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BIOHYDROGEN PRODUCTION FROM CRUDE GLYCEROL, LACTOSE AND HYDROGEN STORAGE POSSIBILTIES WITH METALHYDRIDES FROM FERMENTATION BROTH

DOCTORAL THESIS

Submitted for the degree of Doctor of Biology

Subfield of Microbiology

Supervisor**: *Dr.hab.biol.* UL BF prof. Indriķis Muižnieks**

Scientific consultants: ***Dr. phys*. Jānis Kleperis, *Dr.biol*. Vizma Nikolajeva**

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The thesis will be defended at the public Session of the Doctoral Committee of Biology, University of Latvia at Faculty of Biology, Kronvald blvd. 4, Room , Riga, on _____.__ 201_ at __:__

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Annotation

Thesis title: "Biohydrogen production from crude glycerol, lactose and hydrogen storage possibilties with metalhydrides from fermentation broth"

Hydrogen production by biological processes using fermentative microorganisms is an environment-friendly way to produce renewable energy carrier and for a storage and use of energy, given that renewable substrates are used. Microorganisms are capable to produce hydrogen during fermentation of organic substrates and industrial waste products can be used as a feedstock for hydrogen producing bacteria. Substrates that can be effectively used for microbial hydrogen production are crude glycerol, a by-product from the process of biodiesel production, and lactose containing whey from cheese or milk production process.

Various bacterial isolates and different substrates were tested for hydrogen gas production rates with test-reactor systems constructed in our laboratory. It was established that several of the isolated bacterial strains are suitable for biohydrogen production research and crude glycerol and lactose from cheese whey are perspective substrates for hydrogen production in fermentation process.

To optimize the hydrogen collection method, it is necessary to study properly the hydrogen production kinetics in liquid phase during the fermentation process and find a solution for hydrogen oversaturation. In this work hydrogen-absorbing metal were used to adsorb hydrogen molecules from the solution to its surface, to catalyze them splitting in atomic hydrogen and facilitate the diffusion of it in the crystal lattice volume of metal.

Work was performed at the Department of Microbiology and Biotechnology, Faculty of Biology and in the Institute of Solid State Physics, University of Latvia (2011-2015).

Key words: biohydrogen, fermentation process, crude glycerol, metal hydrides

Anotācija

Darba nosaukums: "Jēlglicerīna, laktozes izmantošana bio-ūdeņraža iegūšanai ar anaerobām mikroorganismu kultūrām un ūdeņraža savākšana no fermentācijas barotnes ar metālhidrīdiem."

Bioloģiska ūdeņraža producēšana, izmantojot anaerobus mikroorganismus, ir alternatīvs, uz atjaunojamiem energoresursiem balstīts, ūdeņraža iegūšanas un uzglabāšanas veids. Šajā darbā pētīta baktēriju spēja veidot ūdeņradi organisko vielu anaerobās fermentācijas procesā. Pārbaudīta dažādu baktēriju kultūru un substrātu ūdeņraža veidošanās produktivitāte, izmantojot mūsu laboratorijā konstruētas testsistēmas, kuras pilnveidotas, lai optimizētu ūdeņraža koncentrācijas mērīšanu šķidrajā un gāzes fāzēs. Izmantotās metodes un izveidotās testsistēmas ļauj salīdzinoši novērtēt substrātu un producentu produktivitāti.

Šajā darbā pierādīts, ka no dabiskiem substrātiem izolētās baktēriju tīrkultūras var izmantot bio-ūdeņraža ieguvei. Konstatēts, ka biodegvielas ražošanas atkritumprodukts - jēlglicerīns, kā arī piena sūkalās esošā laktoze, ņemot vērā to pieejamību, ir perspektīvi substrāti ūdeņraža iegūšanai, izmantojot baktēriju fermentācijas procesu.

Ūdeņraža veidošanās kinētika un koncentrāciju mērījumi gāzveida un šķidrajās fāzēs autores pētījumos parādījuši, ka šķidrajā fāzē fermentācijas sākumā ūdeņradis veido pārsātinātus šķīdumus, tas ir, parciālais ūdeņraža gāzes spiediens virs šķīduma ir mazāks, kā to paredz Henri likums. Lai optimizētu testsistēmu un padarītu procesu rūpnieciski izdevīgāku, tika realizēta alternatīvu metožu izmantošana ūdeņraža savākšanai tieši no barotnes (šķidrās fāzes) ar dažādiem metālhidrīdu sakausējumiem.

Darbs izstrādāts LU Bioloģijas fakultāte Mikrobioloģijas un biotehnoloģijas katedrā, LU Cietvielu fizikas institūta Ūdeņraža enerģētikas materiālu laboratorijā no 2011.-2015.gadam.

Atslēgas vārdi: bioūdeņradis, fermentācijas process, jēlglicerīns, metālhidrīdi

Abbreviations

ADP	Adenosine diphosphate
AG	Analytical glycerol
AM	Anaerobic medium
Ar	Argon (gas)
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
CD	Current density
CFU	Colony forming units
CG	Crude glycerol
CSTR	Continuous stirred tank reactor
CTM	Clark-type microsensors
DC	Direct current
DHA	Dihydroxyacetone
EMP	Embden-Meyerhof-Parnas
ESI	Electrospray ionization
Fd	Ferredoxin
FHL	Formiate hydrogenlyase
FT-IR	Fourier Transform Infrared (Spectrometer)
GGE	Greenhouse gas emissions
GJ	Giga joules
H_2	Hydrogen (gas)
H+	Hydrogen ion
HHV	Higher heating value
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectra
HRT	Hydraulic retention time
KOH	Potassium hydroxide
LB	Luria-Bertrani
LHV	Lower heating value
MEC	Microbial electrolysis cell
MS	Masspectometer

MSCL	Microbial Strain Collection of Latvia
NADH	Nicotinamide adenine dinucleotide reduced
N_2	Nitrogen (gas)
OD	Optical density
PCR	Polymerase Chain Reaction
PFL	Pyruvate:formate lyase
PG	Pure glycerol
POR	Pyruvate:ferredoxin oxidoreductase
PDO	Propanediol
PS	Photosystem
PTA	Phosphotransacetylase
PTFE	Polytetrafluoroethylene
SEM	Scanning electron microscope
TG	Thermogravimetric (measurements)
VE	Voltage efficiency

Content

Introduction ... 9

Approbation of research results ... 10
Original scientific publications: ... 10
Scientific conference reports: ... 11

1. Literature Overiew ... 18
1.1. Alternatives for sustainable energy production and hydrogen as a perspective energy carrier ... 18
1.1.1. Alternative energies - renewable energy resource necessity ... 18
1.1.2. Hydrogen as an environment-friendly energy carrier ... 20
1.2. Biohydrogen production processes ... 23
1.3. Productive bacteria strains and optimal substrates ... 28
1.4. Important physical and chemical fermentation process parameters for biohydrogen production optimal conditions ... 37
1.5. Feasibility of biohydrogen production processes ... 39
1.6. Hydrogen concentration measurements ... 41
1.7. Bioreactors for hydrogen production ... 43
1.8. Metal hydride alloy usage in storing hydrogen ... 43

2. Materials and Methods ... 48
2.1. Hydrogen producing bacteria cultivation, growth media and substrate preparation ... 48
2.1.1. Growth of strains for hydrogen production in bioreactor test-systems (I) ... 48
2.1.2. Growth of strains for hydrogen production in bioreactor test-systems (II) ... 48
2.1.3. Growth of strains for hydrogen production from glycerol-containing substrate ... 49
2.1.4. Growth of strains for hydrogen production from lactose-containing substrate 49
2.1.5. Growth of strains for hydrogen isolation experiments using hydride- forming metals and alloys ... 50
2.2. Construction of strains, plasmids, and cloning ... 51
2.3. Experimental set up for hydrogen production in fermentation process ... 51
2.4. Analytical methods and experimental set up ... 52
2.4.1. Mass Spectrometry (MS) ... 52
2.4.2. Clark-type microsensors (CTM) for liquid phase analysis ... 54
2.4.3. Thermogravimetric weight loss analysis (TG) ... 54
2.4.4. Scanning electron microscopy (SEM) ... 55
Surface structure of the investigated materials before and after contact with microorganisms was studied using SEM EVO 50 (Zeiss, Germany) and SEM Lyra3 (Tescan, Chech Republic) microscope. Prior observation grains were fixed with 98% ethanol on carbon tape and coated with 20 nm Au layer. ... 55
2.4.5. High performance liquid chromatography (HPLC) ... 55
2.4.7. Iodine staining and light microscopy ... 56
2.4. Powdered metals and alloys for hydrogen storage ... 57
2.5. Construction of prototype bioreactors ... 59

3. Results ... 60
3.1. Hydrogen production analysis with experimental test systems and bioreactors, using various, also in Latvia isolated, bacteria strains ... 61

3.1.1. Biological hydrogen production in bioreactor test-systems (Original paper I).. 61
3.1.2. Bioreactor design for hydrogen producing microorganisms – optimization and experiments (Original paper II) ... 63
3.2. Hydrogen production using various, also in Latvia isolated, bacteria strains with glucose, pure glycerol and crude glycerol as substrates *(Orginal paper III and other results)* ... 70
3.3. Hydrogen production from lactose-containing substrate *(Original paper IV)* ... 77
3.4. Substrate conversion dynamic analysis and by-product identification ... 83
3.5. Hydrogen isolation possibilities using hydride- forming metals and alloys ... 86
Hydrogen gas analysis in the headspace and in the hydrogen-absorbing metal and alloys ... 87
4. Discussion ... 93
4.1. Hydrogen production analysis with experimental test systems and bioreactors, using various bacteria strains and organic waste materials ... 93
4.2. Hydrogen isolation possibilities using hydrogen-absorbing metals and alloys ... 99
5. Conclusions ... 102
6. Defense thesis ... 104
Acknowledgements and funding ... 105
References ... 106
APPENDIX ... 124

Introduction

Biological production of hydrogen using bacteria is a promising and advantageous area, especially when hydrogen is gained from a variety of renewable resources. During the conversion of organic wastes, in anaerobic environment, hydrogen gas is produced as a by-product. Substantial factors like availability and cost are highly important in the selection of waste materials to be used in hydrogen production with fermentative bacteria. Industrial and agricultural organic waste used as feedstock for hydrogen producing bacteria is a perspective way for alternative energy production and it noticeably decreases the raw material cost. One of the substrates that can be effectively used for microbial hydrogen production is glycerol, which is a by-product from the process of biodiesel production. Because of large quantities available of crude glycerol and the highly reduced nature of carbon in glycerol per se, microbial conversion is economically and environmentally viable possibility, especially because, over the last several years, the demand and production of biodiesel has remarkably increased. In practice, biohydrogen is collected from in bioreactors from the gaseous state, since dissolved hydrogen tends to be released from the liquid phase and pass into the gaseous phase until the partial pressure of hydrogen is in equilibrium with that liquid accordingly the Henry's law formulated by William Henry in 1803. To optimize the hydrogen collection method, it is necessary to study properly the hydrogen production kinetics in liquid phase during the fermentation process. In this work hydride-forming metal were used to extract the hydrogen molecules from the solution, catalyzing them to split in atomic hydrogen and facilitating the diffusion of it in the atomic lattice of metal. This is one of the first studies for hydrogen collection attempts directly from the nutritional broth using metal hydride alloys to store bacteria produced hydrogen. It has been previously proved that hydrogen over-saturation phenomena in liquid phase during dark fermentation process occur and bacteria produce more hydrogen, when hydrogen from the broth is stored or compulsorily blown out.

The aim of this study is bio-hydrogen production process optimization with isolated anaerobic microorganisms, using organic waste materials as susbtrates and hydrogen storage possibilty demonstration with hydrogen-absorbing metals and alloys.

To achieve the aim, the following tasks are proposed:

1. Hydrogen production analysis, using various, also in Latvia isolated, bacteria stains.
2. Bioconversion of organic waste materials - crude glycerol and lactose from cheese whey - to hydrogen gas with selected bacteria strains.
3. Substrate convertation dynamic analysis and by-product identification.
4. Hydrogen isolation possibilities using hydride- forming metals and alloys.

Approbation of research results

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1. Literature Overview

1.1. Alternatives for sustainable energy production and hydrogen as a perspective energy carrier

1.1.1. Alternative energies - renewable energy resource necessity

Energy has always been the foremost important resource upon which humans have relied for survival and productive activities (Lee et al., 2015). Currently the world's population is more than 7 billion (Worlds population statistics, 2015) and is estimated grow to 9 billion by 2050, and about 10 billion by 2100 (Lee., 2011). The International Energy Agency (IEA) based in Paris has projected that the world's energy demand will increase from about 12 billion ton oil equivalents (t.o.e.) measured in 2009 to 17-18 billion t.o.e. by 2035 and CO_2 emissions are expected to increase from 29 gigatonnes per year to 43 Gt per year (Chu et al., 2012). The most of the energy production is based on fossil fuels, and along with economic issues, pollutant gas emissions from fossil fuels have been raising health and environmental concerns all around the world for many years now (Dincer et. al., 2015). The concentration of CO_2 and other pollutant gases contributes for 55% of the so-called greenhouse effect, the main reason for the Earth's average temperature increase. The trend in global temperature due to the increase of CO_2 in the atmosphere, adjusted considering the rate absorbed by the oceans and the mainland (Nicoletti et. Al., 2015). (Figure 1.1.).

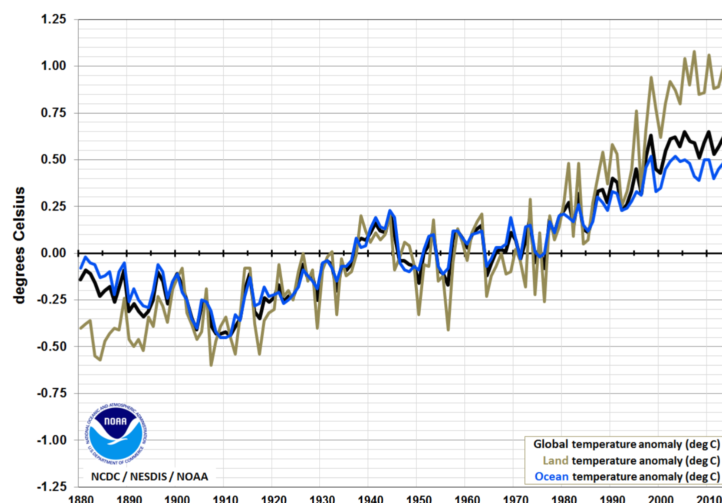

Figure 1.1. Global annual average temperature, with land and ocean components.

(source: NOAA - National Oceanic and Atmospheric Administration).

Advantageously, alternative fuels are no longer a matter for the future, it is a realistic issue of the present as 19,1% of the global final energy consumption was met by renewable fuels in 2013, and the growth continued also through 2014 and 2015. The most rapid growth, and the largest increase in capacity occurred in the power sector and was led by wind, solar PV and hydropower (Global Status Report, 2015). Efforts of researchers worldwide are directed towards the innovations that are environment-friendly and sustainable renewable energy. Renewable energies (Figure 1.2.) are energy sources that are continually replenished by nature and derived directly from the sun (such as thermal, photo-chemical, and photo-electric), indirectly from the sun (such as wind, hydropower, and photosynthetic energy stored in biomass), or from other natural movements and mechanisms of the environment (Ellabban et al., 2014, (Lee et al., 2015).

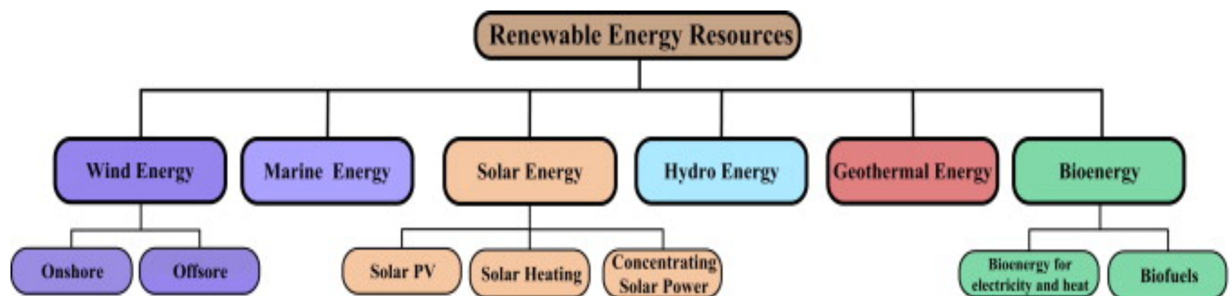

Figure 1.2. Overview of renewable energy sources. (Source: Ellabban et al, 2014).

A diversified portfolio of resources and suppliers for electricity/heat generation in a country decreases the overall risk of energy supply, it does not only reduce vulnerability of supply disruptions from a source, but also it decreases the power of suppliers and risks of higher prices in the market (Mathiesen et al., 2011). Renewable energy has become cheaper over the years, for example, the cost of crystalline silicon photovoltaic (PV) modules has fallen by 99% since 1978 and by 80% since 2008; installation costs for wind power have also dropped, and solar and wind capacity has grown by 40-50 gigwatts each year since 2008, with consumption also rising (Wagner et al., 2015).

In European Union (EU-28) between 1990 and 2012, total electricity generation

from renewables increased by 177%. In 2013, renewable electricity generation accounted for 26% of total gross electricity generation (Figure 1.3) (Eurostat 2015, Energy, transport and environment indicators). Growth has been driven by several factors, including renewable energy support policies and the increasing cost-competiveness of energy from renewable sources (Global Status Report, 2015). In the EU-28, the share of energy from renewable sources in gross final consumption of energy increased from 8.3% in 2004 to 15.0% in 2013. This is evidence of a steady progress towards the Europe 2020 target of 20%. The renewable share in Estonia has been above the 2020 target value since 2011. Sweden reached the 2020 level in 2012. Latvia, along with the Czech Republic, Italy and Romania are less than 1 percentage point from reaching their 2020. targets (Eurostat, 2015).

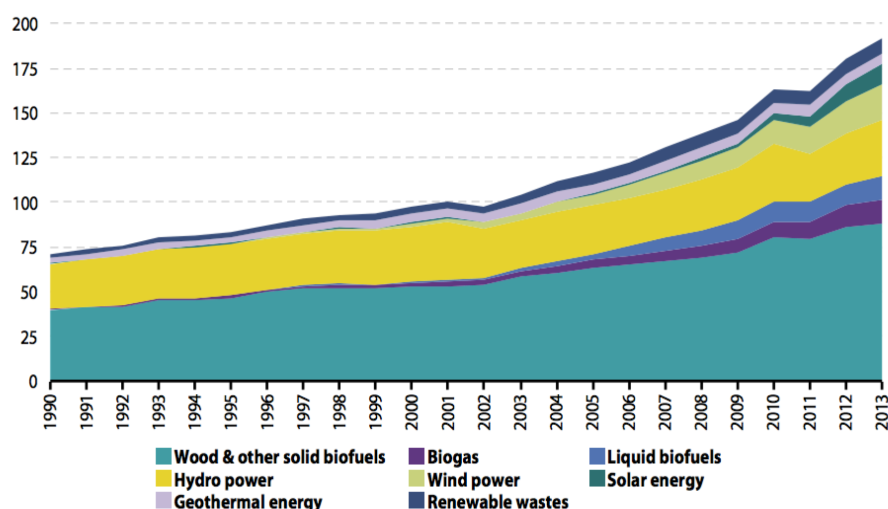

Figure 1.3. Primary production of energy from renewable sources, EU-28, 1990–2013 (million tonnes of oil equivalent) Source: Eurostat (online data code: nrg_110a).

1.1.2. Hydrogen as an environment-friendly energy carrier

Hydrogen is regarded as a sustainable energy carrier for various reasons: (i) high energy conversion efficiencies; (ii) production from water with no emissions; (iii) abundance; (iv) different forms of storage (e.g. gaseous, liquid, or in together with metal hydrides); (v) long distance transportation; (vi) ease of conversion to other forms of energy; (vii) higher heating value (HHV) and lower heating value (LHV) than most of

the conventional fossil fuels. Hydrogen has HHV of 141.9 kJ/g and LHV - 119.9. Comparatively HHV for gasoline is 47.5 kJ/g and LHV 44.5, methane has HHV of 55.5 kJ/g and LHV - 50.0 kJ/g (Acar et al., 2014).

Hydrogen is the most abundant element in the Universe, making up about three quarters of all matter, though scarcely availabl2e on the Earth in a molecular (dihydrogen, H_2) form. The atmosphere contains trace amounts of it (0.07%), while the Earth's surface contains about 0.14% (Goswami et al., 2007). Hydrogen can be found in many substances in nature, for example, in sea/fresh water and biomass. Nevertheless, dihydrogen can be produced from many different sources (e.g. biomass rich in carbohydrates) (Holladay et al., 2009). The worldwide production of H_2 currently exceeds 1 billion m^3/day of which 48% is produced from natural gas, 30% from oil, 18% from coal, and the remaining 4% is produced from water electrolysis currently the most basic industrial process for almost pure hydrogen generation. Most commonly used and the least expensive hydrogen production process is natural gas steam reforming and as it produces heavy greenhouse gas emissions (GGE), more environment friendly mechanisms for hydrogen production are necessary (Kuppam et al., 2015).

Hydrogen usage as an energy carrier is the most important stone in hydrogen economy as it can store the energy from diverse domestic resources (including clean coal, nuclear, and intermittently available renewables) for use in stationary and mobile applications (Hydrogen Tech Roadmap, 2015). Hydrogen is used in the manufacture of two of the most important chemical compounds made industrially - ammonia and methanol. It is also used in the refining of oil, for example in reforming, one of the processes for obtaining high grade petrol and in removing sulfur compounds from petroleum which would otherwise poison the catalytic converters fitted to cars (Essential Chemical Industry, 2015). Technologies of most significance for electrochemical hydrogen production are: 1) polymer membrane (voltage efficiency (VE) 67-82%, with current density (CD) 0.6-2.0 A/cm^2), 2) alkaline membrane (VE 62-82%, CD 0.2-0.4 A/cm^2), and 3) solid oxide electrolysers (VE 81-86%, CD 0.3-1.0 A/cm^2) (Dincer et al., 2015., Bhandari et al., 2014). Apart from electrolysis, different hydrogen production methods have been developed, including photolysis, thermolysis, biophotolysis photoelectrochemical process, photocatalysis and thermochemical water splitting, as well as thermochemical conversion of biomass, gasification, biofuel reforming and

others (Figure 1.4.) (Goswami et al., 2007).

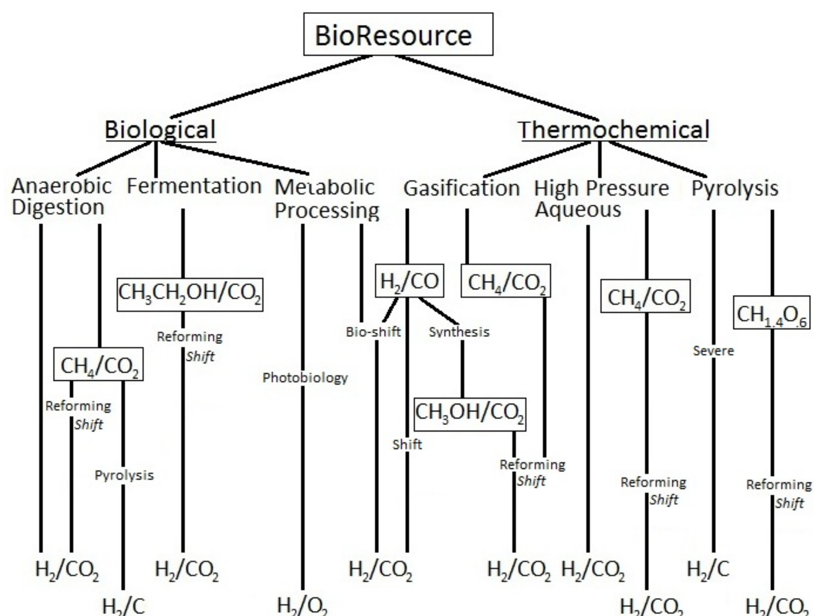

Figure 1.4. Hydrogen production methods (Balat and Kirtay, 2010; Kotay and Das, 2008; Manish and Banerjee, 2008; Hallenbeck and Benemann, 2002).

Every physical or chemical method of hydrogen production requires a powerful electrolytic, thermal or photolytic source of energy, which is not always environment-friendly. On the contrary, biological processes of hydrogen recovery and collection from organic resources such as municipal wastewater and sludge facilitate recycling of sewage and are environmentally benign (Das et al., 2008).

Advantage and critical challenge comparisons of selected hydrogen production methods are listed in table 1.1 Regarding to the cost, several mentioned methods in R&D are more expensive, e.g. photoelectrochemically gained hydrogen costs 10.36 $/kg, less expensive is dark fermentation method - 2.4 $/kg. Historically more developed method costs for hydrogen gas are lower - fossil fuel reforming (0.75 $/kg), plasma are decomposition (0.85 $/kg) and coal gasification (0.92 $/kg) (Dincer et. al., 2015).

Table 1.1. Pros (advantages) and cons (research and development challenges) for selected hydrogen production methods (modified from Holladay et al., 2009., Dincer et. al., 2015., Dimanta 2010., Hydrogen Tech Roadmap, 2015).

