,3-propanediol, dihydroxyacetone). Industrial glycerol also called as raw or crude glycerol, however, is a challenging substrate for microorganisms due to its impurities including alcohol, soaps, salts and metals. Halophiles (the salt-loving microorganisms) require salt for growth and heavy metal resistances have been characterized for numerous halophiles. Therefore, halophiles are potentially useful for the utilization of raw glycerol from biodiesel waste streams without pre-processing.

Another challenge for large-scale microbial bioprocesses is a potential contamination with unfavorable microorganisms. For example, H_2-producing systems tend to get contaminated with H_2-consuming microorganisms. Extremophiles are organisms that have been adapted for life under extreme conditions, such as high salinity, high or low temperature, asidic or basic pH, dryness or high pressure. For extremophilic pure cultures contamination and thus the need to ensure a sterile environment might not be a problem due to the extreme process conditions that efficiently prevent the growth of most other bacteria. In addition, hypersaline environments (above 12 % NaCl) do not support the growth of H_2 utilizing methanogens due to bioenergetic reasons. Halophilic fermentative H_2 producers, on the other hand, have been shown to be active up to near salt saturation.

The aims of the present study can be divided into two categories: 1) to develop an open raw glycerol degrading bioprocess primarily for H_2 (clean and renewable energy carrier molecule) and secondarily for 1,3-PD (component of biopolymers) production employing halophilic pure cultures and 2) to obtain information on the genome of salt-tolerant organisms, halophilic survival strategies, and fermentation pathways via whole-genome sequencing.

In the present study, glycerol fermentation of halophilic *Halanaerobium saccharolyticum* subsp. *saccharolyticum* and *Halanaerobium saccharolyticum* subsp.

senegalense were characterized and H_2 as well as 1,3-PD formation were initially optimized using commercial pure glycerol as a substrate. Growth, as well as the H_2 and 1,3-PD production of *H. saccharolyticum* subsp. *saccharolyticum* were further optimized as small-scale batch experiments for vitamin B_{12} content, process conditions and medium composition. In addition, inhibitory effect of unpurified raw glycerol and the fermentation end-products (H_2, acetate and 1,3-PD) along with contamination risk of an halophilic bioprocess were assessed. The glycerol fermentation and raw glycerol inhibition were compared to non-halophilic microorganisms *Escherichia coli* and/or *Clostridium butyricum*. Eventually, the halophilic H_2 and 1,3-PD production from unpurified raw glycerol were combined in an open (non-sterile) two-stage fermentation process.

The halophilic bacterial subspecies fermented glycerol mainly to H_2, CO_2 and acetate. *H. saccharolyticum* subsp. *saccharolyticum* yielded also 1,3-PD via a vitamin B_{12} dependent pathway. The subsp. *senegalense* grew poorly and after vitamin B_{12} optimization produced H_2 less efficiently, and thus the subsp. *saccharolyticum* was chosen for the further studies. The H_2 and 1,3-PD yielding pathways of *H. saccharolyticum* subsp. *saccharolyticum* were observed to compete, and the H_2 production was remarkably enhanced when the 1,3-PD production was blocked by the unavailability of vitamin B_{12}. The growth limiting factor of the particular raw glycerol fraction used in these studies was observed being methanol or other impurities rather than salt. Hypersaline environment (at and above 12.5 % NaCl) decreased efficiently the risk for contaminations and non-sterile conditions along with communal tap water were successfully applied in a bioprocess employing *H. saccharolyticum* subsp. *saccharolyticum* for production of H_2 and 1,3-PD from unpurified raw glycerol.

The genome of *H. saccharolyticum* subsp. *saccharolyticum* was sequenced and analysed on genetic level for phylogenetic and physiological characteristics, fermentation pathways as well as for resistances for metals, antibiotics and other toxic compounds. The genome sequence analysis revealed four putative hydrogen producing enzymes and a range of putative genes causing resistance for variety of antibiotics, heavy metals and other toxins.

In conclusion, an open anaerobic bioprocess is applicable for halophilic pure cultures with no adverse contaminations. Additionally, *H. saccharolyticum* subsp. *saccharolyticum* is capable for direct utilization of the by-product from biodiesel production process and high-yield production of H_2 and/or 1,3-PD. The genome sequence data provides insight into the relatively little-studied group of halophilic fermentative bacteria on genetic level.

TIIVISTELMÄ

Glyserolia muodostuu suuria määriä biodiesel-tuotannon sivutuotteena (10 kg per 100 kg biodiesel). Sivutuotteet ja jätemateriaalit ovat tyypillisesti edullisia raaka-aineita teollisuuden prosesseihin. Mikrobit voivat yhdistää orgaanisen aineen hajottamisen aineenvaihdunta- sekä muiden solutuotteiden muodostumiseen. Raakaglyserolista esimerkiksi voidaan bioteknisesti tuottaa energiankantajamolekyylejä (vety, metaani, etanoli, butanoli) sekä kemikaaleja (1,3-propaanidioli, dihydroksiasetoni). Raakaglyseroli on kuitenkin haastava substraatti mikroorganismeille sen sisältämien alkoholin, suolan, saippuan ja metallien vuoksi. Halofiiliset (korkeiden suolapitoisuuksien) mikrobit puolestaan vaativat suolaa elääkseen ja niiden sietokykyä raskasmetalleille on kuvattu, minkä vuoksi halofiilit mahdollisesti kykenevät hyödyntämään puhdistamatonta raakaglyserolia hiilen- ja energianlähteenään.

Merkittävä haaste suuren mittakaavan bioprosesseille on prosessin pilaantuminen (kontaminaatio) epäsuotuisilla mikrobeilla. Esimerkiksi vetyä tuottavat prosessit kontaminoituvat helposti vetyä hajottavilla mikrobeilla. Tämä on erityisesti ongelma puhdasviljelmiä käyttäville bioprosesseille, jotka tyypillisesti vaativat suljetun (steriilin) ympäristön. Ekstremofiilit ovat mikro-organismeja, jotka ovat sopeutuneet elämään äärimmäisissä olosuhteissa, kuten korkeassa suolapitoisuudessa, korkeassa tai matalassa lämpötilassa, emäksisessä tai happamassa pH:ssa, kuivuudessa tai korkeassa paineessa. Ekstremofiilisille puhdasviljelmille kontaminaatio ja siten steriiliyden ylläpitäminen ei ole välttämättömyys, koska tavalliset (ei-ekstremofiiliset) mikrobit eivät menesty äärimmäisissä olosuhteissa. Lisäksi korkea suolapitoisuus (yli 12 % NaCl) estää erityisesti vetyä kuluttavien metanogeenien kasvua bioenergeettisistä syistä. Halofiilista fermentoivaa vedyntuottoa on kuitenkin osoitettu tapahtuvan jopa suolan saturaatiota lähenevissä pitoisuuksissa.

Tutkimuksen tavoitteet voidaan jakaa kahteen luokkaan: 1) puhdistamatomatonta raakaglyserolia hyödyntävän avoimen (ei-steriilin), ensisijaisesti vetyä (puhdas ja uusiutuva energiankantajamolekyyli) ja toissijaisesti 1,3-propaanidiolia (biopolymeerien komponentti) tuottavan halofiilisen puhdasviljelmän työllistävän prosessin kehittäminen ja 2) halofiilisen fermentoivan bakteerin genomin emäsjärjestyksen selvittäminen ’suolaa sietävän’ genomin, halofiilisten selviytymismenetelmien ja fermentaatioreittien kuvaamiseksi geenitasolla.

Tutkimuksissa kuvattiin halofiilisten bakteerien *Halanaerobium saccharolyticum* alalaji *saccharolyticum* ja *Halanaerobium saccharolyticum* alalaji *senegalense*

glyserolifermentaatiota sekä vedyn ja 1,3-propaanidiolin tuottoa optimoitiin alustavasti käyttäen kaupallista puhdasta glyserolia raaka-aineena. *H. saccharolyticum* alalaji *saccharolyticum* -bakteerin kasvua sekä vedyn ja 1,3-propanediolin tuottoa optimoitiin edelleen vitamiinin B_{12}, prosessiolosuhteiden ja kasvualustan komponenttien suhteen pienen mittakaavan panoskokeissa. Lisäksi puhdistamattoman raakaglyserolin ja käymisreaktion lopputuotteiden (vety, asetaatti, 1,3-propaanidioli) inhiboivaa vaikutusta sekä halofiilisen bioprosessin kontaminaatioriskiä arvioitiin. Halofiilisen fermentoivan bakteerin glyserolifermentaatiota ja raakaglyseroli-inhibitiota verrattiin ei-halofiilisiin *Escherichia coli* ja/tai *Clostridium butyricum* -bakteereihin. Lopulta halofiilinen vedyn ja 1,3-propaanidiolin tuotto puhdistamattomasta raakaglyserolista yhdistettiin avoimessa kaksivaiheisessa käymisprosessissa.

Tutkimuksen halofiiliset fermentoivat alalajit muodostivat glyserolista pääasiallisesti vetyä, hiilidioksidia ja asetaattia. *H. saccharolyticum* alalaji *saccharolyticum* tuotti lisäksi 1,3-propaanidiolia vitamiinista B_{12} riippuvaisella aineenvaihduntareitillä. Alalaji *senegalense* kasvoi alustavissa tutkimuksissa heikosti ja vitamiinin B_{12} optimoinnin jälkeen tuotti vähemmän vetyä, minkä vuoksi alalaji *saccharolyticum* valittiin jatkotutkimuksiin. *H. saccharolyticum* alalaji *saccharolyticum* -bakteerin vetyä ja 1,3-propanediolia tuottavien reittien havaittiin kilpailevan keskenään ja vedyn tuoton tehostuvan merkittävästi 1,3-propaanidiolia tuottavan reitin ollessa suljettuna vitamiinin B_{12} saatavuuden ollessa estetty. Raakaglyserolin mikrobien aktiivisuutta vähentäväksi komponentiksi tutkimuksissa ei osoittautunut suola vaan metanoli tai jokin muu raakaglyserolin epäpuhtauksista. Korkea suolapitoisuus ($\geq$ 12.5 % NaCl) vähensi tehokkaasti kontaminaatioriskiä ja epästeriilejä olosuhteita sekä kunnallista vesijohtovettä sovellettiin menestyksekkäästi *H. saccharolyticum* alalaji *saccharolyticum* -bakteerin työllistävässä bioprosessissa vedyn ja 1,3-propanediolin tuottoon raakaglyserolista.

H. saccharolyticum alalaji *saccharolyticum* -bakteerin perimän emäsjärjestys selvitettiin, minkä pohjalta fylogeneettisiä ja fysiologisia ominaisuuksia, fermentaatioreittejä sekä metalli-, antibiootti- ja toksiiniresistenttiyttä analysoitiin geenitasolla. Genomianalyysi paljasti neljä putatiivista vetyä tuottavaa entsyymiä ja valikoiman antibiootti-, metalli- ja/tai toksiiniresistenttiyttä aiheuttavia geenejä.

Johtopäätöksenä voidaan todeta avoimen anaerobisen bioprosessin olevan sovellettavissa halofiilisille puhdasviljelmille ilman haitallisia kontaminaatioita. Lisäksi *H. saccharolyticum* alalaji *saccharolyticum* kykenee hyödyntämään puhdistamatonta biodieseltuotannon sivutuotetta ja tuottamaan vetyä ja/tai 1,3-propaanidiolia korkein saannoin. Genomisekvenssidata tarjoaa geenitason näkemystä suhteellisen vähän tutkittuihin halofiilisiin fermentoiviin bakteereihin.

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Tampere, August 2013

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LIST OF PUBLICATIONS

This thesis contains some unpublished material but is mainly based the following original publications (I-VII), refered as Roman numerals in the thesis.

I Kivistö, A., Santala, V., Karp, M. 2010, "Hydrogen production from glycerol using halophilic fermentative bacteria", *Bioresource technology*, vol. 101, no. 22, pp. 8671-8677.

II Kivistö, A., Santala, V., Karp, M. 2011, "Closing the 1,3-propanediol route enhances hydrogen production from glycerol by *Halanaerobium saccharolyticum* subsp. *saccharolyticum*", *International Journal of Hydrogen Energy*, vol. 36, no. 12, pp. 7074-7080.

III Kivistö, A., Santala, V., Karp, M. 2012, "1,3-Propanediol production and tolerance of a halophilic fermentative bacterium, Halanaerobium saccharolyticum subsp. saccharolyticum", *Journal of Biotechnology*, vol. 158, no. 4, pp. 242-247.

IV Kivistö, A., Santala, V., Ciranna, A., Karp, M. "Halophilic biohydrogen production from glycerol-containing by-product of biodiesel manufacturing process", *Biomass and Bioenergy*, Submitted for publication.

V Kivistö, A., Santala, V., Karp, M. "Non-sterile process for biohydrogen and 1,3-propanediol production from raw glycerol", *International Journal of Hydrogen Energy*, vol. 38, no. 27, pp. 11749-11755.

VI Kivistö, A., Larjo, A., Ciranna, A., Santala, V., Roos, C., Karp, M. 2013, "Genome sequence of *Halanaerobium saccharolyticum* subsp. *saccharolyticum* DSM 6643^T, a halophilic hydrogen producing bacterium", *Genome Announcements*, vol. 1, no. 2, e00187-13.

VII Kivistö, A., Larjo, A., Santala, V., Karp, M. "Genome analysis of *Halanaerobium saccharolyticum* subsp. *saccharolyticum* DSM 6643^T provides insight into halophilic fermentative pathways", Manuscript.

Author has contributed as the corresponding author to the following review publications (RI-RII) on the field of this thesis:

RI Kivistö, A.T., Karp, M.T. 2011, "Halophilic anaerobic fermentative bacteria", *Journal of Biotechnology*, vol. 152, no. 4, pp. 114-124.

RII Kivistö, A.T., Ciranna, A., Santala, V.P., Karp, M.T. "Extremophilic microbes in biohydrogen production", *Current Biotechnology*, In Press.

AUTHORS CONTRIBUTION

I Anniina Kivistö wrote the paper and is the corresponding author. She planned and conducted the experimental work and interpreted the results.

II Anniina Kivistö wrote the paper and is the corresponding author. She planned and conducted the experimental work and interpreted the results.

III Anniina Kivistö wrote the paper and is the corresponding author. She planned and conducted the experimental work and interpreted the results.

IV Anniina Kivistö wrote the paper and is the corresponding author. She planned and conducted the experimental work and interpreted the results. A. Ciranna adviced in planning the experiments, interpreting the results and manuscript writing.

V Anniina Kivistö wrote the paper and is the corresponding author. She planned and conducted the experimental work and interpreted the results.

VI Anniina Kivistö and A. Larjo contributed equally to the publication. Anniina Kivistö wrote the paper (80 %) and is the corresponding author. She planned and carried out the wet-lab part and contributed to data analysis. A. Larjo planned and performed dry-lab part and contributed to data analysis and manuscript writing (20 %). A. Ciranna adviced in planning the experiments and interpreting the results. C. Roos adviced in planning the experiments.

VII Anniina Kivistö wrote the paper and is the corresponding author. She planned and conducted the experimental work and interpreted the results. A. Larjo interpretated the statistical results.

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

ABBREVIATIONS

1,3-PD	1,3-Propanediol
3-HPA	3-hydroxypropionaldehyde
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
CoA	Coenzyme A
COD	Chemical oxygen demand
DHA	Dihydroxyacetone
DHAP	Dihydroxyacetone phosphate
DNA	Deoxyribonucleic acid
e^-	Electron
EU	European Union
Fd(ox)	Ferredoxin (oxidized form)
Fd(red)	Ferredoxin (reduced form)
[Fe]-	Iron
[FeFe]-	Iron-Iron
FFA	Free fatty acid
Gap5	Sequence assembly viewer and editor.
GC	Gas chromatography
GlpF	Glycerol fasilitator
H^+	Proton

HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HPLC	High-performance liquid chromatography
IUPAC	International Union of Pure and Applied Chemistry
MATE	Multidrug and toxin extrusion protein
MEC	Microbial electrolysis cell
MIRA	An automated genome assembler
NAD^+	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
[NiFe]-	Nickel-iron
OD_{600}	Optical density (absorbance) at 600 nm wavelength
OH	Hydroxyl group
ORF	Open reading frame
P_i	Orthophosphate
RAST	Rapid annotation using systems technology
RNA	Ribonucleic acid
RND	Resistance-nodulation-cell division protein
spp.	Species
subsp.	Subspecies
w/	with
w/o	without
YE	Yeast extract

1. INTRODUCTION

A biobased economy (Figure 1.1) is defined as an economy "based on production paradigms that rely on biological processes and, as with natural ecosystems, use natural inputs, expended minimum amounts of energy and do not produce waste as all materials discarded by one process are inputs for another process and are reused in the ecosystem" (Buttazzoni 2009). The economy has started to displace a current fossil resource based economy. The benefits of changing into biobased economy include substitution of fossil fuels and oil-based materials, reduced CO_2 emissions, efficient use of resources, and the creation of closed-loop systems in which wastes are recycled to be used as inputs for production of materials that fulfill the needs of the population.

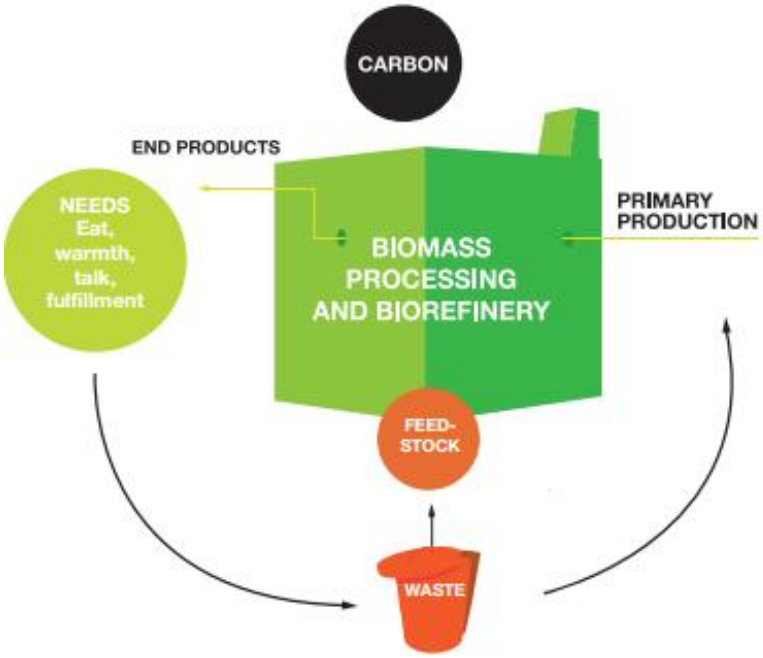

Figure 1.1. Schematic of the principles of a biobased economy. (Adapted from Buttazzoni 2009)

For the efficient use of resources, waste streams such as glycerol, which is produced in large quantities especially as a by-product of biodiesel production, must be utilized. Approximately, 10 kg glycerol is produced in transesterification process for 100 kg biodiesel. The utilization of by-products and waste materials is economical and improves the sustainability of a process. The term sustainability refers to sustainable development defined by the World Commission on Environment and Development (United Nations, 1987) as "development that meets the needs of the present without compromising the ability of future generations to meet their own needs".

In 2008, the World total energy consumption was approximately 142,700 TWh (International Energy Agency (IEA) 2010). Majority of the energy consumed currently originates from fossil fuels, i.e. oil, natural gas, and coal as shown in Figure 1.2. Fossil fuels, however, are non-renewable and have been approximated to diminish by 2112 (Shafiee and Topal 2009). In addition, burning of fossil fuels increases the environmental burden with high CO_2 emissions and thus the usage of fossil fuels with current extent accelerates the green-house effect along with the global climate change especially as the global energy consumption has an uptrend.

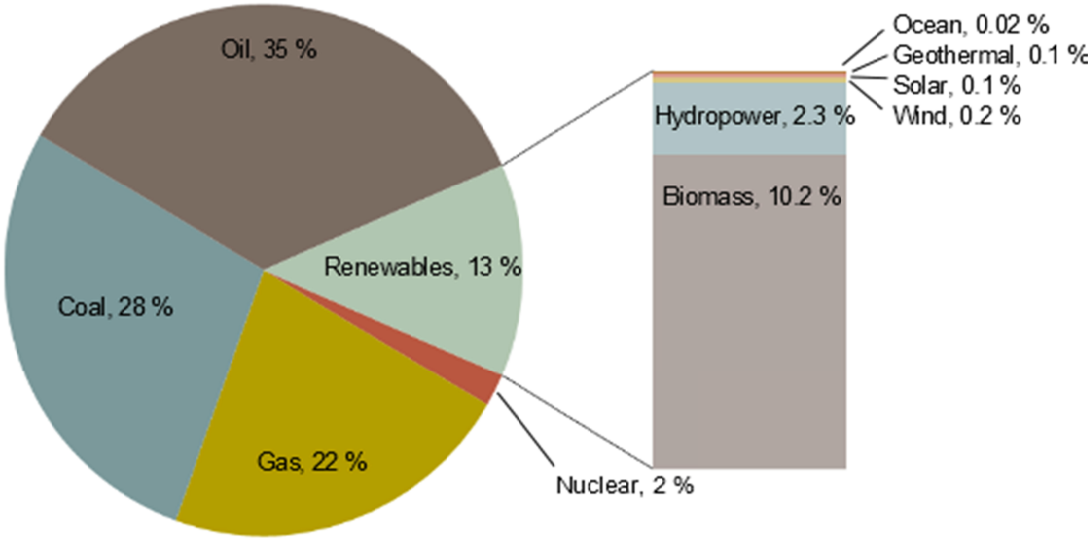

Figure 1.2. Energy sources in total global primary energy supply in 2008. (Modified from Edenhofer et al. 2011)

Public environmental awareness, however, has recently risen, and directions for increasing the proportional energy consumption from renewable sources have been made. European Union, for example, aims to increase the amount of renewable energy consumption from 10 % (2007) to 20 % by 2020. Furthermore, initiatives have been made for the use of renewable energy covering 100 % of the energy consumption in 2050 (Zervos *et al.* 2010).

Hydrogen (H_2) is considered as one of the most potential alternatives for future energy carrier molecules (Das and Veziroğlu 2001). H_2 is environmentally friendly and clean; its combustion yields high amount of energy and water as the sole end product. H_2 is derived chemically from non-renewable material (fossil fuels) or renewable material (electrolysis of water) with high energy input and can be produced biologically from renewable (waste) materials.

2. GLYCEROL ECONOMY

Glycerol (also called as glycerin or glycerine) is a sugar alcohol with molecular formula of $C_3H_8O_3$. It is colourless, odourless and viscous liquid. The IUPAC official name for glycerol is propane-1,2,3-triol implying the structure being a propane molecule which each three carbons have a hydroxyl (OH) group attached. Thanks to the three alcoholic OH groups glycerol is water-soluble.

2.1. Glycerol as an industrial by-product

Glycerol is produced in a variety of industrial processes including oleochemical reactions (hydrolysis/saponification, transesterification) applied in biodiesel and soap industry, yeast fermentation (ethanol production), and hypersaline algal bioprocesses (algal biomass, oil, or β-carotene production) as presented schematically in Figure 2.1. According to the best of my knowledge, the algal processes are on research or pilot level rather than industrial scale applications.

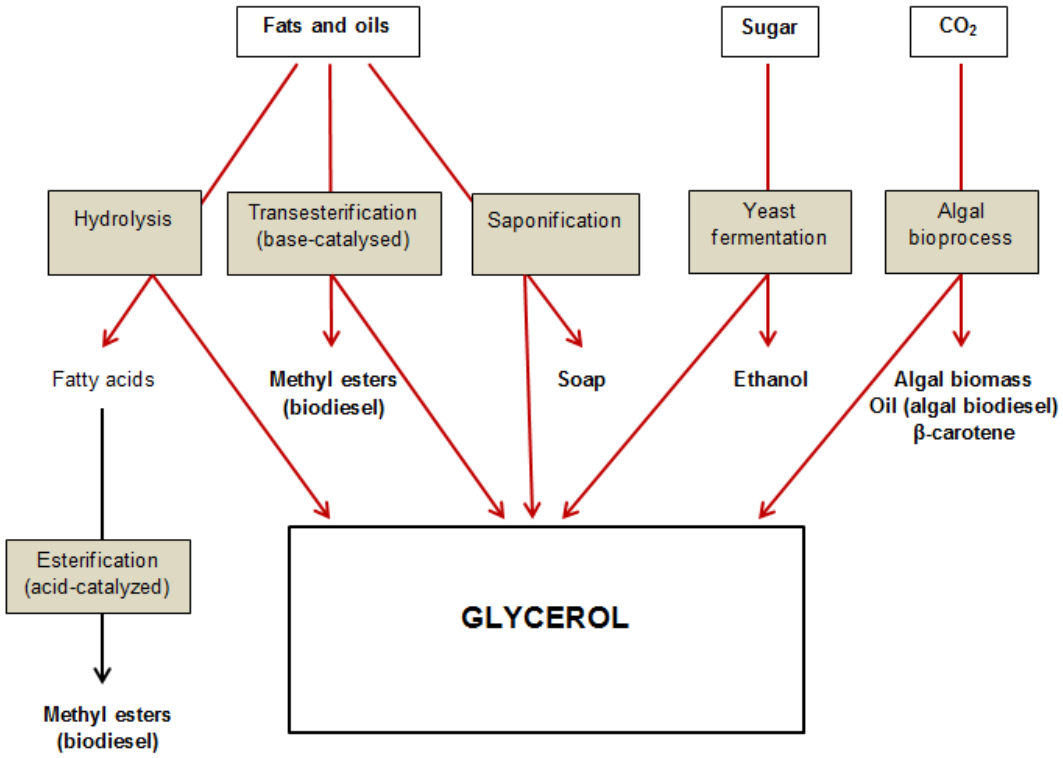

Figure 2.1. Industrial processes yielding glycerol as a by-product. (Modified from Shams Yazdani and Gonzalez 2007).

2.1.1. Biodiesel industry

Among the wide variety of industrial processes yielding glycerol, biodiesel industry is the most significant due to high glycerol yield and large scale of biodiesel production. Biodiesel is defined as vegetable oil or animal fat based mono-alkyl ester fuel (Knothe 2010). The chemical composition of biodiesel is significantly different compared to diesel fuel, as diesel is composed mainly of hydrocarbons, namely alkanes, cycloalkanes and aromatic hydrocarbons (Bacha *et al.* 2007). In some contexts, the term 'biodiesel' is used for any renewable lipid-based fuel such as diesel-type hydrocarbon fuels produced by hydrogenation of vegetable oils or animal fats. According to Knothe (2010), these fuels should, actually, be termed as 'renewable diesel'.

As shown in Figure 2.2, rapid growth of biodiesel industry lately has led to a drop in price of glycerol. Biodiesel refineries have limited options managing glycerol streams, and glycerol might become a waste material. In 2012, Web Greenchemical special report (Bacha *et al.* 2007) approximated the world stock for glycerol as 900 000 t. Therefore, in order to solve the glycerol glut and increase the economical viability of biodiesel production, new glycerol utilizing applications are needed.

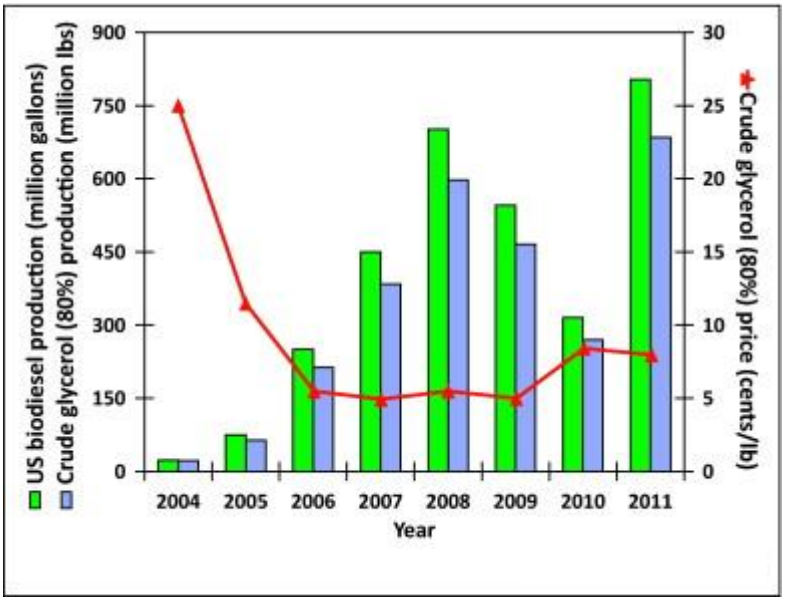

Figure 2.2.US biodiesel and biodiesel-based glycerol production along with crude (80 %) glycerol prices between 2004 and 2011. (Adapted from Clomburg and Gonzalez 2013).

Biodiesel is a renewable alternative for petroleum-based diesel fuel as it is derived from oils and fats of biomass origin and fully compatible with compression-ignition engines (diesel engines). As reviewed by Huang *et al.* (2010) and van Gerben (2005), the benefits of biodiesel are:

- ✓ Markets for excess vegetable oils and animal fats.

- ✓ Decreased dependence of nonrenewable petroleum.
- ✓ Renewability and carbon neutrality.
- ✓ Lower emissions of harmful carbon monoxide, unburned hydrocarbons and particles compared to petroleum. Although, the emission of nitrogen oxides at slightly higher level.
- ✓ No aromatic compounds or other chemicals which are detrimental for environment.
- ✓ Biodiesel has higher flash point and biodegradability compared to diesel fuel.

For reviews on biodiesel process, see van Gerpen (2005), Leung *et al.* (2010), Ma and Hanna (1999), and Meher *et al.* (2006). Transesterification is currently almost exclusively used for biodiesel production. The aim of the production process is to lower the viscosity of oil or fat, which is attained in transesterification with alcohol (Ma and Hanna 1999). In the reaction, as triglyceride and alcohol react, esters and glycerol are formed as is shown in Figure 2.3. Alcohols such as methanol, ethanol, propanol, and butanol can be used in the process but the most commonly used is methanol (Sanli and Canakci 2008). Methanol is an inexpensive chemical and thus advantageous for large scale transesterification processes. Additionally, it is capable for rapid reaction with triglycerides (Sanli and Canakci 2008). Majority of methanol is currently produced from non-renewable natural gas (Methanol Institute 2011c) which decreases the environmental impact of biodiesel. On the other hand, biologically produced methane (biogas) from organic material including glycerol (Methanol Institute 2011a, Voegele 2011) as well as CO_2 originating from geothermal or industrial emission along with H_2 (Methanol Institute 2011b) are suitable renewable ingredients for methanol production.

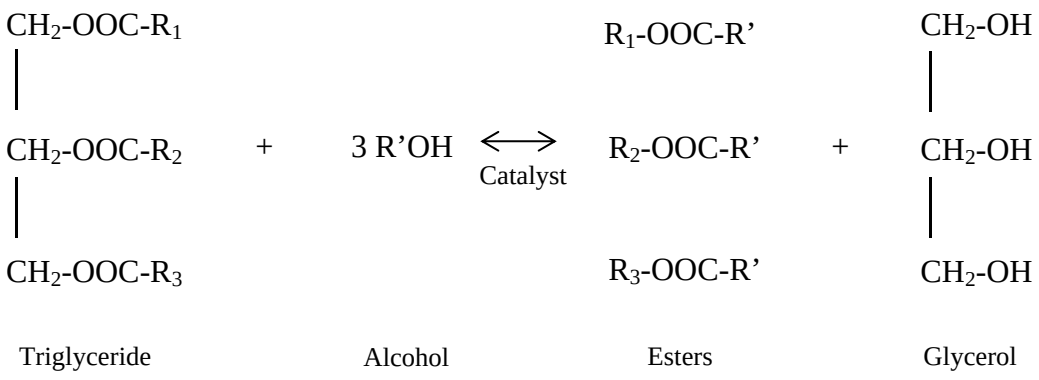

Figure 2.3. Transesterification reaction of triglycerides and alcohol in which esters and glycerol are formed. R1, R2, and R3 contain typically 16 or 18 carbons and from zero to three carbon-carbon double bonds. (Modified from Ma and Hanna 1999)

Transesterification reaction is reversible and alcohol, despite theoretically 3:1 alcohol-triglyceride ratio is enough for a complete reaction, is typically used in excess amount (e.g. 6:1 with alkalicatalyst) in order to shift the equilibrium towards esters and glycerol production (Colucci *et al.* 2005). A transesterification reaction for biodiesel production yields 10 kg of glycerol per 100 kg biodiesel.

The reaction is most commonly catalyzed by an alkaline catalyst, such as sodium and potassium hydroxides and alkoxides (Singh *et al.* 2006). Most industrial scale transesterification processes apply NaOH or KOH due to their low price. Instead of alkaline catalyst, acidic (e.g. sulfuric, sulfonic and hydrochloric acids, zinc stearate) (Al-Widyan and Al-Shyoukh 2002, Jacobson *et al.* 2008, Peng *et al.* 2008, Zheng *et al.* 2006, Zullaikah *et al.* 2005) or enzymatic (e.g. lipase) catalysis is applicable (Dossat *et al.* 1999, Forssell *et al.* 1993, Ghazali *et al.* 1995, Moreira *et al.* 2007, Xu *et al.* 2003). However, acid catalysts are claimed being less rapid and enzymatic catalysts less economical compared to alkaline catalysts (Leung *et al.* 2010, Ma and Hanna 1999). Additionally, a catalyst-free method for transesterification has been invented by Saka and Kushdiana (2001). This method applies supercritical alcohol at high temperature (350-400 ^{o}C) and pressure (45-65 MPa) yielding higher reaction rate compared to alkali-catalyzed reactions. The economic efficiency of the catalyst-free biodiesel process, however, remains unclear due to the highly energy consuming process conditions.

The main factors affecting the transesterification reaction are moisture, free fatty acids, triglyceride-alcohol ratio, type of alcohol, type and amount of catalyst, mixing intensity, and reaction temperature (Colucci *et al.* 2005, Keera *et al.* 2011, Leung and Guo 2006, Ramadhas *et al.* 2005, Rashid and Anwar 2008, Sanli and Canakci 2008). With alkaline catalyst, the water and free fatty acid content of the reactants need to be low in order to prevent unintended saponification and obtain maximum esters yield. Triglycerides with high water or free fatty acid content can be pre-treated by saponification with alkali or esterification with acid catalyst prior to transesterification (Berchmans and Hirata 2008, Ghadge and Raheman 2005, Ma and Hanna 1999).

A typical biodiesel production process by transesterification is schematically shown in Figure 2.4. After transesterification, glycerol fraction is separated from methyl esters either by centrifugation or settling in a tank. The glycerol fraction separated at this stage has glycerol content of approximately 50-60 % and contains significant amounts of soaps, methanol, and catalyst. In many industrial scale biodiesel production processes, the hazardous raw glycerol fraction is further processed in order to remove free fatty acids (FFA) by acidulation and methanol (alcohol) by evaporation. The glycerol fraction after the pretreatment steps has glycerol content of approximately 80-85 % and significantly decreased alcohol and FFA contents. In practice, both the untreated and the

pretreated glycerol fractions are called as crude (raw) glycerol and thus the characteristics of the glycerol fraction vary significantly: According to SRS International (2013) specifications, the glycerol content of biodiesel derived fraction is 40-88 %. In order to differentiate the two glycerol fractions with significantly different grades, the untreated glycerol fraction is termed as raw glycerol and the neutralized and alcohol removed glycerol fraction as crude glycerol in the context of this thesis. Similar classification has been used previously by Posada and Cardona (2010).

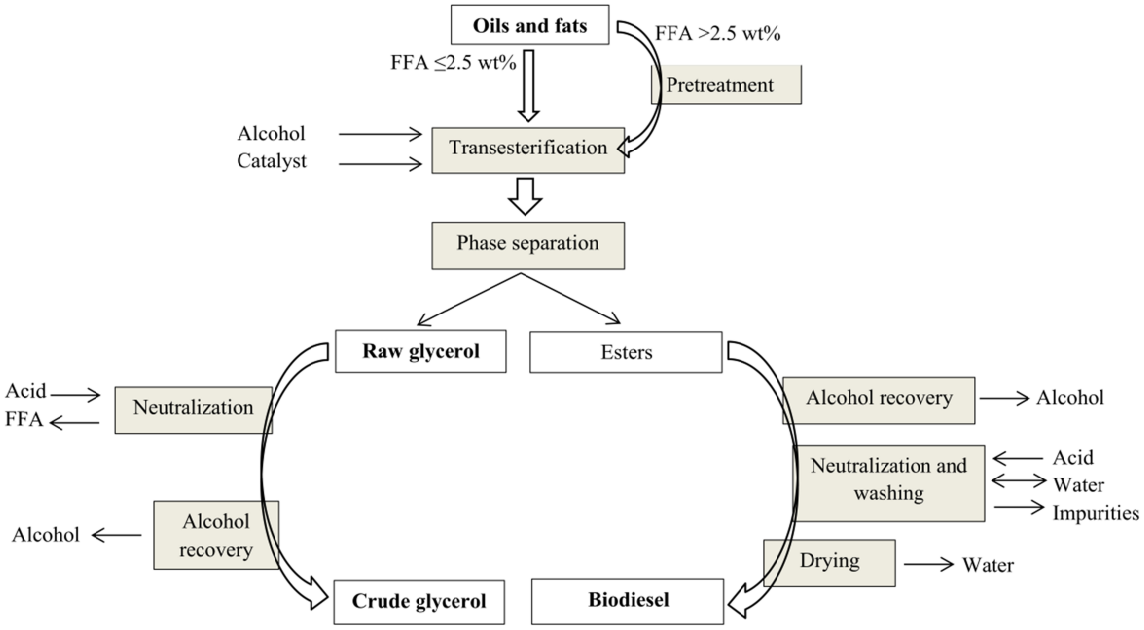

Figure 2.4. Flow chart for biodiesel production by transesterification. (Modified from Leung et al. 2010, Van Gerpen 2005, Waste Management and Research Centre 2006)

After separation of the glycerol fraction, the methyl ester solution is further processed as follows; methanol is removed by stripping, free fatty acids and salts are precipitated by acid treatment and residual catalyst, soap, salts, alcohol and glycerol are washed away with water. Eventually the fraction is dryed (water removal) before the final biodiesel product is collected.

2.1.2. Glycerol fraction contents and purification

Depending on the industrial origin of glycerol, characteristics of raw materials as well as process conditions and layout, the contents of glycerol fractions vary enormously. The major ‘impurities’ of glycerol fractions from different industrial processes are shown in Table 2.1.

Table 2.1.Major components of industrial glycerol fractions.

Industrial process	Glycerol [wt%]	Alcohol [wt%]	Salt [wt%]	Other components	References
Transesterification	56-90	0.01-28	1.3-6	soap, ash	
Raw glycerol[a]	52-62	13-28	1.3-3.1	soap	Pyle *et al.* 2008, **IV-V**[c]
Crude glycerol[b]	81-90	< 0.2	4-6	ash	Metsoviti *et al.* 2013
Saponification	6-20.2	NR	10-64.3	NR	Israel *et al.* 2008, Ooi *et al.* 2004
Yeast fermentation	0.1-0.3	NR[d]	NR	Nutrients	Bideaux *et al.* 2006, Romano *et al.* 1997
Algal bioprosess	0.06	ND	17	small amounts of sugars and lipids	Santos *et al.* 2001

ND, not detected; NR, not reported
[a] Fraction separated from esters fraction with no purifying treatments.
[b] Fraction with alcohol and soaps removed.
[c] Publications of this thesis.
[d] Alcohol is collected from the fraction.

The main impurities of glycerol fraction originating in a transesterification process are methanol, salts, soap, and ash. In addition, depending on the origin of raw material of the biodiesel process, the raw glycerol may contain heavy metals. The characteristics of the glycerol fraction vary significantly due to the differences in the biodiesel processes: Many processes pretreat the glycerol fraction at the biodiesel plant yielding to higher grade crude glycerol (80-90 %) with efficiently removed alcohol and soaps (free fatty acids) but elevated salt content (Metsoviti *et al.* 2013). On the other hand, lower grade raw glycerol fractions (50-60 % glycerol, methanol, soaps) are obtained from the biodiesel plants that do not apply any pretreatment steps for the glycerol fraction (Pyle *et al.* 2008, **IV-V**).

Yeasts produce glycerol as by-product in the formation of ethanol. The glycerol formation decreases the ethanol formation efficiency and therefore the industrial ethanol production processes aim at minimal glycerol production. The final glycerol content of the fermentation broth after ethanol fermentation is 0.1-0.3 % and therefore the glycerol recovery is not economically feasible (Bideaux *et al.* 2006, Romano *et al.* 1997).

In algal cultures, glycerol is produced in order to protect the cells for osmotic pressure. Therefore, glycerol is produced according to the salinity of the environment: The higher the salinity, the higher the glycerol production. However, most of the glycerol produced remain inside the algal cells and is not extensively secreted to the growth medium: The analysis of waste stream from *Dunaliella salina* carotenogenesis revealed low glycerol content but high salt content (Santos et al. 2001). Higher glycerol content is obtainable by lysis of the algal cells.

The impurities of glycerol obtained from transesterification process might cause problems for its usage. If necessary, soaps and alcohol are easily removed as discussed

in Section 2.1.1. Addition of acid causes the dissolved soaps to split to insoluble free fatty acids and salts. The free fatty acids precipitate on top of the glycerol fraction and can be removed. Depending on the acid used, salts either remain soluble in the glycerol fraction or precipitate. Unlike most salts, phosphate salts formed with phosphorous acid treatment are generally poorly soluble. Alcohols have low boiling points and are volatile and therefore alcohols can be removed by evaporation, heating or stripping. From an economical point of view, the less pretreatment steps are needed, the better.

2.2. Glycerol utilization

Glycerol can be used chemically as an intact chemical in various applications or as a reactant in chemical reactions and biologically as a substrate in microbial processes. Since huge amount of glycerol is produced continuously, it has been suggested that both chemical and biological glycerol utilizing applications will be needed in future in order to degrade the glycerol surplus (Johnson and Taconi 2007). When the products of glycerol degradation have higher value than the starting material, large markets and less hazardous nature, the glycerol utilization increases also the economical and environmental impact of the original industrial process.

2.2.1. Chemical applications

Glycerol is used in various industrial products as an intact ingredient. The distribution of glycerol utilization among different products and industries is presented in Table 2.2. Most of glycerol utilization goes into food (23 %), oral care (20 %), cosmetics (13 %), and tobacco (12 %) industries (Biofuels Technology LLC). In food products refined glycerol is used as such as sweetener, thickener, humectant or solvent. Especially in animal food products, glycerol is used as a food additive as it is a good energy source. The oral care industry applies refined glycerol in toothpaste to replace sorbitol. Additionally, glycerol has good moisturizing characteristics and thus it is generally used in cosmetic products such as skin creams, glycerine-soaps, and shampoos. Cigarettes contain 1-5 % glycerol due to the moisture-holding and flavoring characteristics (Carmines and Gaworski 2005).

Table 2.2. Distribution of the use of glycerol among different products and industries. (Data from Biofuels Technology LLC)

Industry	Percent consumption (%)
Food	23
Oral Care	20
Cosmetics	13
Tobacco	12
Drugs	9
Miscellaneous	9
Plastics	8
Alkyds	3
Cellophane and meat casing	2
Explosives	1

The most important products derived chemically from glycerol are propanediols (Johnson and Taconi 2007). 1,2-Propanediol (propylene glycol) have applications as a copolymer in polyester resins and polyurethanes and as a humectant, solvent or preservative in food and tobacco products. Additionally, it is used as an ingredient in cosmetic and pharmaceutical products. The constitutioned isomer of 1,2-propanediol, 1,3-propanediol, on the other hand has increasing markets as a component of biodegradable polymers such as polytrimethylene terephtalate. Other applications include polyesters, films and coatings.

The impurities of raw glycerol set challenges for its usage. For example, when considering glycerol as an additive in animal feed, toxic alcohol needs to be minimized. Additionally, residual potassium ions have shown to cause wet litter or imbalance in dietary electrolyte levels in broilers (Cerrate *et al.* 2006).

2.2.2. Biological applications

A number of microorganisms are able to grow aerobically using glycerol as a sole carbon source including eukaryotic yeasts and molds from genera *Saccharomyces* (Amaretti *et al.* 2012, Jung *et al.* 2011), *Yarrowia* (Papanikolaou *et al.* 2002), *Candida* (Amaretti *et al.* 2012), *Pichia* (Amaretti *et al.* 2012) and *Rhodotorula* (Easterling *et al.* 2009) and prokaryotic bacteria from genera *Klebsiella* (Slininger and Bothast 1985) and *Gluconobacter* (Hu *et al.* 2011). Furthermore, a number of bacteria, belonging to genera of *Citrobacter* (Anand and Saxena 2012, Mangayil *et al.* 2012, Metsoviti *et al.* 2013, Oh *et al.* 2003), *Clostridium* (Biebl 2001, Chatzifragkou *et al.* 2010, Chatzifragkou *et al.* 2011, Forsberg 1987, Gonzalez-Pajuelo *et al.* 2005, González-Pajuelo *et al.* 2006, Heyndrickx *et al.* 1991, Papanikolaou *et al.* 2000, Petitdemange *et al.* 1995, Seppälä *et al.* 2011, Wilkens *et al.* 2012, **I-V**), *Enterobacter* (Ito *et al.* 2005, Reungsang *et al.* 2013, Sarma *et al.* 2013), *Escherichia* (Dharmadi *et al.* 2006, Durnin *et al.* 2009, Murarka *et al.* 2008, Seppälä *et al.* 2011, **I**), *Halanaerobium* (Cayol *et al.* 1994b, Cayol *et al.* 1995, Zhilina *et al.* 1992, **I-V**), *Klebsiella* (Forage and Foster 1982, Huang *et al.*

2002, Liu and Fang 2007, Metsoviti *et al.* 2012, Mu *et al.* 2006, Oh *et al.* 2011) and *Lactobacillus* (Pflügl *et al.* 2012) are able to grow anaerobically on glycerol. Glycerol as a small neutral molecule diffuses readily across cell membranes. However, the passive uptake is typically enhanced (especially at low substrate conditions) by an integral membrane protein, glycerol fasilitator GlpF (Heller *et al.* 1980). Glycerol utilizing bacteria degrade glycerol as substrate of their assimilative metabolism and derive several valuable metabolites and cellular compounds including dihydroxyacetone, 1,3-propanediol, H_2, CH_4 (methane), ethanol and butanol, among others. Examples of aerobic and anaerobic glycerol degradation pathways along with putative higher-value-products are shown in Figure 2.5.

Aerobic dihydroxyacetone (DHA, also called as glycerone) production by *Gluconobacter oxidans* is an example of currently existing industrial glycerol utilizing biological applications (Gupta *et al.* 2001). DHA is widely used in cosmetic industry, for instance, as a component in sunless tanning products. Due to tight safety requirements concerning its chemical production including hazardous chemical reactions, biological production of DHA is more economical compared to the chemical alternative (Hekmat *et al.* 2003). Currently, the DHA production is known as the only biological glycerol degrading industrial application.

Biological production of 1,3-PD is a good alternative for the traditional chemical production process. Glycerol is degraded to 1,3-PD by a group of bacteria along with a route including two enzymatic steps. According to the best of my knowledge, no industrial glycerol degrading 1,3-PD production processes exist, though it is a fascinating application with high production yields. Instead, a genetically modified bacterial process is utilized for biological 1,3-PD production from glucose by DuPont (DuPont 2013; Emptage *et al.* 2003). Biological 1,3-propanediol production is discussed in more detail in Section 4.