Method	Pros (Advantages)	Cons (R&D Challenges)
Water electrolysis	• Well developed technology • Mobile and various size applications • No pollution with renewable energy sources	• High capital costs • Corrosive-resistant membranes, durable and cheap catalysts • Low system efficiency
Biological production	• Sustainable and clean • Self sustaining and variety of waste substrate convertation • Low operation temperature	• Cost • Production rate efficiency is still a challenge • Efficient microorganisms and reactor materials
Photo electroche mical	• Sustainable and clean • Low operation temperature • Compact	• System efficiency • Effective photocatalytic material • Cost effective reactor
Fossil fuel reforming	• Most developed industrial process • Lowest current cost	• Highest GGE • High operation and maintenance costs – many mechanical parts

1.2. Biohydrogen production processes

Bacteria have their own hydrogen fuel cycle. Hydrogen production by dark fermentation is restricted by the incomplete degradation of organic matter into volatile fatty acids, hydrogen and carbon dioxide. In nature, low partial pressure of hydrogen is maintained by the presence of hydrogen consumers in syntrophic association with hydrogen producers (Madigan et al, 2005). Estimates are that microorganisms annually

form approximately 150 million tons of hydrogen. The combustion of 150 million tons of hydrogen yields 18 x 1018 J of energy, that is equivalent to 3.75% of the primary energy consumed by world population (Thauer et al. 2010).

Hydrogen production using microbial fermentation process is acknowledged from 1920-ies (Strickland, 1929), using photosynthesis in microalgae, from 1940-ies (Gaffron & Rubin, 1942). Biological processes for hydrogen production and collection from organic resources such as municipal wastewater and sludge facilitate recycling of sewage are environmentally benign (Das, Veziroglu 2008). Biohydrogen and especially hydrogen generation via dark fermentation process is widely studied due to higher production rates and treatment capacity for organic waste products. Many institutions and universities worldwide are involved in the research of hydrogen production using bacteria or/and algae (Ghimire et al. 2015). In recent years there has been increasing research activity (Figure 1.5.).

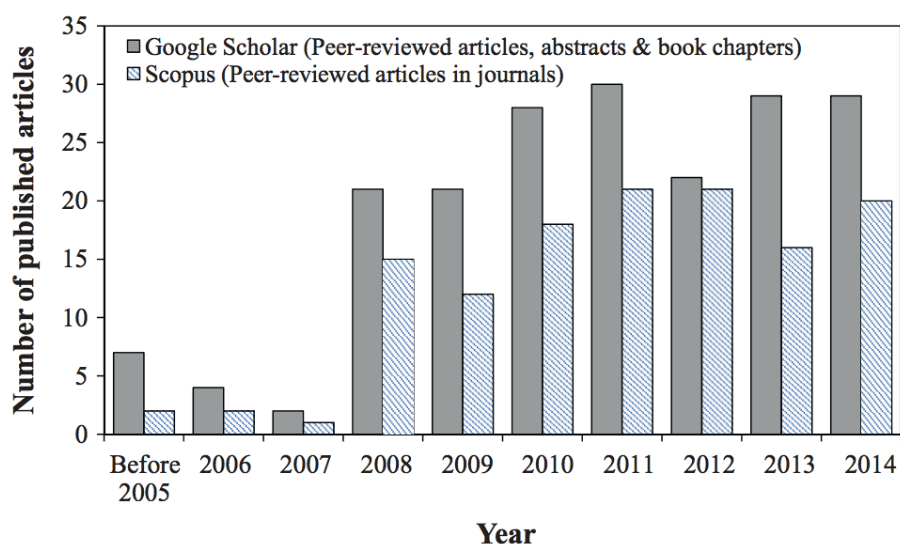

Figure 1.5. Number of peer reviewed publications on biohydrogen generation via dark fermentation process (adapted from A.Ghimire et al. 2015).

Nowadays biohydrogen production is divided in five broader groups: direct photolysis, indirect photolysis, photofermentation, dark fermentation and coupled or hybrid systems and there are vast group of organisms that can produce hydrogen in various environmental conditions (Manish & Banerjee, 2007) More detailed description is shown in Table 1.2.

Table 1.2. Processes of biohydrogen production (modified from Das and Veziroglu, 2001, Hallenbeck and Benemann, 2002, Levin et al., 2004, Maeda et al., 2008.).

Hydrogen production process	**Description**	**Organism**
Direct photolysis	Hydrogen from water and the sun through photosynthesis, genetic modifications necessary for maintaining higher efficiency	Green algae (*Chlamydomonas reinhardtii)*
Indirect photolysis	$6H_2O+6CO_2+light \rightarrow C_6H_{12}O_6 +9O_2$ $C_6H_{12}O_6+6H_2O+light \rightarrow 12H_2+6CO_2$	Cyanobacteria *Anabaena variablis*
Photo-fermentation	Conversion of organic substrate by using light energy with photosynthetic purple non-sulphur bacteria in anaerobic environment.	Photosynthetic bacteria (*Rhodobacter sp.)*
Dark fermentation	By using different substrates, including organic waste in anaerobic environment produces hydrogen	Anaerobic, facultative bacteria *(Enterobacter sp.)*
Hybrid systems	Firstly, via fermentation process acetate, CO_2 and H_2 are produced. Secondly, in a photo-bioreactor, acetate is turned into H_2 and CO_2	Association of photosynthetic and fermentative bacteria

Microorganisms are capable of producing H_2 *via* two main pathways: fermentation and photosynthesis. The processes of biohydrogen production include:

- direct biophotolysis by green algae – the photosynthetic production of hydrogen by splitting water into molecular hydrogen and oxygen using sunlight under

specific conditions;

- indirect biophotolysis by cyanobacteria with specialized cells (heterocysts) that perform nitrogen fixation and contain enzymes (nitrogenase and hydrogenase) directly involved in hydrogen metabolism and synthesis of molecular H_2;
- photo-fermentation by purple non-sulfur bacteria that evolve molecular H_2 catalyzed by nitrogenase enzyme under nitrogen-deficient conditions using the energy of light and organic acids;
- dark-fermentation by anaerobic bacteria grown in the dark on carbohydrate-rich substrates (Das and Veziroglu, 2001; Levin et al., 2004.) All biohydrogen production ways depend on either a hydrogenase or nitrogenase enzyme for hydrogen evolution and derive energy either directly from light energy or indirectly by consuming photosynthetically derived carbon compounds. Each approach has positive and negative aspects, and each has serious technical barriers that need to be overcome before it could become practical (Hallenbeck et al., 2009).

There is a natural consequence of the fact that fermentations have been optimized by evolution to produce cell biomass and not hydrogen. Thus a portion of the substrate is used in both cases to produce ATP giving a product that is excreted. In many anaerobic microorganisms the actual hydrogen generation yields are reduced by hydrogen recycling due to the presence of one or more uptake hydrogenases, which consume the hydrogen that is produced (Rogner et al. 2002).

Hydrogen production by fermentation of carbohydrate-containing substrates (glucose, cellulose, starch and organic waste materials) is frequently preferred to photolysis, because it does not rely on the availability of light sources (Das and Veziroglu, 2001). In the fermentation of glucose by enterobacteria, e.g. *Escherichia coli*, one of the pyruvate oxidation products, alongside with acetyl-CoA, is formate*,* which is produced by pyruvate formate lyase and is the sole source of hydrogen in these bacteria. The formate is split into CO_2 and H_2 by formate hydrogen lyase (FHL) complex, which comprises seven proteins, six of them being encoded *hyc* operon. Five *hyc* operon encoded proteins are membrane-embedded electron transporters. The hycE protein is one of the three *E.coli* NiFe hydrogenases (also referred to as Hyd-3) (Figure 1.6.). Hydrogenases are enzymes that catalyse the oxidation of hydrogen to protons and the

reduction of protons to hydrogen. They can be distributed into three classes: the [Fe]-H2ases, the [NiFe]-H2ases, and the metal-free hydrogenases. Hydrogenases may interact with membrane-bound electron transport systems in order to maintain redox balance, particularly in some photosynthetic microorganisms such as cyanobacteria. (Vignais et al, 2004). One of the pyruvate oxidation products, alongside with acetyl-CoA, is formate*,* which is produced by pyruvate formate lyase and is the sole source of hydrogen in these bacteria. The formate is split into CO_2 and H_2 by formate hydrogen lyase (FHL) complex, which comprises seven proteins, six of them being encoded *hyc* operon. Five *hyc* operon encoded proteins are membrane-embedded electron transporters. The hycE protein is one of the three *E.coli* NiFe hydrogenases (also referred to as Hyd-3) (Maeda et al., 2007).

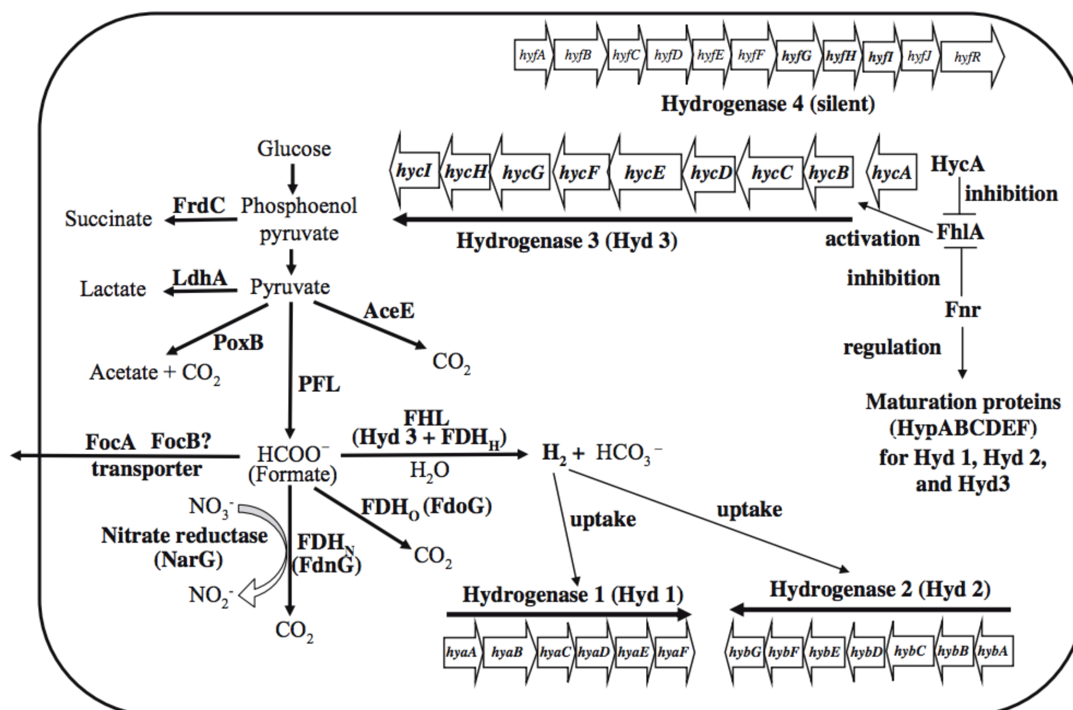

Figure 1.6. Fermentation process and formate hydrogenlyase complex in bacteria *E.coli* (after Maeda et al., 2007).

The hycE and FDH-H components of FHL complex are soluble periplasmic proteins. The hydrogen evolved from FHL is consumed by *E.coli* uptake hydrogenases Hyd-1 and Hyd-2. In contrast to enterobacteria, strictly anaerobic fermenters, e.g. *Clostridia*, use a reduced ferredoxin (required to oxidize pyruvate to acetyl-CoA) for H_2 production by the hydrogenase that generates ferredoxin in the oxidized form and releases electrons as

molecular hydrogen (Nath and Das, 2004; Das and Veziroglu, 2001; Hallenbeck and Benemann, 2002; Maeda et al. 2008, Wang, J., and Wan, 2009). Glucose fermentation by enteric bacteria yields the maximum of 2 mol H_2/ mol glucose (Nath et al., 2008). To enhance the hydrogen production and utilize the substrate in the full measure for complete conversion, synergy of biological processes (two-stage/hybrid ones) should be applied (Sander, 1999). Anaerobic fermentations with *Escherichia coli* and *Clostridia* sp. are the most thoroughly investigated hydrogen bioprocesses, which offer hydrogen yields from glucose up to 2 or 4 moles per mol of glucose (Nath and Das, 2004). The end products of fermentation process are mainly volatile organic acids and spirits that are produced depending on microbial metabolic pathways and substrate used (Figure 1.7.).

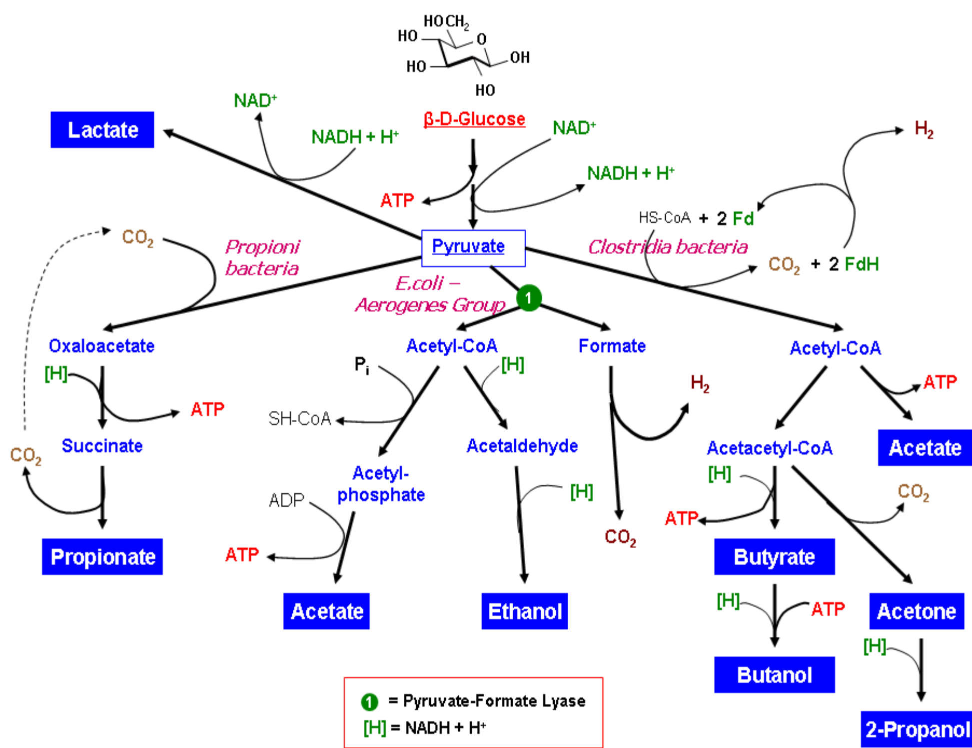

Figure 1.7. Fermentation products of various bacteria groups (Source: E.Schmid, 2006).

1.3. Productive bacteria strains and optimal substrates

Various microorganisms have the ability to produce hydrogen under certain conditions (Fig. 1.8.). Starting from microalgae that use light energy, cyanobacteria, but the major hydrogen yielding biocatalysts are heterotrophs in the fermentation process –

facultative and obligate anaerobes. Here are listed some of optimal hydrogen producers: Green algae (*Scenedesmus obliquus, Chlamydomonas reinhardii, C. moewusii),* Cyanobacteria Heterocystous (*Anabaena azollae, Anabaena CA, A. variabilis, A. cylindrical, Nostoc muscorum N. spongiaeforme, Westiellopsis prolifica),* Cyanobacteria Nonheterocystous (*Plectonema boryanum, Oscillotoria Miami BG7, O. limnetica, Synechococcus sp, Aphanothece halophytico, Mastidocladus laminosus, Phormidium valderianum),* Photosynthetic bacteria (*Rhodobater sphaeroides, R. capsulatus, R. sulidophilus, Rhodopseudomonas, sphaeroides, R. palustris, R. capsulate, Rhodospirillum rubnum, Chromatium sp. Miami PSB,* 1071, *Chlorobium limicola, Chloroflexu aurantiacus, Thiocapsa roseopersicina, Halobacterium halobium)* and Fermentative bacteria (*Enterobacter aerogenes, E. cloacae, Clostridium butyricum, C. pasteurianum, Desulfovibrio vulgaris, Magashaera elsdenii, Citrobacter intermedius, Escherichia coli)* (Das and Veziroglu, 2001, Das, 2009, Hallenbeck et al 2012, Boichenko et al. 2004).

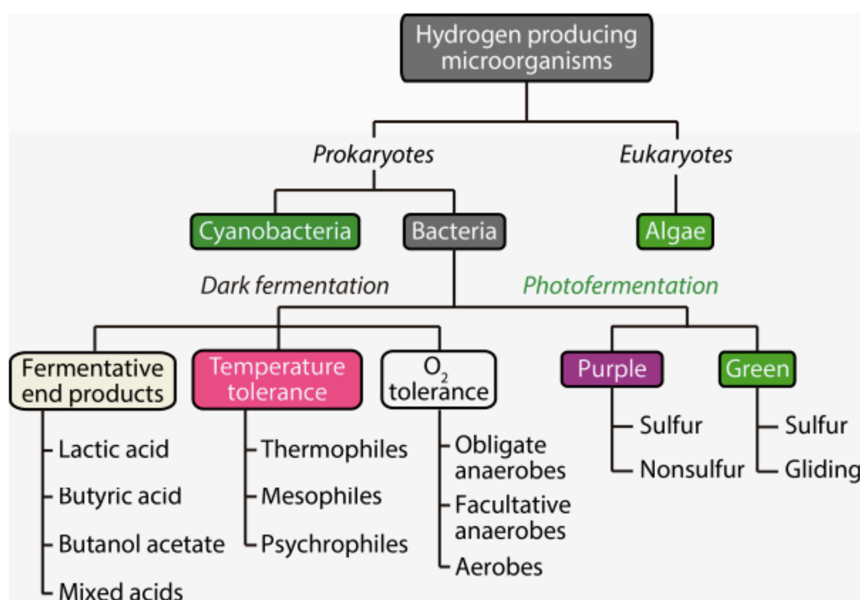

Fig.1. 8. Diversity of hydrogen producing organisms (adapted from Chandrasekhar et al, 2015).

Thermophiles are the most closest for hydrogen production to the theoretical yield by overwhelming thermodynamic barrier, but these strains have to be cultivated at elevated temperature with high energy requirememets (Das, 2009). In the most recent biohydrogen review (Chandrasekhar et al., 2015) hydrogen research strategies organism-

wise stated that productive pure cultures were used with defined substrate as the carbon source, but mixed cultures are preferred for operational ease and process stability. When wastewater or agricultural waste used as the substrate, a mixed microbial population is more favorable and practical (Venkata et al., 2013). No matter what te research goal is, the key requirement for every biological hydrogen production research is a proper choice of hydrogen production system and understanding of the biochemical and biophysical characteristics of this system (Angenent et al., 2004, Wang and Wan, 2009). The substrate plays an important role in the H_2 yield, H_2 production rate and the overall economy of the process. Carbohydrate rich substrates have been extensively used in biohydrogen production studies (Ghimire et al. 2015). Glucose and sucrose are the most commonly used pure substrates in both batch and continuous processes due to their simple structures and ease of biodegradability (Kapdan and Kargi, 2006). Renewable biohydrogen production requires the substrate or feedstock to come from renewable resources. Second generation biomass sources, such as waste biomass, are abundant and thus can support the supply of renewable substrates and fermentation process offers biological treatment of the organic waste (Ghimire et al. 2015). In order to reach sustainable production and also waste management, various agriculture and industrial waste materials as feedstock may be used (Fig.1.9). (Hawkes et al., 2002).

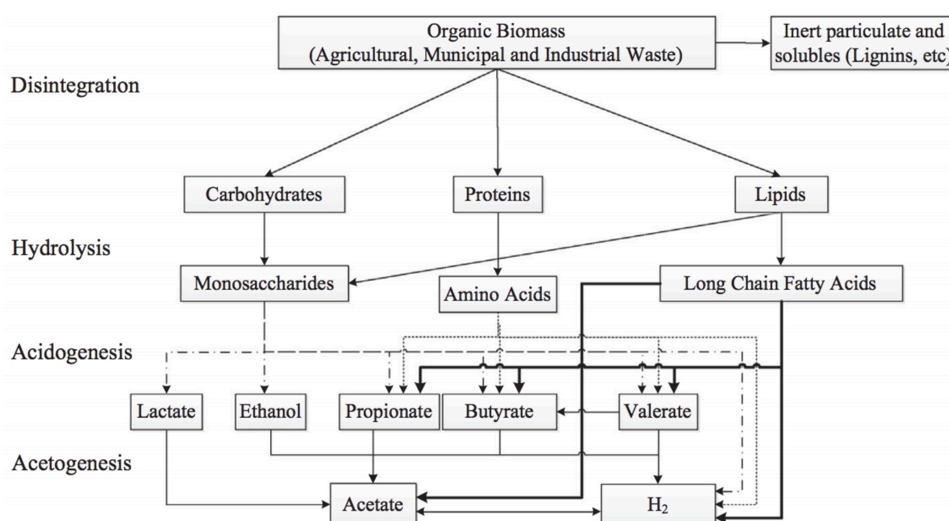

Figure 1.9. Biodegradation and microbiological pathways involved in the fermentative breakdown of waste biomass (adapted from Ghimire et al. 2015).

Substantial factors like availability and cost are highly important in the selection of

waste materials to be used in hydrogen production with fermentative bacteria (Kapdan and Kargi, 2006).

Crude glycerol fermentation process

One of the substrates that can be effectively used for microbial hydrogen production is crude glycerol (Varrone et al., 2013), which is a by-product from the process of biodiesel production (Fig. 1.10.).

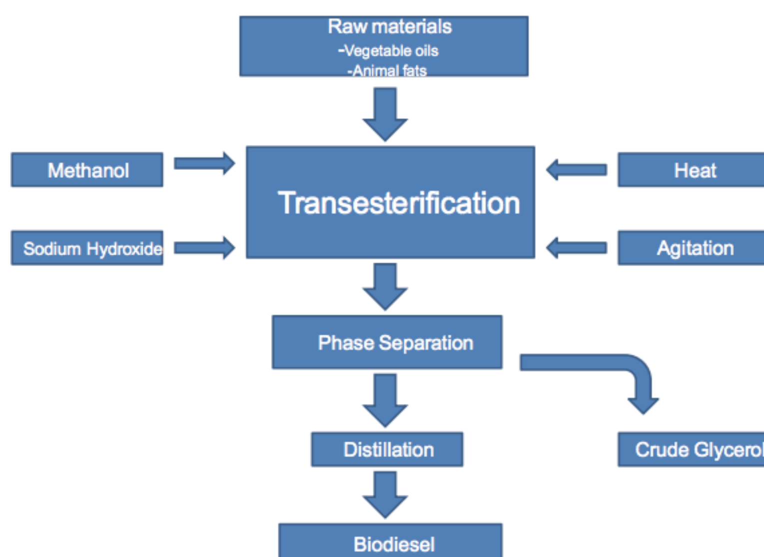

Fig.1.10. Biodiesel production process using transesterification of vegetable oil and fats (from Sarma et al., 2012).

Because large quantities of crude glycerol are available and the highly reduced nature of carbon in glycerol per se, microbial conversion of it in hydrogen is economically and environmentally viable possibility, especially because, over the last several years, the demand and production of biodiesel has remarkably increased (Raghunandan et al., 2014, Adhikari et al., 2009, Ito et al., 2005). Glycerol ($C_3H_8O_3$) is a trihydroxyl (sugar alcohol) compound, energy content of pure glycerol is 19.0 MJ/kg, but for crude glycerol energy content is 25.30 MJ/kg that is most probably due to presence of methanol and traces of biodiesel. Energy content of crude glycerol indicates its high potential to be an effective substrate for biological hydrogen production, especially because it does not require additional pre-treatment to make it available for the hydrogen-producing microorganisms (Sarma et al., 2012). About 10 billion liters (L)

of crude glycerol per year are a by-product of biodiesel generation from plant oils, giving 1 L of glycerol for every 10 L of biodiesel (Adhikari et al., 2009, Mane and Rode, 2013). Biodiesel is mainly produced from the transesterification of vegetable oils or animal fats with methanol catalyzed by alkalis such as NaOH and KOH. After the transesterification process, two layers are formed: the top layer is the desired product, i.e., biodiesel, and the bottom layer is the raw/unrefined crude glycerol. (Hu et al., 2012, Hajek et al., 2010). One of the major challenges for the utilization of crude glycerol is the inconsistency of its composition since it varies with the feedstocks, production processes, and post-treatments involved in the biodiesel production. Upgrading or refining crude glycerol to technical grade glycerol (>98% glycerol content) makes its composition more consistent, but currently this is not economically viable, especially for small and/or medium-sized biodiesel plants (Manosak et al., 2011).

Various ways for crude glycerol usage have been managed and studied, e.g. direct burning as heating oil, purification for commercial pure glycerol, steam reforming to make hydrogen, and microbial conversion into hydrogen. If direct combustion is used – it requires off-gas treatment to control the emission of toxic gases. Auto-thermal reforming results emissions of greenhouse gases. And as crude glycerol is contaminated with chemicals, purification for sale grade is not economically viable option. Microbial fermentation has therefore been identified as a valuable alternative, considering use of optimal conditions and microorganisms. (Jitrwung and Yargeau, 2015, Adhikari et al. 2009).