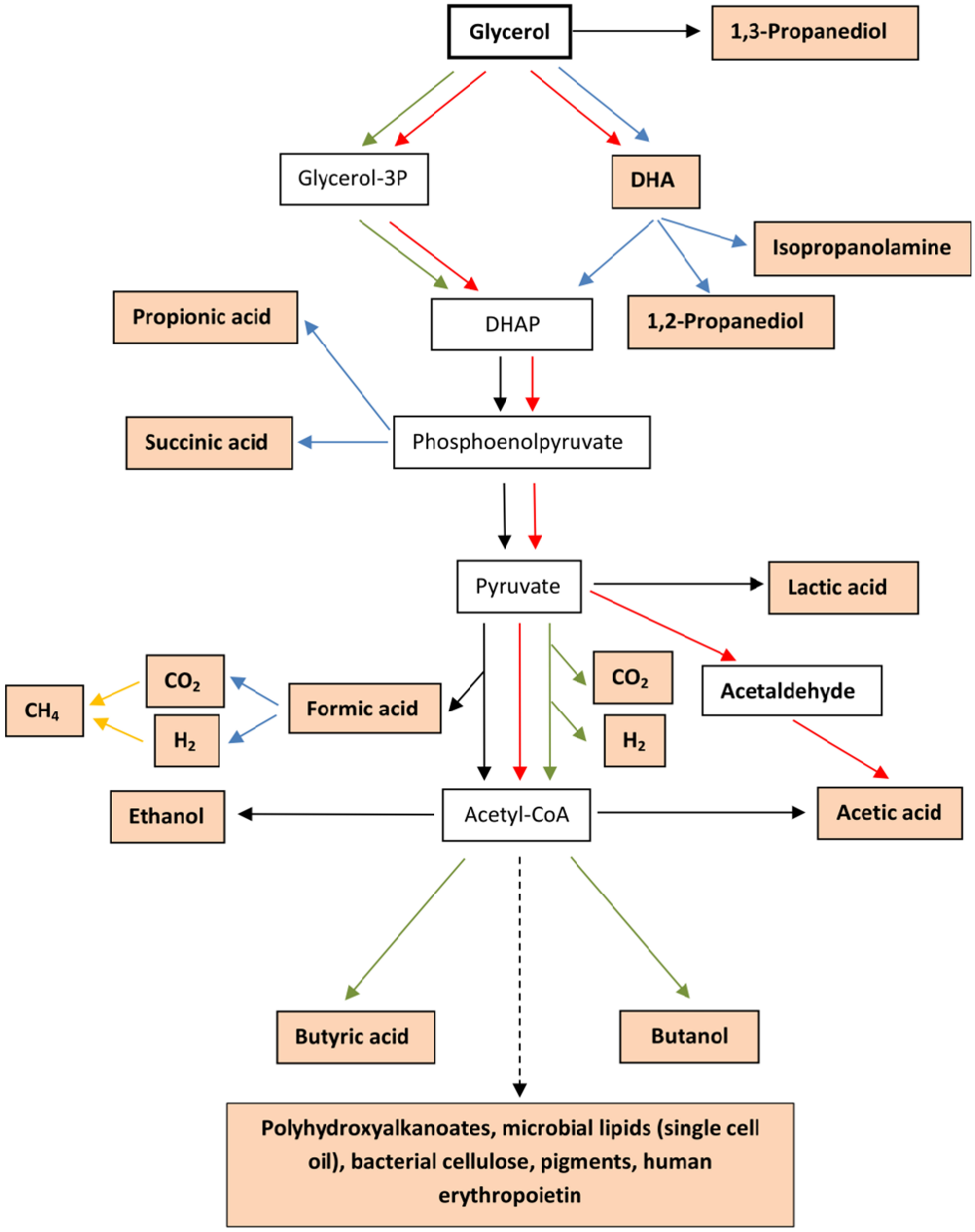

Figure 2.5. Putative end-products and routes for aerobic and anaerobic glycerol degradation. The putative aerobic routes are shown with red arrows (Richhardt et al. 2012), whereas the fermentative enteric pathways (Murarka et al. 2008) as blue arrows and clostridial (Saint-Amans et al. 2001) as green arrows, and methanogenic as orange arrows. The common fermentative routes for both clostridial and enteric microorganisms are shown as black arrows. The broken arrow implies other potential degradation products. (Modified from da Silva et al. 2009, Mattam et al. 2013)

Biologically produced ethanol and butanol are relevant alternatives as biofuels. The costs of a biodiesel derived glycerol-based bioethanol process have been calculated being 40 % less than a corn-based process (Yazdani and Gonzalez 2007). Crude glycerol (concentration in experiment 25 g/l; purity 80 %) has been fermented to 27 g/l (yield 0.8 mol/mol) and 25 g/l (yield not reported) ethanol by *Kluyvera cryocrescens* mutant strain (Choi *et al.* 2011) and *Klebsiella pneumonia* mutant strain (Oh *et al.* 2011), respectively. Furthermore, Ito *et al.* (2005) reported ethanol production at yield

0.85 mol/mol from raw glycerol using *Enterobacter aerogenes*. Recently a special interest has risen for butanol due to its superior characteristics compared to ethanol (Green 2011). *Clostridium pasteurianum* has proven to be efficient butanol producer with production level of 17 g/l and 0.4 mol/mol on pure glycerol (Biebl 2001) and 12.6 g/l and 0.3 mol/mol on crude glycerol (Jensen *et al.* 2012).

After the boost of biodiesel industry in the beginning of the 21st century and the consequencing glycerol glut, plenty of research efforts have been made concerning various putative glycerol utilizing applications including biodegradable polyesters (polyhydroxyalkanoates) for bioplastics (Ashby *et al.* 2011), amino acids (Rittmann *et al.* 2008), 1,2-propanediol (propylene glycol) (Altaras and Cameron 1999, Clomburg and Gonzalez 2011, Jung *et al.* 2008, Jung *et al.* 2011), microbial lipids (also called as single cell oils) e.g. for biodiesel production (André *et al.* 2010, Meesters *et al.* 1996, Papanikolaou *et al.* 2008, Papanikolaou and Aggelis 2009), pigments (Kusdiyantini *et al.* 1998, Tao *et al.* 2005), bacterial cellulose (Carreira *et al.* 2011) and human erythropoietin (Çelik *et al.* 2008).

3. DARK FERMENTATIVE HYDROGEN PRODUCTION

Review **RII** is the main basis of this chapter. Hydrogen (H_2) is considered as one of the most potential alternatives for future energy carrier molecules (Das and Veziroğlu 2001). It is is environmental friendly and clean; its combustion yields high amount of energy and water as the sole end product. The sustainability of H_2 as an energy molecule, however, depends on the origin of the energy H_2 molecule carriers, i.e. the H_2 production process. The chemical methods either derive H_2 from non-renewable material (fossil fuels) or require high energy input (water splitting) and thus are considered un-sustainable. Biological methods derive H_2 from renewable sources such as biomass or sunlight in processes employing microorganisms. The biological methods are either light-dependent or light-independent. The light-dependent methods include biophotolysis and photofermentation. Biophotolysis by microalgae or cyanobacteria utilizes sunlight in order to split water whereas photofermentation by purple photosynthetic bacteria uses sunlight along with organic substrate for H_2 production. The methods, however, face several challenges including low light-conversion efficiencies and costly photobioreactor systems, and potentially require years of research in order to become economically feasible in large scale (Hallenbeck *et al.* 2012). The light-independent process is suggested being the most promising choice for sustainable large-scale H_2 production in the near future (Hallenbeck *et al.* 2012). This light-independent method, dark fermentation, employs bacteria which in their anaerobic metabolism naturally derive H_2 from organic material. The fermentative bacteria can combine H_2 production with degradation of organic wastes, e.g. food waste, sewage sludge, agricultural residues or industrial waste materials (Bala-Amutha and Murugesan 2013, Mangayil *et al.* 2012, Pott *et al.* 2013, Reungsang *et al.* 2013, Sarma *et al.* 2013, Scoma *et al.* 2013, Singh *et al.* 2013, Zhou *et al.* 2013).

Several bacteria are able to produce H_2 as a part of their anaerobic metabolism. The bacteria include facultative anaerobes as well as strict anaerobes, non-extremophiles as well as extremophiles. In nature a typical bacterial community contains both H_2 producers and H_2 utilizers and thus the H_2 gas does not accumulate in the ecosystem. A typical scheme for dark fermentative H_2 production is shown as Figure 3.1. The fermentative bacteria combine H_2 production with degradation of organic material, e.g. plant biomass (energy crops), organic waste materials (food waste, sewage sludge, agricultural residues or industrial organic waste), or industrial by-products (glycerol). Energy crops as substrates have rose discussion concerning the ethicality of utilizing land area applicable for food production. Waste materials, on the other hand, have no

such ethical contradictions. The waste materials most suitable for economical bioprocesses have good availability, low or negative cost, high carbohydrate content, and good biodegradability (Kapdan and Kargi 2006). Depending on the raw material and the characteristics of microbial culture in the fermentation, the organic material requires pretreatment including mechanical grounding (plant biomass), chemical or enzymatic hydrolysis (plant biomass), detoxification (plant biomass, crude glycerol) and nutritional balancing (industrial waste waters) (Kapdan and Kargi 2006). Pretreatment steps, however, require electricity and are considered as fossil fuel dependent decreasing the positive environmental effect of biohydrogen production. In addition, pretreatment increases the costs of the production process. Thus, the aim is to reduce the economical and environmental effect of the pretreatment e.g. by search for microorganisms that can degrade cellulose/hemicellulose without the need for separate hydrolysis, or that remain active in harsh waste streams without need for waste detoxification.

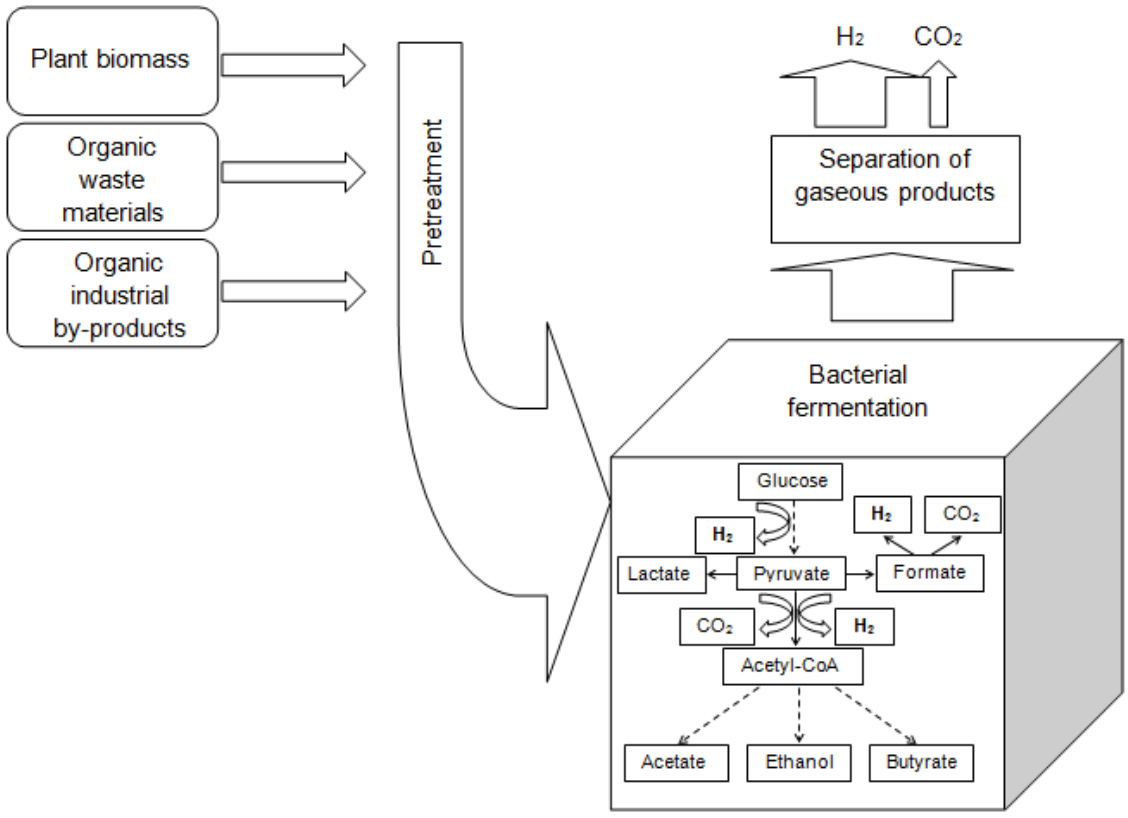

Figure 3.1. Dark fermentative H_2 production process.

Most often microbial H_2 production is connected with anaerobic metabolism of pyruvate which is a typical intermediate product formed in cellular catabolic routes from most organic substrates. During the degradation of organic substrates to pyruvate, NAD^+ (nicotinamide adenine dinucleotide, oxidized form) is used as coenzyme leading

to formation of its reduced form, NADH. Two molecules of NADH are produced per one molecule of glucose degraded to pyruvate (pyruvic acid):

$$\text{Glucose} + 2\ NAD^+ \rightarrow 2\ \text{Pyruvate} + 2\ \text{NADH} + 2H^+ \quad (3.1)$$

Pyruvate is further metabolized to acetyl coenzyme A (acetyl-CoA) in reactions catalyzed by puryvate-formate lyase (Eq. 3.2) and pyruvate-ferredoxin oxidoreductase (Eq. 3.3) yielding also either formate (formic acid) (Eq. 3.2) or reduced ferredoxin (fd (red)) and CO_2 (Eq. 3.3):

$$\text{Pyruvate} + \text{CoA} \rightarrow \text{acetyl-CoA} + \text{formate} \quad (3.2)$$

$$\text{Pyruvate} + \text{CoA} + 2\ \text{Fd (ox)} \rightarrow \text{acetyl-CoA} + 2\ \text{Fd (red)} + CO_2 \quad (3.3)$$

Oxidation of formate (to CO_2), reduced ferredoxin (to oxidized ferredoxin) or NADH (to NAD^+) yields electrons for the reduction of protons to molecular H_2 catalyzed by hydrogenases (Crable *et al.* 2011):

$$2\ H^+ + 2\ e^- \leftrightarrow H_2 \quad (3.4)$$

In general, facultative anaerobic (enteric-type) fermentation derives hydrogen from formate, whereas strictly anaerobic (clostridial-type) fermentation from ferredoxin- and NADH-bound electrons. Clostridial-type H_2 production has an important role in disposing excess reducing equivalents in anoxic conditions whereas the main role of enteric-type H_2 production is pH control (Hallenbeck 2009).

Dark fermentative H_2 producing bioprocess yields not pure H_2 but a mixture of gases containing mainly H_2 and CO_2 but potentially also methane (CH_4), hydrogen sulfide (H_2S) and/or carbon monoxide (CO) (Levin *et al.* 2004). The maximum H_2 yield as well as the ratio of H_2 to CO_2 depends on the metabolic characteristics of the microbial culture and process conditions. Theoretically, a complete oxidation of hexose sugar would release 12 moles H_2 per hexose mol:

$$\text{Glucose} + 6\ H_2O \rightarrow 12\ H_2 + 6\ CO_2 \quad (3.5)$$

However, in dark fermentative metabolism most of the hydrogen from substrate is bound to by-products like acetate, butyrate, ethanol, butanol, propionate, or lactate decreasing the H_2 yield significantly. The formate-based H_2 production by facultative anaerobes has been predicted to yield a theoretical maximum of 2 mol H_2/mol hexose (Hallenbeck 2009):

$$\text{Glucose} + 2\ \text{CoA} + 2\ NAD^+ \rightarrow 2\ \text{Acetyl-CoA} + 2\ \text{Formate} + 2\ \text{NADH} + 2\ H^+ \quad (3.6)$$

$$2\ \text{Formate} \rightarrow 2\ H_2 + 2\ CO_2 \quad (3.7)$$

Higher yields are obtainable by strict anaerobes as a theoretical maximum of 4 mol H_2/mol hexose can be reached when acetate (acetic acid; Eq. 3.8)) is the sole liquid end-product. When butyrate (butyric acid; Eq. 3.9) is produced as the only liquid metabolite the maximum yield is 2 mol H_2/mol hexose (Levin *et al.* 2004):

$$\text{Glucose} + 2\ H_2O \rightarrow 2\ \text{Acetate} + 4\ H_2 + 2\ CO_2 \quad (3.8)$$

$$\text{Glucose} + 2\ H_2O \rightarrow \text{Butyrate} + 2\ H_2 + 2\ CO_2 \quad (3.9)$$

The maximum yields are given for a hexose sugar substrate. If considering glycerol as a substrate, the theoretical H_2 yield obtainable by dark fermentation is 3 mol H_2/mol glycerol with acetate as the sole liquid metabolite (Thauer *et al.* 1977):

$$\text{Glycerol} + H_2O \rightarrow \text{Acetate} + 3\ H_2 + CO_2 \quad (3.10)$$

Production of reduced molecules, such as alcohols, short chain fatty acids (butyrate, propionate), organic acids (lactate) and amino acids (alanine), as main fermentation products results in lower H_2 yields. In practice, microbes tend to produce mixtures of liquid metabolites leading to decreased H_2 yields. The microbial metabolism can be directed towards the most favorable pathways via optimizing process conditions (pH, partial H_2 pressure, etc.) or metabolic engineering (Hallenbeck *et al.* 2012, Mathews and Wang 2009). When considering a H_2 producing mixed culture, the metabolic variety is generally even greater compared to a pure culture and the metabolic engineering is challenging. The function of mixed culture can be improved by process conditions inhibiting the adverse microbial activity.

3.1. Hydrogenases

The enzymes catalyzing reversible proton reduction (Eq. 3.4) are classified based on the main function of the enzyme and metal atoms attached to the active site into four groups: nitrogenases, [FeFe]-, [NiFe]-, and [Fe]-hydrogenases. Instead of H_2 production, the main function of nitrogenase is nitrogen fixation and H_2 is produced at certain conditions as a by-product. Dark fermentative H_2 production using electrons from formate and reduced ferredoxin/NADH are catalyzed generally by [NiFe]- and [FeFe]-hydrogenases, respectively. Therefore, the [NiFe]-hydrogenase plays the main role in facultative anaerobes and the [FeFe]-hydrogenase in strict anaerobes though [NiFe]-hydrogenases are found being encoded by some strict anaerobes (Schut and Adams 2009) and [FeFe]-hydrogenases by few facultative anaerobes (Mishra *et al.* 2004). The turnover rate of [FeFe]-hydrogenase is approximately ten times the turnover

rate of [NiFe]-hydrogenase (Frey 2002) and, in general, the role of [FeFe]-hydrogenase is to evolve H_2 whereas the role of [NiFe]-hydrogenase is to consume H_2.

Although most NiFe-hydrogenases rather degrade than generate H_2, a small group of membrane-bound H_2 evolving [NiFe]-hydrogenases (Ech-hydrogenases) have been identified from several bacteria including both enteric and clostrial representatives. Unlike most [NiFe]-hydrogenases, the Ech-hydrogenase generates H_2 using ferredoxin-derived electrons. The structure of the hydrogenase along with genetic organization of Ech-genes (A-F) is shown in Figure 3.2. The Ech-hydrogenase comprises of six subunits (A-F) and the genes for subunits of Ech-hydrogenase are typically clustered into an operon in genomes.

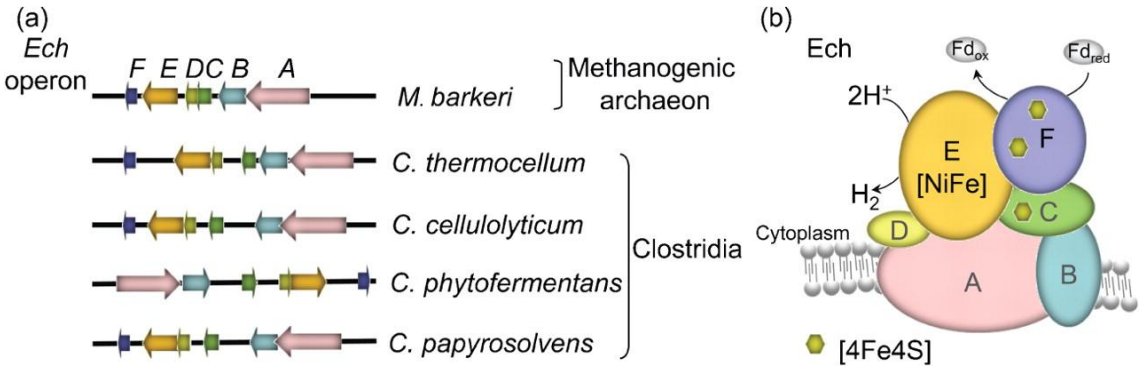

Figure 3.2. Schematic structure of Ech-hydrogenase (b) and genetic organization of Ech-operon in Methanosarcina barkeri, Clostridium thermocellum, Clostridium cellulolyticum, Clostridium phylofermentans and Clostridium papyrosolvens (a).(Modified from Calusinska et al. 2010)

As reviewed by Calusinska *et al.* (2010), several types of [FeFe]-hydrogenases have been characterized. They occur either as monomers, dimers, trimers, or tetramers. As is the case for [NiFe]-hydrogenases, the genes for [FeFe]-hydrogenase subunits are generally clustered into an operon. The molecular structure of the catalytic subunit differs between the different types of [FeFe]-hydrogenases. The catalytic core, termed as H cluster is present in the catalytic subunit of hydrogenase, along with numerous iron-sulfur cluster (2[4Fe4S], [4Fe4S], [2Fe2S], etc.) binding domains located in the catalytic subunit and/or accessory subunits.

The electrons for H_2 formation by [FeFe]-hydrogenases are obtained from reduced ferredoxin and/or NADH. In 2004, an NADH-dependent [FeFe]-hydrogenase (Figure 3.3) was characterized for *Thermoanaerobacter tengcongensis* (Soboh *et al.* 2004), although the mechanism for the endergonic reaction catalyzed by the hydrogenase remains unclear. In 2009, a bifurcating hydrogenase oxidizing both reduced ferredoxin and NADH synergistically was characterized for *Thermotoga maritima* (Schut and Adams 2009). In the same publication, the NADH-hydrogenase of *T. tengcongensis* is suggested actually being bifurcating which would explain its reaction mechanism. The accessory subunits of the putative bifurcating trimeric hydrogenase of *T. maritima*

include NuoF-domain protein (subunit β) and thioredoxin-like protein (subunit γ). The putative tetrameric bifurcating/NADH hydrogenase of *T. tengcongensis* has three subunits similar to the trimeric hydrogenase and additionally another thioredoxin-like protein (subunit δ).