Glycerol content in the crude glycerol varies from 40% - 90% (Mane and Rode, 2013). The crude glycerol has an acidic (pH 2.4) to alkaline pH (pH 12), pure glycerol (>98%) has an acidic pH (pH 5.5), soluble in water (>500 g/l, at 20 °C) (Trchounian and Trchounian, 2015). Crude glycerol economic value is approximately $0.1/kg, due to the presence of various impurities such as methanol, soap, fatty acid methyl esters and alkaline catalyst residues. (Santibanez et al., 2011, Hu et al., 2012). Concentration of impurities in crude glycerol may vary. Rossi et al. (2011) used crude glycerol with glycerol concentration 80.8%, that contained also 6,4% ash and 6,6% NaCl. Ito et al. (2005) used crude glycerol supplied from a biodiesel manufacturing factory in Hiroshima prefecture, Japan that was chemically produced with potassium hydroxide as the alkali catalyst and it contained 41% (w/w) glycerol and the amount of total organic

carbon (TOC) was 540 g/l, of which 524 g was soluble. The impurities were mainly composed of ash (8%, w/v) and methanol (25%, w/w), 0.04% (w/w) diacylglycerol and 0.01% (w/w) monoacylglycerol and water. Reungsang et al. (2013) for hydrogen production with *Enterobacter aerogenes* KKU-S1 used crude glycerol with glycerol concentration of 441.3 g/l, methanol 230 g/l, NaCl 10g/l. Mangayil et al. (2012) used Savon Siemen Oy manufactury glycerol, that contained 45% (v/v) glycerol and 30% (v/v) metanol. Valuable compounds can be produced from crude glycerol such as hydrogen, and 1,3-propanediol as main products, methane, ethanol and succinic acid in smaller amounts (Mangayil et al., 2012). Hydrogen production comparing to production of 1,3-propanediol is more valuable, hydrogen has higher energy content (142.9 kJ/g) and it results in higher yield and productivity (Sarma et al., 2012). Various bacterial strains are considered promising for glycerol utilization (Table 1.3.) because of possibility to ferment crude glycerol, and H_2 is one of the end-products of this process (Ito et al., 2005). The most productive microorganisms that grow anaerobically on glycerol as the sole carbon and energy source:

- *Rhodopseudomonas palustris*
- *Citrobacter freundii*
- *Klebsiella pneumoniae*
- *Clostridium pasteurianum*
- *Clostridium butyricum*
- *Enterobacter agglomerans*
- *Enterobacter aerogenes*
- *Escherichia coli*
- *Thermotoga neapolitana*

(Sarma et al., 2012, Ito et al. 2005).

Table 1.3. Maximum hydrogen production by different inocula using pure and crude glycerol as feedstock. (adapted from Sarma et al., 2012, Anniina et al, 2010, Escapa et al. 2009, Selembo et al. 2009, Ken-Jer et al. 2011, Seifert et al. 2009, Yohei et al. 2009, Ito et al, 2005., Ngo et al. 2011, Priscilla et al. 2009, Kim e al. 2004, Selembo et al. 2009, Sakai et al. 2007, Guillaume et al. 2009).

Substrate Analytical glycerol (AG) Crude glycerol (CG)	Inocula	Experiment	Maximum hydrogen yield
AG	*Halanaerobium saccharolyticum*	Batch process	0.62 ± 0.02 mol-H_2 mol^{-1}glycerol
AG	*Klebsiella* sp. HE1	Batch process	0.345 mol-H_2 mol^{-1}glycerol
AG	Anaerobic sludge	Batch process	0.41 mol-H_2 mol^{-1} glycerol
CG	*E. aerogenes* HU-101	Continiouss flow	80 mmol-H_2 l^{-1} h^{-1}
CG	*Thermotoga neapolitana* DSM 4359	Batch process	2.73 ± 0.14 mol-H_2 mol^{-1}glycerol
CG	*E. aerogenes* HU-101	Continiouss flow	63 mmol-H_2 l^{-1} h^{-1}
CG	*E. aerogenes* NBRC 12010	Batch process	0.77 mol-H_2 mol^{-1} glycerol
CG	Sewage assosciations	Batch process	0.41 ± 0.1 m^3-H_2 m^{-3} d^{-1}
CG	*Rhodopseudomonas palustris*	Batch process	6 mol-H_2 mol^{-1} glycerol

There are two ways of glycerol metabolism – oxidative and reductive. In the reducing pathway glycerol is converted in 1,3 – propanediol. In the oxidative pathway glycerol is firstly converted in dihydroxyacetone using catalytic activity by glycerol dehydrogenase, then dihydroxyacetone is phosphorylated by the glycolytic enzyme dihydroxyacetone kinase and the phosphorylated product is metabolized through glycolysis. Pyruvate may be further converted to various endproducts depeding on microorganism. In most of glycerol bioconversion pathways hydrogen is produced along with other metabolites (e.g. butyrate, ethanol, butanol, acetate, acetone, lactate) (Figure

1.11.). (Sarma et al, 2012).

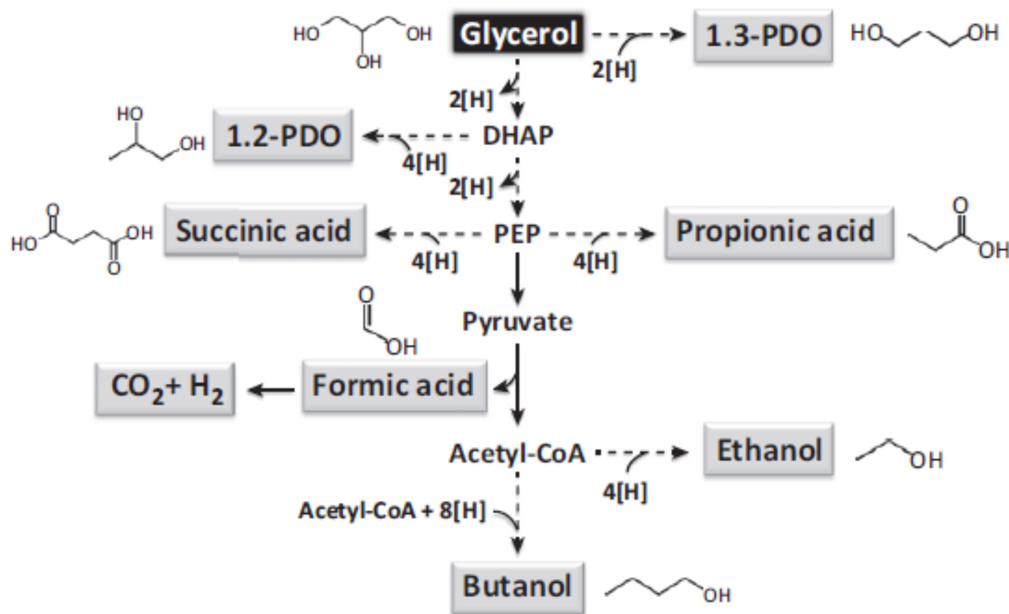

Figure 1.11. Anaerobic fermentation of glycerol (adapted from Clomburg, Gonzalez 2013). Abbreviations: DHAP - dihydroxyacetone-phosphate; PEP - phosphoenolpyruvate; 1.2-PDO - 1,2 - propanediol; 1.3-PDO - 1,3 – propanediol.

Mangayil et al (2012) found optimal conditions (pH 6.5; 40°C) for hydrogen production using crude glycerol with microbial consortium mainly dominated by *Clostridium* species. Environmental conditions like medium pH and temperature are the major parameters to be controlled in the hydrogen production, because they affect bacterial produced gas qualitative and quantitative content and hydrogen yield and rate. Hydrogen production using glycerol is 1.5-fold higher at pH 5.5 than at pH 6.5 (Trchounian et al., 2011) using *E.coli*.

Anaerobic conditions have to be maintained during the hydrogen production process, which are ensured by barbotage media with reducing agents such as argon or nitrogen (Kapdan and Kargi, 2006). Guillaume and Patrick (2009) have reported hydrogen production using photo fermentation of pure and crude glycerol by *Rhodopseudomonas palustris* with maximum of 6 mol of hydrogen per mole of glycerol, that is 75% of theoretical maximum 8 mol hydrogen production per mole glycerol, meaning that intermediate products such as acetic acid, ethanol and butyric acid were

further metabolized to hydrogen, which is otherwise accumulated during dark fermentation (Sarma et al., 2012). *Enterobacter aerogenes* have an ability to convert glycerol in a fermentative process to yield hydrogen and ethanol as the main by-products. Yields of 0.86 mol H_2/mol glycerol and 0.75 mol ethanol/mole glycerol were reported by Jitrwung and Yargeau (2015). Dharmadi et al. (2006) reported that glycerol can be fermented also by *E. coli* at slightly acidic pH (pH 6.3). Bruna et al. (2010) have reported production of hydrogen from curde glycerol with anaerobic sludge.

Milk/cheese way fermentation

Lactose is a content of cheese whey and milk whey. Cheese why contains ca. 5% lactose that can be successfully used for hydrogen production via fermentation process (Ferchichi et al., 2005). Collet et al. (2004) reported 3 mol H_2 / mol lactose, using *Clostridium thermolacticum* in continuous flow bioreactor.

For the enterobacteria it proved to be necessary to simplify their branched fermentation pathway, in order to channel the catabolic flux towards hydrogen production. Recently, that was achieved by construction of a strain of *E. coli*, containing seven knock-out mutations, disabling the alternative catabolic branches (Maeda et al., 2007). The septuple mutant strain showed a 4.6-fold increase of hydrogen production from glucose and a twofold increase of the hydrogen yield, from 0.65 to 1.3 mol H_2 / mol glucose. Lactose, the main carbohydrate constituent of the cheese whey, is known to be a much cheaper alternative. Cheese whey is a waste product of dairy industry, and its conversion to bioethanol (Viitanen et al. 2003) or to other added value products (Rosales-Colunga et al. 2010) is an important current focus in the bioprocessing of renewable resources. In *E. coli*, the genes necessary to metabolize lactose are coded by the *lac* operon. Rosales-Colunga *et al*. (2010) demonstrated that if the lactose repressor gene (*lacI*) was deleted, in order to express constitutively the *lac* operon and to increase the lactose consumption rate, a substantial improvement of lactose conversion could be reached. In the present work we have improved the lactose conversion in the strain reported by Maeda *et al*. (Maeda et al. 2007), by overexpressing the lactose permease gene (*lacY*), and analyzed some physiological consequences of adaptation to increased lactose uptake.

1.4. Important physical and chemical fermentation process parameters for biohydrogen production optimal conditions

Process parameters that affect hydrogen production include anaerobic environment; fermentation broth is barbotaged with inert gas (namely, nitrogen or argon) (Penfold et al., 2003), as well as optimal temperature and pH are maintained. Hydrogen production with fermentative bacteria requires pH 4,5-6,5 and optimal temperature varies in between 30°C-80°C (Das et al., 2008). Here is more detailed description for affecting parameters:

Temperature

It has been previously investigated that moderate increment of the temperature in fermentation process could increase overall hydrogen yield, while higher temperature levels can suppress it (Wang and Wan, 2009). Optimal temperature depends on the organism used and productive hydrogen production has been demonstrated in mesophilic (around 37°C) and thermophilic (around 55°C) (Li and Fang, 2007). Hydrogen yields increase with higher temperatures as the thermodynamics of hydrogen formation becomes more favourable (Hallenbeck, 2005), yielding even 4 moles of hydrogen per mole of glucose in extreme environment (Zeidan and van Niel, 2009). Other end-product formation is also effected, Wang and Wan, 2008 reported that concentration of either ethanol or acetic acid increased with temperature level changes from 20°C to 35°C and decreased with temperature level changes from 35°C to 55°C. Regarding to propionic acid and butyric acid – production decreased on changes in temperature levels from 20°C to 55°C.

pH

One of the most important factors that influence the activities of hydrogen-producing bacteria is pH. With mixed bacteria cultures indirect effect was investigated, since activity of hydrogen-consuming microbes (methanogens) are inhibited at low pH conditions. In bioreactor systems pH control is important regulator factor, hence hydrogen production by fermentative bacteria is also accompanied by the formation of organic acids as metabolic end products (e.g. lactate, acetate, butyrate) whom formation lower the pH. Generated organic acids accumulate during the fermentation process, causing a sharp drop in the pH and generating an inhibition of bacterial hydrogen

production (Oh et al, 2003, Fabiano and Perego, 2002). Although it has been often suggested that pH may affect hydrogenase enzyme activity, this has never been directly proven with experimental data, as well as it is difficult to rationalise this fact since the internal pH should remain constant depending of the pH control of the medium (Hallenbeck et al., 2012).

Hydrogen partial pressure

Hydrogen solubility in 37°C is 1,4 mg/kg water or 700 - 800 µmol per one litre of water at normal atmosphere pressure. Henri law states that at constant temperature the dissolved gas in a given volume of liquid is directly proportional to the partial pressure of the gas in the atmosphere above the liquid;) $p=k_H*c$, where p- partial pressure, k_H - constant, c- concentration of dissolved substance. (Laidler, Meiser, Physcial Chemistry, 1999.) Hydrogen partial pressure in the liquid phase is very important factor that affects fermentative hydrogen production per se. Because building up of the product – hydrogen- depending on the thermodynamics involved, further conversation of substrate to hydrogen decreases, as hydrogen production via hydrogenase enzymes is a reversible process. In nature mixed anaerobic cultures accumulate very little hydrogen, as its production is normally balanced by rapid hydrogen consumption of methanogens. Capturing hydrogen before the interspecies hydrogen transfer from natural habitats in practise would be hard, because of relatively high affinity of hydrogen consumers for hydrogen and the slow mass transfer of hydrogen gas from liquid to gas phase (Hallenbeck, 2012). Hydrogen partial pressure change various depending on hydrogenase reduction pathway, namely, hydrogen gas production from reduced ferredoxin is much more favourable thank hydrogen production from NADH, meaning less sensitivity to this effect. NADH driven hydrogen gas production proceeds at low hydrogen pressures, following H_2 synthesis decreases and metabolic pathways shift towards to production of more reduced substrates, including lactate, ethanol, acetone, butanol (Hallenbeck, 2005). It is experimentally well described and showed fact, that decrease in hydrogen partial pressure in liquid phase, increases overall hydrogen yield. In order to decrease hydrogen partial pressure, physical or chemical methods are used, involving gas spraging with inert gas, to reduce hydrogen partial pressure in the liquid phase. Importantly, that process that affects hydrogen partial pressures by dilution, i.e. barbotage with inert has, would create a diluted hydrogen stream, adding extra hydrogen

concentration costs to the production system. (Hallenbeck, 2012). Mizuno et al. (2000) showed that lowering dissolved hydrogen by barbotage with nitrogen gave a 68% increase in overall hydrogen yield in the fermentation process, using bioreactor with enriched mixed microflora with 10g/l glucose concentration. Alternative methods have been applied for decrease of hydrogen partial pressure. Liang et al. (2002) used hollow silicone rubber membrane in order to reduce hydrogen partial pressure, resulting in 15% increase in hydrogen yield.

Fermentation broth – necessary nutrients

Nitrogen is an essential component of proteins, nucleic acids, and other cellular components, therefore its availability greatly affects the growth of hydrogen producing bacteria and therefore hydrogen generation possibilities within the fermentation process (Bisaillon et al., 2006). Fixed nitrogen in the form of ammonium salt is the most widely used nitrogen source for the fermentative hydrogen producers. As hydrogenase enzymes contain iron, therefore ferrous (Fe^{+2}) is the most widely described and investigated metal ion in regards to fermentative hydrogen production (Wang and Wan, 2008). Important inorganic nutrient is phosphate as it is an essential element in microbial nutrition, as well as offering buffering capacity. Many studies have shown that regulated C/N and C/P are essential for hydrogen generation process. Recommended CP for anaerobic digestion is 130:1, for carbohydrate 350:1 - COD:P. (Wang et al., 2008).

1.5. Feasibility of biohydrogen production processes

In order to achieve higher overall volumetric hydrogen production co-culture employing gives a possibility to maintain anaerobic conditions for strict anaerobes and eliminate the need for a reducing agent – facultative anaerobes consume oxygen in medium (Elsharnouby et al., 2013). Biohydrogen price could compete with current fuel prices, when fermentation process outcome is 10 H_2 moles per glucose mol (it is close to maximally theoretically possible outcome – 12 moles) and glucose price is approximately 0,05 USD for one glucose dry matter pound. Dark fermentation - 2,4 $/kg. Comparatively, more developed method costs for hydrogen gas – fossil fuel reforming (0.75 $/kg), plasma arc decomposition (0.85 $E/kg) and coal gasification (0.92 $/kg) (Dincer and Acar 2015, Kotay et al., 2008).

Nath et al. (2005) studied the combined dark and photofermentation for biohydrogen production using glucose as the substrate. For dark fermentation *Enterobacter cloacae* strain DM11 was used, followed by photofermentation by *R. sphaeroides* strain O.U.001 using the spent medium from *Enterobacter cloacae* strain DM11, which mainly contained acetic acid. The combined hydrogen yield was higher than a single biohydrogen system, i.e. 1.86 mol H_2/mol glucose in dark fermentation and 1.5– 1.72 mol H_2/mol acetic acid in the photofermentation. Similarly, combining the two fermentation processes, Chen et al. (2010) attained a total yield of 10.25 mol H_2/mol sucrose and 6.63 mol H_2/mol sucrose. Also hybrid processes with subsequent methane production or electrofermentation are considered as valuable possibilties to increase overall process effciency (Hallenbeck et al, 2009).

Vast investigation recently is done on microbial electrolysis cells (MECs), a technique that is used to produce H_2 from a wide variety of substrates. MECs are adapted microbial fuel cells (MFCs), which have been broadly investigated, for few years also in the Institute of Solid State physics (University of Latvia). Using the MECs as an alternative electrically driven hydrogen production process results in the conversion of a wide range of organic substrates into hydrogen under applied external potential (Chandrasekhar et al., 2015). The MEC technology resembles an MFC in which the primary difference is the necessity of a small input of external voltage. Based on thermodynamics, a potential higher than 0.110 V, in addition to that generated by a microorganism (−0.300 V), will produce H_2 The MECs are capable of more than 90% efficiency in the production of H_2. The performance of MECs is determined by the type of microorganism, electrode materials, type of the membrane used, applied potential range, composition and concentration of the substrate, and design of the MEC (Cheng and Logan, 2007). Babu et al. 2013 reported usage of two-stage process integrating the MEC process with the dark fermentation in order to convert the acid-rich dark fermentation effluents into substrates for additional hydrogen gas production. MECs were operated with a small range of varying applied potential (0.2, 0.5, 0.6, 0.8, and 1.0 V) using an anaerobic mixed consortium as a biocatalyst. The process produced hydrogen with rate 0.53 mmol/h and high substrate conversion efficiency (90%) (Fig. 1.12.).

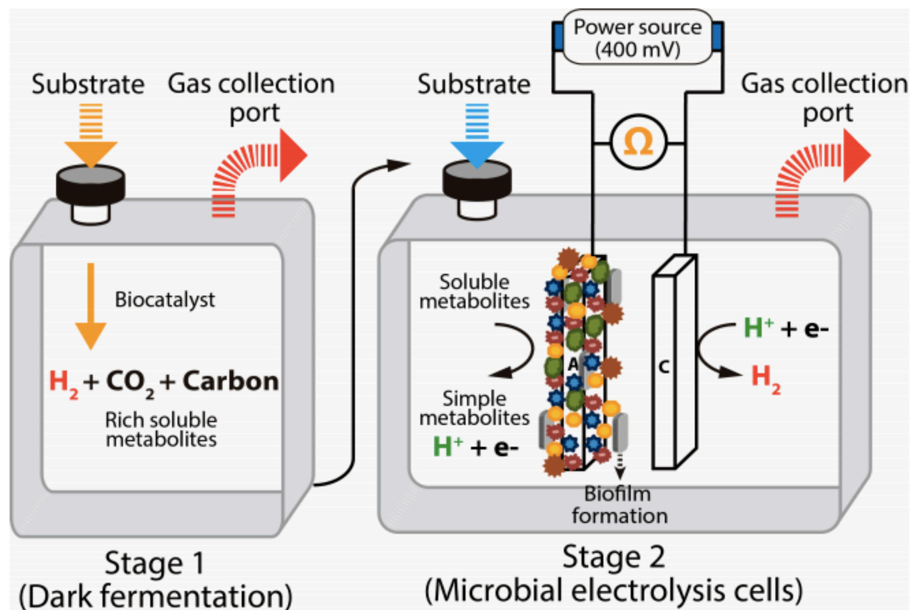

Fig. 1.12. Two-stage process integrating the MEC process with the dark fermentation Green, orange, brown, and blue symbols represent a mixed microbial population. In stage 1 complex substrates are used for H_2 production in dark fermentation, and in stage 2 acid-rich effluents were used as substrates in MECs for further H_2 production. (adapted from Babu et al. 2013 and Chandrasekhar et al., 2015).

1.6. Hydrogen concentration measurements

Gaseous hydrogen is formed in liquid media during bacterial fermentation. Hydrogen gas hardly dissolves in aquatic solutions (Sander, 1999), accordingly various methods are required to discharge hydrogen into atmosphere and in so doing to escape oversaturation (Mandelis and Christofides, 1993). In the atmosphere the parameters of hydrogen gas are measured by classical volumetric, mass-spectrometric and chromatography methods, or using chemical gas sensors (Pauss et al, 1990). Wilkins et al. (1974) described a method for measuring gas production by microorganisms using a platinum electrode and a reference Calomel ($Hg–Hg_2Cl_2$) electrode. To measure hydrogen gas concentration in liquid a hydrogen electrode is usually used (Pt or another noble metal – gold, rhodium, palladium, etc.). The hydrogen H^+ ions and the molecular hydrogen H_2 set the equilibrium potential in compliance with the reaction: $H_2 \Leftrightarrow 2H^+ + 2e^-$ (Mills, 2006). This reaction proceeds very fast, so in its course the equilibrium state

remains stable; in electrochemistry this electrode is adopted as zero reference (with zero potential). In microbiology, to measure dissolved oxygen and hydrogen gases the micro-respiration Clark electrodes are used (Ghirardi et al., 1997). In a Clark's electrode the cathode polarized versus an internal Ag/AgCl anode is placed behind an electrically insulating silicone rubber membrane, which is extremely permeable to oxygen. The flow of electrons from the anode to the oxygen-reducing cathode reflects linearly the partial oxygen pressure around the sensor tip and is in the pA range. The same principle holds for a hydrogen Clark-type sensor: the environmental hydrogen is driven by the external partial pressure and penetrates through the sensor tip membrane to be oxidized at the platinum anode surface (Revsbech and Jørgensen, 1986; Unisense, 2007) Flynn *et al.* (2002) used chemochromic sensors for screening in order to identify positive (i.e. hydrogen-producing) algal colonies. A chemochromic sensor film, which is normally transparent, turns blue in the presence of hydrogen gas. Hydrogen gas is produced during the bacterial fermentation process in anaerobic conditions. In practice, hydrogen is collected in the gaseous state, since dissolved hydrogen tends to become gas. To optimize the hydrogen collection methods it is necessary to study properly the hydrogen production kinetics in liquid phase during the fermentation process. The experimental test system for bacterial hydrogen production and micro-sensors (Unisense, Denmark) were used to determine the hydrogen gas concentrations in liquid; the mass-spectrometry method was employed for measurements in the hydrogen-containing head space. It is well known fact, that reducing the partial pressure of gases in the reactor can increase the overall hydrogen production. Hydrogen can be reduced using vacuum or spraging with inert gas or other methods (Logan et al. 2002, Tanisho et al. 1998). In batch tests continuous versus intermittent release of gas pressure increased hydrogen production by 43% (Logan et al. 2002). Park et al (2005) investigated enhancement by removal of headspace CO_2 and gained 2.0 mol H_2 / mol glucose (241 mL of H_2 / g glucose) comparing to 1,4 mol H_2 / mol glucose (175 mL of H_2 / g glucose). Author used dewatered and thickened sludge from wastewater treatment and chemical scavenging of CO_2 using KOH (30% wt).

1.7. Bioreactors for hydrogen production

Fermentative hydrogen production is a very complex process and influenced by many physical, chemical and biological factors and also fermentation reactor type. Therefore the design and optimisation of industrial fermentation processes requires the experimental investigation in small-scale laboratory test-systems (Nath and Das, 2004). However commercial laboratory test-systems and bioreactors vary a lot and are designed for many different scientific purposes, they are quite expensive and often have some disadvantages for specific experiments and are not multifunctional. Instead of buying a new appropriate commercial test-system each time we meet specific drawback with existing system we should consider using and developing cheap multifunctional test-systems that could be rapidly modified if need. Most of the studies on biohydrogen production via fermentation process have been made in batch mode regarding its simplicity and ease of control (Hallenbeck, 2012). The continuous stirred tank reactor (CSTR) has been widely used for continuous measurements (Hawkes et al., 2002; Hallenbeck et al., 2009). Immobilized cell reactors provide an attractive alternative to conventional CSTR because they are capable of maintaining higher biomass concentrations and can operate at short HRTs. Biomass immobilization can be achieved through formation of granules, biofilms or by using gel entrapment (Li and Fang, 2007).

1.8. Metal hydride alloy usage in storing hydrogen

Research activity in hydrogen storage field has increased substantially due to practical need for hydrogen storage possibilities. Hydrogen is a low density gas in atmospheric pressure, for example, 1 kg of hydrogen occupies a volume of $11m^3$. In order to achieve practical densities for the purpose of storage, hydrogen can be cooled to cryogenic temperatures (20 K) and stored as liquid, or compressed to high pressures and stored as a gas at near ambient temperature (Zuttel, 2003). Current state of the art storage tanks in light passenger vehicles (Toyota, Hyundai) hold hydrogen at pressures up to 70MPa, which allows the storage of 5.4 kg of hydrogen in a 260 L tank and travel 600 km without refilling. Hydrogen storage in solid-state materials is an alternative way to store hydrogen at low pressure and ambient temperatures, and may be a very

promising potential solution. Reversible hydrogen storage materials tend to be hydrides or microporous materials. Alternatively, it can be stored in non-reversible chemical form in hydrogen-rich liquids or solids, or in molecular or atomic form absorbed or adsorbed in solid state materials. Regarding to liquids, hydrogen storage possibilities includes boron-based compounds, such as sodium tetrahydroborate ($NaBH_4$) and ammonia borane (NH_3BH_3). The advantage of this solution can be seen in the high achievable gravimetric and volumetric storage densities, e.g. ammonia borane stores 19.6 wt% and 0.145 kg/L.

Palladium is white silver like metal that soaks up hydrogen like a sponge - at room temperature and atmospheric pressure, palladium can absorb up to 900 times its own volume of hydrogen; it was first noted by T. Graham in 1866. Former researches tend to focus on metal-hydrogen system properties, whether thermodynamic, magnetic, crystallographic and so forth. Metal hydrides are formed from metallic elements or compounds that react with hydrogen to produce binary or higher hydrides (Broom, 2011). Material that can reversibly absorb or adsorb hydrogen in atomic (H) or molecular (H_2) form is used to compress hydrogen (chemically or physically) to high storage densities. (Fig.1.13.)

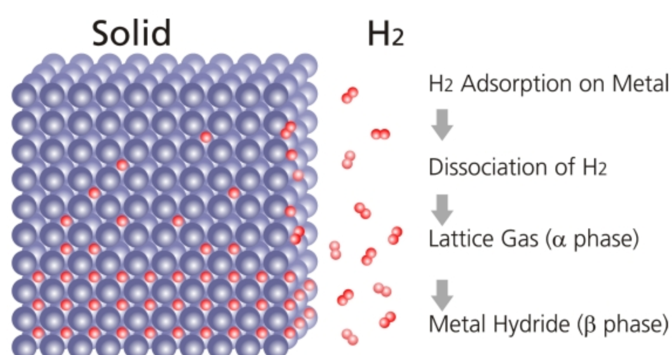

Fig. 1.13. Metal hydride formation (Fraunhofer IFAM Dresden, 2015)

Hydrogen atom (H) coordination can be different due to various metal hydride structures (Banerjee, Mukhopadhyay 2007). The advantage is the density to which the

hydrogen atoms can be compressed in crystal structures of metals (Figure 1.14.). Archetypal metal hydrides such as $LaNi_5H_x$ and PdH_x, for example, can store hydrogen at an atomic density far greater than that seen in its liquid form, without the need for cryogenic temperatures and the associated liquefaction. (Broom, 2011).

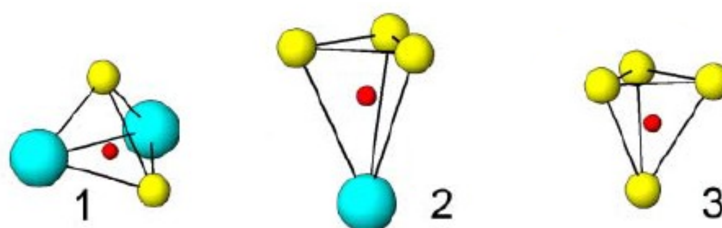

Figure 1.14. Hydrogen coordination in the crystal structures ABH_2, AB_5H_6, BH: H – red, A – blue, B - yellow (Yartys et al. 2008).

In standard practices biohydrogen is synthesized in the fermentation medium, but collected from the headspace of the flask or fermenter after diffusion into gaseous phase. Theoretically dissolved hydrogen is in equilibration with the gas phase and tends to release from the liquid phase if partial pressure of H_2 decreases (Pray, 1952). Nevertheless during dark fermentation biohydrogen over-saturation in the liquid phase takes place (Klepere et al., 2011) and inhibits further synthesis of the gas. To optimize biohydrogen production, it must be removed from the liquid phase by, e.g., barbotage N_2 or Ar through the fermentation medium, and afterwards purified, collected and stored from gaseous phase. Absorption of biohydrogen by alloys and metals that can form hydrides is widely used, safe and efficient method to collect the gas. Metal hydrides $LaNi_5H_x$ and PdH_x and various hydride forming alloys specifically and reversibly absorb or adsorb hydrogen in atomic or molecular form securing high storage densities (Table 1.4.).

Table 1.4. Hydrogen weight and volume capacity in different storage systems (Hirscher et al., 2010).

Material	*H- atoms/cm^3 (x 10^{22})*	*Mass %, of hydrogen*
H_2 gas, 200 bar (2850psi)	0,99	100*
$H_{2\ liquid}$, 20 K (-253 C)	4,2	100*
$H_{2\ solid}$, 4.2 K (-269 C)	5,3	100*
MgH_2	6,5	7,6
Mg_2NiH_4	5,9	3,6
$FeTiH_2$	6	1,89

These materials can accumulate hydrogen at an atomic density far greater than that can be obtained in liquid form, without the need for cryogenic temperatures and the associated liquefaction. Metals and alloys can be "charged" with hydrogen under pressure, they release hydrogen when heated or subjected to evacuation (Hirscher et al., 2010; Kleperis and Grīnberga, 2008). It is reported that the presence of moisture in the hydrogen gas decreases the efficiency of its sorption by metals and alloys, e.g. $LaNi_5$ in 300 ppm of water vapor loses its hydrogen absorption capacity (Sandrock and Goodell, 1980). $LaNi_5$ is one of the most widely used intermetallic alloys for hydrogen storage, due to its sorption capacity, reversibility of absorption and desorption and quick charge–discharge kinetics under room temperature and atmospheric pressure. The $LaNi_5$ alloy can absorb hydrogen in α-phase (low concentration of hydrogen in the metal lattice, $LaNi_5H_{0\text{-}0.5}$), β-phase (hydride $LaNi_5H_3$), γ-phase (hydride $LaNi_5H_6$) and so-called trapped hydrogen that is attached to typical structural defects (Gajek et al., 2008).

Nevertheless already for decades various hydride-forming alloys are used as the cathode material in Ni/Me hydride batteries (see, for example, Kleperis et al., 2001). Hydrogen ion (proton) is inserted in metal hydride cathode through overvoltage applied to the battery in the process of charging and is ejected due to the difference of electrode potentials in the battery. Our innovative idea in this research is that absorption of the biohydrogen directly from the medium of cultivation would increase the efficiency of the gas production due to (i) removing of its inhibitory over-saturated concentration from the medium; (ii) more simple construction of the fermenter since the inert gas

barbotage appliances will become superfluous; (iii) collection of the gas would occur directly during the fermentation process, not afterwards with additional treatment of the gas phase. The purpose of the study was to explore if the hydride-forming metals and alloys are able to adsorb and desorb biohydrogen directly from the fermentation solution. Thermogravimetric weight loss (TG) method was used to estimate the amount of gases and volatile substances absorbed in powdered Pd and $LaNi_5$, AB_5, AB_2 alloys; vacuum extraction and mass-spectrometry were used to characterize the composition of the absorbed gases. Results firstly were announced in the 11th International Symposium on Systems with Fast Ionic Transport (ISSFIT) conference in Gdansk (Poland) in June 25-29, 2014; and is accepted for publication in IJHE, 2016.

Only one similar study found, Nobuyuki Nishimiya et al (2014) recently introduced (IDHE meeting, Nantes France, 2014) their first results with ZrVFe as effective material to recover hydrogen from bio-hydrogen mixtures produced by anabaena in argon atmosphere. They investigated that total amounts of hydrogen increased 6-7 times compared to normal hydrogen production in the absence of the alloy.

2. Materials and Methods

2.1. Hydrogen producing bacteria cultivation, growth media and substrate preparation

2.1.1. Growth of strains for hydrogen production in bioreactor test-systems (I)

Escherichia coli strain MSCL 332 (i.e. from Microbial Strain Collection of Latvia) was grown on Luria-Bertani (LB) nutrient agar plates (5 g/l yeast extract, 10 g/l tryptone, 10 g/l sodium chloride, 15 g/l Bacto agar (Atlas, 2004). *E.coli* from single colonies on the agar plates were inoculated in 2 x 150 ml flasks containing LB liquid medium. The flasks were aerobically shaken at 37°C for 12 hours at 120 rpm using a multi-shaker PSU-20 (BioSan, Latvia). The bacteria cell number in the overnight culture was titrated at 10^{-6} dilution. The amount of bacterial cell protein was calculated assuming that one *E.coli* cell contains 1.54×10^{-13} g of protein (Madigan and Martinko, 2006). The overnight culture in LB liquid medium was mixed (1:1) with phosphate buffer saline (PBS) pH 7.3 (0.8 g/l NaCl, 0.2 g/l KCl, 1.43 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4 (Penfold et al., 2003) in a vessel sterilized for measurements. The PBS contained a complex trace element medium pH 6.5 (0.039g/l $Fe(NH_4)_2{\cdot}SO_4{\cdot}6H_2O$, 0.172 mg/l Na_2SeO_3, 0.02 mg/l $NiCl_2$, 0.4 mg/l $(NH_4)_6Mo_7O_{24}$ (Maeda et al., 2008). Argon gas (99.99 % purity) barbotage through the media was used to sustain anaerobic environment. Barbotage is a process of gas penetration through a layer of liquid as a flow of dispersed small gas bubbles (Prokofiev, 2014). Glucose (3.3 mM, final concentration, sterilized through membrane 0.2µm filter) was added at the start of experiment.

2.1.2. Growth of strains for hydrogen production in bioreactor test-systems (II)

Two *Escherichia coli* (*E. coli*) strains were used for first test experiments *E. coli 332* and modified *E. coli* BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE* that has up to 4.6 times increased hydrogen production capability (Maeda et al. 2007). Cultivation was identical to chapter 2.1.1. described method. Optical density (OD) calibration curve was used to find out number of cells in 1mL of culture (Widdel, 2007). In sterile test-system 250 mL of culture was mixed with 250 mL of phosphate buffer, used also in chapter 2.1.1. Glucose was used as a model substrate to observe dynamic of hydrogen production in

test-system. 2 mL of 50% glucose was added to culture in test-system. System was placed in thermostat at 37 °C.

2.1.3. Growth of strains for hydrogen production from glycerol-containing substrate

Seven facultative anaerobic bacteria strains (*Aneurinibacillus aneurinilyticus* MSCL 1018*, Enterobacter aerogenes* MSCL 758, *Enterobacter asburiae* MSCL 839 isolated from Latvia's dental clinic pacient tooth channels)*, Escherichia coli* MSCL 332*, Enterobacter cloacae* MSCL 778 (isolated from Latvia's dental clinic pacient tooth channels), *Kluyvera ascorbata* MSCL 732*, Paenibacillus pabuli* MSCL 1006*)*, one strictly anaerobic bacterium strain (*Clostridium sporogenes* MSCL 764 (ATCC 19404)*)* and one modified strain (*E. coli* BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE::kan*). Cultivation was identical to chapter 2.1.1. described method with the exception of *Clostridium sporogenes* (cultivated in the Thioglycollate resazurin broth (Bio-Rad, France) for two days without shaking). The bacteria concentration in the fresh culture was 10^7 CFU/mL. Crude glycerol (40%, determined with HPLC analysis) from biodiesel fuel production was used as substrate, final concentration of glycerol used was 240 mM. Glycerol and glucose were sterilized through 0.2 µm membrane filters.

2.1.4. Growth of strains for hydrogen production from lactose-containing substrate

E. coli strains were grown on LB medium (10 g/l tryptone, 5 g/l yeast extract and 5 g/l NaCl), supplemented with either 3 % glucose or lactose as the carbohydrate substrates. Growth medium was sterilized by filtration through membrane filters with 0.2 µm pore size. In addition, growth medium for all strains (except JM109) contained 30 ug ml^{-1} kanamycin, and that for the plasmid-bearing strains – also 100 ug ml^{-1} ampicillin. Strains were cultivated overnight aerobically in shaken flasks, and the obtained cultures were further used to inoculate the anaerobic fermentations for the study of hydrogen production. All cultivation experiments were carried out in three independent repeats. Representative results from one cultivation experiment of each type are shown in Figure 2.1.

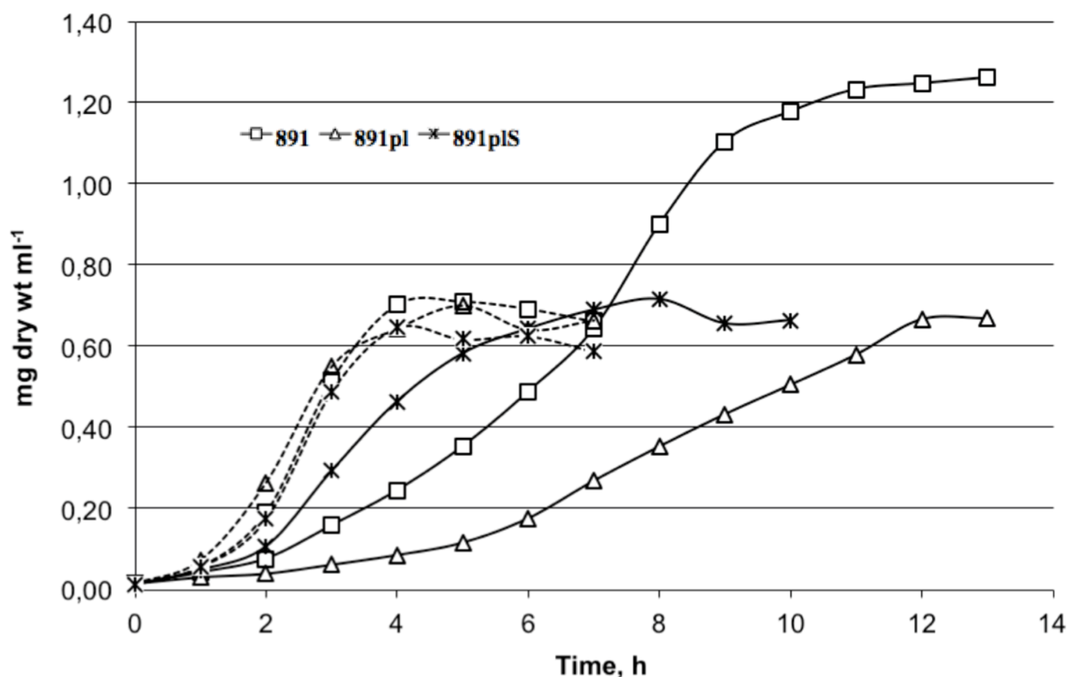

Figure 2.1. Aerobic batch growth of *E. coli* strains on LB medium, supplemented with 3 % of lactose (continuous lines) or glucose (dashed lines); representative data of cultivation from a series of three experiments are shown.

2.1.5. Growth of strains for hydrogen isolation experiments using hydride- forming metals and alloys

Enterobacter aerogenes (MSCL 758) from Microbial Strain Collection of Latvia and *Escherichia coli* BW25113 *hyaB hybC hycA fdoG frdC aceE ldhA::kan* (kindly provided by prof. T.K. Wood, Texas A&M University, USA) were used for fermentation experiments. Strains were grown on modified anaerobic growth medium (AM) (g/l): tryptone 1.0, yeast extract 2.5, cysteine 0.5, crude glycerol 5.0. Crude glycerol (40% glycerol content) was obtained from the biofuel factory in Naukseni, Latvia. Nutritional medium was sterilized by autoclaving for 15 min at 121 °C. Bacterial strains were cultivated overnight aerobically in Petri dishes on solid Luria-Bertani (LB) medium at 37°C. The overnight cultures were adjusted to 1 OD (600 nm) in distilled water and the OD-adjusted cultures used as inoculum for further cultivation anaerobically in 50 mL serum bottles (Supelco Analytical, USA) containing 35 mL AM media and 1 mL bacteria suspension. Inoculated serum bottles were closed with butyl

rubber stoppers sealed with aluminum caps and flushed with argon gas to obtain anaerobic environment. The serum bottles were incubated in Biosan RS-24 programmable rotator in a thermostat (Binder, Germany), at 37±2.

2.2. Construction of strains, plasmids, and cloning

The septuple knock-out mutant *Escherichia coli* BW25113 *hyaB hybC hycA fdoG frdC aceE ldhA::kan* (here - strain *E. coli* 891, a gift from prof. T.K. Wood, Texas A&M University, USA) was used as the parent strain. The strain is defective in the large subunits of hydrogenase 1 and hydrogenase 2, defective in repressor of formate hydrogen lyase, in the alfa-subunit of formate dehydrogenase-O, in lactate dehydrogenase, in fumarate reductase, and in pyruvate dehydrogenase. The host strain *E. coli* 891 was transformed with the expression vector pTrcHis (Invitrogen), carrying the gene *lacY* inserted between *Nhe*I and *Hind*III restriction sites of the vector MCS. The *lacY* gene was amplified, using the genomic DNA of *E. coli* JM109 as the template.
The oligonucleotides
AAAAA<u>GCTAGC</u>ATGTACTATTTAAAAAACACAAACTTTTGGATG (*Nhe*I restriction site underlined) and
AAAAA<u>AAGCTT</u>ATTGATTGCTTAAGCGACTTCATTCACCTGA (*Hind*III restriction site underlined) were used as the forward and reverse primer, respectively.
After amplification of *lacY* with *Pfu* polymerase, the resulting 1.27 kb PCR product was digested with both restrictases and ligated in the vector, downstream of trc promoter, yielding the plasmid pTrcHislacY. This plasmid was transformed in *E. coli* JM109, using the standard $CaCl_2$ protocol (Kalnenieks et al. 2008). After cloning and purification of pTrcHislacY, the insertion of *lacY* gene was confirmed by sequencing, and the obtained plasmid construct was transformed in the strain 891, yielding the plasmid-containing recombinant strain *E. coli* 891pl. Qiagen kits were used for purification of plasmids and PCR products, and all other DNA manipulations were performed, as described previously (Helm et al. 1991).

2.3. Experimental set up for hydrogen production in fermentation process

Measurements of hydrogen production were carried out in two experimental systems – in 30 ml serum bottles with working volume of 15 ml, allowing to study several small culture volumes in parallel, and in a prototype bioreactor system for

hydrogen gas production, coupled to mass spectrometer.

Serum bottles were closed with butyl rubber stoppers and aluminium caps. Before use, the bottles were flushed with argon gas and autoclaved for 60 min at 121°C. The evolved gas from serum bottles was collected with a syringe, and subsequently injected in the mass-spectrometer RGAPro-100 (HyEnergy, Setaram, France) for hydrogen gas measurement. Gas samples with syringe were taken every hour from each bottle of a series of parallel cultivations. Dissolved hydrogen concentrations were measured with Clark-type microsensor (Unisense, Denmark).

The lab-scale bioreactor was used for larger scale cultivation experiments. The total volume of bioreactor was 930 ml, with 330 ml headspace for gas.

Along with the gas samples for hydrogen determination, it enabled to take culture samples for analysis, without significant perturbing of the system. The gas from the headspace of the bioreactor was taken to the mass-spectrometer in 20 cm^3 aliquots, while argon gas (99.99 % purity) was continuously flushed through a diffuser to maintain anaerobic environment. During the experiments, the bioreactor, as well as the serum bottles were kept in a water bath (Precisterm 2-110, 2L), in order to maintain temperature around 37±2 ºC.

2.4. Analytical methods and experimental set up

2.4.1. Mass Spectrometry (MS)

On experiments with glycerol and lactose usage, the gas mixture from the headspace of the serum bottles was collected with a syringe and injected in the mass-spectrometer RGAPro-100 input node (HyEnergy, Setaram, France). The data were analyzed by RGA 3.0 Software for SR Residual Gas Analyzer. On experiments with bioreactor test-systems (Original paper I and II): the gas from the headspace of liquid bacterial culture in the test vessel was taken to an RGAPro-100 mass-spectrometer to analyse its components. The gas from an argon balloon through a diffuser was let in the test vessel with bacteria culture to sustain the anaerobic environment and put in a water bath (Biosan) to maintain a temperature of 37±2 °C. Argon gas was bubbled through the liquid for 15 min (flow 13 L/h) and gas measurements were made with 30 min intervals. The total time of measurements was six hours. There was 200 mL headspace for gas in the test-system. The gas from the headspace of the bioreactor was taken to the mass

spectrometer in 20 cm3 aliquots. During the mass-spectrometric analysis, simultaneous measurements with a hydrogen microsensor were taken in order to make unbiased comparison of mass-spectrometric and hydrogen microsensor analyses (Fig. 2.2.).

Fig. 2.2. Masspectometer RGAPro-100 connected with bioreactor and microsensors in water bath (photo Dimanta, 2014).

The concentration of dissolved hydrogen gas was measured with a microsensor; the mass-spectrometric analyses were made for the atmospheric composition in the headspace of the sample bacteria culture and nutrients. Concentration of hydrogen in gaseous phase was calculated using volumetric data from masspectrometer and simple calculations Eq. below. End-result units were mmol/l.

$$[H] = \left(\frac{V(\%) \cdot 0,01}{22.4} \right) \cdot 1000$$

On experiments hydrogen-absorbing materials: MS of the gases released during the anaerobic fermentation of glycerol in the media containing hydrogen absorbing materials and thermogravimetric (TG) measurements were performed in separate experiments, since at sampling of the Pd or alloy grains for TG the gas volume and the partial pressure of H_2 in the headspace was changing. In masspectrometric (MS) measurements for the H_2 concentration measurements the gas mixture from the headspace of the serum bottles was collected with a syringe and injected in the MS.

Gases absored by the alloy AB_5 were identified after vacuum extraction of the alloy powder in the 8 cm^3 hydrogen adsorption/desorption reactor. The temperature was elevated gradually up to 200°C, measurements were made continuously for two hours and simultaneously for seven gases: argon, carbon dioxide, ethyl alcohol, hydrogen, nitrogen, oxygen and water vapor. The mass-spectrometer was connected to the reactor with PTFE tubing for volumetric gas analysis; argon gas was used for reactor cleaning between experiments.

2.4.2. Clark-type microsensors (CTM) for liquid phase analysis

The hydrogen and oxygen concentrations were measured with CTM (Unisense, Denmark) in the sample liquid phase. The microsensors were connected with the signal amplifier − a pico-ammeter and an A/D current converter connected to PC using USB port. Before the measurements, both oxygen and hydrogen microsensors were calibrated in a liquid culture medium (similar to the sample measured by 15 min barbotage Ar) for zero concentrations and hydrogen gas (99.999% supplied from AGA Ltd. or using a proton exchange membrane electrolyser from Fuelcellstore.com) and clean air for 100% dissolved H_2 and O_2 concentrations (730 and 760 μmol/l, accordingly for 30° C temperature) (Unisense, 2007). The system is able to work independently when measurements are made in one sample. If there are several samples at a time, it is necessary to move CTM manually and to sterilize the sensor tip using 96% ethanol, 0.1 *M* NaOH and distilled water every time when it is taken out from the sample (Unisense DK, 2007). The experimental results of CTM measurements were analyzed using *Sensor Trace Basic* and *MicOX* (A/S Unisense) programs, and processed by *Microsoft Office Excel 2007*.

2.4.3. Thermogravimetric weight loss analysis (TG)

Differential TG of the Pd or alloy grains upon heating after the incubation in the fermentation media was performed using DTG-60 device (Shimadzu). The amount of gas absorbed by the hydrogen-absorbing materials was tested by removing 1,5 - 2,0 cm^3 medium containing 5-15 mg in of the Pd or alloy grains from the bottle with the large gauge syringe. Before the TG measurements the grains were washed with 98% ethanol and dried at room temperature for 24 hours in argon atmosphere. The temperature of the

crucible in TG experiments was increased with constant rate, 10°C per minute.

2.4.4. Scanning electron microscopy (SEM)

Surface structure of the investigated materials before and after contact with microorganisms was studied using SEM EVO 50 (Zeiss, Germany) and SEM Lyra3 (Tescan, Chech Republic) microscope. Prior observation grains were fixed with 98% ethanol on carbon tape and coated with 20 nm Au layer.

2.4.5. High performance liquid chromatography (HPLC)

The utilization of glycerol at bacterial fermentation was measured by HPLC on Agilent *1290 Infinity* device (Agilent Technologies, Germany) using the *Atlantis dC18 3 µm, 2,1x15 0mm* (Waters) column. Standard of analytical glycerin was purchased from Stanlab (Lublin, Poland). Stock solutions of the standards (analytical, crude glycerin) at a concentration of 1.0 mg/ml were prepared in acetonitrile (Sigma-Aldrich, gradient grade) and were stored at 4 °C. Working solutions of mixtures of all the standards were prepared immediately before analyses by diluting the stock solution with acetonitrile, to attain the required concentrations for calibration measurements. The analyses were performed on the modular UPLC system using acetonitrile – water gradient in 0.05% formic acid. High resolution mass spectra (HRMS) were taken on *Agilent 6230 TOF LC/MS* (Agilent Technologies, Germany) with electrospray ionization (ESI): positive ionization mode, drying gas 10 ml/ min at 325°C, fragmentor ionization 90 V. Internal reference mass 121.050873 m/z for all sample analyses were used. The aliquots from the fermentation media were passed through 0.45 µm filters (Nonpyrogenic Sterile-R, Sarstedt) and 1 µl of the solution was injected into the liquid chromatography system. All the experiments were performed in triplicate. The relative standard deviation was determined to be less than 1%. Data analyses were performed using MassHunter version B05.00 software (Agilent Technologies).