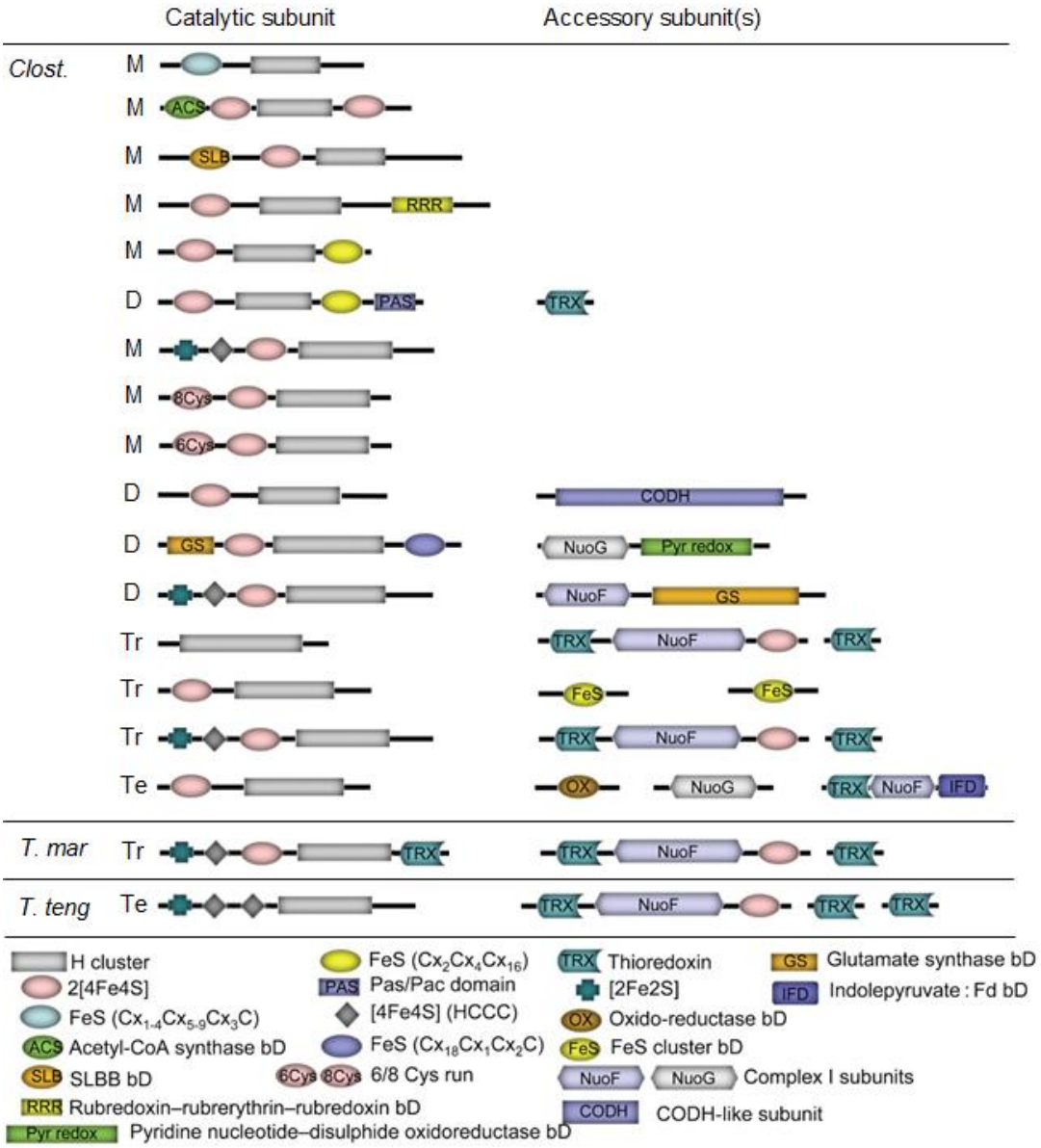

Figure 3.3. The catalytic subunit structure and accessory subunits of [FeFe]-hydrogenases in genus Clostridium (Clost.) and putative bifurcating hydrogenases in Thermotoga maritima (T. mar) and Thermoanaerobacter tengcongensis (T. teng). Hydrogenases occur either as monomers (M), dimers (D), trimers (Tr) or tetramers (Te). Abbraviation: bD, binding domain. (Modified from Calusinska et al. 2010)

3.2. Extremophilic biohydrogen production

Extremophiles are microorganisms isolated from harsh environments that are not suitable for survival of most living organisms. Such extreme environments include hot springs, polar sites, dry deserts, salt or alkaline lakes and sulphuric pools, among others.

The cellular systems of extremophiles have adapted to the harsh conditions and the extreme living conditions are typically not only tolerated but actually required for survival. A significant biotechnological advantage of extremophiles in general is a potential for active functioning in harsh conditions bringing possibilities for utilization of complex raw materials including hot (thermophiles), salty (halophiles), acidic (acidophiles) or basic (alkaliphiles) waste streams. The enzymes produced by extremophiles might also be directly used in harsh industrial processes that are challenging for conventional enzymes.

A significant challenge in microbial bioprocesses is the contamination with unfavorable microorganisms. For example, the H_2 producing systems tend to contaminate with H_2 utilizing bacteria. This is especially a challenge for bioprocesses utilizing pure cultures that, in general, require closed (sterile) systems, whereas mixed cultures are applied at open (non-sterile) systems (Abreu *et al.* 2012, Show *et al.* 2011). Mixed cultures, however, are typically less efficient production systems due to wide range of active metabolic pathways yielding undesired products with few options for metabolic engineering (Antonopoulou *et al.* 2007, Masset *et al.* 2012). Contamination might not be a problem and thus sterility a non-necessity for extremophilic pure cultures in which the extreme process conditions prevent efficiently the growth of most other bacteria.

A special benefit for hypersaline H_2 producing bioprocess is a disabled growth of H_2 utilizing methanogens. Due to bioenergetic reasons the upper salt limitation for H_2 utilizing methanogenesis is approximated being 12 %, whereas halophilic fermentative bacteria are able to function at higher salinities, up to near salt saturation (Oren 1999, Oren 2001, Oren 2002). On the other hand the kinetics of H_2 production is most favourable at high-temperature conditions. Therefore, thermophiles have recently been extensively studied for H_2 production and are, out of extremophilic H_2 producers, by far the most studied group.

3.3. Future directions

Currently, inefficient H_2 production is a challenge for economically feasible dark fermentative process. Out of theoretical 12 moles of H_2 per mole hexose the natural dark fermentative pathways are limited to 4 moles as most of the H_2 is bound to reduced by-products. The increasing knowledge on enzymatic activities and tools for genetic engineering opens up possibilities for designing new metabolic activities possibly

circumventing the limitations of natural microbial metabolism. In addition, the economic significance of dark fermentation can be increased without need for synthetic biology by combining the process with utilization of reduced by-products for generation of H_2 or other valuable (energy) molecules. Acetate and butyrate, for example, are degradable for H_2 production via photofermentation (Srikanth *et al.* 2009) and electrohydrogenesis (Lalaurette *et al.* 2009). The photofermentation by purple photosynthetic bacteria uses sunlight along with organic substrate for H_2 production; however, this method faces challenges including low light-conversion efficiency and costly photobioreactor systems. Electrohydrogenesis is a method in which small electric currency is applied in order to convert the organic acids to H_2 in microbial electrolysis cells (MEC) (Lalaurette *et al.* 2009). The technology has been studied extensively during the recent years and promising development has been obtained, however, more research is needed to prove its practical potential (Hallenbeck and Ghosh 2009, Hallenbeck *et al.* 2012). Another attractive choice for improvement of dark fermentation efficiency is acetate utilizing methanogenesis, which combined to dark fermentative process results in co-generation of two energy molecules, H_2 and CH_4.

Extremophiles are fascinating microorganisms applicable for bioprocesses in complex conditions. On the other hand, the creation of extremophilic environment, if not obtained from a waste stream, demands energy input decreasing the economical and environmental impact of the process. Thus, the use extremophilic applications aiming for biohydrogen production will most prominently be limited to certain circumstances in which the process significantly benefits of the use of specific microbes. The greatest benefits of extremophiles are the decreased risk for adverse contaminations and capability for utilization of harsh waste streams as substrates, thus decreasing the need for hygienisation and pretreatment of the substrate. The extreme process conditions, however, set challenges for combined bioprocesses described above as the metabolic variety in extreme environments is narrower compared to non-extremophilic environments. The more narrow nature of metabolic activities is partially due to incomplete studies of extremophilic environments – new extremophilic species are characterized continuously but also metabolic limitations arising from bioenergetic reasons are known to occur (Oren 2001, Oren 2002).

4. BIOLOGICAL 1,3-PROPANEDIOL PRODUCTION

The chemical, 1,3-propanediol (1,3-PD; IUPAC name propane-1,3-diol; other names 1,3-dihydroxypropane trimethylene glycol) is a three-carbon diol with a molecular formula of $C_3H_8O_2$. It is a colourless, odourless and viscous liquid with good miscibility with water. 1,3-PD is a component widely used in polymer industry as a building block of polyethers, polyesters and polyurethanes.

Chemically 1,3-PD is produced from petroleum derivatives, including toxic intermediates and requiring a step where high hydrogen (H_2) pressure is applied (Sullivan 2000). An environmentally friendly alternative is to derive 1,3-PD biologically from glycerol. A group of bacteria, including species from genera *Klebsiella* (Mu *et al.* 2006), *Enterobacter* (Barbirato *et al.* 1995), *Citrobacter* (Anand and Saxena 2012), *Lactobacillus* (Pflügl *et al.* 2012), *Ilyobacter* (Stieb and Schink 1984), *Trichococcus* (van Gelder *et al.* 2012), *Clostridium* (Papanikolaou *et al.* 2000) (**I-II, V**), and *Halanaerobium* (**I-III, V**) are able to naturally produce 1,3-PD from glycerol. The biological production route, as presented in Figure 4.1, includes glycerol dehydration to 3-hydroxypropionaldehyde (3-HPA) by glycerol dehydratase and 3-HPA reduction to 1,3-PD by 1,3-propanediol dehydrogenase. The intermediate, 3-HPA, is a toxic compound. However, its turnover to 1,3-PD is rapid and no accumulation tends to occur (Malaoui and Marczak 2001). Glycerol is produced in high amounts as a by-product in biodiesel industry, which makes glycerol an inexpensive substrate for processes.

Fermentative 1,3-PD production has a long history as it was discovered already in 1881 by August Freund (Freund 1881). Few bacteria, such as *Escherischia coli* (Murarka *et al.* 2008), *Halanaerobium saccharolyticum* subsp. *senegalense* (**I-II**), and *Thermotoga neapolitana* (Ngo *et al.* 2011, Ngo and Sim 2012) have been shown to ferment glycerol in a non-1,3-PD-producing manner. Currently, glycerol is the sole substrate for 1,3-PD production by natural strains, i.e. though glycerol generation from glucose is known to occur, no natural bacteria coupling the reactions for glycerol generation and degradation to 1,3-PD have been characterized. On the other hand, such bacteria have been created using the tools of systems biology. For example, as glycerol was an expensive compound compared to sugars, DuPont developed a genetically modified *E. coli* strain that derives 1,3-PD from glucose with high titer (Emptage *et al.* 2003). Furthermore, since 2006, DuPont, Tate and Lyle have been biologically producing 1,3-PD, bio-PDO™, using glucose extracted from corn biomass as a substrate for a recombinant

production organism (DuPont 2013). Nowadays though, the price of glycerol is low (Figure 2.2) and glycerol has become an economically competitive substrate.

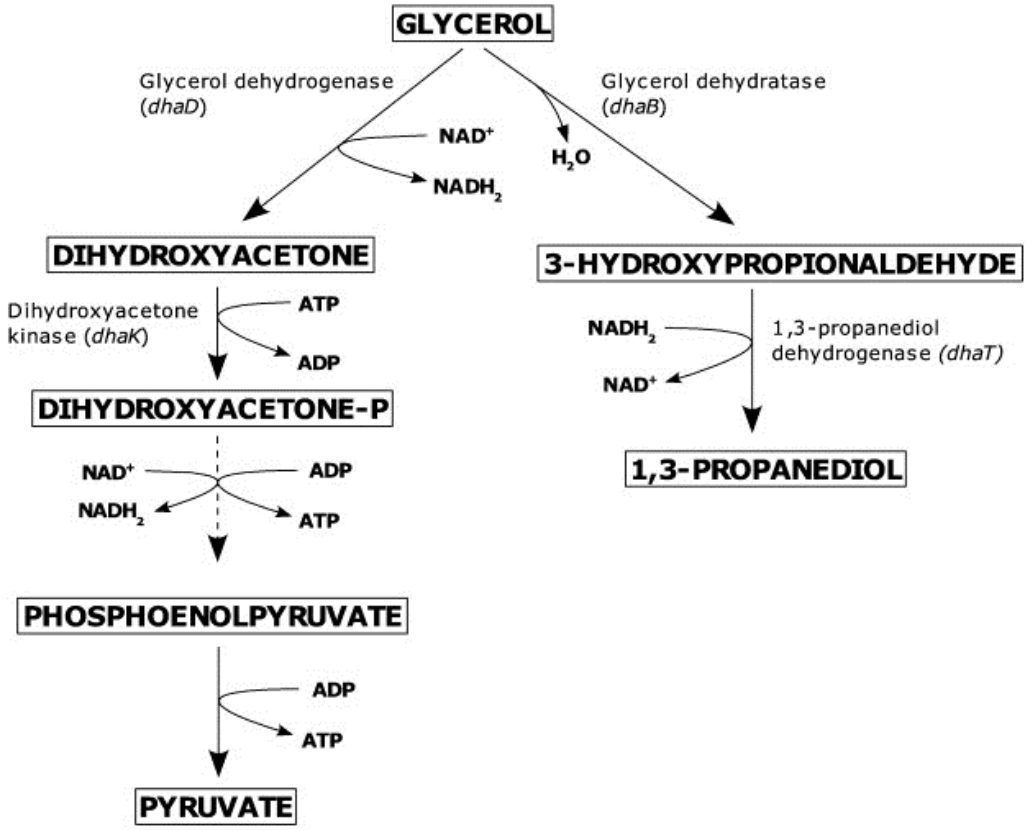

Figure 4.1.Pathway for biological 1,3-propanediol production. (Adapted from da Silva et al. 2009)

As the Figure 4.1 indicates, glycerol fermentation solely to 1,3-PD is not possible due to redox-balance: The NADH oxidized on 1,3-PD pathway need to be reduced via glycerol oxidizing pathway leading to pyruvate and further to volatile fatty acid or alcohol formation. Additionally, biomass formation from glycerol yields NADH. According to the calculations by Zeng (1996), the maximum 1,3-PD yield of 0.72 mol/mol glycerol is obtained with acetate as the sole product of the oxidizing pathway. The most studied natural 1,3-PD producers are *Clostridium butyricum* and *Klebsiella pneumoniae* which degrade glycerol efficiently to 1,3-PD. As high 1,3-PD production as 93.7 g/l (0.52 g/g) for *C. butyricum* (Wilkens *et al.* 2012) and 58.8 g/l (0.44 g/g) for *K. pneumoniae* (Mu *et al.* 2006) have been reported for refined glycerol. Slightly lower titers, 76.2 g/l for *C. butyricum* and 51.3 g/l for *K. pneumoniae*, were obtained with biodiesel derived glycerol as a substrate (Mu *et al.* 2006, Wilkens *et al.* 2012). Recently, Chatzifragkou *et al.* (2011) and Metsoviti *et al.* (2012, 2013) exploited successfully non-sterile crude glycerol fermentation for non-extremophilic *C. butyricum*, *Klebsiella oxytoca* and *Citrobacter freudii* with respective 1,3-PD titers of 67.9 g/l (0.55 g/g), 41.3 g/l (0.47

g/g) and 68.1 g/l (0.4 g/g). The recombinant *E. coli* strain developed by DuPont yielded a 1,3-PD titer of 129 g/l from glucose (Emptage *et al.* 2003).

Biological production of 1,3-PD is either vitamin B_{12} dependent or independent determined by the vitamin B_{12} dependence characteristics of glycerol dehydratase that catalyzes glycerol dehydration to 3-HPA. Most characterized 1,3-PD producers encode for vitamin B_{12}-dependent glycerol dehydratase, whereas the enzyme of *C. butyricum* was characterized as vitamin B_{12}-independent. The structure of the two types of glycerol dehydratases vary significantly; B_{12}-dependent enzyme is encoded by three genes (dhaB1, dhaB2, dhaB3), whereas the B_{12}-independent enzyme by two genes (dhaB1, dhaB2). The organization of the glycerol dehydratase genes in genomes of *K. pneumoniae* and *C. butyricum* are shown in Figure 4.2. In the genome of *C. butyricum*, the vitamin B_{12}-independent glycerol dehydratase subunits dhaB1 and dhaB2 are strongly suggested to form an operon with 1,3-propanediol dehydrogenase gene dhaT (Raynaud *et al.* 2003). The genes dhaS and dhaA, encoding a putative two-component signal transduction system, were identified upstream the operon (Raynaud *et al.* 2003).

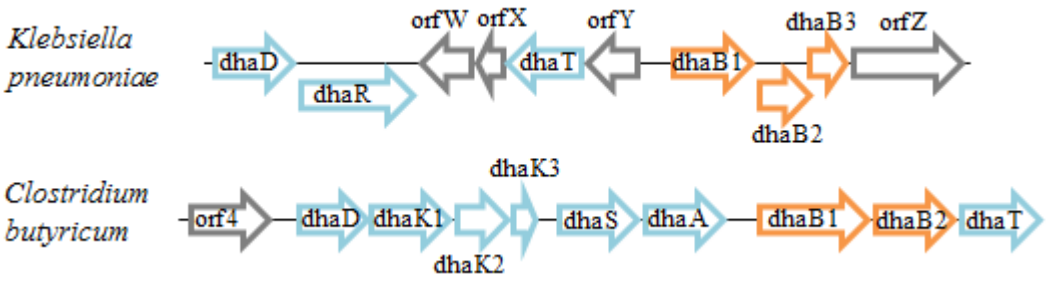

Figure 4.2. The organization of glycerol dehydratase genes in genomes of Klebsiella pneumoniae and Clostridium butyricum. DhaB1B2, dhaB1B2B3, structural subunits of glycerol dehydratases; dhaD; glycerol dehydrogenase, dhaR, regulatory protein; dhaT, 1,3-propanediol dehydrogenase; dhaK1K2K3, dihydroxyacetone kinase; dhaAS, a two-component signal transduction system (Modified from Sun et al. 2003).

5. HALOPHILIC ANAEROBIC FERMENTATIVE BACTERIA

Review **RI** is the main basis of this chapter. Halophiles are microorganisms that require high salt concentration for growth. They are found among all kingdoms of life: bacteria, eukarya, and archaea. According to the salt concentration required for optimal growth, halophiles are categorized as halotolerant, moderately halophilic and extremely halophilic microorganisms. The halotolerant microorganisms can survive and grow even in relatively high concentrations of salt but prefer to live in the absence of it, whereas the moderate halophiles achieve their optimal growth at salt concentrations from 25 g/l (0.5 M) to 150 g/l (2.5 M) and the extreme halophiles at salt concentrations over 150 g/l up to near saturation (340 g/l) (Joo and Kim 2005, Ventosa *et al.* 1998).

Halophilic microorganisms are advantageous for biotechnology because hypersalinity suppresses the growth of most other organisms and therefore, sterilization costs can be reduced. Biotechnological applications of halophilic archaea include production of bacteriorhodopsin, polymers, enzymes, and compatible solutes (Ventosa and Nieto 1995). While much of the interest in halophilic bacteria has been focused on their enzymes, putative compatible solutes, and biodegradation of residues and wastes, fermentative bacteria can also produce useful fermentation products such as hydrogen, ethanol, and 1,3-propanediol (Dan *et al.* 2003, Kapdan and Erten 2007, Ventosa and Nieto 1995, **I-V**).

Various aspects of halophilic anaerobic fermentative bacteria have been reviewed as follows: halophilic bacteria by Lowe *et al.* (1993), Madigan and Oren (1999), Mesbah and Wiegel (2008), Ollivier *et al.* (1994), Oren (2006), and Kivistö and Karp (**RI**) taxonomy by Rainey *et al.* (1995), phylogenetic and metabolic diversity by Oren (2002, 2008), survival strategies by Litchfield (1998), halophilic proteins by Mevarech *et al.* (2000), Fukuchi *et al.* (2003), and Joo and Kim (2005), bioenergetics in hypersaline environments by Oren (1999, 2001), and halophilic potential for biotechnology by Margesin and Schinner (2001), and Ventosa and Nieto (1995).

5.1. Taxonomy and Phylogeny

Most of the halophilic fermentative bacteria belong phylogenetically to the order Halanaerobiales which is considered as the order of halophilic fermentative bacteria and

which, until recently, comprised solely of halophilic fermentative bacteria. The order Halanaerobiales belongs to the phylum of Firmicutes and the class of Clostridia, and is further divided into two families, the Halanaerobiaceae and the Halobacteroidaceae (Mavromatis *et al.* 2009, Oren 2006). Altogether, the order Halanaerobiales currently includes 27 fermentative species or subspecies. Recently, the order Halanaerobiales have increased with three non-fermentative species, *Halanarsenatibacter silvermanii* (family Halanaerobiaceae) (Switzer Blum *et al.* 2009), *Selenihalobacter shriftii* (family Halobacteroidaceae) (Switzer Blum *et al.* 2001), and *Fuchsiella alkaliacetigena* (family Halobacteroidaceae) (Zhilina *et al.* 2012). The non-fermentative species characterized were observed to grow by anaerobic respiration of wide range of compounds including arsenic (*Halarsenatibacter*) and selenium (*Selenihalobacter, Fuchsiella*) compounds (Switzer Blum *et al.* 2001, Switzer Blum *et al.* 2009, Zhilina *et al.* 2012).

Although the majority of the halophilic fermentative bacteria belong to the order Halanaerobiales, there are few exceptions: A halothermophilic fermentative bacterium *Thermohalobacter berrensis* was assigned to the order Clostridiales and the family Clostridiaceae, and a haloalkalithermophilic bacterium *Natranaerobius thermophilus* to a novel order Natranaerobiales and a family Natranaerobiaceae based on characteristics and similarities in 16S ribosomal RNA sequences (Cayol *et al.* 2000, Mesbah *et al.* 2007). The taxonomic structure with order, family, genera, species, and subspecies of the halophilic fermentative bacteria is shown in Table 5.1.

Table 5.1. Taxonomic structure of halophilic fermentative bacteria.

Order	Family	Genus	Species	Subspecies
Halanaerobiales	*Halanaerobiaceae*	*Halanaerobium*	*H. acetethylicum*	
			H. alcaliphilum	
			H. congolense	
			H. fermentans	
			H. hydrogeniformans	
			H. kushneri	
			H. lacusrosei	
			H. praevalens^T	
			H. saccharolyticum	*saccharolyticum*
				senegalense
			H. salsuginis	
		Halocella	*Hc. cellulosilytica*^{T}	
		Halothermothrix	*Ht. orenii*^{T}	
	Halobacteroidaceae	*Acetohalobium*	*A. arabaticum*^{T}	
		Halanaerobacter	*Han. chitinovorans*^{T}	
			Han. lacunarum	
			Han. salinarius	
		Halanaerobaculum	*Hab. tunisiense*^{T}	
		Halanaerocella	*Hac. petrolearia*^{T}	
		Halobacteroides	*Hb. elegans*	
			Hb. halobius^T	
		Halonatronum	*Hn. saccharophilum*^{T}	
		Natroniella	*N. acetigena*^{T}	
		Orenia	*O. marismortui*^{T}	
			O. salinaria	
			O. sivashensis	
		Sporohalobacter	*Sp. lortetii*^{T}	
Clostridiales	*Clostridiaceae*	*Thermohalobacter*	*T. berrensis*^{T}	
Natranaerobiales	*Natranaerobiaceae*	*Natranaerobius*	*Nt. thermophilus*^{T}	

^{T} Type species of the genus.

The family Halanaerobiaceae contains species of the genus *Halanaerobium*, *Halothermothrix*, *Halocella*, and *Halarsenatibacter* (non-fermentative) whereas the family Halobacteroidaceae contains species of the genus *Acetohalobium, Fuchsiella* (non-fermentative), *Haloanaerobacter, Halanaerobaculum, Halanaerocella, Halobacteroides, Halonatronum, Natroniella, Orenia, Selenihalobacter* (non-fermentative), and *Sporohalobacter* (Cayol *et al.* 1994a, Cayol *et al.* 2000, Gales *et al.* 2011, Hedi *et al.* 2009, Liaw and Mah 1992, Oren 1983, Oren *et al.* 1984, Oren *et al.* 1987, Simankova *et al.* 1993, Switzer Blum *et al.* 2001, Switzer Blum *et al.* 2009, Zeikus *et al.* 1983, Zhilina and Zavarzin 1990, Zhilina *et al.* 1996, Zhilina *et al.* 2001, Zhilina *et al.* 2012). The most recently characterized genus among the halophilic fermentative bacteria is *Halanaerocella*, which was isolated from deep subsurface oil-bearing sandstone core and characterized in 2011 (Gales *et al.* 2011). The phylogenetic tree showing the phylogenetic position of the halophilic fermentative bacterial genera based on 16S rRNA gene analysis is presented in Figure 5.1. For comparison, the relationship of these bacteria to the newly identified non-fermentative members of the

order Halanaerobiales, selected other members of the phylum Firmicutes, Proteobacteria, and Actinobacteria is shown.