2.4.6. FT-IR spectroscopic analysis

Bacterial cells were sampled, washed with distilled water, and 10-15 μl of water suspension dried at T<50 °C on a 384 well silicon plate. FT-IR spectra were recorded on a VERTEX 70 coupled with the microplate reader - HTS-XT extension (BRUKER, Germany). Transmission spectra were collected over the range of 4000 - 600 cm^{-1}, and displayed as absorbance spectra. Spectra were acquired at a resolution of 4 cm^{-1}, and 64 spectra were co-added. Baseline was corrected by the rubber band method, CO_2 bands excluded. Only spectra fitting in the absorption limits between 0.25-0.80 (where the concentration of a component was proportional to the intensity of the absorption band) were used for data analyses and at least two analytical replicates were evaluated. Data were processed using OPUS 6.5. software and the second derivative spectra were evaluated. Several spectral regions were considered for identification of specific macromolecular components of the biomass: the lipid region at 3050-2800 cm^{-1}, where peaks originate from the vibrations of the CH_2 and CH_3 groups of fatty acids, the protein region at 1750-1500 cm^{-1}, dominated by the Amide I and II (N-H and C=O vibrations) and peptide bands, the mixed region at 1500-1200 cm^{-1} containing vibrations coming from fatty acids, proteins and nucleic acids, and polysaccharide and carbohydrate region at 1200-900 cm^{-1} (Helm and Naumann, 1995, Naumann, 2000, Grube et al. 2002). Quantitative analysis of carbohydrates, nucleic acids, proteins and lipids was carried out as in (Grube et al. 2012). Lactose concentration in the growth medium was monitored at region 993-986 cm^{-1} using the 2nd derivative spectra (Vikso-Nielsen, 2002).

2.4.7. Iodine staining and light microscopy

Glycogen staining with iodine was performed following the routine of Vikso-Nielsen *et al*. (14). In brief, *E. coli* cells were harvested by centrifugation, washed with distilled water and stained with I_2/KI solution (1:10, final concentration 0.08 g/l). Unbound iodine was washed by centrifugation. Cell suspensions were smeared and heat-fixed on slides. Samples were analyzed and photographed by Olympus BX51 microscope equipped with DP71 camera. Cell F software was used for image processing. All pictures were taken in phase contrast regimen, 1000 x magnification.

2.4. Powdered metals and alloys for hydrogen storage

Pd and $LaNi_5$, AB_5, AB_2 alloy powders that were tested for the capacity to remove bio-hydrogen from the liquid phase in fermentation are listed in table 2.1.

Table 2.1. Materials tested for hydrogen absorption capacity from the liquid phase of fermentation.

No	Metal or alloy	Origin	Composition, grain size
1	Pd	Aldrich	Microcrystalline powder, <10 µm
2	$LaNi_5$	Treibacher Industrie AG, Austria	Crystalline powder, <500 µm
3	AB_5	Gesellschaft fur Elektrometallurgie; Nuremberg; Germany	Microcrystalline powder <75µm; A= Mm=La,Ce,Nd,Pr B=Co, Mn, Al ; $Mm(Co,Mn,Al)_5$
4	AB_2	Gesellschaft fur Elektrometallurgie; Nuremberg; Germany	Microcrystalline powder<75µm; A=Zr, Y ; B=Ni, Al, Fe, Mn $(Zr,Y)(Ni,Al,Fe,Mn)_2$

10-12 g of powders were activated before use: simultaneously heated up to 170 °C and evacuated up to ~0.01 bar in ca 8 cm^3 cylindrical stainless steel hydrogen adsorption/desorption reactor built in the Institute of Solid State Physics (University of Latvia), then exposed to hydrogen gas (2 bar) for 30 min and cooled down to room temperature in hydrogen atmosphere in the reactor; this process was repeated three times until rapid hydrogen absorption in alloy powder was observed (Fig. 2.3.) (Kleperis, Grinberga 2008).

Fig. 2.3. Activation of metal hydide powder in cylindrical stainless steel hydrogen adsorption/desorption reactor (photo Dimanta, 2014).

Finally the absorbed hydrogen was removed by simultaneous heating and evacuation process. Argon gas was used to preserve activated alloys until their contact with the fermentation medium. 1 g of each alloy and 0.5 g of Pd powder was used for 30 ml of fermentation medium. Serum bottles with microorganisms, medium and metal hydride powder were rotated for 18, 24 and 36 hours in 37°C temperature (Fig. 2.3.). Metal hydride powders were washed with 96% ethanol and dryed in 4°C temperature for at least 12 hours.

Fig. 2.4. Serum bottles rotation with microorganisms, medium and metal hydride powder (photo Dimanta, 2014).

2.5. Construction of prototype bioreactors

Various materials were used to build up the prototype of bioreactors (shematics of bioreactors available in section 3.1.2.). Hardened glass vessels of different volumes (0.75 l – 3 l) were purchased together with top sealing lids from stainless steel. For stirring common 4.5V DC motors sealed in hermetic housing were used. Special porous, thermostable (up to 200 °C) silicon rubber was used for gaskets and as sealing material. Sensor probe holders, valves, gas inlets and outlets were made from Ertacetal H-TF because it is resistant to chemicals, mechanical degradation, easy to handle within machining process and is stable up to 175 °C (that is far enough for sterilization in autoclave at 120 °C).

3. Results

The results are presented here as original publications and as unpublished results. The author's (birth name – Klepere) contribution to the enclosed original publications:

Original paper I

I.Klepere, I.Muiznieks, J.Kleperis „A bacterial hydrogen production test system for measuring H2 concentrations in liquids and gases." *Latvian Journal of Physics and Technical Sciences* Vol. 2 p.60-68. (2010) ISSN 0868-8257

Contribution: performed experimental work, partly performed the result analysis; prepared graphical information and wrote the manuscript.

Original paper II

Gruduls A., Dimanta I., Dirnena I., Muiznieks I., Kleperis J. „Simple bioreactor design for hydrogen and methane gas producing microorganisms – optimization and experiments" ISJAEE (International Scientific Journal for Alternative Energy and Ecology) 09, 2012, p.32-38. ISSN-1608-8298

Contribution: experimental set up for hydrogen production experiments with bacteria, performed bacteria cultivation and hydrogen production experiments, partly wrote the results discussion part.

Original paper III

I.Dimanta, V.Nikolajeva, A.Gruduls, I.Muižnieks "Assessment of bio-hydrogen production from glycerol and glucose by fermentative bacteria." Power Engineering Vol 59 (3), 2013. ISSN 0235-7208

Contribution: performed the experimental work, partly performed the result analysis**;** wrote the manuscript.

Original paper IV

M.Grube, I.Dimanta, M.Gavare, I.Strazdina, J.Liepins, T.Juhna and U.Kalnenieks "Hydrogen-producing Escherichia coli strains, overexpressing lactose permease: FT-IR analysis of the lactose-induced stress'', Biotechnology and Applied Biochemistry, 2014 Mar-Apr;61(2): 111-7. ISSN:0885-4513

Contribution: perfomed work decribed in 2.2. (Growth media, cultivation and

monitoring of hydrogen production) that were used for result chapter 3.1. (Growth of the strains with glucose or lactose) and chapter 3.2. (Hydrogen production with lactose), partly perfomed experimental work described in chapter 2.1. (Construction of strains, plasmids, and cloning,), prepared text and graphical information for chapter 3.2.

Manuscript

I.Dimanta, J.Kleperis, I.Nakurte, S.Valucka, V.Nikolajeva, Z.Ruktovska, I.Muiznieks "Metal hydride alloys for storing hydrogen produced by anaerobic bacterial fermentation" (manuscript submitted to *Internation Journal of Hydrogen Energy in 2015).*

Contribution: prepared experimental set-up, partly perfomed experimental work, performed result analysis, text and graphical information, partially wrote the manuscript.

3.1. Hydrogen production analysis with experimental test systems and bioreactors, using various, also in Latvia isolated, bacteria strains

3.1.1. Biological hydrogen production in bioreactor test-systems *(Original paper I)*

Hydrogen output was measured for seven hours after the beginning of fermentation process; increase in the hydrogen concentration was observed starting from the second hour after adding glucose. The constancy of oxygen concentration in the measurements evidences that the system had reliable anaerobic conditions (Figure 3.1.). As is seen from this figure, the concentration of dissolved hydrogen stopped increasing after 5–10 h as glucose exhausted (similar results are reported Nanti et al 2001). The maximum rate of hydrogen formation in the test system was 612 µmol/ l/20 min or 1.4 mmol [2.4 mg] /l per h for 43 mg protein mass (i.e. 32.6 µmol/mg protein mass). The maximum concentration of dissolved hydrogen (2481 µmol/l or 2.5 mmol/l) is reached in the fourth hour of fermentation as is seen in Figure 3.2. This concentration at least three times exceeds the maximum thermodynamically allowed concentration of dissolved hydrogen in water (730 um/l (Mandelis and Christofides, 1993)).

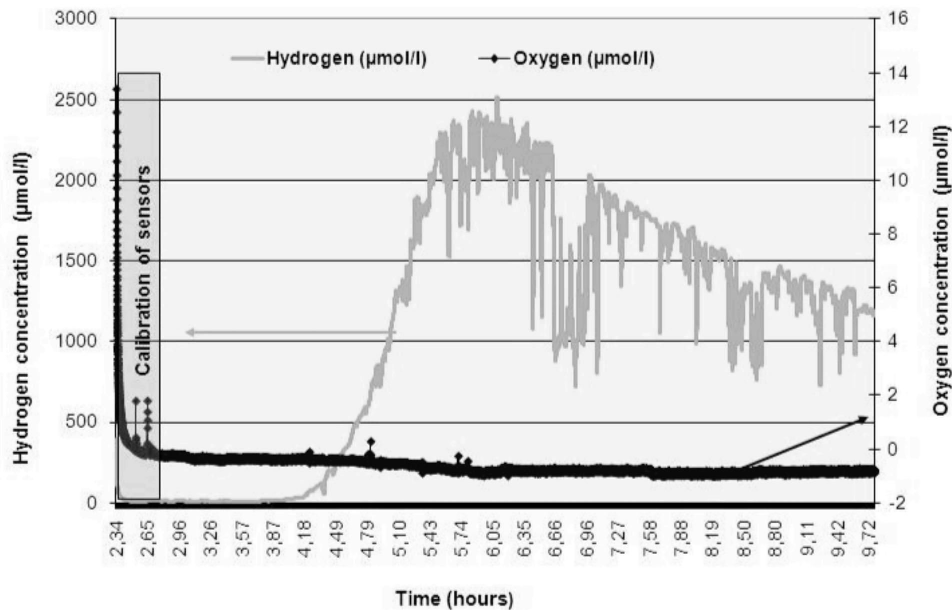

Figure 3.1. Microsensoric fermentation measurements on the sample with *E.coli.*

To demonstrate that the hydrogen production began only after glucose had been added various glucose concentrations were tested. The correlation between the glucose concentration and the hydrogen output is shown in Figure 3.2.

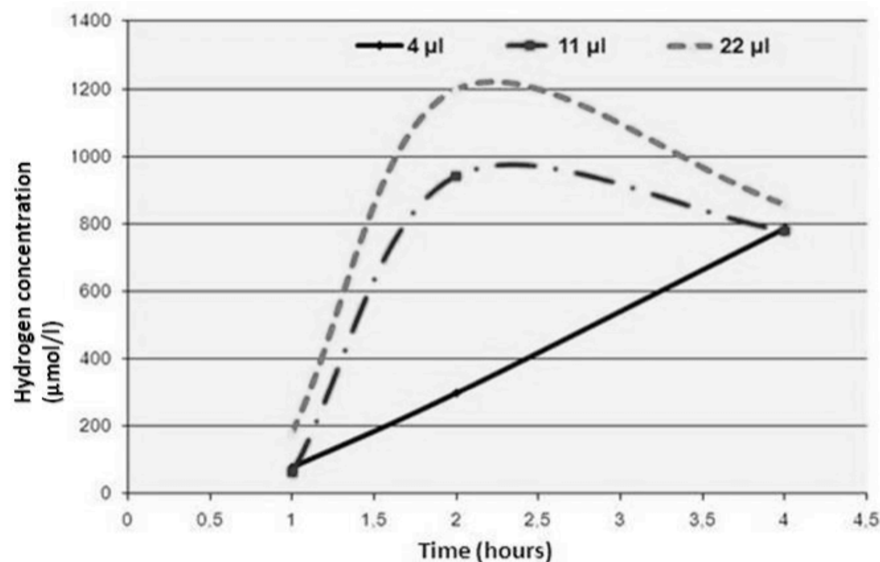

Figure 3.2. Time dependence of hydrogen output at different glucose concentrations (microsensoric measurements on the sample with *E.coli*).

To calculate the partial pressure of hydrogen in the headspace Henry's law was used. The calculations were done using the measured dissolved hydrogen concentrations in the

test system (2481 μmol/l after 4 h fermentation). At room temperature and normal atmospheric pressure the Henry constant is:

$$k_{\mathrm{H}} = 1282.05 \frac{l}{\mathrm{atm} \cdot \mathrm{mol}},$$

therefore p_{H} = 1282.05·c_{H} (atm) and in our case p_{H} = $1282.05 \cdot 2481 \cdot 10^{-6} = 3.18$ atm, which obviously does not fit the experimental results obtained in the mass-spectrometric analysis. As is seen from Figure 3.3., the partial pressure of hydrogen of 3.18 atm above the test system's headspace is inadequate to the concentrations determined by mass-spectrometric analysis – only $6 \cdot 10^{-3}$ atm or 0.6% vol.

3.1.2. Bioreactor design for hydrogen producing microorganisms – optimization and experiments *(Original paper II)*

Most of the studies on fermentative hydrogen production are conducted in batch type mode due to its simple operation and control (Maeda et al., 2007; Das & Veziroglu, 2008; Wang & Wan, 2009). In these studies mostly batch type reactors without stirring or inert gas barbotage are used. In our work we examined the influence of stirring and inert gas s parging on hydrogen production efficiency. Our first reactor test-system (Figure 3.3.) was made to find out effects of these two factors.

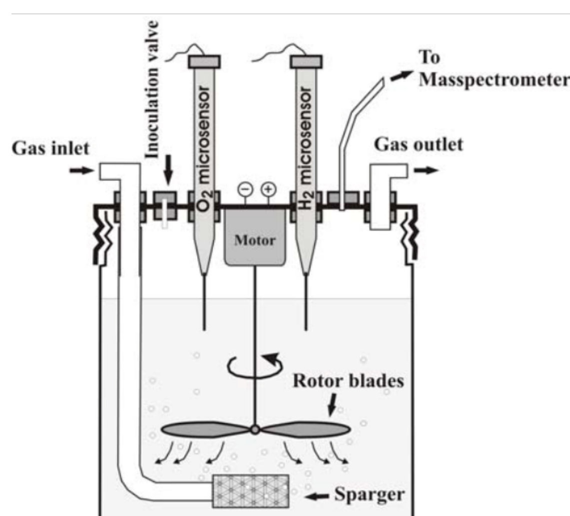

Figure 3.3. Schematic diagram of our first constructed anaerobic cultivation test system (design by Gruduls A.).

Henri law equation Eq. (3.1.) shows close connection between dissolved hydrogen concentration and partial pressure of hydrogen in gaseous state above the liquid, where p – partial pressure, k_H – constant, c – concentration of dissolved gas (Laidler et al. 1999):

$$p = k_H c. \qquad (3.1.)$$

An inert gas heavier than hydrogen can be used to extract hydrogen from liquid phase to increase its concentration in gaseous phase. Experiment results showed that stirring and gas barbotage has significant impact on hydrogen production efficiency and will be discussed later. Optimization of first system was carried out reflecting on results of various experiments, compatibility requirements with commercial sensors and test probes, reducing build-up and maintenance and future predictions on prototype reactor operation. Our constructed laboratory scale bioreactor system consists of two reactors: one for biohydrogen production and second for biogas production. However each reactor can be used as separate unit. It is reported that hydrogen-utilizing methanogens are critical factor for the anaerobic bio-hydrogen production due to the rapid H_2 uptake during fermentation (Kim et al., 2004). Therefore it could be possible to increase biogas outcome by adding extra bio-hydrogen to reactor. System was made to be hermetic and compatible with different size commercial sensor probes using a specially developed fast coupling system. Test-system can be modified and can be used to analyse different fermentative processes using various microorganisms. System allows monitoring of hydrogen or oxygen concentration simultaneously in liquid phase and gaseous phase. It is possible to monitor gas composition with masspectrometer during fermentation processes.

Importance of stirring and inert gas barbotage

Hydrogen production efficiency for wild type *E. coli* 332 strain (Figure 3.4.) is lower than that of modified *E. coli* BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE* (Figure 3.5.) strain. Maximal hydrogen concentration that was achieved using wild type strain was 1.32 mmol/l in liquid phase and 0.40 mmol/l in gaseous phase. However as for modified strain these concentrations went up to 4.0 mmol/l in liquid phase and 6.9 mmol/l in gaseous phase. Stirring and inert gas barbotage has significant

effect on effectiveness of hydrogen production in lab scale test-systems (Figure 3.5., Figure 3.6.). These results show that stirring and inert gas barbotage increased hydrogen concentration in liquid phase from 4.0 mmol/l to 9.0 mmol/l and from 6.9 mmol/l to 8.1 mmol/l in gaseous phase. Maeda (2007) concluded that, using modified *E. coli* BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE* strain, highest achieved hydrogen production efficiency is 44 µmol H_2/mg of protein. Our achieved efficiency is 47 µmol H_2/mg of protein. The concentration alteration in liquid phase is greater than that in gaseous phase. This could be explained due to gradual hydrogen accumulation in headspace above the medium with culture. Thus the partial pressure of gaseous hydrogen increases. As showed in Henri equation (Eq. 3.1.) increase of partial pressure in gaseous phase increases the concentration of dissolved gas. Produced hydrogen had no opportunity to exit system and it accumulated in liquid phase, thus probably slowing down entire production.

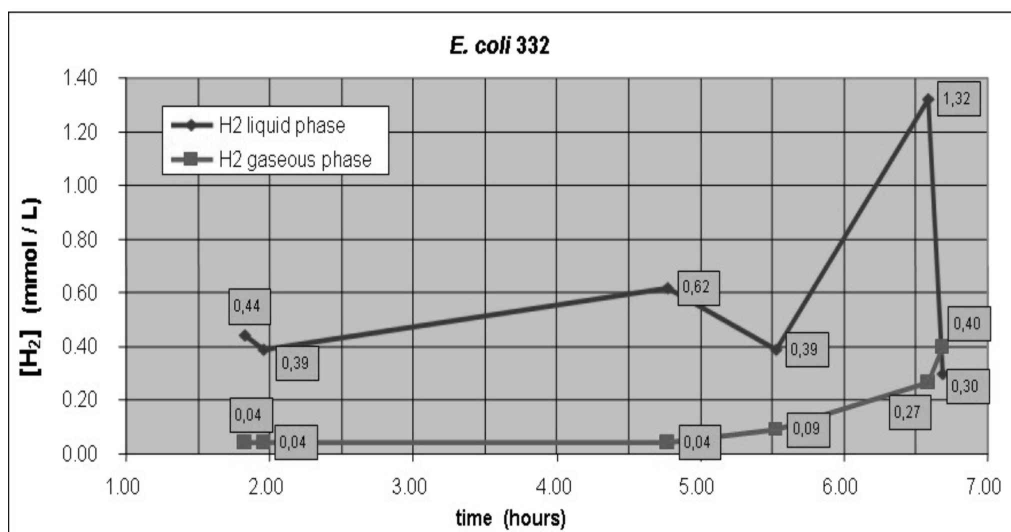

Figure 3.4. Comparison of H_2 production by *E. coli 332* strain in dissolved and gaseous phases in anaerobic system with stirring and argon barbotage.

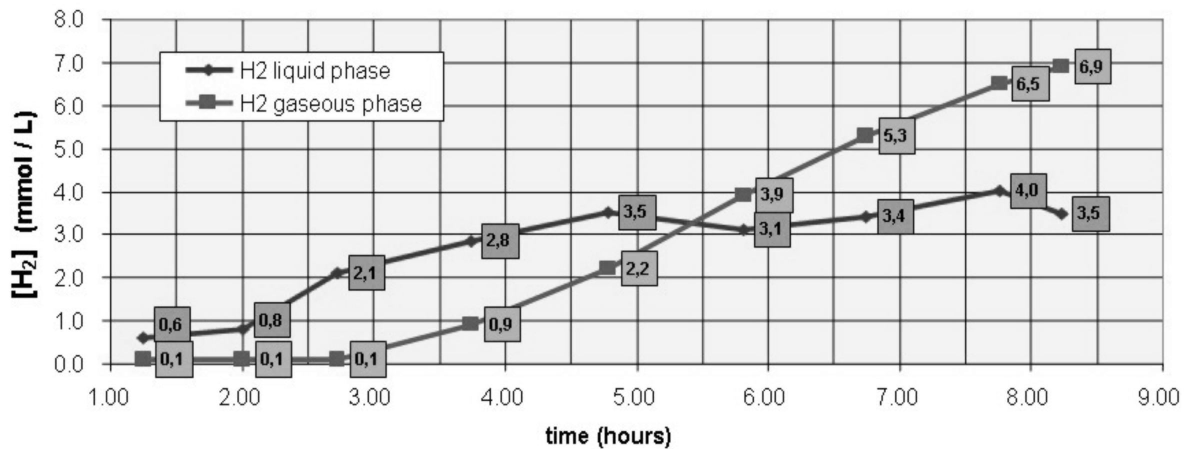

Figure 3.5. Comparison of H2 concentration produced by *E. Coli BW25113* in dissolved and gaseous phase. Anaerobic system without stirring and argon barbotage.

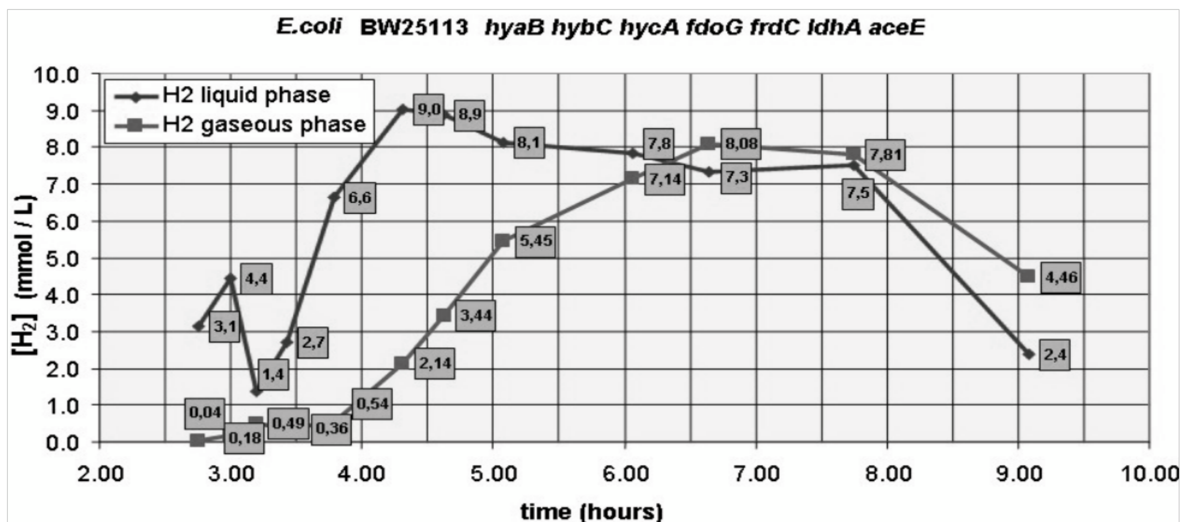

Figure 3.6. Comparison of H2 concentration produced by *E. coli BW25113* in dissolved and gaseous phase. Anaerobic system with stirring and argon barbotage.

An additional experiment was carried out with discontinuous stirring and discontinuous gas barbotage (Figure 3.7.) to explore how each of these two factors influence hydrogen production effectiveness.

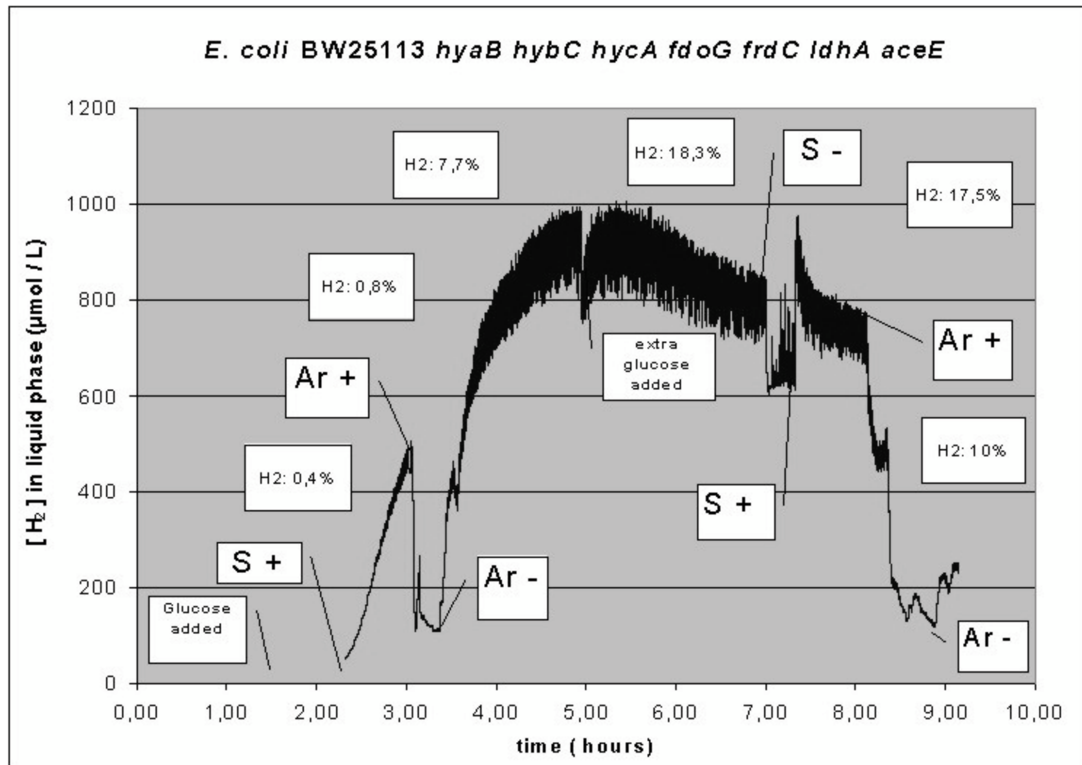

Figure 3.7. Changes in hydrogen concentration in dissolved and gaseous state depending on barbotage and stirring effects: Ar+ - barbotage with argon; Ar- - barbotage turned off; S+ stirring on; S- stirring off; H_2 hydrogen concentration in gaseous state (volume %).