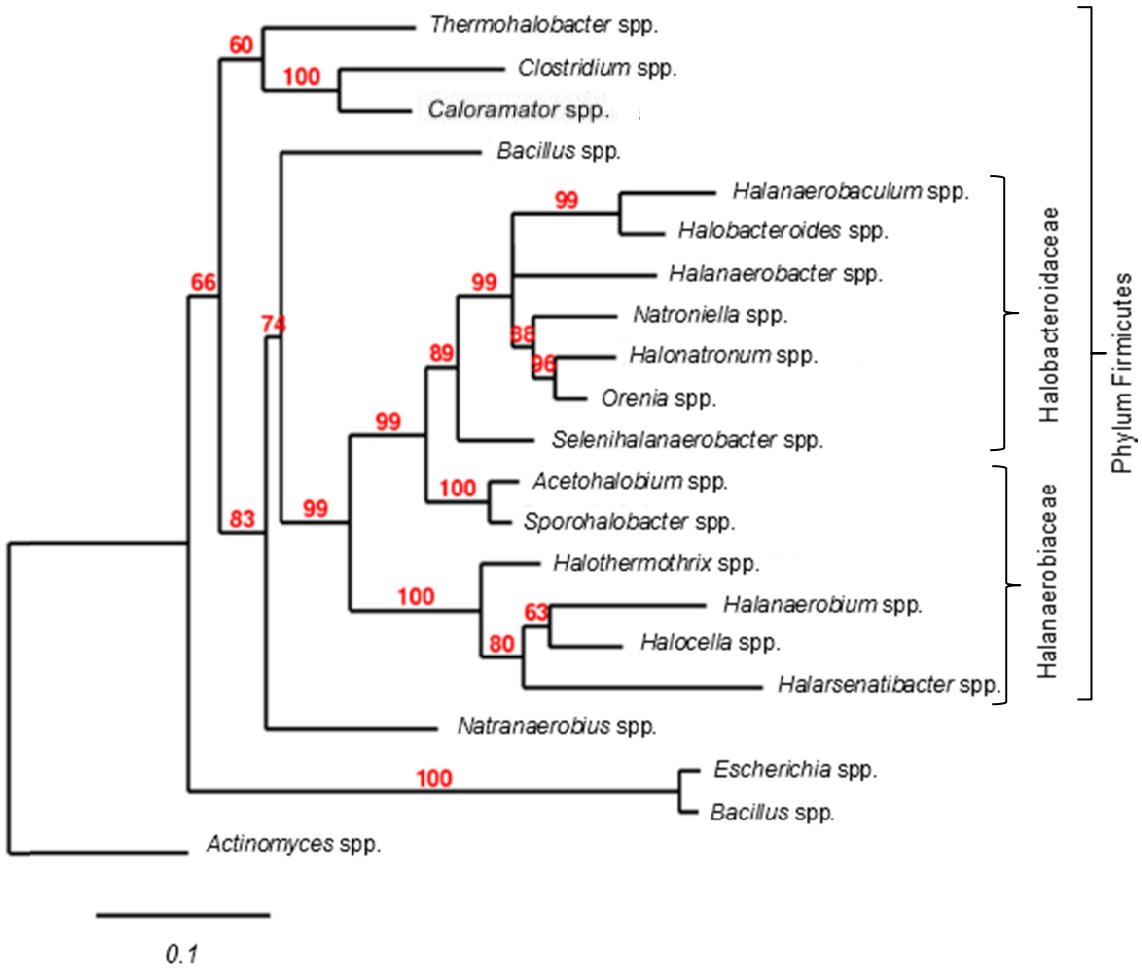

Figure 5.1. A phylogenetic tree based on 16S rDNA sequence similarities between the halophilic fermentative genera, the newly identified halophilic non-fermentative genera of the order Halanaerobiales, selected other genera of the Firmicutes phylum (Bacillus spp., Caloramator spp., and Clostridium spp.), and selected members of proteobacteria (Escherichia spp. and Citrobacter spp.). The tree was rooter with Actinomyces spp. as an out-group organism.

5.2. Characteristics

Halophilic fermentative bacteria have been isolated from a wide range of hypersaline environments including hypersaline lagoons and lakes, high-temperature or alkaline hypersaline lakes, soda lakes, saltern ponds and solar salterns, hypersaline sulphur springs, oil brines and fields, and food products (Bhupathiraju *et al.* 1994, Bhupathiraju *et al.* 1999, Cayol *et al.* 1994a, Cayol *et al.* 1994b, Cayol *et al.* 1995, Cayol *et al.* 2000, Gales *et al.* 2011, Hedi *et al.* 2009, Kobayashi *et al.* 2000a, Liaw and Mah 1992, Mouné *et al.* 1999, Mouné *et al.* 2000, Oren 1983, Oren *et al.* 1984, Oren *et al.* 1987, Ravot *et al.* 1997, Rengpipat *et al.* 1988, Simankova *et al.* 1993, Tsai *et al.* 1995, Zeikus *et al.* 1983, Zhilina and Zavarzin 1990, Zhilina *et al.* 1991, Zhilina *et al.* 1996, Zhilina *et al.* 1997, Zhilina *et al.* 1999). The species of the order Halanaerobiales are obligate

anaerobes but have not been reported being extremely oxygen-sensitive (Oren 2006). Most of the halophilic fermentative bacteria are moderate halophiles growing optimally at salt contents between 50 g/l and 150 g/l. However, few extremely halophilic representatives occur; *Halanaerobium lacusrosei*, *Halanaerobaculum tunisiense*, and *Acetohalobium arabaticum* with optimal salt content for growth 200 g/l, 200-220 g/l, and 150-180 g/l, respectively. Many of the halophilic fermentative bacteria are slowly growing with doubling times from 2 h to 8 h (Cayol *et al.* 1994a, Hedi *et al.* 2009, Liaw and Mah 1992, Mesbah and Wiegel 2008, Oren 1983, Zeikus *et al.* 1983, Zhilina *et al.* 2001). However, few representatives are able to grow with doubling time under 1 h including *Thermohalobacter berrensis* (0.46 h), *Orenia marismortui* (0.67 h), and *Halobacteroides halobius* (0.92 h) (Cayol *et al.* 2000, Oren *et al.* 1984, Oren *et al.* 1987).

5.2.1. Physiology

Characteristics of the halophilic fermentative genera are shown in Table 5.2. The halophilic fermentative bacteria share many common characteristics. For example, the cells are typically rod-shaped with the length of rods varying remarkably from strain to strain and even from cell to cell in the same cultivation. The cells occur singly, in pairs or in groups of several cells. Most of the halophilic fermentative bacterial species are Gram-negative, with few exceptions. The newest members of the Halanaerobiales, *Hb. tunisiense* and *Hac. petrolearia*, stained Gram-positive. Most of the halophilic fermentative bacteria are motile and flagellated peritrichously. The production of heat-resistant endospores of some members of Halanaerobiales has been reported. The sporeforming bacteria belong to the family Halobacteroidaceae and further to genera *Halonatrum*, *Natroniella*, *Orenia*, and *Sporohalobacter* (Oren 1983, Oren *et al.* 1987, Zhilina *et al.* 1996, Zhilina *et al.* 2001). In addition, rare spore formation in *A. arabaticum* cells have been reported (Zhilina and Zavarzin 1990).

Table 5.2. Characteristics of halophilic fermentative bacteria.

Characteristic	*Halanaerobium praevalens*	*Halocella cellulosilytica*	*Halothermothrix orenii*	*Thermohalobacter berrensis*	*Natranaerobius thermophilus*	*Acetohalobium arabaticum*	*Halanaerobacter chitinovorans*
Cell morphology	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Cell size (µm)	0.9-1.1 x 2.0-2.6	0.4-0.6 x 3.8-12.0	0.4-0.6 x 10-20	0.5 x 3.0-8.0	0.2-0.4 x 3-5	0.7-1 x 2-5	0.5 x 1.4-8
Gram stain	Negative	Negative	Negative	Negative	Positive	Negative	Negative
Motility	-	+	+	+	-	+	+
Spores	-	-	-	-	-	Rare	-
Temp. range (^oC)	5-60	20-50	45-68	45-70	35-56	NR[d]-47	23-50
Optimal temp. (^oC)	37	39	60	65	53	38-40	30-45
pH range	6.0-9.0	5.5-8.5	5.5-8.2	5.2-8.8	8.3-10.6	5.8-8.4	NR[d]
Optimal pH	7.0-7.4	7.0	6.5-7.0	7.0	9.5	7.4-8	7.0
NaCl range (g/l)	20-300	50-200	40-200	20-150	90-190	100-200	29-292
Optimal NaCl (g/l)	125	150	100	50	100-135	150-180	117-176
Doubling time (h)	4.0	NR[b]	5.3	0.46	3.5	NR[d]	2.47
G+C (mol%)	27	29	40	33	40	34	35
Substrates utilized	Fructose, glucose, n-acetyl glucosamine, mannose, pectin	cellulose, cellobiose, mannose, galactose, glucose, sucrose, sorbitol, starch	arabinose, cellobiose, fructose, galactose, glucose, melibiose, mannose, starch, ribose, xylose	cellobiose, fructose, glucose, maltose, mannose, mannitol, sucrose, glycerol, N-acetylglucosamine, starch, pyruvate, bio-Trypticase	fructose, cellobiose, ribose, trehalose, trimethylamine, puryvate, casamino acids, acetate, xylose, peptone	betaine, formate, puryvate, lactate, casamino acids, histidine, trimethylamine	glucose, fructose, mannose, acetylglucosamine, sucrose, maltose, cellobiose, chitin[e]
End-products[a]	Butyrate, acetate, propionate, H_2, CO_2	acetate, ethanol, H_2, CO_2[b]	acetate, ethanol, H_2, CO_2	acetate, ethanol, H_2, CO_2	acetate, formate[c]	acetate	acetate, isobutyrate, H_2, CO_2
Reference	Zeikus et al. 1983	Simankova et al., 1993	Cayol et al., 1994b	Cayol et al., 2000	Mesbah et al., 2008	Zhilina and Zavarzin, 1990	Liaw and Mah 1992

[a] End-products in glucose unless otherwise mentioned
[b] End-products in cellulose
[c] End-products in sucrose
[d] Not reported
[e] The type strain utilizes chitin as a substrate
[f] Cell wall gram-negative-like

Table 5.2. Continued.

Characteristic	*Halanaerobaculum tunisiense*	*Halanaerocella petrolearia*	*Halobacteroides halobius*	*Halonatronum saccharophilum*	*Natroniella acetigena*	*Orenia marismortui*	*Sporohalobacter lortetii*
Cell morphology	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Cell size (µm)	0.7-1 x 4-13	0.8-1.2 x 8-15	0.5 x 10-20	0.4-0.6 x 3.5-10	1-1.2 x 6-15	0.6 x 3-13	0.5-2 x 2.5-15
Gram stain	Positive[f]	Positive	Negative	Negative	Negative	Negative	Negative
Motility	-	-	+	+	+	+	+
Spores	-	-	-	+	+	+	+
Temp. range (^oC)	30-50	25-47	30-47	18-60	28-42	25-50	25-52
Optimal temp. (^oC)	42	40-45	37-45	36-55	37	36-45	37-45
pH range	5.9-8.4	6.2-8.8	NR[d]	7.7-10.3	8.1-10.7	NR[d]	NR[d]
Optimal pH	7.2-7.4	7.3	NR[d]	8-8.5	9.7-10.0	NR[d]	NR[d]
NaCl range (g/l)	140-300	60-260	70-187	30-170	100-260	29-176	41-146
Optimal NaCl (g/l)	200-220	150	88-146	70-120	120-150	29-117	82-99
Doubling time (h)	2.1	3.5	0.92	2.5	ND[d]	0.67	8
G+C (mol%)	34	33	31	34	32	30	32
Substrates utilized	glucose, sucrose, galactose, mannose, maltose, cellobiose, pyruvate, starch	cellobiose, fructose, glucose, galactose, lactose, maltose, mannose, sucrose, xylose, pyruvate, mannitol	glucose, fructose, sucrose, galactose, mannose, maltose, raffinose, puryvate, starch	glucose, fructose, sucrose, maltose, starch, glycogen, N-acetyl-D-glucosamine, peptone, yeast extract	lactate, pyruvate, glutamate, ethanol, propanol	glucose, fructose, mannose, sucrose, starch, glycogen	glucose, fructose, maltose, sucrose, starch
End-products[a]	acetate, butyrate, lactate, H_2, CO_2	lactate, formate, ethanol, acetate, H_2, CO_2	ethanol, acetate, H_2, CO_2	acetate, formate, ethanol, H_2, CO_2	acetate	acetate, ethanol, butyrate, formate, H_2, CO_2	acetate, n-butyrate, isobutyrate, isovalerate, propionate, H_2
Reference	Hedi et al., 2009	Gales et al., 2011	Oren et al., 1984b	Zhilina et al., 2001	Zhilina et al., 1996	Oren et al., 1987	Oren, 1983

[a] End-products in glucose unless otherwise mentioned
[b] End-products in cellulose
[c] End-products in sucrose
[d] Not reported
[e] The type strain utilizes chitin as a substrate
[f] Cell wall gram-negative-like

5.2.2. Polyextremophiles among halophiles

Polyextremophiles are extremophiles that thrive in multiple extreme environments. Most of the halophilic fermentative bacteria characterized thus far are mesophilic, with optimal temperature for growth between 35 ^{o}C and 45 ^{o}C, and neutrophilic, with optimal pH for growth between 6 and 8. However, halophilic fermentative bacterial species grow in double or even triple extreme environments.

Ht. orenii isolated from Tunisian salt lake and *T. berrensis* isolated from a solar saltern in France are halothermophilic bacteria having optimal temperature for growth 60 ^{o}C and 65 ^{o}C, respectively. In addition, few strains have been characterized growing in hypersaline environments with high alkalinity: *N. acetigena* and *H. alcaliphilum* (Tsai *et al.* 1995, Zhilina *et al.* 1996). *N. acetigena* is a homoacetogenic bacterium that was isolated from the soda depositing Lake Magadi (Zhilina *et al.* 1996). The pH optimum of *N. acetigena* for growth is between 9.7 and 10.0. *H. alcaliphilum*, isolated from hypersaline Great Salt Lake, Utah, is an alkalitolerant bacterium and achieves its optimal growth rate at neutral pH but is capable for growing up pH 10.0 (Tsai *et al.* 1995). Not only halophilic fermentative alkaliphilic bacteria but also halophilic fermentative alkalithermophilic bacterium has been characterized: *Nt. thermophilus* was isolated from soda lake Wadi An Natrum, Egypt and characterized in 2007 (Mesbah *et al.* 2007). The bacterium is capable for fermentative growth and has optimal pH for growth of 9.5, optimal temperature of 53 ^{o}C, and optimal salt content between 100 g/l and 135 g/l. When considering halophilic alkalithermophiles, all the three extremes discussed are combined and special adaptation of the cells is required.

5.2.3. Metabolism

There are three kinds of fermentative metabolic routes for the members of the order Halanaerobiales according to the substrate utilized. The metabolic routes of halophilic anaerobic bacteria have been relatively unexplored thus far but the ones of halophilic archaea have been characterized in more detail (Falb *et al.* 2008).

Most of the members are able to utilize a wide range of carbohydrates including a variety of sugars. Typical fermentation products of carbohydrate metabolism are acetate, ethanol, hydrogen, and carbon dioxide. However, not all strains have been reported to produce ethanol (Cayol *et al.* 1994b, Ravot *et al.* 1997, Zhilina *et al.* 1992). In addition, fermentation products may include butyrate, lactate, propionate, formate, valerate, and 1,3-propanediol (from glycerol) (Hedi *et al.* 2009, Liaw and Mah 1992, Oren 1983, Oren *et al.* 1987, Simankova *et al.* 1993, Zeikus *et al.* 1983, Zhilina *et al.* 2001, **I-V**).

Some species utilize amino acids as substrate of fermentation. For example, *Sp. lortetii*, a spore-forming bacterium, uses primarily amino acids as substrates instead of sugars which are utilized only poorly (Oren 1983). Besides this, some strains are able to ferment mixtures of amino acids using the Stickland reaction i.e. a reaction in which one amino acid is used as an electron donor and another as an electron acceptor resulting in production of organic acids, NH_3, and CO_2 (Nisman 1954). For example, *Halanaerobacter salinarius* is able to grow using the Stickland reaction with serine as the electron donor and glycine-betaine as the acceptor (Mouné *et al.* 1999).

The third fermentative route of metabolism includes production of acetate from organic substrates such as lactate, ethanol, pyruvate, glutamate, propionate, and glycine-betaine by homoacetogens. Homoacetogenic members of the Halanaerobiales are *A. arabaticum* and *N. acetigena* (Zhilina and Zavarzin 1990, Zhilina *et al.* 1996). The main difference between the two homoacetogenic representatives is the ability of *A. arabaticum* for growing on hydrogen and carbon dioxide or carbon monoxide (Zhilina and Zavarzin 1990).

5.3. Survival methods

To survive in such extreme hypersaline conditions, halophiles need to have specific strategies for balancing osmotic pressure caused by extremely high ion concentration of the environment. Basically, there are two means, termed as ‘salt-in’ and ‘organic solutes-in’, for handling the high extracellular salt concentrations. In the ’salt-in’ strategy, cells maintain high intracellular salt concentrations and in the ‘organic solutes-in’ strategy cells accumulate organic compatible solutes e.g. glycerol, glycine betaine, trehalose, and sucrose (Roberts 2005). Glycerol is bioenergetically the cheapest compatible solute to produce and accumulate, however, special adaptation for a cell wall is needed and thus only eubacterial halophiles are able to accumulate glycerol for survival (Oren 1999). Since all biological membranes are permeable for water, the main idea of both strategies is to maintain isoosmotic conditions within the cell. Roughly classified, the ‘salt-in’ strategy is used by extremely halophilic aerobic archaea and halophilic fermentative bacteria, whereas the ‘organic solutes-in’ strategy is used by halophilic alga, halophilic methanogenic archaea as well as halotolerant and halophilic aerobic bacteria.

The Halanaerobiales, as halophilic fermentative bacteria, typically cope with the extreme living conditions by balancing the osmotic pressure with high intracellular ion concentrations. High intracellular ion content of *Halanaerobium acetethylicum, H. praevalens,* and *Hb. halobius* has been reported and no reports for organic compatible solutes being accumulated by halophilic fermentative bacteria exist (Oren *et al.* 1997, Oren 2001, Rengpipat *et al.* 1988). Typically the salt accumulated in molar

concentrations consists of K^+ and Cl^- ions. However, in some cases, especially in the stationary phase of growth, the main inorganic cation of the cytoplasm has been observed to be Na^+ (Oren *et al.* 1997, Rengpipat *et al.* 1988, Roberts 2005). Although, Cl^- is by far the most relevant anion accumulated by halophiles using the 'salt-in' strategy, also high concentrations of sulfate have been observed in some halophilic microorganisms (Ede *et al.* 2004).

Energy in the 'salt-in' strategy is typically used for extruding Na^+ and accumulation of Cl^- ions. Uptake of K^+ is often considered as passive process but might also be an active energy requiring process in some microorganisms e.g. in halophilic aerobic archaea *Haloferax volcanii* (Oren 2001). K^+ ions typically pass through cell membrane via uniport system as a response to membrane potential, whereas chloride ions might be transferred via symport along with Na^+ (Oren 2001). Although life at hypersaline environment is energetically expensive, the 'salt-in' strategy used by halophilic fermentative bacteria is energetically remarkably cheaper compared to the production and accumulation of organic compatible solutes. According to Oren (2001), the reason why halophilic fermentative bacteria prefer 'salt-in' strategy is their loss of affordability for the strategy of organic solutes.

The intracellular systems of microorganisms using 'salt-in' strategy are exposed to high salt concentrations and thus need to be well adapted in order to maintain their functionality and stability. For example, the non-halophilic proteins tend to aggregate and become rigid at high salt, whereas the halophilic proteins are able to stay soluble and functional (Mevarech *et al.* 2000). The proteomic studies on halophilic microorganisms have mainly been focused on halophilic archaea thus far, and only little is known about halophilic eubacterial representatives. The proteins of halophilic microbes maintaining high intracellular ion concentrations typically denature at salt concentrations under 1 M (Fukuchi *et al.* 2003, Mevarech *et al.* 2000). For a long time, the salt-tolerant proteins were thought being rich in acidic amino acid residues and thus negatively charged. Most recent genomic analyses, however, question this hypothesis (Elevi Bardavid and Oren 2012a, Elevi Bardavid and Oren 2012b). The salt-tolerant nature of the proteins remains thus unrevealed. Halothermophilic *Ht. orenii* was the first completely sequenced member of the halophilic fermentative bacteria. At the moment, genome information of *H. praevalens* (Ivanova *et al.* 2011), *Halanaerobium hydrogeniformans* (Brown *et al.* 2011), *A. arabaticum* (Sikorski *et al.* 2010) and *H. saccharolyticum* subsp. *saccharolyticum* (**VI**) are also available in genome databases.

5.4. Potential for Biotechnology

According to the best of my knowledge, no commercial applications for halophilic fermentative bacteria are currently available and all the studies are at experimental

phase. The few studies on the potential of halophilic fermentative bacteria for biotechnology are listed in Table 5.3.

Table 5.3. The potential biotechnological applications studied for halophilic fermentative bacteria.

Putative application	Bacterium	Substrate	Level	Reference
Biodegradation of nitro-substituted aromatic compounds	*Halanaerobium praevalens*	Nitro-aromatic compounds	100 % degradation [a]	Oren *et al.* 1991
	Sporohalobacter marismortui	Nitro-aromatic compounds	100 % degradation [a]	Oren *et al.* 1991
Biological COD removal	*Halanaerobium lacusrosei*	Synthetic saline waste water	94 % of COD removed [b]	Kapdan and Erten 2007
Biohydrogen production	*Halanaerobium saccharolyticum* subsp. *saccharolyticum*	Pure glycerol	2.71 mol H_2/mol glycerol	**I-II**
		Waste glycerol	3.0 mol H_2/mol glycerol	**IV-V**
	Halanaerobium saccharolyticum subsp. *senegalense*	Pure glycerol	1.61 mol H_2/mol glycerol	**I**
	Halanaerobium hydrogeniformans	Sugars	2.42 mol H_2/mol hexose	Begemann *et al.* 2012
		Alkalitreated biomass	2.93 mmol H_2/l/h	
Biological 1,3-PD production	*Halanaerobium saccharolyticum* subsp. *saccharolyticum*	Pure glycerol	0.63 mol 1,3-PD/mol glycerol	**I, III**
		Waste glycerol	0.66 mol 1,3-PD/mol glycerol	**V**

[a] The initial concentrations of nitro-substituted aromatic compounds 50-100 mg/l
[b] The initial concentration of COD 1900 mg/l

Halophilic fermentation is reasonable when sustainable substrates are being used and high-value products being produced. Renewable substrates, waste materials, and by-products are often considered as sustainable and economical substrates. Most of the halophilic fermentative species are able to utilize starch, an energy-storing polysaccharide of green plants, as a substrate (Table 5.2). Additionally, cellulose, a polysaccharide used as a main component of cell wall by green plants, is considered as a prominent substrate. Only one species, *Halocella cellulosilytica*, among the halophilic fermentative bacteria identified thus far is able to degrade cellulose. However, several strains are capable for biodegradation of cellobiose, the main structural unit of cellulose.

The price of glycerol has decreased significantly recently, and thus glycerol has become an economical substrate for applications. Industrial glycerol often contains salts and heavy metals which are putative growth inhibiting factors for most microorganisms. Halophilic bacteria might possess potential for industrial glycerol utilizing applications as high heavy metal tolerances of several halophilic bacteria have been reported and salts cause no problem for halophiles (Nieto *et al.* 1989). Glycerol is reported being utilized by three halophilic fermentative species, *Halanaerobium saccharolyticum*

subsp. *saccharolyticum*, *Halanaerobium saccharolyticum* subsp. *senegalense*, and *Halanaerobium lacusrosei* (Cayol *et al.* 1994b, Cayol *et al.* 1995, Zhilina *et al.* 1992).

The fermentation products of halophilic fermentative bacteria include H_2, CO_2, acetate, butyrate, ethanol, and 1,3-PD (from glycerol), among others. H_2 gas is considered as a potential future energy carrier molecule. Few halophilic fermentative bacteria, *H. saccharolyticum* subsp. *saccharolyticum* and subsp. *senegalense* as well as *H. hydrogeniformans*, have been assessed for biohydrogen production from carbohydrates or waste streams (Begemann *et al.* 2012, **I-V**). The liquid products are considered industrially as follows: Acetic acid might have applications in chemical industry, ethanol is highly valued as a motor fuel or fuel additive and currently one of the most commonly used renewable fuels, and 1,3-propanediol has large markets for the use of polymer industry.

In addition to the end-metabolites, the putative valuable products of halophilic fermentation include salt-tolerant proteins and enzymes. The enzymes of the halophilic fermentative bacteria are salt-tolerant and, actually, salt-requiring due to the high intracellular ion concentrations maintained for balancing the osmotic pressure in hypersaline environment. The active form of the proteins and enzymes are achieved at high salt, and the denaturation occurs at low salt. The salinity requirement is a challenge for the production and handling of the proteins but also a possibility for using enzymes in processes containing high salt concentrations. The highest interest among the halophilic enzymes is on isomerases and hydrolases including amylases that catalyze the bioprosessing of starch and β-galactosidases that catalyze the bioprosessing of lactose. Salt-requirering enzymes have been cloned and produced as inactive forms in *Escherichia coli* and thereafter successfully activated with increase of salt concentration (Cendrin *et al.*, 1993).

Halophilic (halotolerant) fermentation is taken into advantage in production of salt-containing food. The fermentation products give taste, aroma, and flavor. In addition, acetate, a fermentation product lowers the acidity in foods and thus serves as preservative in foods for e.g. yeast spoilage (Margesin and Schinner 2001). Halophilic or halotolerant fermentative bacteria are involved in production of a wide variety of food products, such as fermented fish, shrimp, meat, fruits, and vegetables (pickles), Asian fish and meat sauces, rice noodles and flours, and Indonesian soy sauce (Margesin and Schinner 2001). Most of the bacteria reported being involved in food production are halotolerant including species among genera *Lactobacillus*, *Halobacterium*, *Halococcus*, *Bacillus*, *Pediococcus*, and *Tetragenococcus* (Margesin and Schinner 2001, Röling and Van Verseveld 1996, Tanasupawat and Komagata 1995), however halophilic fermentative species from genus *Halanaerobium* including

H. fermentans have been isolated from fermented food products (Kobayashi *et al.* 2000a, Kobayashi *et al.* 2000b).

Potential of the halophilic anaerobic fermentative bacteria for use in anaerobic treatment of saline waste waters has been reported (Kapdan and Erten 2007). Halophilic fermentative bacteria have few advantages over conventional biological treatment systems. They are usable at high salt concentrations, might have tolerance for several heavy metals, and degrade a wide range of organic compounds (Bhupathiraju *et al.* 1994, Cayol *et al.* 1995, Nieto *et al.* 1989, Tsai *et al.* 1995, Zeikus *et al.* 1983). According to Oren *et al.* (1992), halophilic microorganisms are in major role in biodegradation of pollutants in hypersaline environments. The potential of two halophilic fermentative bacteria, *H. praevalens* and *O. marismortui*, for biodegradation of nitro-substituted aromatic compounds including nitrobenzene, o-nitrophenol, m-nitrophenol, p-nitrophenol, nitroanilines, 2,4-dinitrophenol, and 2,4-dinitroaniline with complete degradation of the most compounds tested (initial concentrations 50-100 mg/l) was observed by Oren *et al.* (Oren *et al.* 1991).

6. HYPOTHESES AND OBJECTIVES

The aim of this study is to access halophilic fermentative bacteria for degradation of raw glycerol in order to produce H_2 and/or 1,3-PD.

The more specific goals are categorized as follows:

1. Characterization of the halophilic fermentative glycerol metabolism.
2. Optimization of H_2, 1,3-PD and/or biomass production.
3. Development of an open bioprocess for H_2 and/or 1,3-PD production from unpurified raw glycerol.
4. Determination of the genome sequence of a halophilic fermentative bacterium chosen for the studies.

Ultimately, the project is aimed to enable the economical utilization of glycerol produced as main by-product in biodiesel industry. The utilization of by-product for production of valuable environmentally friendly product(s) will decrease the costs and environmental burden of biodiesel production process.

7. SUMMARY OF MATERIALS AND METHODS

Detailed information concerning the materials and methodology used in the studies are given in original publications **I-VII**.

7.1. Introduction to the studies conducted

All the ‘wet-lab’ experiments (microbial incubations; papers **I-V**) were performed as small scale (culture volume 10-170 ml) batch experiments. Computer-based ‘dry-lab’ experiments were carried out for genome sequence assembly and analysis (**VI** and **VII**). The experiments conducted are introduced in Table 7.1. Sterile working methods and process conditions along with ultrapure MQ water (basis of the medium) were used in fermentation characterization, bioprocess optimization and substrate/end-product tolerance studies whereas experiments on contamination risk at increasing salt content (paper **V**) as well as H_2 and 1,3-PD production from raw glycerol (paper **V**) were accomplished under nonsterility using communal tap water as basis of the medium.

Table 7.1.Experiments conducted, substrate(s) used, and microbial strains employed along with reference to original publication(s) of this thesis.

Experiment	Substrate(s)	Strain(s)	Ref.
Characterization of glycerol fermentation	Pure glycerol	*H. saccharolyticum* subsp. *saccharolyricum* *H. saccharolyticum* subsp. *senegalense* *C. butyricum* DSM 2478 *E. coli* BW25113	**I**
Characterization of glucose fermentation	Glucose	*H. saccharolyticum* subsp. *saccharolyricum* *H. saccharolyticum* subsp. *senegalense*	**I**
Effect of yeast extract	Pure glycerol+YE	*H. saccharolyticum* subsp. *saccharolyricum* *H. saccharolyticum* subsp. *senegalense*	**I**
Optimization of pH, substrate, salt	Pure glycerol	*H. saccharolyticum* subsp. *saccharolyricum* *H. saccharolyticum* subsp. *senegalense*	**I**
H_2 production at optimized pH, substrate and salt content	Pure glycerol	*H. saccharolyticum* subsp. *saccharolyricum* *H. saccharolyticum* subsp. *senegalense*	**I**
Effect of vitamin B_{12} on glycerol fermentation	Pure glycerol	*H. saccharolyticum* subsp. *saccharolyricum* *H. saccharolyticum* subsp. *senegalense* 'C. butyricum'	**II, III**
Effect of vitamin B_{12} on glucose fermentation	Glucose	*H. saccharolyticum* subsp. *saccharolyricum*	**II**
H_2 production at optimized pH, substrate, salt and vitamin B_{12}	Pure glycerol	*H. saccharolyticum* subsp. *saccharolyricum*	**II**
Effect of temperature on 1,3-PD production	Pure glycerol	*H. saccharolyticum* subsp. *saccharolyricum*	**III**
Acetate tolerance	Pure glycerol	*H. saccharolyticum* subsp. *saccharolyricum*	**III, IV**
1,3-PD tolerance	Pure glycerol	*H. saccharolyticum* subsp. *saccharolyricum*	**III**
The effect of H_2 partial pressure on H_2 production	Pure glycerol	*H. saccharolyticum* subsp. *saccharolyricum*	**IV**
Growth optimization for enhanced H_2 production	Pure glycerol	*H. saccharolyticum* subsp. *saccharolyricum*	**IV**
H_2 production at optimized conditions with N_2 flushing	Pure glycerol Raw glycerol	*H. saccharolyticum* subsp. *saccharolyricum*	**IV**
Raw glycerol tolerance	Pure glycerol Raw glycerol	*H. saccharolyticum* subsp. *saccharolyricum* 'C. butyricum'	**IV, V**
Contamination of the bioprocess at increasing salt contents	Pure glycerol Glucose	NA[a] (Tap water-, chemical-, and air-borne contaminants)	**V**
Non-sterile H_2 and 1,3-PD production	Raw glycerol	*H. saccharolyticum* subsp. *saccharolyricum*	**V**
Genome sequencing and analysis	NA[a]	*H. saccharolyticum* subsp. *saccharolyricum*	**VI, VII**

[a] Not applicable.

7.2. Microbial strains

Three halophilic strains, *H. saccharolyticum* subsp. *saccharolyticum* DSM 6643^T, *H. saccharolyticum* subsp. *senegalense* DSM 7379^T and *H. lacusrosei* DSM 10165^T, were chosen for the studies based on their ability for glycerol degration and H_2 production as reported in the strain descriptions (Cayol *et al.* 1994b, Cayol *et al.* 1995, Zhilina *et al.* 1992). *H. lacusrosei* showed problematic growth in the lab environment and thus was omitted from the studies. Non-halophilic control strains included *Escherichia coli* BW25113 (Datsenko and Wanner 2000), *Clostridium butyricum* DSM 2478, and '*C.*

butyricum' strain that was isolated from an H_2 producing bioreactor (Koskinen *et al.* 2007, Seppälä *et al.* 2011).

7.3. Substrates

Glucose, pure commercial glycerol (Sigma-Aldrich Co.) and raw glycerol were used as substrates in the experiments. Raw glycerol, a by-product from biodiesel manufacturing process was kindly provided by Savon Siemen Oy (Iisalmi, Finland). The main contents of un-purified raw glycerol fraction were 56 % m/v glycerol, 23 % m/v methanol and 1.8 % m/v K^+. The density of raw glycerol fraction was 1.079 g/cm^3.

7.4. Experimental set-up

7.4.1. Process optimization

The halophilic bioprocess for H_2 and/or 1,3-PD production from glycerol was optimized for several parameters including medium components and cultivation conditions. The parameters, as well as their range in the studies, main objective (H_2, 1,3-PD, and/or growth) of optimization and a reference to original papers of the Thesis were collected in Table 7.2.

Table 7.2. Parameters of process optimization, range studied, objective of optimization and the reference to original papers of this thesis.

Parameter	Range	Objective	Reference
Substrate (pure glycerol)	2.5-20 g/l	H_2, 1,3-PD	I
Substrate (pure glycerol + YE)	5 g/l + 0-10 g/l	H_2, 1,3-PD	I
pH	6.6-8.2	H_2, 1,3-PD	I
Salt (NaCl)	50-200 g/l	H_2, 1,3-PD	I
Vitamin B_{12}	0-64 µg/l	H_2	II,
	0-500 µg/l	1,3-PD	III
Temperature	30-40 ^oC	1,3-PD	III
Gas-to-liquid ratio	0.7-4	H_2	IV
Inorganic nitrogen (NH_4Cl)	0-4 g/l	Growth, H_2	IV
Tryptone	0-4 g/l	Growth, H_2	IV
Buffer (HEPES/phosphate)	0-50 mM	Growth, H_2	IV
Glycine-betaine	0-50 mM	Growth, H_2	IV
Substrate (raw glycerol)	2.5-40 g/l	H_2, 1,3-PD	V

7.4.2. Tolerance to end-metabolites and substrate

Substrates and end-products may cause inhibition for the fermentation process. Therefore, the effects of substrate (pure and raw glycerol) and metabolites (H_2, 1,3-PD, acetate) on halophilic fermentation were studied individually for tolerance or inhibition as summarized in Table 7.3.

Table 7.3. Studies on tolerance/inhibition to substrates and end-metabolites.

Parameter	Range	Objective	Reference
Substrate (pure glycerol)	2.5-20 g/l	H_2, 1,3-PD	I
Substrate (raw glycerol)	2.5-40 g/l	H_2, 1,3-PD	V
pH_2 (Gas-to-liquid ratio)	0.7-4.0	H_2	IV
1,3-PD	0-57 g/l	1,3-PD, growth	IV
Acetate	0-58 g/l	1,3-PD, growth	III

7.4.3. Contamination at increasing salinity

The contamination risk with increasing salt content was studied at 0-150 g/l NaCl using non-sterile working conditions and medium; tap water, microbiology laboratory environment and chemicals were the sources of bacteria. The contamination risk with glucose and pure glycerol as substrates were compared.

7.4.4. Non-sterile H_2 and 1,3-PD production

Biomass and 1,3-PD production were combined to H_2 production in a two-stage process, in which supplementation of excess vitamin B_{12} (500 µg/l) directs the bacterial culture for enhanced biomass and 1,3-PD production in the first phase, followed by collection of biomass and a second phase aiming for efficient H_2 production without vitamin B_{12} supplementation. In addition, one-stage processes for separate production of H_2 (w/o vitamin B_{12}) and 1,3-PD (w/ vitamin B_{12}) were included as well as negative growth (w/o inoculum) and substrate control (w/o substrate). The experiment was accomplished using non-sterile working techniques. Unpurified raw glycerol (5 g/l) was used as a substrate and the growth medium supplemented with raw glycerol was additionally filtered prior to inoculation in order to enable optical density measurements. The pH of the cultures was adjusted daily to 7.4 and N_2 flushing was applied to renew the headspace as the H_2 production was observed to decrease.

7.4.5. Genome analysis

The sequenced genome of *H. saccharolyticum* subsp. *saccharolyticum* was analysed for fermentation pathways (glucose and glycerol), hydrogenases, tolerance towards antibiotics, metals and other toxins, and halophilic survival strategies.

7.5. Analytical methods and calculations

Bacterial cultivations were analysed for substrate utilization, liquid and gaseous end-metabolites, pH, and biomass. The genome of *H. saccharolyticum* subsp. *saccharolyticum* was sequenced, assembled, manually edited, annotated and analysed.

The methods used for cultivation and genome sequencing and analyses are listed in Table 7.4.

Table 7.4. Analytical methods used for analysis of bacterial cultivations and genome.

	Analysis	**Method**	**Paper**	**References**
Cultivation analyses	Gaseous compounds; *H_2, CO_2, CH_4*	Gas chromatography	I-V	
	Liquid compounds; *glucose, lactate, glycerol, formate, acetate, 1,3-PD, propionate, ethanol, methanol, and butyrate*	High-performance liquid chromatography	I-V	
	pH	pH electrode	I-V	
	Biomass; *Optical density* *Dry cell weight*	Spectrophotometer (600 nm) Freeze-drying + weighing	I-V V	
	Cell morphology	Phase-contrast microscope	V	
Genome analyses	Genome sequencing; *Sequencing*	 Illumina paired-end, 454 technologies	VI	
	Assembly	MIRA		Chevreux *et al.* 1999
	Manual edition	Gap5		Bonfield and Whitwham 2010
	Autoannotation	RAST server		Aziz *et al.* 2008
	Manual annotation revision	Blast analysis		Altschul *et al.* 1990
	Phylogenetic analysis; *Multiple sequence alignment* *Alignment curation* *Tree construction* *Tree visualization*	 MUSCLE Gblocks PhyML Treedyn	VII	 Edgar 2004 Castresana 2000 Guindon and Gascuel 2003 Anisimova and Gascuel 2006
	Metabolism reconstruction	RAST server Blast analysis KEGG reaction database	VII	Chevenet *et al.* 2006 Aziz *et al.* 2008
	Operon prediction; *Rho-dependent termination* *Rho-independent termination*	 Operon ARNold	VII	Altschul *et al.* 1990 Ermolaeva *et al.* 2000 Naville *et al.* 2011

8. SUMMARY OF RESULTS AND DISCUSSION

8.1. Characterization of fermentation

H. saccharolyticum subsp. *saccharolyticum* fermented glucose to H_2, CO_2, acetate, and ethanol (trace amount) and glycerol with vitamin B_{12} supplementation to H_2, CO_2, acetate, 1,3-PD, ethanol (trace amount) and butyrate (trace amount). According to the end-metabolites, the glycerol fermentation of *H. saccharolyticum* subsp. *saccharolyticum* is similar to clostridial metabolism (Figure 2.5; Section 2.2.2.). The main difference between glycerol fermentation of *H. saccharolyticum* subsp. *saccharolyticum* and subsp. *senegalense* is the formation of 1,3-PD as the subsp. *senegalense* derived glycerol to H_2, CO_2, acetate and an unidentified compound (trace amount).

Only a small group of microorganisms are known to produce 1,3-PD and the production in halophilic microorganisms has not been reported prior to studies of this thesis. Biological production of 1,3-PD is either vitamin B_{12} dependent or independent depending on the characteristics of glycerol dehydratase that catalyzes the first step of the 1,3-PD production route. The 1,3-PD formation by *C. butyricum* is vitamin B_{12} independent (Raynaud *et al.* 2003, Saint-Amans *et al.* 2001) whereas most other 1,3-PD producing microorganisms require vitamin B_{12} for the activity of the 1,3-PD yielding metabolic route.

8.2. Process optimization

8.2.1. Individual parameter optimization

The effects of pure glycerol, yeast extract, salt (NaCl), pH and vitamin B_{12} on fermentation were studied for both *H. saccharolyticum* subsp. *saccharolyticum* and *senegalense*, whereas the rest of the process optimization was exploited on the subsp. *saccharolyticum* only due to better growth and hydrogen production. The results of *H. saccharolyticum* subsp. *senegalense* are discussed in the original publications **I-II**.

Optimized parameter values and/or a range of parameter values that resulted in the highest production or yields for biomass, H_2 or 1,3-PD by *H. saccharolyticum* subsp. *saccharolyticum* are shown in Table 8.1. The standard deviations in the optimization (10 ml culture volume) experiments were observed in many cases as relatively large (especially with vitamin B_{12} supplementation), which resulted in relatively large optimal

parameter ranges in case the parameter did not have a remarkable effect on fermentation.

Table 8.1. Parameter values resulting the highest H_2 and 1,3-PD production and yields.The data shown is the parameter value resulting the highest average production or yield, and, in parenthesis, a parameter range determined by taking into account the standard deviations.

Parameter	**Parameter value for the highest**					**Ref**
	H_2 yield	**H_2 production**	**1,3-PD yield**	**1,3-PD production**	**Growth (OD_{600})**	
Pure glycerol [g/l][a,b,c]	2.5 (2.5-20)[a]	2.5 (2.5-20)[a]	2.5[a]	2.5 (2.5-5)[a]	10 (2.5-20)[a]	I
	20 (10-20)[b]	10 (2.5-40)[b]	5[c]	10 (5-10)[c]	NA	V
Raw glycerol [g/l][b,c]	5 (5-20)[b]	5 (2.5-20)[b]	5[c]	5 (2.5-10)[c]	NA	V
Yeast extract [g/l][a,d]	NA	10[d]	NA	0	NA	I
NaCl [g/l][a]	150 (150-200)	100 (100-150)	100 (100-150)	100 (100-150)	100	I
pH[a]	7.4 (6.6-7.8)	7.4	7.8 (7.8-8.2)	7.8 (7.8-8.2)	8.2 (7.4-8.2)	I
Vitamin B_{12} [µg/l]	0 (0-2)	0	64 (≥ 64)	64 (≥ 64)	480 (≥ 64)	II, III
Temperature [^oC]	37 (36-37)	36 (36-37)	30 (30-31; 34-35)	30 (30, 34)	40[e]; 30-36[f]	III
Gas-to-liquid ratio	4 (2.1-4)	4 (2.1-4)	NA	NA	4	IV
NH_4Cl [g/l]	NA	5 (1;5)	NA	NA	5[e]; 1[f]	IV
Tryptone [g/l]	NA	1 (1-2)	NA	NA	5[e]; 2[f]	IV
Buffer:						
HEPES[mM]	NA	50	NA	NA	50 (25-50)	IV
Phosphate [mM]	NA	50	NA	NA	50 (25-50)	IV
Glycine-betaine [mM]	NA	0	NA	NA	50	IV

NA, not analyzed
[a] Slight (5 µg/l) vitamin B_{12} supplementation
[b] Excess (500 µg/l) vitamin B_{12} supplementation
[c] Without vitamin B_{12} supplementation
[d] Background metabolite production from YE un-subtracted
[e] Fastest growth
[f] Highest OD value

Pure glycerol (Papers **I** and **V**) had a remarkable effect on 1,3-PD production and yield, whereas the effect on H_2 and biomass production was observed as insignificant. With slight (5 µg/l) vitamin B_{12} supplementation, the highest H_2 and 1,3-PD production and yields were obtained with 2.5 g/l and 1,3-PD production decreased as glycerol content increased (Paper **I**). On the other hand, with excess (500 µg/l) vitamin B_{12} supplementation the 1,3-PD production increased up to 10 g/l pure glycerol and decreased at higher concentrations (Paper **V**).

Raw glycerol (Papers **IV-V**), the unpurified glycerol fraction from a biodiesel process, was observed suitable for halophilic fermentation process. Highest H_2 and 1,3-PD production as well as yields were obtained at initial raw glycerol 5 g/l (Paper **V**). Raw glycerol inhibition at high concentrations is discussed later in Section 8.3.

Yeast extract (Paper **I**) contains peptides, free amino acids, purine and pyrimidine nucleobases, trace elements and water soluble B vitamins. The increasing amount of

yeast extract along with constant amount of pure glycerol as a substrate suppressed glycerol utilization and 1,3-PD production whereas enhanced H_2, CO_2 and acetate formation were observed. The highest H_2, CO_2, and acetate production was obtained at 10 g/l YE. However, the calculations for carbon recovery (from glycerol to metabolites) at 10 g/l remarkably exceeds 100 % (being 280 % excluding biomass formation) implies the metabolite production originating mostly from the YE rather than glycerol. Therefore, inclusion of high YE content is not favorable for bioprocess aiming not only for high metabolite production but also for degradation of waste glycerol. The highest H_2 production at small YE contents (0-1 g/l) was obtained at 0 g/l YE. The results indicate that YE is eagerly used as a carbon source instead of glycerol. Since yeast extract utilization was not analyzed, no reliable yields were obtained.

Halophilic microorganisms have certain requirements for salt content in their habitats. Though according to a strain description (Zhilina *et al.* 1992) the NaCl range for growth of *H. saccharolyticum* subsp. *saccharolyticum* is 30-300 g/l, the bacterial type strain did not grow at 50 g/l NaCl (Paper **I**). The optimum growth was obtained at 100 (100-150) g/l as well as the highest H_2 production, 1,3-PD production and 1,3-PD yield. The highest H_2 yield was obtained at slightly higher salinity, 150 (150-200) g/l.

Fermentation is typically remarkably affected by the process pH. The initial pH (Paper **I**) of 7.4 was the most favorable for H_2 production and yield, whereas 1,3-PD production was highly stimulated by an alkaline pH, the optimum being 7.8 (7.8-8.2). Similar response of 1,3-PD production to pH has been reported by Barbirato *et al.* (1998) and Forsberg (1987). This sensitivity might be explained by the influence of pH on the activity of 1,3-PD dehydrogenase, an enzyme responsible for 1,3-PD production from 3-hydroxypropionaldehyde (Barbirato *et al.* 1997). The increase in 1,3-PD production was accompanied by a decrease in H_2 production. This observation can be explained by the clostridial metabolic route for 1,3-PD production, in which H_2 and 1,3-PD producing routes are competing (Saint-Amans *et al.* 2001).

Biological production of 1,3-PD is either vitamin B_{12} dependent or independent determined by the vitamin B_{12} dependence characteristics of glycerol dehydratase that catalyzes glycerol dehydration to 3-HPA. In the studies with *H. saccharolyticum* subsp. *saccharolyticum* (Papers **II** and **III**) no 1,3-PD was produced without vitamin B_{12} supplementation whereas 64 µg/l vitamin was enough for maximal production. As expected, the control bacterium, *C. butyricum*, produced 1,3-PD even in the absence of vitamin B_{12}. Therefore, the 1,3-PD production of *H. saccharolyticum* subsp. *saccharolyticum* is vitamin B_{12}-dependent in contrast to 1,3-PD production of *C. butyricum*. In clostridial bacteria, 1,3-PD is produced via a route in which glycerol is first reduced to 3-HPA catalyzed by glycerol dehydratase enzyme, of which active functioning is often dependent on vitamin B_{12}, and then further to 1,3-PD catalysed by 1,3-propanediol dehydrogenase (Saint-Amans *et al.* 2001). H_2 is produced in clostridial

bacteria on glycerol oxidizing route leading to production of acetate, ethanol, and/or butyrate instead of the reducing pathway leading to production of 1,3-PD (Saint-Amans *et al.* 2001).