Our results showed that constant stirring increase overall hydrogen concentration in liquid phase. If stirring is stopped concentration decreases, concentration alterations in gaseous phase are not very obvious until saturation is not reached. Fast increase in hydrogen concentration in gaseous phase can be achieved due to inert gas barbotage thus decreasing concentration in liquid phase and promoting overall hydrogen fermentative production. However argon is quite expensive gas. Gas barbotage should be used only periodically as hydrogen concentration in liquid phase has reached saturation. Thereby decreasing systems maintenance costs.

Large scale bioreactors have some drawbacks on even substrate diffusion due to their great volume (Enfors et al., 2001). Small scale-down test-systems could be used to simulateand solve these problems (Delvigne et al., 2006). Our new multifunctional bioreactor prototype schematic (designed by co-author Arturs Gruduls) is shown in Figure 3.8. It can be used as an adjustable laboratory scale test-system for various

fermentation experiments. Medium inlet valve and culture outlet valve makes it possible to use this system also as a continuous flow hemostat. With external automatics it is possible to control different parameters for fermentation process. Heating is provided with external thermostat. Central processing unit (CPU) processes incoming data from pH probe (Figure 3.9.) and automatically regulates pH of medium. When hydrogen concentration in liquid phase reaches saturation, CPU triggers gas inlet valve to open. Barbotage with argon is the initiated until hydrogen concentration in medium is greatly reduced. This conformation of system is a typical application for simple fermentation experiments. Composition of headspace gas can be measured with masspectrometer. For quantitative analysis produced gas is bubbled trough sodium hydroxide to get rid of excess carbon dioxide. It is accumulated in a special vessel.

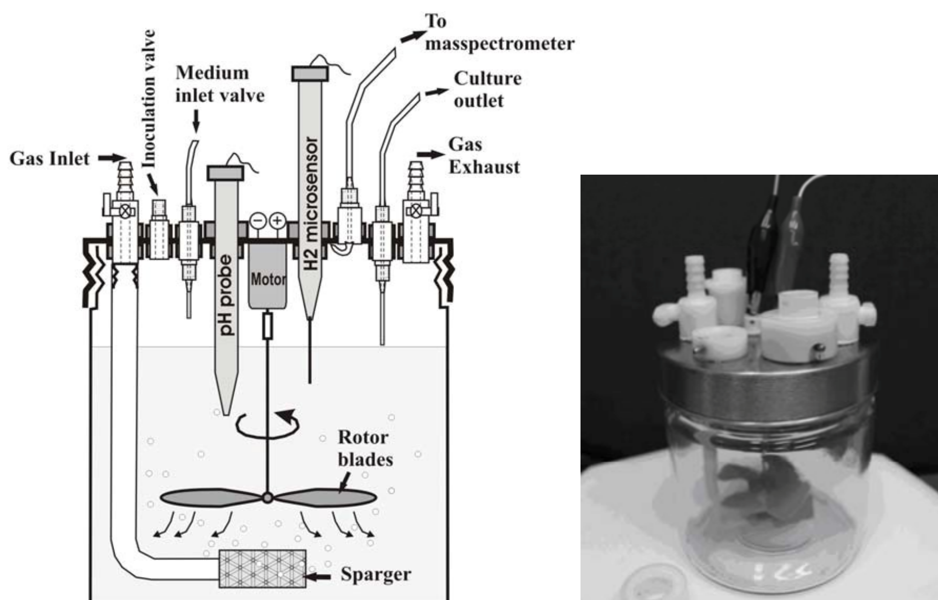

Figure 3.8. Schematic diagram of new bioreactor prototype single reactor unit under construction process biogas (design by Gruduls A.)

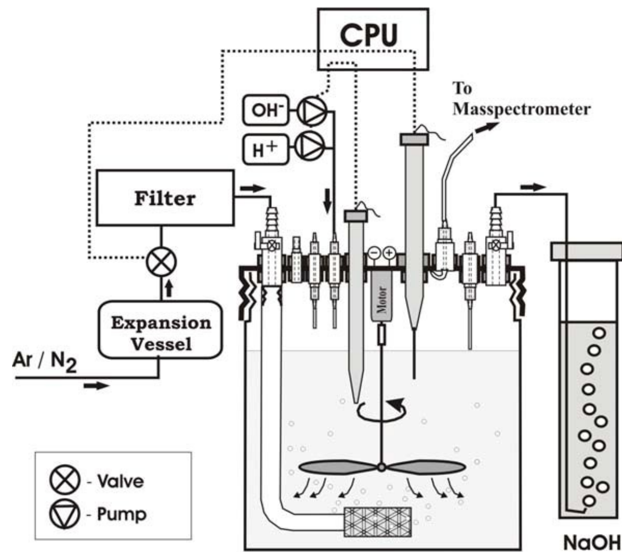

Figure 3.9. Typical application for hydrogen and methane production experiments biogas (design by Gruduls A.)

Our constructed bioreactor system for biogas production experiments consists of two separate reactors (Figure 3.10.). First stage is used for biohydrogen production. Unpurified biohydrogen is then passed trough microbial filter to second stage reactor. In second stage methanogenic bacteria are used for biogas production. CO_2 sensor measures the intensity of fermentation while CH_4 sensor measures concentration of methane in exhaust gas. More accurate results are achieved when masspectrometer is used. We expect that in continious experiments delivering unrefined biohydrogen gas from first reactor into biogas reactor will result in increase in CH_4 outcome.

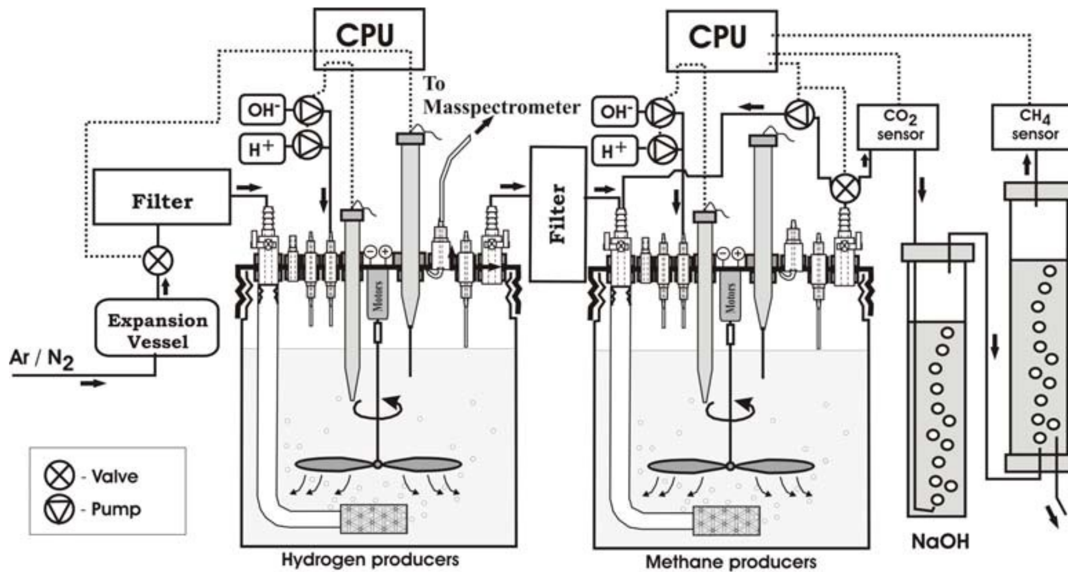

Figure 3.10. Bioreactor system prototype for biohydrogen and biogas (design by Gruduls A.).

3.2. Hydrogen production using various, also in Latvia isolated, bacteria strains with glucose, pure glycerol and crude glycerol as substrates *(Orginal paper III and other results)*

Bacterial strains that have appropriate metabolic pathways for hydrogen production from glycerol substrates were chosen for this study. *Enterobacter aerogenes*, *Enterobacter asburiae, Enterobacter cloacae* (isolated from Latvia's dental clinic pacient tooth channels), *Aneurinibacillus aneurinilyticus, Kluyvera ascorbata, Paenibacillus pabuli* and *Clostridium sporogenes* have been investigated for hydrogen production by many authors (Koskinen et al. 2008, Maeda et al., 2007, Kotay and Das, 2008, Collet et al. 2004, Klepere, 2013). These strains have been isolated in Latvia and the goal from this and further investigations is to use crude glycerol as substrate from Latvia's biodiesel production leftovers.

Experiments were started using model substrate – analytical or pure glycerol with concentration 15 g/l and selected strains were used for fermentation process and hydrogen gas was analyzed in both gaseous and liquid phase. *C. sporogenes* and *E. cloacae,* didn't produce hydrogen gas up to sixth fermentation hour. However, in 48th hour of fermentation these cultures showed remarkable increase, reaching maximally

7.11 and 9.52 mmol H_2/L (Figure 3.11.). *C. sporogenes* and *E. cloacae* grew slower than *E.aerogenes* (changes measured with optical density) and *E. asburiae* didn't show any hydrogen production in this study. *E.aerogenes* hydrogen concentration persistent increase was measured after 48 fermentation hours - 4.03 mmol H_2/l.

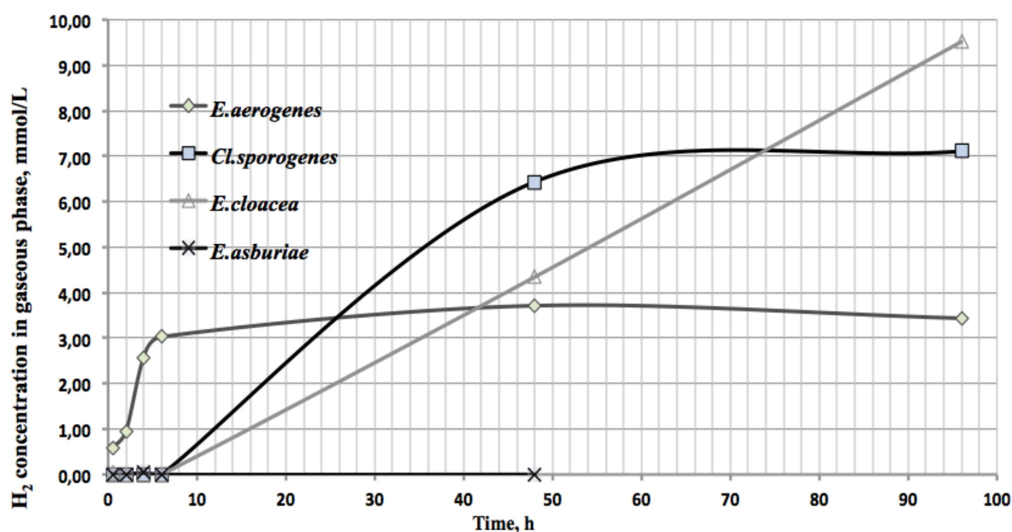

Figure 3.11. Hydrogen production in gaseous phase using 15g/l pure glycerol, various bacteria strains.

Regarding to crude glycerol usage, the most hydrogen substrate was detected using *Enterobacter aerogenes* in the gaseous phase. After 48th hour of fermentation, 7 mmol/L hydrogen concentration was reached, after 96 hours hydrogen concentration in the gaseous phase was still growing. While *E. cloacae*, *C. sporogenes* and *E. asburiae* showed comparatively small hydrogen concentration measures in the gaseous phase using crude glycerol (Figure 3.12.).

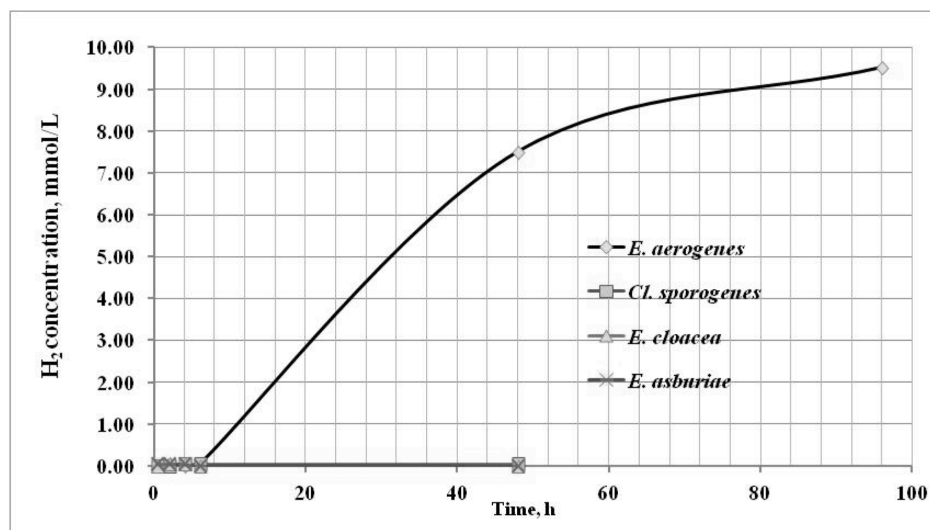

Figure 3.12. Hydrogen production in gaseous phase from 7 g/l crude glycerol, various bacteria strains.

As investigated afterwards with HPLC analysis, crude glycerol contained 40-50% of pure glycerol in the study samples; therefore 7g/l concentration was used for the result analysis, though experiments were planned with the same concentration for both substrates. Liquid phase hydrogen concentration was measured after the fermentation (96th hour) for all cultures using hydrogen microelectrode (Unisense A/S, Denmark) (Table 3.1., Appendix 1).

Table 3.1. H_2 concentration (average) in liquid phase (fermentation 96th hour) by cultures of hydrogen fermentation on both glycerol substrates.

Substrate	Genus taxonomy	Average H_2 concentration in liquid phase, mmol/L
Crude glycerol, 7g/l	*E. aerogenes*	2.32
	C. sporogenes	0.03
	E. cloacae	1.82
	E. asburiae	0.02
Pure glycerol, 15g/l	*E. aerogenes*	1.511
	C. sporogenes	1.315
	E. cloacae	1.707
	E. asburiae	0.036

Our findings show that there was hydrogen dissolved in the liquid phase for *E.aerogenes* (2.3 mmol/L) with crude glycerol (CG) and 1.5 mmol/L - with pure glycerol (PG). For *E.cloacae* similar amount in liquid phase was detected with both substrates: 1.82 mmol/L (CG), 1.7 mmol/L (PG), though there was no hydrogen in samples with CG in the gaseous phase. Regarding to *C.sporogenes,* hydrogen in liquid phase was detected in samples with PG – 1.7mmol/L. There was no additional liquid mixing or stirring provided in this experimental set up, though partial pressure was changed by regular manual injection of 0.2 ml of headspace gas from the culture serum bottles that were used for gas content analysis.

Rate is an important factor for understanding hydrogen productivity using selected strains and substrates. Table 3.2. demonstrates hydrogen production rates for various strains with both substrates - pure and crude glycerol. Highest rate was measured for *Enterobacter aerogenes* - 1,7 H_2 mmol/l/h using crude glycerol. While using pure glycerol with twice as higher concentration *E.aerogenes* produced hydrogen with slower rate - 0,831 H_2 mmol/l/h. With pure glycerol the highe rates were measured with *Enterobacter cloacae* - 1,223 H_2 mmol/l/h and *Clostridium sporogenes* - 1,084 H_2 mmol/l/h.

Table 3.2. Biohydrogen production rate using 15g/l pure and 7 g/l crude glycerol (only results with H_2% higher than 0.3 mmol/l/h are shown) in gaseous phase.

Substrate crude glycerol (CD) 7g/l/ pure glycerol (PG) 15g/l	Genus taxonomy	H_2 production rate (max), mmol/l/h	H_2 production rate (average), mmol/l/h
CG	*Enterobacter aerogenes*	1.700	1.063
CG		0.831	0.736
CG	*Clostridium sporogenes*	0.019	0.014
PG		1.084	0.546
CG	*Enterobacter cloacae*	0.938	0.260
PG		1.223	0.620
CG	*Enterobacter asburiae*	0.009	0.006
PG		0.013	0.011

Our findings with HPLC analysis demonstrated unefficient substrate utilization within 96 hours of fermentation of analytical glycerol, namely, 33-51% (Table 3.3.). Comparatively, in the study with crude glycerol (conc. 7 g/l) 90% substrate consumption was measured with strain *E.aerogenes.* In the study for hydrogen yield analysis our data demonstrate highest yield with *E.aerogenes* using crude glycerol - 0.36 H_2 mol/mol $glycerol_{consumed}$ (concentration 6g/l), with pure glycerol this strain yielded 0.24 H_2 mol/mol $glycerol_{consumed}$. Regarding to *C. sporogenes* un *E.cloacae* simillar amount was demonstrated using pure glycerol: 0.22 and 0.24 mol/mol $glycerol_{consumed}$.

Table 3.3. Hydrogen yield and substrate consumption mesured with High performance liquid chromatography (HPLC) in dark fermentation using pure and crude glycerol as substrates with various bacteria.

Genus taxanomy	HPLC measured pure glycerol (PG) or crude glycerol (CG) concentration (g/l)	Substrate (glycerol) consumption (%)	Produced H_2 concentration (96h) mmol/L	H_2 yield (mol H_2/ mol $glycerol_{consumed}$)
E. aerogenes	11.77 PG	50.50	15.27	0.24
E. aerogenes	6.00 CG	90.0-	21.05	0.36
E. cloacae	10.42 PG	48.00	13.84	0.24
C. sporogenes	16.81 PG	32.90	13.48	0.22

Effect of crude glycerol concentration on hydrogen gas production with *Enterobacter aerogenes* was investigated (Fig. 3.13.) Although the best hydrogen gas production results were with concentration 6 g/l, statistically significant differences were

only with the samples without glycerol in the medium, for concentrations 3 g/l and 12 g/l, hydrogen gas production resulted in 14.3 - 14.7% hydrogen of total gas volume. Also total gas production by *E. aerogenes* (ml/ 100 ml medium) in media was measured, resulting with highest amount of produced gas using crude glycerol concentration 6g/l (Fig. 3.14).

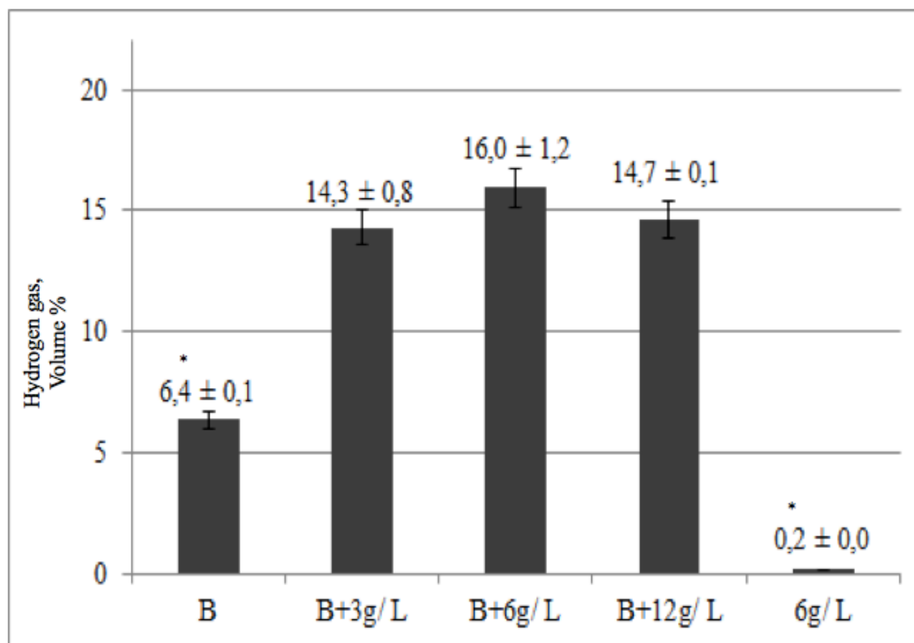

Figure 3.13. Amount of H_2 produced by *E. aerogenes* in the gas phase sample (%) of the medium without crude glycerol (B), with crude glycerol concentration 3 g/ L (B + 3 g/ L), 6 g/ L (B + 6 g/ L), 12 g/ L (B + 12 g/ L) and crude glycerol solution in water with concentration of 6 g/ L (6 g/ L). * Significantly different ($p < 0,05$) from other media. (Valucka, 2015).

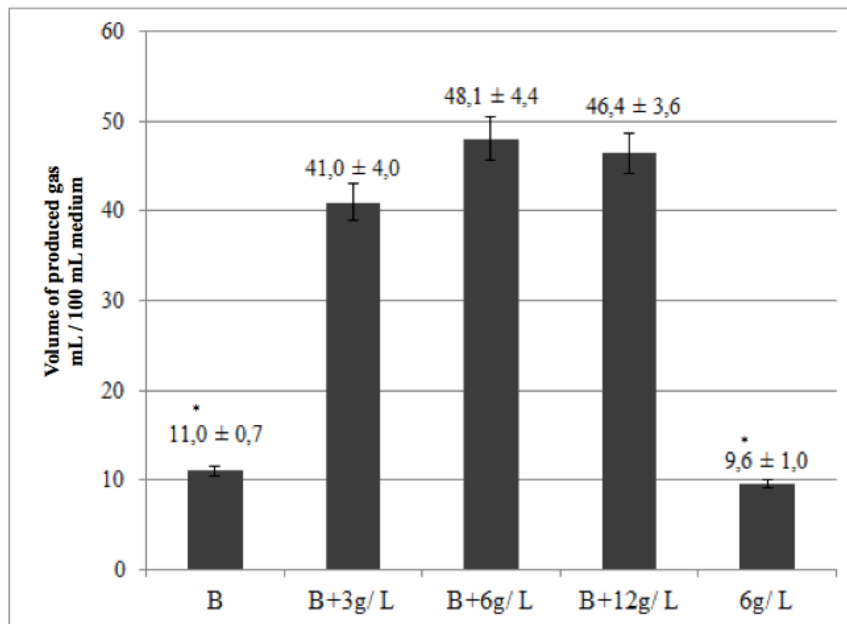

Figure 3.14. Average gas production by *E. aerogenes* (mL/ 100 mL medium) in media without crude glycerol (B), with crude glycerol concentration 3 g/ L (B + 3 g/ L), 6 g/ L (B + 6 g/ L), 12 g/ L (B + 12 g/ L) and crude glycerol solution in water with concentration of 6 g/ L (6 g/ L). * Significantly different ($p < 0,05$) from other media (S.Valucka, 2015).

Optimum pH value for hydrogen production with this study's most productive produce *Enterobacter aerogenes* based on literature data is 6-7 pH, but growth is possible from pH 4,4 – pH 9(Atlas, Bartha 1998). In this study pH was not adjusted during the experiments, but was measured in the beginning and after the fermentation process. (Fig. 3.15., App. 2.)

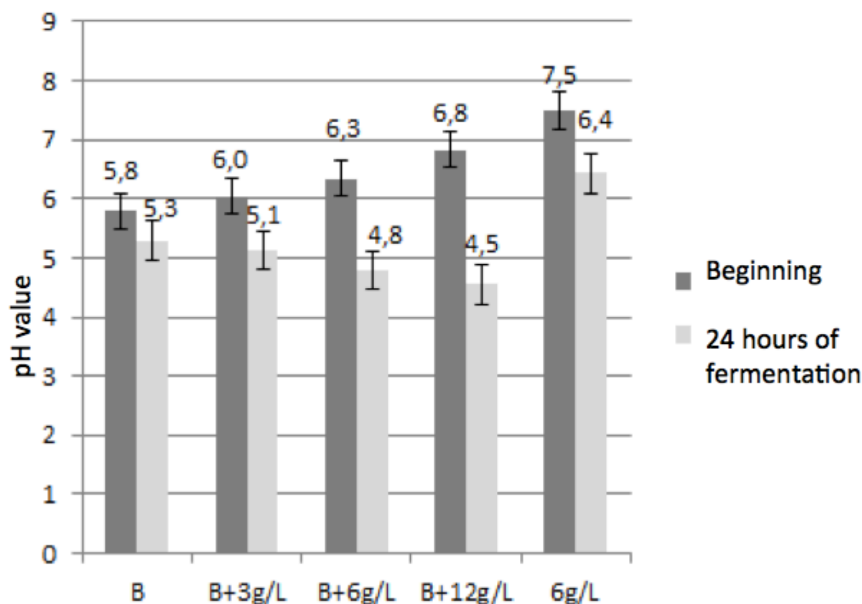

Fig. 3.15. pH value of media with *E. aerogenes* without crude glycerol (B), with crude glycerol concentration 3 g/ L (B + 3 g/ L), 6 g/ L (B + 6 g/ L), 12 g/ L (B + 12 g/ L) and crude glycerol solution in water with concentration of 6 g/ L (6 g/ L) at the beginning of the cultivation and after 24 h of cultivation.