Without vitamin B_{12} supplementation, the H_2 production and yield were enhanced significantly (Papers **II** and **III**). This phenomena is most prominently due to the blocked 1,3-PD route forcing the fermentation towards the competing route on glycerol oxidizing branch of metabolism. Cells produce reduced metabolites such as 1,3-PD, H_2, butyrate, and ethanol in order to maintain the electron balance. In the absence of 1,3-PD production the metabolism has to adapt and the electrons must be transferred to other reduced compounds. Our findings indicate that by controlling the nutrient supply, certain metabolic routes can be blocked or induced easily without need for genetic engineering. This opens up opportunities for metabolic engineering, with certain limitations, of the bacteria for which genetic manipulation systems have not yet been established.

The highest cumulative 1,3-PD production was obtained at 30 ^{o}C temperature (Paper **III**). The differences in 1,3-PD production were not remarkable. On the other hand, the growth was fastest at 40 ^{o}C, and therefore, though no kinetic analyses were accomplished, the 1,3-PD production rate might be faster with higher temperatures. The highest H_2 production and yield was obtained at 36-37 ^{o}C.

Several studies on H_2 production indicate that H_2 partial pressure inhibits H_2 production and cause changes in metabolism (Bastidas-Oyanedel *et al.* 2012, Ciranna *et al.* 2011, Nguyen *et al.* 2010). The H_2 sensitivity of *H. saccharolyticum* subsp. *saccharolyticum* was studied by creating an experimental setup with different head-space to culture (gas-to-liquid) volume ratios in which different H_2 partial pressure levels are obtained as the H_2 starts to evolve. The results indicated that the H_2 production of *H. saccharolyticum* is sensitive to H_2 partial pressure: as the gas-to-liquid volume ratio was increased, the final H_2 partial pressure decreased and a remarkable increase in H_2 production was observed. In addition, lower H_2 partial pressure promoted growth, and slightly enhanced the H_2 yields. The increase in H_2 partial pressure typically leads to changes in metabolic fluxes towards more reduced metabolites such as lactate, ethanol, acetone, butanol, or alanine (Levin *et al.* 2004). However, no alternative metabolic routes were observed in *H. saccharolyticum*, instead, the bacterium grew scarcely under higher H_2 pressures. This indicates that when 1,3-PD production is blocked by the lack of vitamin B_{12} supplementation, the only efficient way for the bacterium to balance reduction and oxidation reactions is the H_2 production.

One significant challenge for an efficient bioprocess concerning halophilic fermentative bacteria is the slow growth rate most probably due to the bioenergetically expensive life in hypersaline environment. The halophilic fermentative bacteria need to constantly

balance the osmotic pressure by accumulating ions inside the cells. Therefore, the cumulative metabolite production remains low. Glycine betaine (betaine) is a common compatible solute accumulated by halophilic aerobic bacteria for balancing the osmotic pressure and survival in hypersalinity. Compatible solutes act as osmoprotectants also for non-halophilic cells and biomolecules. Recently genes for betaine uptake were found in the genome of *Halothermothrix orenii*, a halothermophilic fermentative bacterium, suggesting that betaine could play a role also in fermentative bacteria which almost exclusively accumulate ions instead of organic compounds for survival (Mavromatis *et al.* 2009). The results (Paper **III**) from *H. saccharolyticum* subsp. *saccharolyticum* showed betaine to slightly increase growth and decrease the final H_2 concentrations. The analysis of liquid end-metabolites further indicated a significant increase (100 %) in acetate yield and carbon recoveries (biomass production excluded) exceeding 100 % (110 % with 10-50 mM betaine). The results suggest that the bacterium could be able to utilize betaine and H_2 for acetate production. The reaction in which an amino acid (e.g. serine) or H_2 is used as an electron donor and betaine as an acceptor, known as Stickland reaction (Nisman 1954), has been reported being active also in other halophiles (Mouné *et al.* 1999, Tsai *et al.* 1995). The slight increase in growth might be due to lower H_2 partial pressure as shown above.

As all living organisms, bacteria require nitrogen source for building blocks of amino and nucleic acids. The halophilic growth medium used in the studies contains two nitrogen sources, ammonium chloride (NH_4Cl) and tryptone. The studies (Paper **III**) indicate that *H. saccharolyticum* subsp. *saccharolyticum* does not require NH_4Cl for growth in the presence of tryptone as a nitrogen source and no significant effects on growth were observed with 0-2 g/l NH_4Cl. On the other hand, the highest OD was achieved with concentration of 5 g/l, however, combined to a prolonged lag phase. The long lag phase for 5 g/l NH_4Cl suggest that high NH_4^+ might act as an inhibitory factor for growth. The highest H_2 production was observed with 1 g/l and 5 g/l NH_4Cl, respectively. Tryptone is another source for nitrogen and also for amino acids. The results (Paper **III**) indicated that tryptone is required for growth and has an enhancing effect on biomass production. Thus, despite the presence of NH_4Cl as inorganic nitrogen source, the bacterium requires peptides or free amino acids from tryptone for growth. The maximum growth rate achieved increased together with the tryptone content, however only slight differences were observed in maximal optical densities. The highest OD was measured with 2 g/l tryptone. H_2 production (background metabolite production subtracted; increased H_2 production in substrate controls) along with glycerol degradation had slightly decreasing trends as tryptone content increased from 1 to 5 g/l due to increased utilization of tryptone as a carbon source. The H_2 production was approximately at the same level with 1 g/l and 2 g/l tryptone.

Buffers are being used for preventing or minimizing the effects of acid production on pH during bacterial fermentations. The increased concentrations of phosphate and

HEPES buffers increased growth and especially H_2 production of *H. saccharolyticum* subsp. *saccharolyticum*. The observation together with the previous results of the effect of pH indicates that both the growth and H_2 production are pH sensitive. The final pH was increased from 5.5 to 6.4 with 50 mM phosphate buffer and to 6.7 with 50 mM HEPES buffer. Both the buffers were well suited for maintenance of neutral pH providing similar results in terms of growth and H_2 production. The use of phosphate buffering is preferable for its economical convenience.

8.2.2. Optimized bioprocesses

The optimized parameters were applied to *H. saccharolyticum* subsp. *saccharolyticum* H_2 producing bioprocess either in groups (salt, pH, and glycerol; buffer, tryptone, NH_4Cl and pH_2) or individually (vitamin B_{12}). Optimized parameter values were chosen as follows:

1. Optimal parameter for H_2 yield
2. If 1) is not applicable, optimal parameter for growth

Exception made if the parameter has a negative effect on H_2 production

According to the guidelines above, the optimal parameters were applied from Table 8.1 with the following exceptions or clarifications; 2.5 g/l pure glycerol, 0 g/l YE, 50 mM phosphate buffer, and 0 mM glycine-betaine. Yet another exception is pH_2, for which gas-to-liquid ratio 2.1 was used and additionally N_2 sparging was shortly applied in order to renew the headspace after the H_2 formation had stopped. The results for optimization of H_2 producing halophilic bioprosess are presented in Figure 8.1.

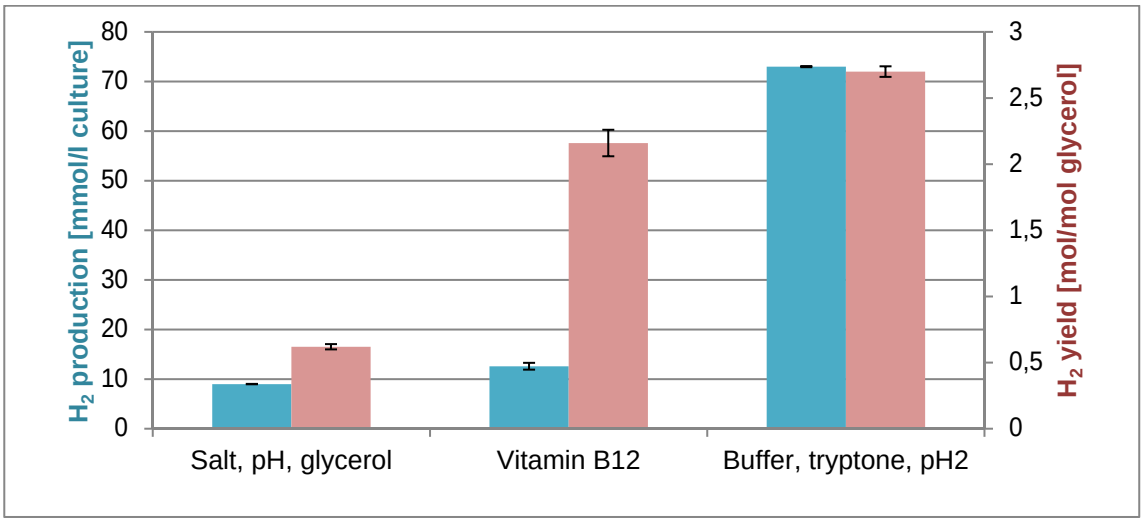

Figure 8.1. Stepwise optimization of H_2 production.

The H_2 production was slightly increased from 9 to 13 mmol H_2/l culture, whereas the H_2 yield was enhanced significantly from 0.6 to 2.2 mol H_2/mol glycerol as the salt, pH

and glycerol optimized conditions were further optimized for vitamin B_{12} (Paper **I** and **II**). Furthermore, the H_2 production was increased remarkably up to 73 mmol H_2/l culture and yield up to 2.7 mol H_2/mol glycerol as buffer, nitrogen source, gas-to-liquid ratio and periodic N_2 sparging were applied to bioprocess (Paper **IV**). The optimization experiments were accomplished with pure glycerol as a substrate but the final optimized conditions were also successfully exploited with unpurified raw glycerol (2.5 g/l). H_2 production and yield comparable for pure glycerol fermentation were obtained with raw glycerol, 71 mmol H_2/l culture and 2.70 mol H_2/mol glycerol (Paper **IV**).

As the main product of the study of this thesis was H_2, 1,3-PD production was not optimized in the same extent. The 1,3-PD production was increased from 5.4 to 14 mmol 1,3-PD/l culture and yield from 0.49 to 0.63 mol 1,3-PD/mol glycerol as the pH optimized (pH=7.8) conditions were further optimized for glycerol (2.5 g/l), NaCl (100 g/l) and vitamin B_{12} (64 µg/l). The effect of pH (7.0, 7.4 and 7.8) for 1,3-PD production was also studied in the above mentioned optimized conditions with daily pH adjustment (Kivistö *et al.*, unpublished results). The 1,3-PD production was doubled up to 29 mmol/l culture with pH adjustments. No differences were obtained between 1,3-PD production at pH 7.4-7.8 in which the production was fastest at the beginning of the experiment. However, the highest final 1,3-PD production was obtained at pH 7.0, in which initially the production was slightly lower compared to higher pHs tested, but on the other hand, the production activity remained for a longer period of time. No differences in 1,3-PD yields were obtained.

8.3. Tolerance to end-metabolites and substrate

The effect of H_2 on growth, glycerol fermentation and H_2 production has been described in Section 8.2.1. To conclude, H_2 inhibits growth and H_2 production of *H. saccharolyticum* subsp. *saccharolyticum*. Since also growth is inhibited by pH_2, H_2 formation is suggested to be essential for the halophilic fermentative bacterium in order to dispose excess reducing equivalents while the 1,3-PD producing route is closed. The end-product inhibition of hydrogenase can be circumvented by periodic N_2 flushing as shown in Section 8.2.2 and Paper **IV**.

The results of this thesis (Paper **III**) indicate that acetate has an inhibitory effect on growth. Remarkably decreased growth rate and/or early flocculation of cells were obtained at initial acetate at and above 1.5 g/l. However, a long lag phase without initial acetate was observed. No significant effects on 1,3-PD production of *H. saccharolyticum* subsp. *saccharolyticum* were observed with acetate concentration up to 10 g/l. High initial acetate (29 g/l - 58 g/l) was observed to cause minor decrease in 1,3-PD concentrations produced but no effects on 1,3-PD yields (mol/mol glycerol). As has been previously reported by Heyndrickx *et al.* (1991), acetate acts in some bacteria as a cosubstrate for fermentation.

Not only acetate but also 1,3-PD inhibited the growth of *H. saccharolyticum* subsp. *saccharolyticum* (Paper **III**). When initial 1,3-PD concentration was raised from 1 g/l to 57 g/l, a decrease of 12 % to 75 %, respectively, in the highest optical density was observed. The inhibitory effect of 1,3-PD on cell growth and glycerol uptake have been previously studied in *C. butyricum* (Colin *et al.* 2000, González-Pajuelo *et al.* 2004, Papanikolaou *et al.* 2000, Petitdemange *et al.* 1995) and *K. pneumoniaea* (Huang *et al.* 2002). An inhibitory effect of 1,3-PD at some level was observed in all the studies conducted.

No significant differences were observed in H_2 production of *H. saccharolyticum* grown either with (fermentation directed towards 1,3-PD) or without (fermentation directed towards H_2) vitamin B_{12} at 2.5-40 g/l pure glycerol and 2.5-5 g/l raw glycerol (Paper **V**). The raw glycerol contents of 10-40 g/l and 20-40 g/l inhibited the glycerol fermentation directed for H_2 (w/o B_{12}) and 1,3-PD (w/ B_{12}) production, respectively. Similarly, raw glycerol concentrations of 20-40 g/l inhibited the activity of non-halophilic *C. butyricum*. Previous optimization studies (Paper **I**) indicated a decrease in 1,3-PD production as glycerol content increases. In the further study with optimized vitamin B_{12} contents, the phenomena were observed for *H. saccharolyticum* and *C. butyricum* using both pure and raw glycerol as substrates. Additionally, the 1,3-PD production of *H. saccharolyticum* using raw glycerol as a substrate was at lower level compared to pure glycerol.

The raw glycerol inhibition most likely originates from the methanol or soaps present in the raw glycerol fraction instead of ionic compounds. In previous studies accomplished by Ngo *et al.* (2011) and Srinophakun *et al.* (2012), unpurified raw glycerol (approximately 1 % w/v glycerol) was reported to inhibit *Thermotoga neapolitana* and pretreatment (alcohol and solids removal) to be crucial, whereas for the growth of *Escherichia coli* the crude glycerol impurities (75.5 % w/w glycerol, 13.3 % w/w soap, 3.5 % w/w soap and 1-2 % w/w methanol) had a positive effect. The consistency of biodiesel derived glycerol varies depending on the biodiesel production process and thus the results with different glycerol stocks are hardly comparable. Using the same raw glycerol fraction (56 % w/v glycerol, 23 % m/v methanol, 1.8 % m/v K^+) as in this thesis, Mangayil *et al.* (2012) recently examined glycerol fermentation in a mixed culture and observed positive effect of raw glycerol impurities at glycerol content 1 g/l, above which the inhibitory effect was shown as decreased metabolic activity.

8.4. Contamination at increasing salinity

Bioprocess contaminants generally utilize either substrate or product for unintended metabolite production. Both the cases result in the decreased product yields. Tap water is more economical matrice for bioprocesses compared to ultrapure water. Without further sterilization, tap water acts as a significant source of contaminants. At

extremophilic process conditions, however, the chance for survival of the contaminants is suggested being non-existent. In the experiment accomplished, the highest NaCl concentration for the growth of contaminants was 0 g/l (glycerol as a substrate) and 100 g/l (glucose as a substrate).

The upper salt content for contaminant growth in glucose medium is relatively high, however, below the salt content (150 g/l) well applicable for fermentative processes by halophilic bacteria. Furthermore, the use of glycerol instead of glucose as a carbon source protects the process effectively for contaminants due to a fact that glycerol is used as a carbon source by a limited group of microorganisms whereas glucose is, in general, readily degraded by nearly all microorganisms.

8.5. Non-sterile H_2 and 1,3-PD production

The main challenge in halophilic H_2 production is low biomass production and thus low volumetric H_2 production. The vitamin B_{12} consisting process conditions were observed to induce 1,3-PD production and enhance biomass production by *H. saccharolyticum* (Papers **II** and **III**). The two-stage process for combined H_2 and 1,3-PD production aimed in not only combining the two valuable but competing end-products into one process but also providing the H_2 producing phase for higher biomass content via biomass harvesting after the 1,3-PD and biomass producing phase.

The highest biomass in the experiment was obtained in the 2-stage culture shortly after the biomass (cell) collection and the beginning of the 2nd phase (Paper **V**). However, the cells collected flocculated and the metabolic activity was decreased shortly after inoculation of the 2nd phase. Another remarkable phenomena was the retained activity for 1,3-PD production in the early 2nd stage despite the omitted vitamin B_{12} in the growth medium which lead to decreased H_2 production efficiency. Besides biomass, the 2-stage process resulted in slightly higher cumulative H_2 and 1,3-PD production (74 mmol H_2/l culture, 31 mmol 1,3-PD/l culture) compared to the separate 1-stage processes (70 mmol H_2/l culture, 30 mmol 1,3-PD/l culture).

A theoretical H_2 yield (3 mol/mol glycerol) was calculated for the 1-stage process directed for H_2 production, whereas yields of 1.2 and 0.61 mol H_2/mol glycerol were obtained in the 2-stage process for combined 1,3-PD and H_2, and the 1-stage process directed for 1,3-PD production, respectively. The highest 1,3-PD yield of the experiment (0.66 mol/mol glycerol) was obtained in the 1-stage process for 1,3-PD production in which the bacteria were incubated in the presence of vitamin B_{12} throughout the experiment. The 1,3-PD yield in the 2-stage process was 0.50 mol/mol glycerol. The dark-fermentative theoretical maximum H_2 yield (3 mol/mol glycerol) obtained and the electron recovery exceeding 100 % (102 % - 105 %) implies on a small amount of non-glycerol component usable as a substrate in the raw glycerol solution.

The experiment proved tap water being suitable component for bioprocess employing *H. saccharolyticum*. In addition, the non-sterile process remained uncontaminated, i.e. no growth, substrate utilization or metabolite production was observed in negative growth controls throughout the experiment, giving yet another indication for the significantly decreased risk for contamination in hypersaline process conditions.

The results of the non-sterile H_2 and 1,3-PD production in tap water medium were collected in Table 8.2 along with previously reported biodiesel derived glycerol degrading H_2 production results. Despite low yields of the enteric bacteria (0.5-1 mol/mol), higher volumetric H_2 production and H_2 production rates are achieved. Cumulative H_2 production of 118 mmol/l was obtained by *K. pneumoniae* with H_2 rate of 17.8 mmol/l/h (Liu and Fang 2007). Even a higher rate, 63 mmol/l/h, has been achieved by *E. aerogenes* (Ito *et al.* 2005). For *H. saccharolyticum* the cumulative H_2 production was 74 mmol/l and maximum production rate 0.41 mmol/l/h. Additionally, the 1,3-PD titer obtained, 2.4 g/l (31 mmol/l), is low compared to previously reported 76.2 g/l for *C. butyricum* and 51.3 g/l for *K. pneumonia* (Mu *et al.* 2006, Wilkens *et al.* 2012), which however, result in inefficient H_2 production. H_2 yields up to 2.73 mol/mol glycerol has been reported for dark fermentative processes (Ngo *et al.* 2011), and according to the best of my knowledge, the H_2 yield of 3 mol/mol glycerol obtained by *H. saccharolyticum* is the highest yield reported.

Table 8.2. Dark-fermentative H_2 production from biodiesel derived glycerol.

Bacterial culture	Substrate	Type of fermentation	Biomass [g/l]	H_2 yield [mol/mol]	H_2 production [mmol l^{-1}]	H_2 rate [mmol $l^{-1}h^{-1}$]	1,3-PD yield [mol/mol]	1,3-PD production [mmol l^{-1}]	Ref.
H. saccharolyticum subsp. *saccharolyticum*	Raw glycerol	Batch	0.09-0.24	0.61-3.0	28-74	0.14-0.41	0-0.66	0-31	**V**
E. aerogenes	Crude glycerol	Batch	NR[a]	0.85-0.95	NR[a]	NR[a]	NR[a]	NR[a]	Jitrwung and Yargeau 2011
E. aerogenes	Raw glycerol	Batch Continuous	NR	1.12 NR[a]	NR[a]	NR[a] 63	0.22 NR[a]	NR[a]	Ito *et al.* 2005
E. coli HB41	Pretreated[b] raw glycerol	Batch	0.025[c]	NR[a]	3.8[c]	NR[a]	NR[a]	NR[a]	Yuwa-amornpitak 2012
K. pneumoniaea	Raw glycerol	Batch	NR[a]	0.53	117.8	17.8	NR[a]	88[d]	Liu and Fang 2007
T. neapolitana	Pre-treated[e] raw glycerol	Batch	0.97	2.73	77	NR[a]	NR[a]	NR[a]	Ngo *et al.* 2011
T. neapolitana	Raw glycerol Pre-treated[b] raw glycerol	Batch	0.71 NR[a]	1.3 NR[a]	20[f] 27[g]	NR[a]	NR[a]	NR[a]	Ngo and Sim 2012
Mixed culture (heat-treated)	Crude glycerol	Batch	NR[a]	0.31	8	NR[a]	0.59	13[c]	Selembo *et al.* 2009
Mixed culture	Raw/crude glycerol	Batch	NR[a]	6[h]	NR[a]	NR[a]	NR[a]	NR[a]	Fernandes *et al.* 2010

[a] Not reported.
[b] Acid pretreatment, salts removal
[c] Approximated from a figure.
[d] 6.7 g/l
[e] Alcohol and solids removed.
[f] 456 ml/l
[g] 620 ml/l
[h] mmol/gCOD

The cultivation studies of this thesis were all preliminary small scale studies. The recommended future studies include scale-up and experiments in a continuous mode. Additionally, the use of marine water as a matrix would decrease the dependence of the process on fresh water and therefore might be worth future studies. The developed bioprosesses at present are most prominently not economically viable due to slow growth and low productivity of the halophilic fermentative bacterium. Additionally, despite the relatively high H_2 and/or 1,3-PD yields, a lot of carbon and electrons are bound to the liquid by-product, acetate. In order to improve the economic viability of the process, acetate could be further transformed into higher-value (energy) products, such as H_2 via photofermentation (Srikanth *et al.* 2009) or electrohydrogenesis (Lalaurette *et al.* 2009) and CH_4 via acetotrophic methanogenesis (Luo *et al.* 2011).

8.6. Genome analysis

The whole-genome shotgun project (Papers **VI** and **VII**) of *Halanaerobium saccharolyticum* subsp. *saccharolyticum* has been deposited at DDBJ/EMBL/GenBank under the accession no. CAUI00000000. The version described in this thesis is the first version, CAUI01000000. The "improved high-quality draft" (**VI**) genome is 2,873,865 bp in size, comprising 24 contigs (≥1 kb). The genome was predicted to contain 2,664 coding sequences and which 72 are for RNAs. The maximum contig length in the assembly is 900,505 bp, and the N_{50} is 723,182 bp. The GC content of the genome is 32.3 %. The genome properties and comparison to previously sequenced *Halanaerobium praevalens* (Ivanova *et al.* 2011), *Halanaerobium hydrogeniformans* (Brown *et al.* 2011), *Halothermothrix orenii* (Mavromatis *et al.* 2009) and *Acetohalobium arabaticum* (Sikorski *et al.* 2010) is presented in Table 8.3.

Table 8.3. Genome statistics of Halanaerobium saccharolyticum subsp. saccharolyticum (H. sacch) compared to genomes of Halanaerobium praevalens (H. praev), Halanaerobium hydrogeniformans (H. hydr), Halothermothrix orenii (Ht. oren), and Acetohalobium arabaticum (A. arab).