3.3. Hydrogen production from lactose-containing substrate *(Original paper IV)*

Lactose permease gene (*lacY*) was overexpressed in the septuple knock-out mutant of *Escherichia coli*, previously engineered for hydrogen production from glucose. It was expected that raising the lactose transporter activity would elevate the intracellular lactose concentration, inactivate the lactose repressor, induce the lactose operon, and in result, stimulate the overall lactose consumption and conversion. However, overexpression of the lactose transporter caused a considerable growth delay of the recombinant strain on lactose, resembling to some extent the 'lactose killing' phenomenon, described by Dykhuizen and Hartl (Nath and Das, 2004). Therefore, the recombinant strain was subjected to selection on lactose-containing media. The strains *E.coli* 891 and *E.coli* 891pl showed closely similar growth pattern when cultivated aerobically in shaken flasks on LB medium supplemented with 3 % glucose. With lactose the growth proceeded slower, and the strains differed more between themselves. The strain 891 grew significantly slower on lactose than on glucose, yet for some reason

its growth continued longer, and higher biomass concentrations were reached. Notably, the recombinant strain 891pl demonstrated a particularly slow growth on lactose medium (Figure 3.16.), with a prolonged lag phase. In order to improve the performance of 891pl, a two-step selection procedure was carried out. At first, *E. coli* 891pl was plated on LB medium containing 1.5 % lactose. One of the first colonies, which appeared on the 1.5 % lactose medium, was further plated on 3 % lactose. The largest colony on the 3 % lactose plate was taken for further examination and named *E. coli* 891plS.

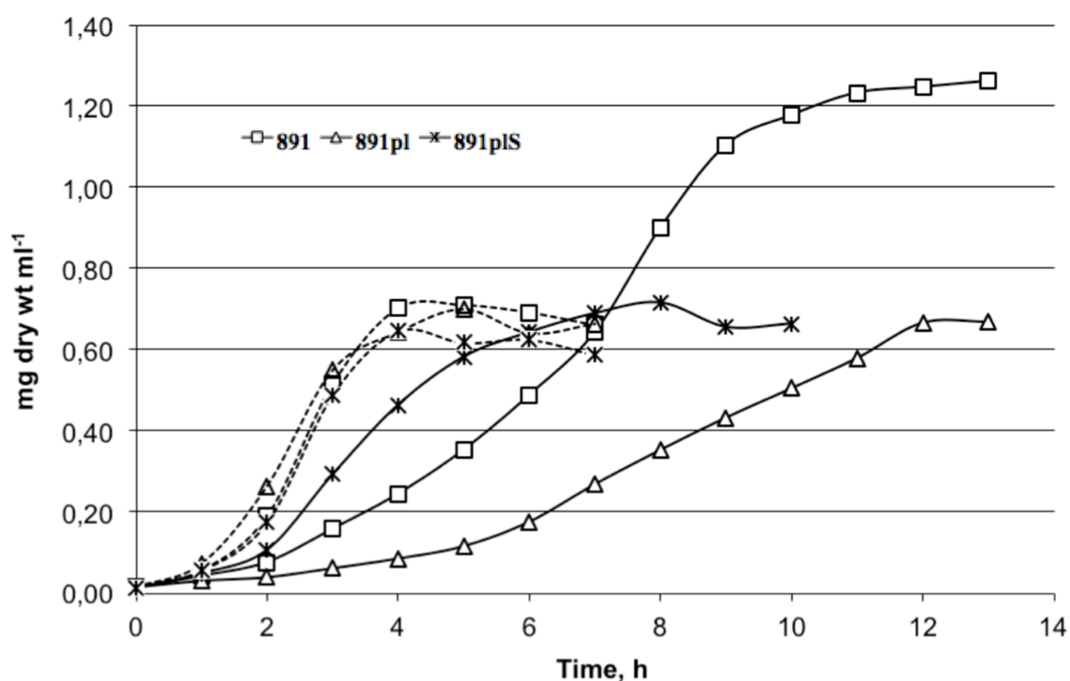

Fig. 3.16. Aerobic batch growth of E. coli strains on LB medium, supplemented with 3% of lactose (continuous lines) or glucose (dashed lines); representative data of one cultivation from a series of three experiments are shown.

As seen from the figure 3.16. strain 891plS demonstrated a much higher growth rate during exponential phase, and shorter lag phase on lactose, than both other strains. Its growth kinetics on lactose approached to that on glucose. In contrast to strain 891, the biomass concentration of 891plS, reached at the early stationary phase, was the same as on glucose. At the same time, 891plS showed the highest rate of lactose consumption: after its aerobic cultivation overnight, lactose concentration in the medium had

decreased from 3 % to 2.15 %. For the strains 891pl and 891, which reached the same as that of 891plS or even higher biomass concentrations, the remaining lactose concentration was found to be 2.75 % and 2.45 %, respectively (not shown). This indicated that in 891plS a larger fraction of lactose was being diverted to synthesis of catabolic end-products, than in other strains.

Hydrogen production with lactose

After an overnight cultivation aerobically in shaken flasks, the cultures of all three strains were used as inocula for anaerobic fermentations on LB medium with 3 % lactose, in order to compare their ability to produce hydrogen. The conditions of equilibria between the hydrogen concentrations in the solution and in the gas phase were different in each of the two experimental systems, due to different modes of gassing with argon (see Methods). We monitored the hydrogen in the gas phase for both experimental systems, therefore, the presented time-course of hydrogen production in serum bottles differed from that, obtained in the bioreactor. Although not comparable between themselves at a strictly quantitative level, the results in both systems showed a clear advantage of the strain 891plS over the other two (Figure 3.17.). In the serum bottle system strain 891plS produced more hydrogen gas at the early stage of anaerobic fermentation, H_2 reaching 4.6 % of the gas phase in 3 hours, exceeding the hydrogen concentration in the cultures of strains 891 and 891pl by factors of 1.5 and 2, respectively. In the prototype bioreactor system the advantage of 891plS was more evident at a later stage of fermentation. Hydrogen concentration in the efflux gas reached a maximum of 4.1 % at the 6th fermentation hour, and in contrast to both other strains, did not decrease during the next couple of hours

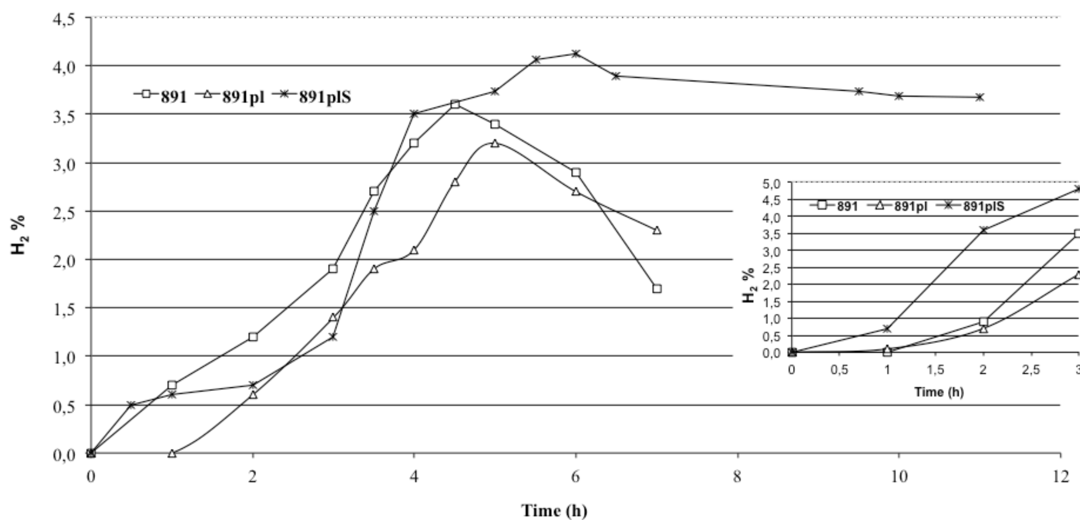

Figure 3.17. Hydrogen gas production using 3 % lactose with various *E. coli* strains 891, 891pl, and 891plS in a prototype bioreactor system (inserted graph - Hydrogen gas production measurements with serum bottle experimental system using *E. coli* strains 891, 891pl, and 891plS; substrate 3 % lactose).

Hydrogen yield per mole of consumed lactose was estimated for the strain 891plS during anaerobic fermentation in serum bottles. For this purpose, we summarized the amount of hydrogen, accumulated in the gas phase with the amount of hydrogen, measured by the Clark-type microsensor in the liquid. Typically, during the period between the 2nd and the 4th hour of fermentation, around 0.04 millimoles of H_2 were released in the gaseous phase, while approximately 0.01 millimole was accumulated in the 15 millilitres of the culture medium, containing growing cells in the concentration range between 0.05 and 0.1 mg dry wt ml^{-1} (not shown). The corresponding amount of consumed lactose was close to 0.020 – 0.021 millimoles, hence the molar yield of hydrogen was between 2.4 and 2.5 moles of H_2 per mole of lactose. This range of yield values is close to the value 2.74, reported by Rosales-Colunga et al. (2010). At the end of fermentation the plasmid pTrcHislacY was isolated from the strains and its relative amounts were compared by electrophoresis in agarose gel (Figure 3.18.). Notably, 891plS contained less plasmid DNA than 891pl. Using the Qiagen plasmid isolation protocol, strain 891pl yielded 34 ng DNA per µl, while the selectioned strain yielded only 19 ng per µl (according to standard spectrophotometric

assay at 260 nm).

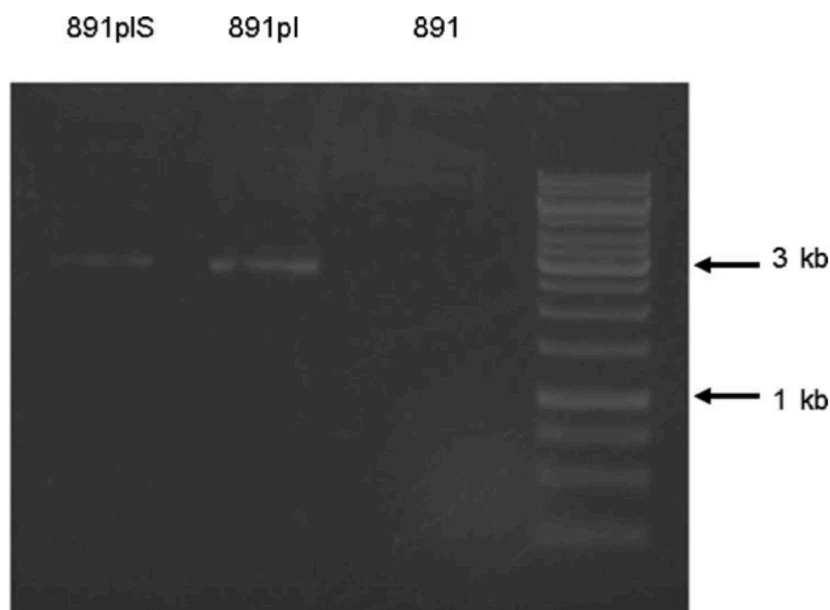

Figure 3.18. Plasmid pTrcHislacY isolated from *E. coli* strains, fermenting lactose under anaerobic conditions. 5.3 ml cell suspension of each strain (with OD_{600}=1) were taken for plasmid isolation with Qia prep Spin miniprep kit, and 15 μl of the purified plasmid preparations were transferred on 0.8% agarose gel.

Macromolecular composition of biomass

To determine the response of cells to the presence of lactose in the growth medium, the raw FT-IR spectra were compared, and also the second derivative spectra analyzed to quantify the observed changes. During growth on LB, or LB medium supplemented with glucose, the spectra of the three *E. coli* strains showed only subtle differences in the content of carbohydrates, proteins and lipids (not shown). During growth on lactose, the spectra of the strain 891 and the recombinant 891pl were quite similar. However, the spectrum of the lactose-grown *E. coli* 891plS distinctly differed by the peak around 1152 cm^{-1} and a shoulder in the region of 1060-985 cm^{-1} (Figure 3.19.). Since broad spectral bands often occur as a result of overlapping of several separate bands, they could in principle hide specific peaks of particular compounds that could be identified in the 2nd derivative spectrum. Thereby, in the 2nd derivative spectra

of *E. coli* 891plS grown on lactose, two distinct minimum peaks at 1154 and 1026 cm^{-1} were identified (Figure 3.20.). Remarkably, these minimums were observed only for the selected recombinant strain 891plS, and only when it was cultivated on medium with lactose. According to previous reports, they indicate accumulation of polysaccharides (Moen et al. 2005, Perfeito et al. 2011). The estimated concentration of total carbohydrates in the lactose-grown strain 891pl was 7.91 % dry weight (dw), in the strain 891 it was 8.98 % dw, while in the strain 891plS it was significantly higher – 14.43 % dw. Accordingly, the fraction of protein was larger in 891 and 891pl – 56.07 % and 55.22 % dw, respectively, and only 49.57 % dw in 891plS. At the same time, the concentration of lipids in 891 and 891pl was just slightly lower (8.09 and 7.81 % dw, respectively), than in the selectioned strain 891plS (8.84 % dw).

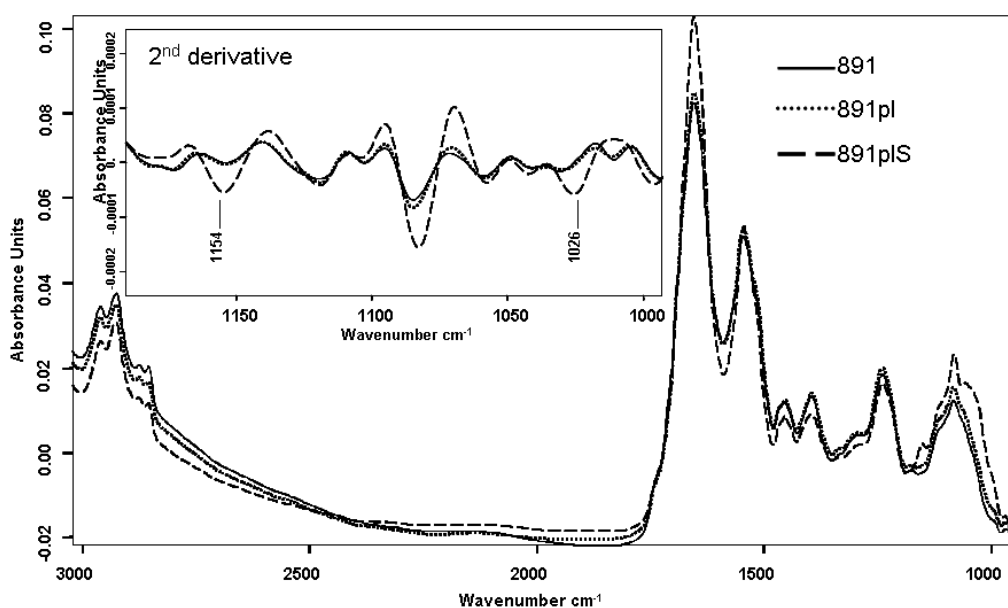

Figure 3.20. Raw (vector normalized) FT-IR spectra of *E. coli* 891, 891pl and 891plS strains grown on LB medium with 3 % lactose and the 2nd derivative spectra of the carbohydrate region (1200-900 cm^{-1}).

Iodine-stained cells of all three strains were subjected to microscopic analysis, in order to monitor intracellular glycogen accumulation. Lactose-grown cells of 891plS (Figure 3.21.) had accumulated massive amounts glycogen, seen as dark inclusion

bodies, occupying large part of intracellular space in almost all cells. Neither strain 891pl, nor strain 891 (not shown) accumulated glycogen to a comparable extent.

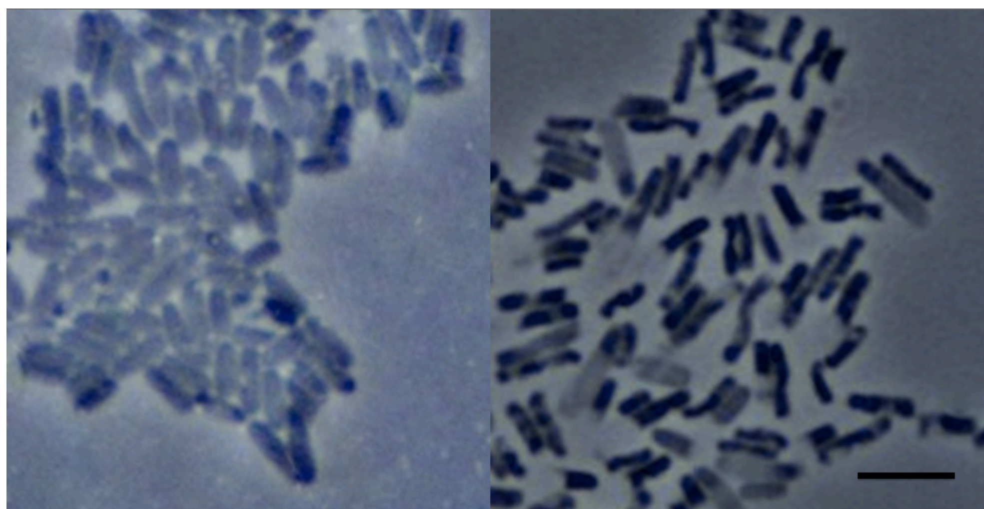

Figure 3.21. Iodine-stained *E. coli* cells, cultivated aerobically on lactose-containing medium: strain 891pl (on the left) and strain 891plS (on the right). Scale bar corresponds to 1 micrometer.

3.4. Substrate conversion dynamic analysis and by-product identification

Results for substrate usage (analytical and crude glycerol) analysis with High Performance Liquid Chromatography (HPLC)

Glycerol fermentation beginning concentration and substrate conversion dynamics were measured periodically in intervals during the fermentation process using HPLC. Glycerol fermentation beginning concentration measures were necessary due to crude glycerol heterogeneity. We measured that crude glycerol (Naukseni factory, Latvia) contained 40% of glycerol. Mangayil et al (2012) used Savon Siemen Oy manufactury glycerol, that contained 45% (v/v) glycerol, 30% (v/v) metanol. Substrate conversion dynamics were analyzed and our experiment results indicated that liquid chromatography directly combined with electrospray-ionization high-resolution mass spectrometry could be used to selectively separate and detect glycerin in fermentation broth extracts (Figures 3.22, 3.23.)

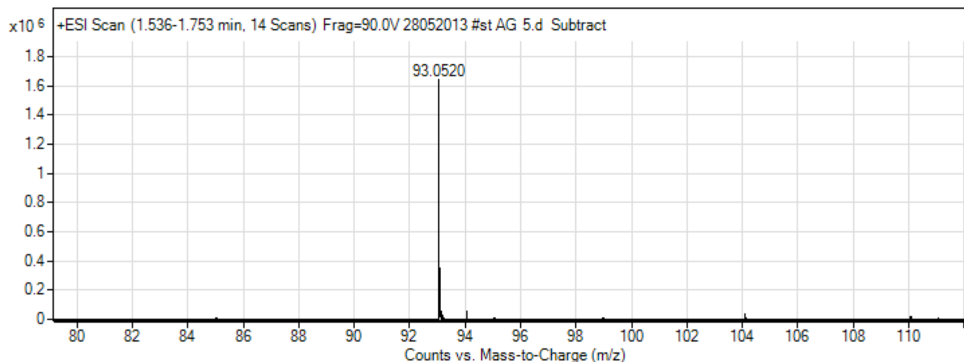

Figure 3.22. Normalized LC-UPLC TOF EIC Chromatograms and high resolution masspectrum of glycerine standard (A) and sample (B) solutions.

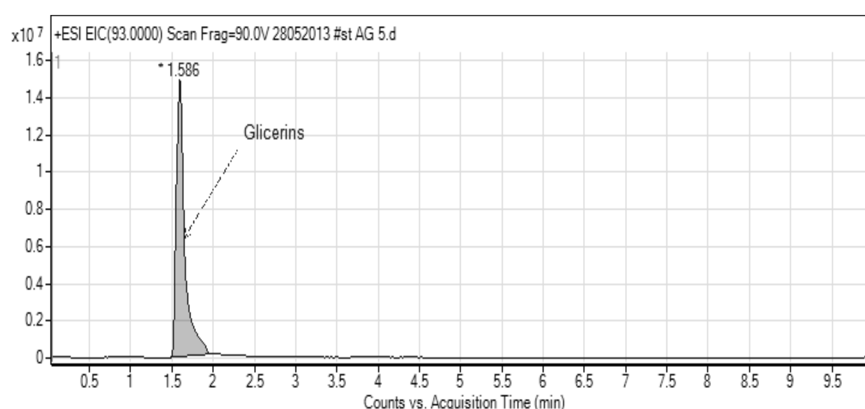

Figure 3.23. Chromatogram of glycerol standart dilution.

To determine whether usage of hydride-forming metal powders or alloys affect substrate utilization in bacteria fermentation process, we measured glycerol concentration dynamics with all samples of tested hydride-forming materials. Results of HPLC-mass spectrometry show that utilization of the crude glycerol by bacteria was not influenced by the presence of the any of the hydride- forming metal powders or alloys in the fermentation medium. HPLC-mass spectrometry measurements demonstrated that glycerol was degraded completely within 24 hours of fermentation with or without alloy grains in the medium (more in Result section Nr. 3.5).

Other fermentation by-product identification

The mass-spectrometric analysis revealed the presence of different volatile substances − the end products of *E. coli* MSCL 332 glucose fermentation process: acetate, carbon dioxide, ethanol, acteone, and hydrogen gas (Figure 3.24.). Argon gas (that was used for anaerobic condition maintenance) decrease was due to gas content changes: more fermentation end-products appeared and gas content equilibrium correspondingly adjusted (Table 3.4.). In table R glycerol fermentation end products that were detected in gaseous phase with mass-spectrometry above 0.1% are shown. Nitrogen (N_2) and O_2 appearance represents air presence and is caused by sampling procedure (experimental system set-up). Hydrogen and carbon dioxide are the main fermentation products detected in gaseous phase and our results show continuous increase (Figure 3.24.).

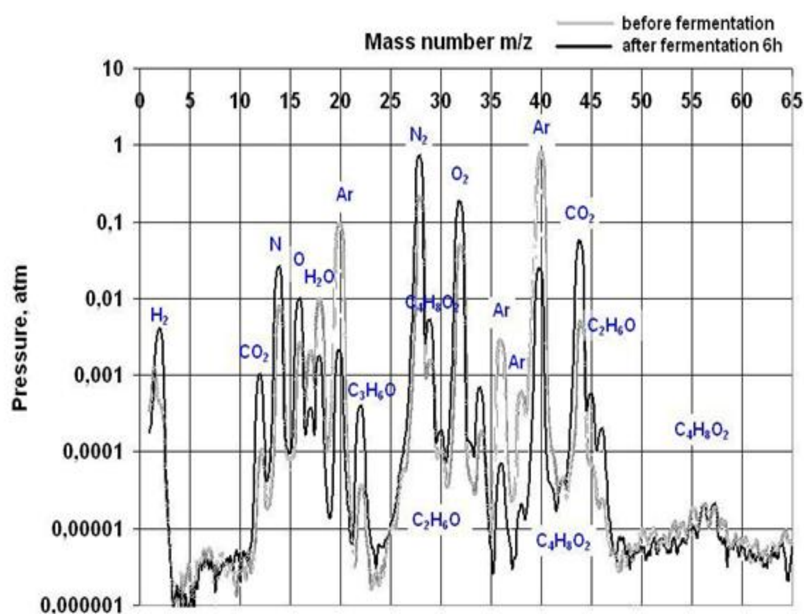

Figure *3.24.* Mass-spectrometric analysis of the sample with bacteria *E. coli* MSCL 332 before fermentation (light gray curve) and after 6h (black curve).

Table 3.4. Mass-spectrometric analysis of the sample with *E.aerogenes* and *E.coli* BW25113 crude glycerol fermentation.

Sample	Gas content%					
	Ar	CO_2	N_2	O_2	H_2O	H_2
Control	97	0.6	1.9	0.3	0.3	0
Control saturated with hydrogen gas	22	0.6	2.7	0.2	0.5	75
E.coli	82	7	3.1	0.5	0.5	6.7
E.aerogenes	51	24	0.2	0.1	0.5	25.2

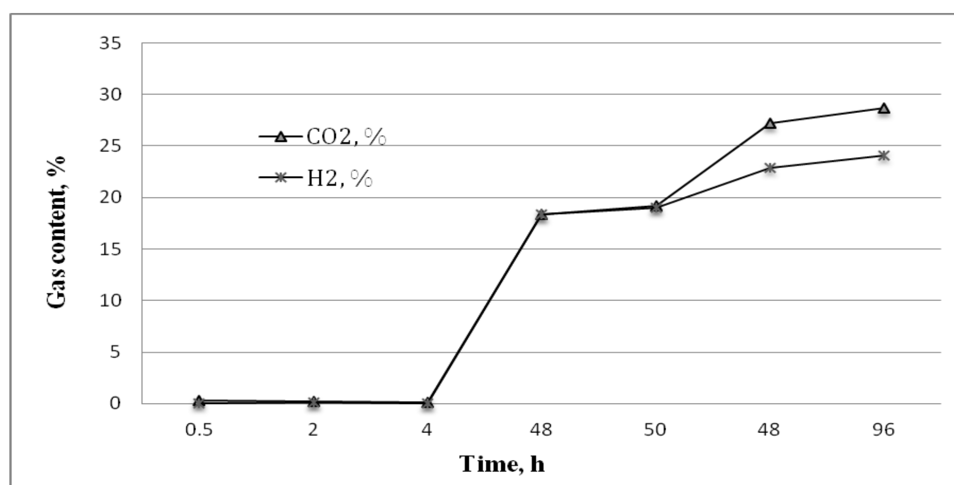

Figure 3.25. Mass-spectrometric analysis of the sample with bacteria *E.aerogenes* and crude glycerol for 96 hour fermentation process in anaerobic serum bottles.