Genome characteristic	*H. sacch*	*H. praev*	*H. hydr*	*Ht. oren*	*A. arab*
Genome sequence	Draft	Complete	Complete	Complete	Complete
Contigs	24	1	1	1	1
Genome size	2,873,865	2,309,262	2,613,116	2,578,146	2,469,596
GC-content	32.3	30.3	33.1	37.9	36.6
Genes	2736	2180	2457	2457	2443
Protein coding sequences	2664	2068[a]	2295	2372	2353
Proteins with function prediction	1888	1694	1839[a]	1965	1873
Proteins w/o function prediction	776	486	456[a]	407	480[c]
Genes for rRNAs	15[b]	12	12	11	15
Genes for tRNAs	57	55[a]	57[a]	56	67
Genes for other RNAs	-	4[a]	4[a]	18	9
Reference	**IV**	Ivanova *et al.* 2011	Brown *et al.* 2011	Mavromatis *et al.* 2009	Sikorski *et al.* 2010

[a] Data retrieved from GenBank genome database.
[b] Five complete, 10 partial genes.
[c] Calculated from the results

According to 16S rDNA multiple sequence alignments (**VII**) presented in Figure 8.2 as a phylogenetic tree, the closest relatives of *H. saccharolyticum* subsp. *saccharolyticum* are *H. saccharolyticum* subsp. *senegalense* followed by *Halanaerobium praevalens*, *Halanaerobium kushneri*, and *Halanaerobium acetethylicum*.

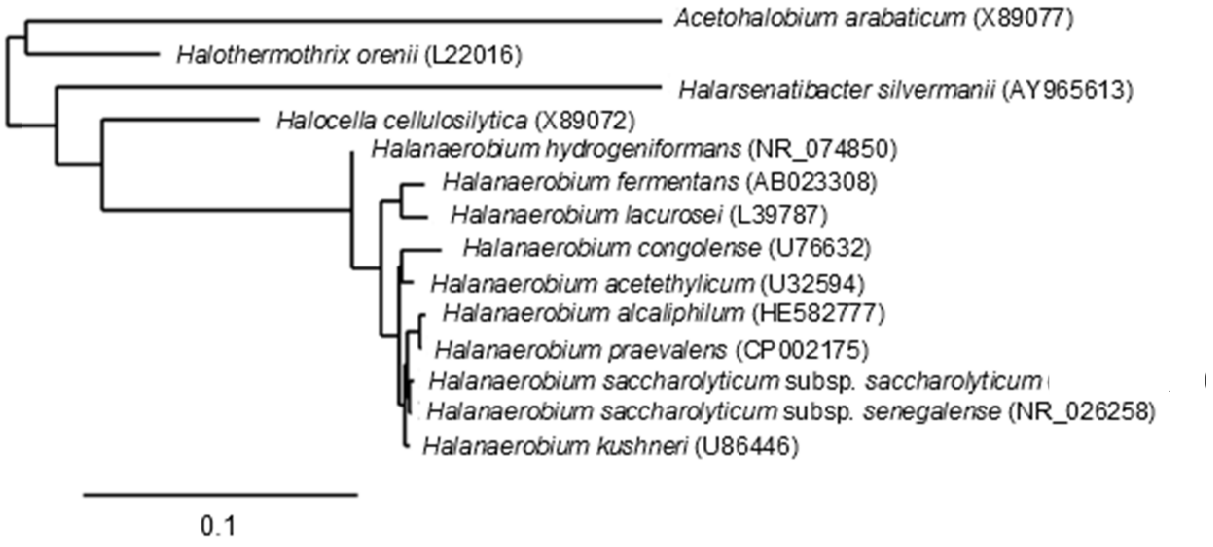

Figure 8.2. Phylogenetic relations of Halanaerobium saccharolyticum subsp. saccharolyticum (ORF HSACCH_r00015) to Halanaerobium spp. and other members of family Halanaerobiaceae.

Reconstruction of glycerol fermentation pathways of *H. saccharolyticum* revealed H_2, CO_2, acetate, butyrate, butanol, ethanol, lactate, malate, formate, oxaloacetate, and 1,3-propanediol (vitamin B_{12} dependent route) as potential fermentation products (Figure 8.3). The fermentation pathways were shown to be highly similar with the pathways of *C. butyricum* (Saint-Amans *et al.* 2001). The main differences were observed in glycerol oxidation and phosphorylation to dihydroxyacetone phosphate (DHAP) and glycerol dehydration to 3-hydroxypropanal. The genome of *H. saccharolyticum* encodes for genes catalyzing two alternative pathways for glycerol oxidation and phosphorylation to DHAP. The first alternative pathway phosphorylates glycerol using ATP to glycerol-3P which is further oxidized to DHAP in either NAD^+ or FAD^+ dependent reaction. The second alternative route oxidizes glycerol to DHA with NAD^+ as a coenzyme, followed by phosphorylation to DHAP in a reaction transforming phosphoenolpyruvate to pyruvate. *C. butyricum* has been reported to degrade glycerol along with the second alternative route described, however, using ATP as the origin of phosphate group instead of PEP (Saint-Amans *et al.* 2001). Another main difference is found in the genes for glycerol dehydratase. The putative glycerol dehydratase of *H. saccharolyticum* consists of three subunits (DhaB1, DhaB2, DhaB3) and thus is suggested being cobalamin dependent. In contrast, two subunits (DhaB1, DhaB2) form the glycerol dehydratase of *C. butyricum* characterized as cobalamin independent.

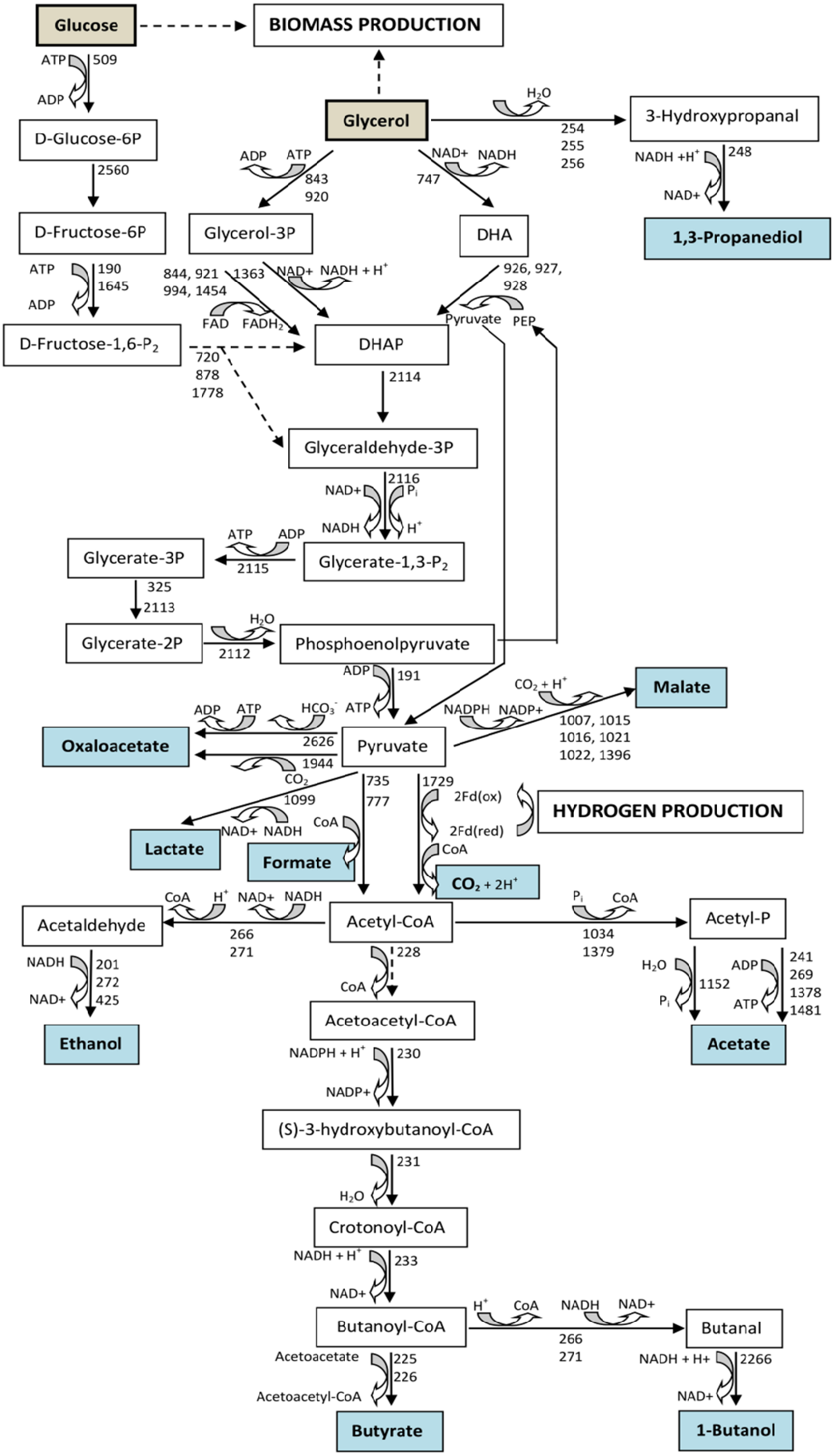
Glucose
BIOMASS PRODUCTION
ATP
509
ADP
D-Glucose-6P
2560
D-Fructose-6P
ATP
190
1645
ADP
D-Fructose-1,6-P2
720
878
1778
Glycerol
H2O
254
255
256
3-Hydroxypropanal
NADH +H+
248
NAD+
1,3-Propanediol
ADP ATP
843
920
Glycerol-3P
NAD+ NADH
747
DHA
844, 921
994, 1454
1363
NAD+ NADH + H+
FAD FADH2
926, 927,
928
Pyruvate PEP
DHAP
2114
Glyceraldehyde-3P
2116
NAD+ Pi
NADH H+
ATP ADP
2115
Glycerate-1,3-P2
Glycerate-3P
325
2113
Glycerate-2P
H2O
2112
Phosphoenolpyruvate
ADP
191
CO2 + H+
Malate
NADPH NADP+
1007, 1015
1016, 1021
1022, 1396
ADP ATP
HCO3-
2626
1944
CO2
Oxaloacetate
ATP
Pyruvate
1099
735
777
1729
2Fd(ox)
2Fd(red)
HYDROGEN PRODUCTION
NAD+ NADH
CoA
Lactate
Formate
CoA
CO2 + 2H+
CoA H+ NAD+ NADH
266
271
Acetaldehyde
Acetyl-CoA
228
Pi CoA
1034
1379
Acetyl-P
NADH
201
272
425
NAD+
Ethanol
CoA
Acetoacetyl-CoA
H2O
1152
Pi
ADP
ATP
241
269
1378
1481
Acetate
NADPH + H+
230
NADP+
(S)-3-hydroxybutanoyl-CoA
231
H2O
Crotonoyl-CoA
NADH + H+
233
NAD+
H+ CoA NADH NAD+
266
271
Butanoyl-CoA
Butanal
Acetoacetate
225
226
Acetoacetyl-CoA
Butyrate
NADH + H+
2266
NAD+
1-Butanol

Figure 8.3. Putative re-constructed glucose and glycerol fermentation pathways in H. saccharolyticum. The numbers associated with reactions (marked primarily on right side or below an arrow) refer to locus tags (e.g. No. 789 refers to HSACCH_0789) for ORFs of putative catalyzing enzymes or enzyme subunits in the genome. Substrates of fermentation pathways are marked as bold border boxes and end-metabolites as gray boxes. Solid arrow, equal amount of reactant and product; dash arrow, unequal amount of reactant and product: One molecule of D-Fructose-1,6-P2 yields one molecule of DHAP, one molecule of glyceraldehyde-3P eventually yielding two molecules of glyceraldehyde-3P, two molecules of Acetyl-CoA yields one molecule of acetoacetyl-CoA. Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; CoA, coenzyme A; DHA, dihydroxyacetone; DHAP, dihydroxyacetonephosphate; FAD, flavin adenine dinucleotide (oxidiced form); $FADH_2$, flavin adenine dinucleotide (reduced form); Fd(ox), ferredoxin (oxidized form); Fd(red), ferredoxin (reduced form); H^+, proton; HCO_3^-, bicarbonate; NAD^+, nicotinamide adenine dinucleotide (oxidized form); NADH, nicotinamide adenine dinucleotide (reduced form); $NADP^+$, nicotinamide adenine dinucleotide phosphate (oxidized form); NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); P, phosphate; P_2, diphosphate; P_i orthophosphate; PEP, phophoenolpuryvate.

The regulon containing glycerol dehydratase is commonly called as dihydroxyacetone kinase (dha) regulon because it typically contains not only glycerol dehydratase but also dihydroxyacetone (dha) kinase. However, this is not the case in the draft genome of *H. saccharolyticum* subsp. *saccharolyticum* as the dihydroxyacetone kinase genes are located in another contig. The dha cluster for *H. saccharolyticum* encoding for putative cobalamin dependent glycerol dehydratase is presented in Figure 8.4.

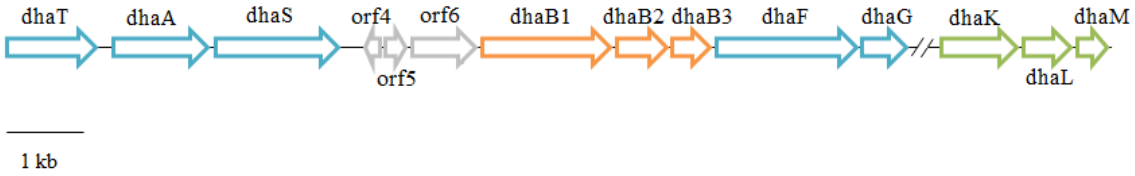

Figure 8.4. Dha regulon and related genes in H. saccharolyticum genome. Abbreviations: dhaKLM, dihydroxycetone kinase; dhaSA, a two-component signal transduction system; dhaB1B2B3, cobalamin dependent glycerol dehydratase; dhaT, 1,3-propanediol dehydrogenase; dhaFG, glycerol dehydratase re-activation factor; orf4, hypothetical protein; orf5, orf6, putative ethanolamine/1,2-propanediol utilization proteins.

In the genome of *H. saccharolyticum*, four [FeFe]-hydrogenases are found, two of which are putative bifurcating hydrogenases requiring both reduced ferredoxin and NADH. No [NiFe]-hydrogenases were identified. The hydrogenase gene operons (A) and putative molecular structures of the catalytic subunits (α, HydA, HydB) as well as accessory subunits (B) are presented in Figure 8.5. The cluster M1/M2 contains two putative monomeric hydrogenases of which M1 contains PAS/PAC sensor domain. The PAS/PAC domains are typically involved in proteins with light, oxygen, or redox-sensing abilities (Taylor and Zhulin 1999). However, the function of this type of hydrogenase remains uncharacterized (Calusinska *et al.* 2010). Cluster Tr (Figure 8.5) encodes for a putative trimeric bifurcating hydrogenase characterized for the first time in *Thermotoga maritima* (Schut and Adams 2009). The cluster Te (Figure 8.5) has nearly identical structure with tetrameric putative bifurcating (or NADH dependent) [FeFe]-hydrogenase of *Thermoanaerobacter tengcongensis* (Soboh *et al.* 2004).

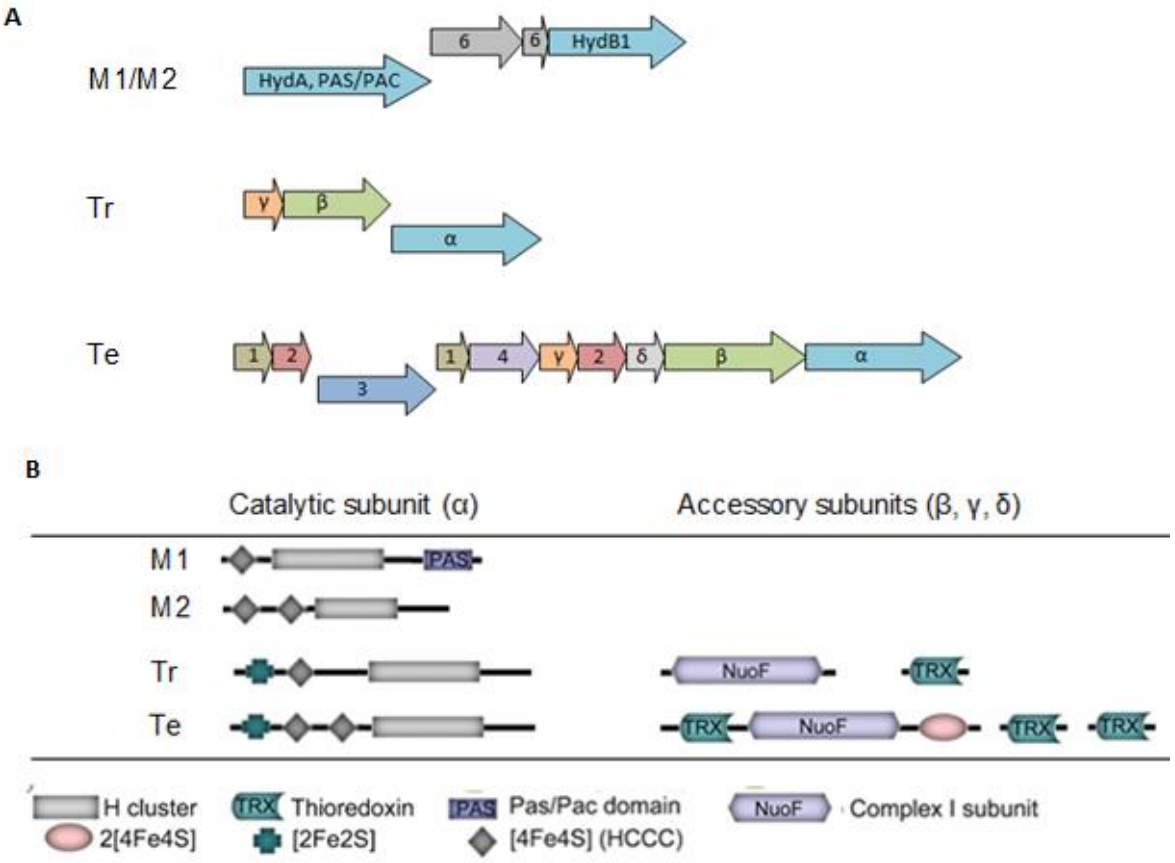

Figure 8.5. The hydrogenase clusters in the genome of H. saccharolyticum (A) and putative molecular structures of the catalytic subunits as well as the putative accessory subunits (B). The putative bifurcating hydrogenases (Tr, Te) comprise of subunits α (hydrogenase A), β (NADH-ubiquinone oxidoreductase chain F like protein), γ (NADH-ubiquinone oxidoreductase chain E like protein; thioredoxin-like protein) and δ (Te; thioredoxin-like protein). 1, DRTGG-domain containing protein; 2, ATP binding region; 3, FeS cluster domain protein; 4, PHP domain protein; 6, Hypothetical protein.

The hydrogen production pathways of *H. saccharolyticum* presented in Figure 8.6 were reconstructed according to the encoded hydrogenases (Figure 8.5). The two putative monomeric hydrogenases derive H_2 using the electrons from reduced ferredoxin formed during acidogenesis (Figure 8.3), whereas the putative trimeric and tetrameric hydrogenases utilize NADH formed during glycerol degradation and reduced ferredoxin synergistically. Balance between NADH and ferredoxin is maintained by a putative membrane-bound electron transfer chain-complex (rnf) that couples the reactions with Na^+-extrusion. Na^+ extrusion is typical for halophilic salt-in bacteria as they commonly accumulate K^+ ions instead of cytotoxic Na^+ for survival in highly saline environment.

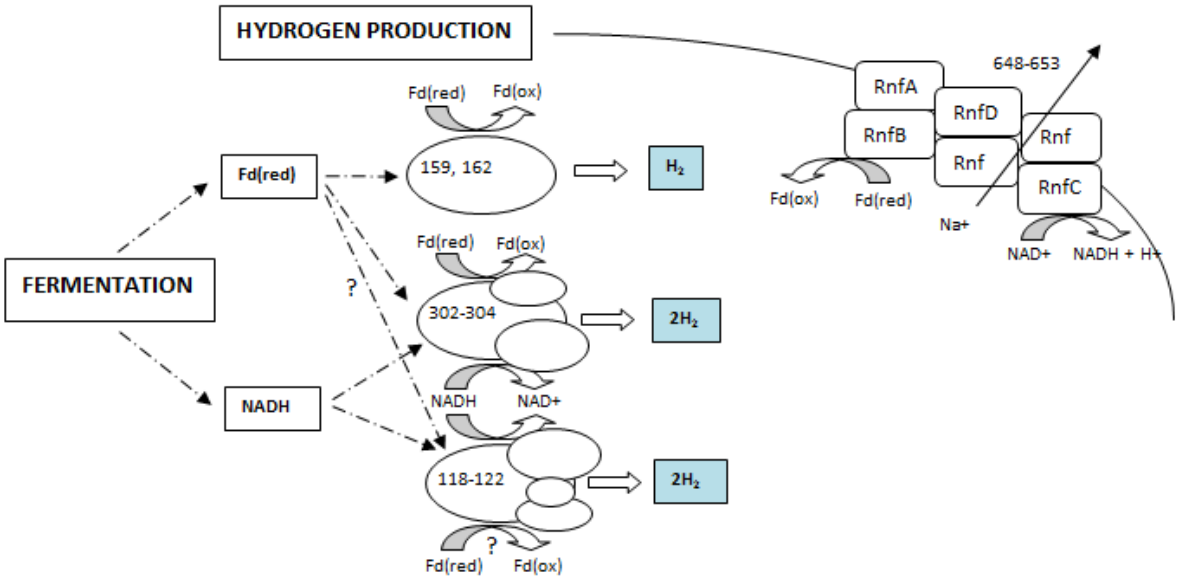

Figure 8.6. The reconstructed H_2 production pathways of H. saccharolyticum.

Two types of multidrug efflux pumps were identified in the genome of *H. saccharolyticum*: multidrug and toxin extrusion proteins (MATE) and resistance-nodulation-cell division proteins (RND). The MATE proteins are antiporters that couple the extrusion of metabolites, drugs and toxins with Na^+ intrusion. The RND system of *H. saccharolyticum* consist three subunits; an inner membrane efflux protein, a membrane fusion protein, and an outer-membrane efflux protein. Additionally, the genes for β-lactamase, mercuric reductase, copper translocating ATPase, and cobalt-zinc-cadmium resistance protein suggest *H. saccharolyticum* being resistant to wide variety of antibiotics and toxic compounds including heavy metals. The resistance to heavy metals and other toxic compounds is beneficial for tolerance and degradation of impure waste streams and industrial by-products.

9. CONCLUDING REMARKS

- Glycerol fermentation of *H. saccharolyticum* subsp. *saccharolyticum* yielded H_2, CO_2, acetate and 1,3-PD.
- 1,3-PD production is vitamin B_{12} dependent and the unavailability of B_{12} yields the highest H_2 production.
- High-yield H_2 production was obtained by proper buffering and periodic N_2 flushing.
- H_2 production was enhanced from 9 to 13 mmol/l culture with optimized salt, pH and glycerol concentration, and further to 73 mmol/l culture with optimized buffer, nitrogen source and gas-to-liquid ratio along with periodic N_2 sparging.
- Similarly, H_2 yield was increased from 0.6 to 2.2 mol/mol glycerol and further to 2.7 mol/mol glycerol.
- The process remains uncontaminated despite the nonsterile methods.
- Regular tap water can be used in the process instead of expensive ultra-pure MQ water.
- Unpurified raw glycerol was proven as a suitable substrate for the fermentation of *H. saccharolyticum* subsp. *saccharolyticum*. A maximum H_2 yield of 3.0 mol/mol and a 1,3-PD yield of 0.66 mol/mol were obtained.
- 1,3-propanediol production can be combined with hydrogen production in a two-step process. The combined bioprocess yielded 74 mmol H_2/l culture and 31 mmol 1,3-PD/l culture.
- Genome analysis provides insight into the halophilic salt-in bacteria and especially into the halophilic metabolic routes.
- Future studies could reveal the functionality of *H. saccharolyticum* subsp. *saccharolyticum* in larger scale bioreactor studies, examine the possibilities for transformation of acetate to higher-value products and evaluate marine water as a matrix. The recommended genome analysis studies will focus on revealing the characteristics of the salt-tolerant proteins.

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