3.5. Hydrogen isolation possibilities using hydride- forming metals and alloys

We studied hydrogen sorption from bacterial fermentation media containing crude glycerol by the powdered Pd and alloys that are capable to form hydrides, $LaNi_5$, AB_5, and AB_2. Mass changes of the powders after incubation in the fermentation media were measured by differential thermogravimetry; composition and concentrations of the gases accumulated during the fermentation and absorbed by the Pd or alloy powders were determined by mass spectrometry.

Hydrogen gas analysis in the headspace and in the hydrogen-absorbing metal and alloys

MS was undertaken for H_2 concentration measurements during glycerol fermentation in the headspace gas of serum flasks with or without hydride-forming materials. Maximum H_2 concentration was observed after 24 h of fermentation in the control samples without alloys – 28.7 vol% for *E. aerogenes* and 21.5 vol% for *E. coli.* The presence of hydride-forming materials decreased H_2 concentration in the headspace. Meanwhile, crude glycerol utilization by bacteria was not impaired by alloy grains in the medium. HPLC-MS demonstrated that glycerol was degraded completely within 24 h of fermentation with or without alloy grains in the medium. (Figure 3.26.).

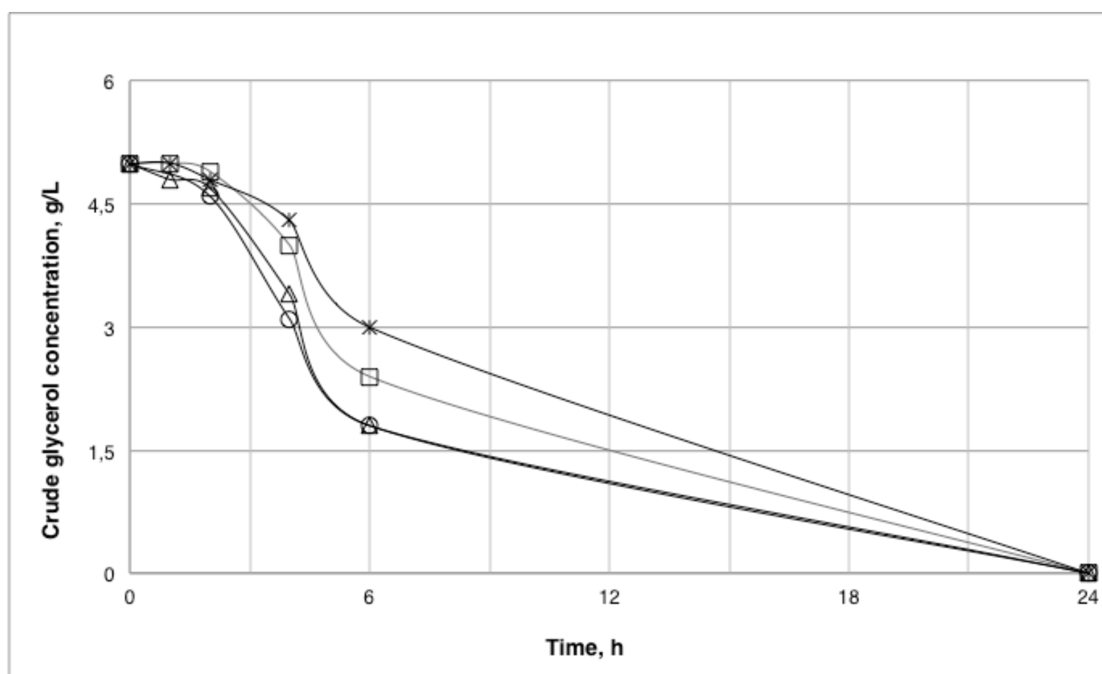

Figure 3.26. Crude glycerol utilization by *E. coli* during fermentation in 35 mL of cultivation medium containing 1 g of hydride-forming material alloys $LaNi_5$ (✳), AB_5 (△), AB_2 (□) with 7 g/l crude glycerol and control samples without hydride-forming material (O). Glycerol concentration was measured directly by HPLC combined with electrospray-ionization high-resolution MS.

H_2 sorption by the materials tested in our experiments was confirmed by MS analysis of the gases released at vacuum extraction from AB_5 alloy after incubation in *E. coli* fermentation broth. Measured gases above the background level included: Ar

(carrier gas) - 87%, H_2 - 2.8%, CO_2 - 0.2%, O_2 - 0.4%, H_2O - 0.5%. The controls, with prior H_2-saturated samples, showed similar H_2 level - 2.5%, Ar - 89%, CO_2 - 0.2%, O_2 - 0.2% and H_2O - 0.5%. Samples with AB_5 alloy in fermentation broth without bacteria had H_2 - 0%, Ar - 96%, CO_2 – 0%, O_2 - 0.1% and H_2O - 0.1%.

H_2 sorption in hydride-forming materials

Since the MS data proved that H_2 sorption occurred in hydride-forming materials during incubation in fermentation medium with bacteria, we undertook TG to approximate it (Table 3.5.). The calculations presume that weight loss of the hydride-forming materials observed in TG was due to H_2 desorption in the 30-70°C range.

In the presence of $LaNi_5$, AB_2 and AB_5, total volumes of obtained H_2 were increased in comparison to the results in control samples without any alloy added.

$LaNi_5$ was chosen as reference material to compare hydrogenation and fermentation:

(i) 50 mg of $LaNi_5$ saturated with H_2: hydrogenation was performed at up to 2 bar pressure and 170°C temperarture, followed by cooling to room temperature for 2 h and measurement in aluminum foil inserted in a ceramic crucible);

(ii) 50 mg of $LaNi_5$ from the liquid fermentation medium after 72-h fermentation of glycerol with *E. coli* (measured in aluminum foil inserted in a ceramic crucible).

The amount of stored H_2 was deduced from weight loss measured by TG in a 50-mg aluminium crucible (Figure 3.27.). H_2 release from hydride alloys started at 40° C. The alloys showed similar temperature-dependent, weight-losing patterns with all hydrogenation methods explored.

Various hydride-forming materials were compared for their H_2 absorption capacity from fermentation broth with H_2-producing *E. coli.* TG measurements disclosed that H_2 was absorbed most efficiently by powdered Pd, followed by AB_5, AB_2 and $LaNi_5$ alloys (Figure 3.28., Table 3.5.).

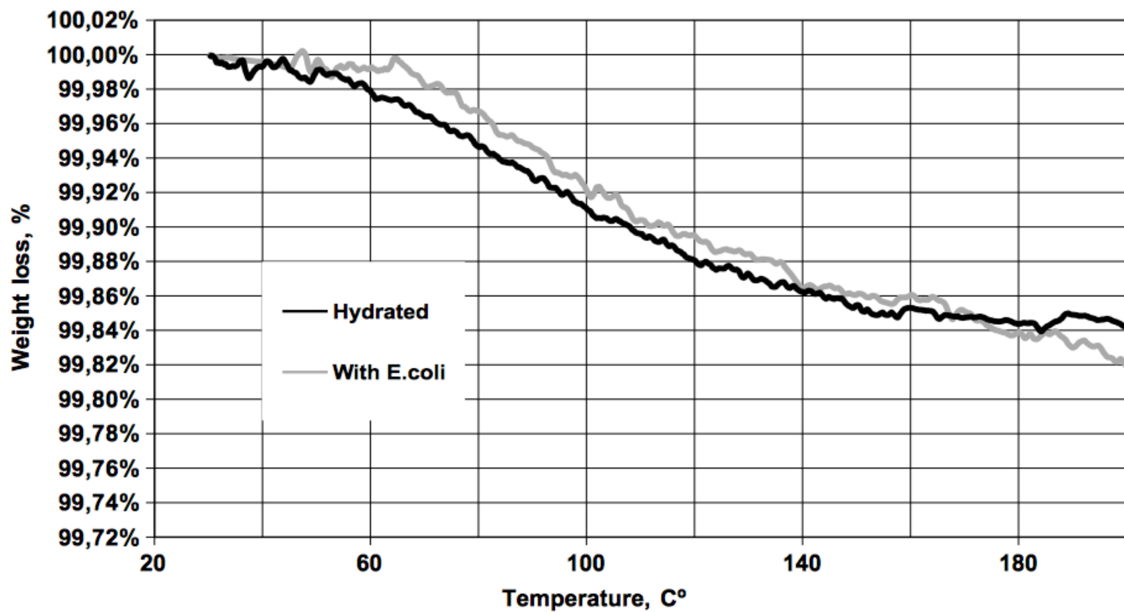

Figure 3.27. TG assessment of H_2 release from $LaNi_5$ hydride by different hydrogenation methods (black line: hydrogenation in H_2 atmosphere under 2 bar pressure at 170^oC; grey line: 72-h anaerobic crude glycerol fermentation by *E. coli.*

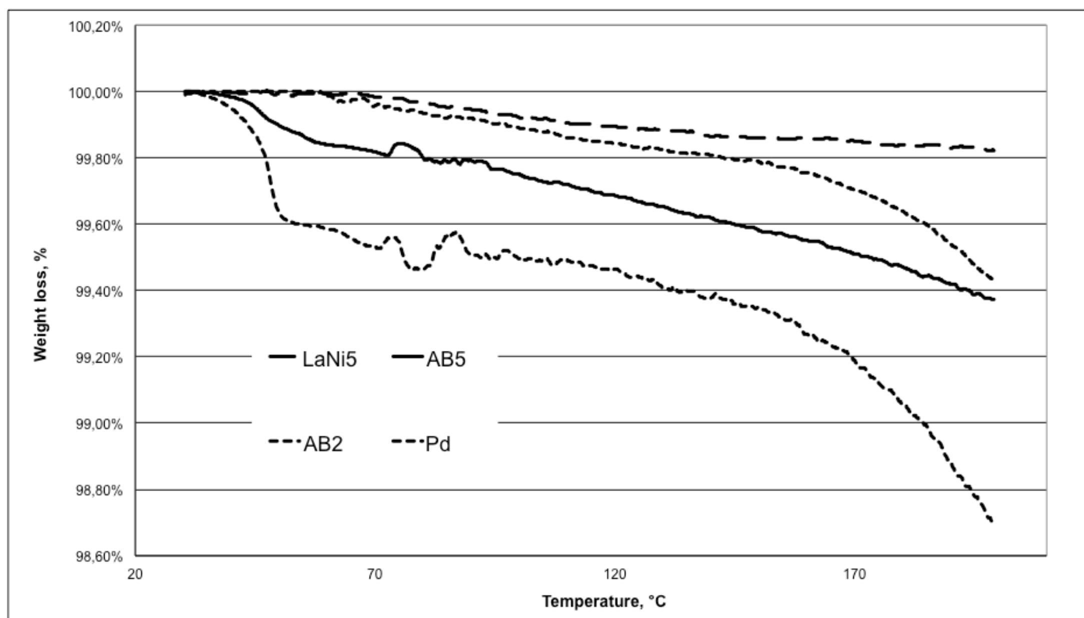

Figure 3.28. TG assessment of H_2 release from different hydrides formed during 72-h incubation in *E. coli* fermentation medium containing crude glycerol.

Table 3.5. Calculated amount of H_2 absorbed by Pd and alloy powders after 72-h incubation in *E. coli* fermentation medium containing 6 g/l of crude glycerol. Gas volume was calculated for normal conditions, TG data average error ± 10% *Calculated for 1 g for standardization purposes.

No.		Pd*	$LaNi_5$	AB_2	AB_5
1	Material sample mass in crucible, mg	24	66	56	119
2	Theoretical maximum H_2 absorption capacity, wt%	0.8	1	1.5	1
3	Theoretical maximum H_2 weight in sample, mg	0.19	0.66	0.84	1.19
4	Theoretical maximum H_2 volume in sample, cm^3	2.21	7.59	9.66	13.7
5	Observed weight loss of sample, wt%	0.4	0.1	0.2	0.3
6	Observed weight loss of sample, mg	0.10	0.07	0.11	0.36
7	Respective calculated volume of desorbed H_2, cm^3	2.20*	0.76	1.29	4.10
8	Calculated volume of desorbed H_2 per g Pd or alloy, cm^3	46*	11.5	23.0	34.5
9	H_2 in headspace gaseous phase (cm^3/vol%). Without any metal hydride – 4.2/21.5 cm^3/vol %)	1.6/8.1	3.6/18.1	4.2./21.3	0.9/4.3

The results of headspace measurements after 24 h of fermentation process show contribution of metal hydrides in the samples (Table 3.5., row 9): without any metal or alloy – 4.2 cm^3 of H_2 was produced in the gaseous phase. Comparatively metal hydride presence revealed 0.9-3.6 cm^3 H_2 in the gaseous phase, except for samples containing AB_2 alloy, where a similar amount was observed – 4.2 cm^3 in the gaseous phase.

H_2 absorption by alloys during fermentation was analyzed by TG in separate experiments by removing 5-15 mg of granulated material from the serum flask at regular intervals. Three parallel tests were conducted. Table 3.6. illustrates that the highest amount of H_2 was absorbed after 18 h of fermentation.

Table 3.6. H_2 absorption by AB_5 alloy during 42 h *E. aerogenes* fermentation of crude glycerol.

Time after the beginning of fermentation, h	Weight loss (30-100ºC), wt%
6	0.41±0.07
18	1.61±0.72
24	0.38±0.16
36	0.12±0.01

Alloy surface after contact with bacteria

The surfaces of alloy grains after incubation in fermentation medium were examined by SEM. Figure 3.29. shows that bacteria cells are present on the surfaces of alloy grains, thus being in close contact with H_2-absorbing material. It also appeared that contact with alloy grains stimulates bacteria growth. More colony forming units (cff · 10^7/ mL medium) of *E. aerogenes* were investigated in medium with of AB_5 alloy than without it (Fig. 3.30, App. 2.). Although this field with metal hydride usage for bacteria produced hydrogen storage is novelty, it has been investigaed that ability to grow on special carrier or substrate stimulates hydrogen production (Wongthanate, Polprasert 2015).

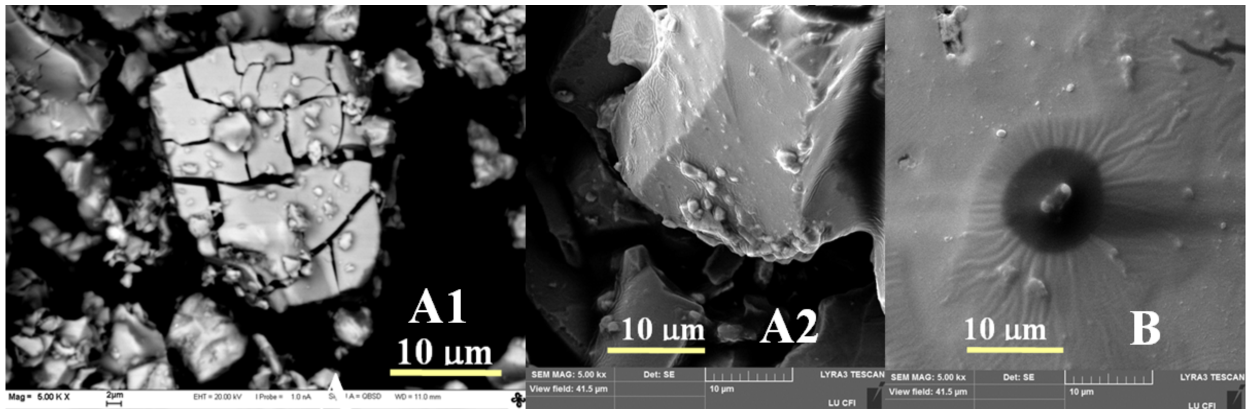

Figure 3.29. SEM of $LaNi_5$ alloy surface: A1 – before contact with bacteria; A2 – after contact with absorbed bacteria: B – contact with *E. coli* cell.

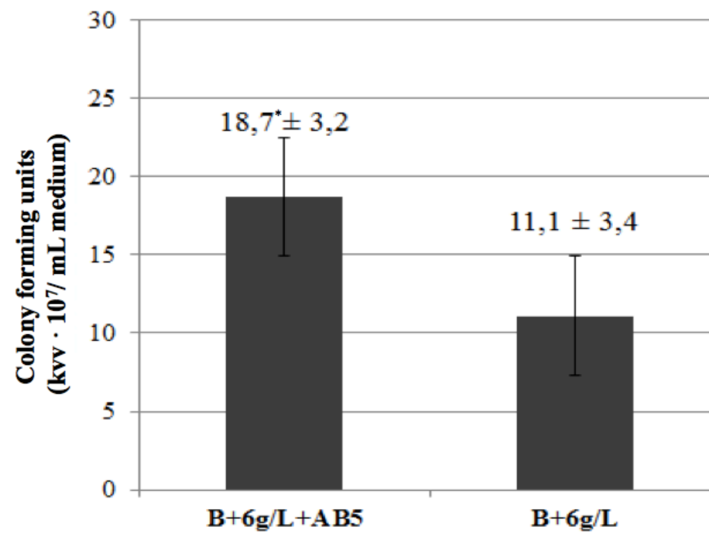

Figure 3.30. Average colony forming units (kvv · 10^7/ mL medium) of *E. aerogenes* in media with AB_5 alloy (B +6 g/ L + AB5) and without it (B + 6 g/ L). * Significantly different ($p < 0,05$) from media without AB_5 alloy.

4. Discussion

The aim of the promotion work was to address hydrogen production possibilities from feedstocks, characterize bacteria available in Latvia and investigate hydrogen storage possibility from broth with hydrogen-absorbing metals and alloys. In order to succsesfully conduct this research, various test systesms were used, built and optimized, also prototype bioreactors were built in our laboratory that enabled monitoring and revealed some problems, as well as challanges that were investigated in further experiments, including hydrogen sorption from bacterial fermentation media using hydrogen-absorbing materials. Here I present the most important discussion matters within the research.

4.1. Hydrogen production analysis with experimental test systems and bioreactors, using various bacteria strains and organic waste materials

Our developed lab-scale test system (prototype bioreactor model with hydrogen microelectrode and connection with masspectometer for gas analysis) allowed optimal estimation of hydrogen production process and showed that medium stirring and inert gas barbotage has a significant impact on hydrogen production efficiency per se (Klepere et al., 2011). As reported in scientific literature (Battino and Clever, 1966), in experiments with dissolved and gaseous hydrogen a low liquid-to-gas mass transfer was observed. Despite hydrogen being a poorly-soluble gas in anaerobic environment, it can become over-saturated in liquids (Kraemer and Bagley, 2006). Concentrations of dissolved gas are higher than theoretically possible in the anaerobic processes where gases are formed in the liquid phase and tend to reach the gas phase. Such over-saturation could be associated with biological processes: lower pH due to the formation of gases (e.g. CO_2, H_2S) in anaerobic processes; besides, a negative thermodynamic effect is caused by inhibator gases - e.g. H_2, since the hydrogen synthesising enzymes are sensitive to H_2 concentrations and are subject to the end-product inhibition. As concentrations of hydrogen increase, its synthesis rate decreases: the evolved H_2 is consumed by *E.coli* uptake hydrogenases Hyd-1 and Hyd-2 (Pauss et al., 1990; Levin et al., 2004; Maeda et al., 2008). As mentioned before, the hydrogen synthesising enzymes

are sensitive to the end product − the hydrogen gas concentration. As this concentration increases the synthesis rate decreases, with formation of mixed-acid hydrogen-containing fermentation products (ethanol, acetate, butane) (Levin et al., 2004). To enhance the hydrogen gas output, the bacterial metabolism has to be switched from alchocol and acid formation to volatile fatty acids (Pauss et al., 1990). This can be facilitated by the system's optimization, for example, using continuous barbotage with inert gas to reduce the partial pressure of hydrogen in the liquid phase thereby increasing its formation in the gaseous phase. To enhance hydrogen formation, very delicate barbotage/mixing procedures should be applied, since in our measurements, with intense barbotage and mixing by a magnetic stirrer, the least hydrogen increase in the headspace was observed. However, Oh Y-K *et al*. (2002) report that the barbotage with argon or nitrogen increases the production of hydrogen in fermentation processes as compared with bioreactors where no barbotage occurs.

Regarding to developed (chapter 3.1.1. Simple bioreactor design for hydrogen and methane gas producing microorganisms – optimization and experiments) bioreactor maintenance: in order to reduce overall costs, gas barbotage should be periodic, instead of continuous, started when hydrogen gas concentration in liquid state (measured with hydrogen microelectrode) has reached saturation (Gruduls et al. 2012). Also, as mentioned in ours and other author studies, despite hydrogen being a poorly soluble gas in an anaerobic environment, it can become over-saturated in liquids (Kraemer et al 2006, Klepere et al. 2011). We suggest that alternative methods should be employed for the hydrogen collection directly from the fermentation broth. This particular topic was further investigated in dissertation chapters 3.3. and 4.3. (Hydrogen isolation possibilities using hydride- forming metals and alloys).

Glucose is very convenient substrate for fermentative bacteria metabolism. Isolated microorganisms can use glucose for fermentation up to 99.3% efficiency and reaching up to 64% hydrogen concentration in gaseous phase (Fang and Liu, 2002), bacterial strains with genetic modifications show higher results but grow comparatively slower (Maeda et al. 2007). *E. coli* BW25113 *hyaB hybC hycA fdoG frdC ldhA* aceE::kan (from prof. T.K. Wood, USA) previously has showed a 4.6-fold increase of hydrogen production from glucose (Maeda et al. 2007). Our results (33 μmol hydrogen/mg protein/h) are comparable to previous research by Maeda (2007) where the

same bacterial strain *E.coli* BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE::kan* produced 32±6 μmol hydrogen/mg protein/h from glucose.

For hydrogen to be renewable, it must come from renewable feedstocks (Show, 2012). As Elsharnouby et al. (2013) concluded in their most recent review for hydrogen production possibilities - biodiesel waste have significant potential as sustainable feedstock in the near future.

We did a study with various fermentative bacteria for hydrogen production possibilities using pure glycerol and crude glycerol. It is well known that substrate concentration is crucial parameter for an optimal hydrogen production with fermentative bacteria. Many publications reflect optimal substrate concentration investigations for hydrogen production matters, though there are only few made for optimal crude glycerol concentration findings (Mangayil et al., 2012.; Marques et al., 2009). Firstly we used 15g/l crude glycerol concentration, that was chosen based on the literature studies. During research we investigated that specifically for *Enterobacter* genus smaller concentrations work better. Subsequently, we used 6 g/l glycerol concentration with this genus for the flowing studies (chapter 3.5. about hydrogen isolation possibilities using hydride- forming metals and alloys).

We measured that crude glycerol (Naukseni factory, Latvia) contained 40% of glycerol. Mangayil et al (2012) used Savon Siemen Oy manufactury glycerol, that contained 45% (v/v) glycerol, 30% (v/v) metanol. Our investigations with crude glycerol from Naukseni (Latvia) biodiesel factory showed that between 3g/l, 6g/l and 12g/l, the most efficient concentration in terms of hydrogen production was 6g/l for *E.aerogenes* pure (single strain) culture fermentation process. Marques et al (2009) study was with *E.aerogenes* and they showed that crude glycerol concentration of 10g/l was more efficient. While Mangayil et al. (2012) reported that hydrogen yield increased with crude glycerol concentration only 1g/l for microbial consortium, which was mainly dominated by *Clostridium* species. The results between all authors may differ due to pure culture (single strain) or consortium used, also due to various strains and due to substrate production in different factories, thereby having different glycerol concentration and containing various impurities. We propose that using described pure cultures for hydrogen production via glycerol fermentation, the begining concentration for glycerol should be lower than 15g/l.

Two of our investigated bacterial strains (*C. sporogenes* and *E.aerogenes*, chapter 3.2.) appeared to be good candidates for further investigations of bio-hydrogen production from crude glycerol. In search for optimal hydrogen producer from glycerol here I present a summary of strains investigated within our hydrogen production research using glycerol as substrate:

C.sporogenes: This strictly anaerobic bacterium was found to be an optimal hydrogen producer with pure glycerol, but within this study we were not able to produce any hydrogen with the strain from crude glycerol. These findings show that crude glycerol impurities are essential inhibitor for hydrogen production with *C.sporogenes.* Comparatively Mangayil et al. (2012) used crude glycerol with microorganism associations, mainly dominated by *Clostridium* sp., and authors succeeded in producing hydrogen. Kinetic experiments on H_2 production from pure and crude glycerol indicated the absence of any inhibitory effects from the impurities present in crude glycerol. We propose, that factor of associations instead of pure culture is needed for successful production or usage of specifically *C.sporogenes* strain CL3.

E.aerogenes: Strain produced hydrogen from both - pure and crude glycerol. Almost twice as more hydrogen with *E.aerogenes* could be gained using crude glycerol. This effect closely resembles to substrate impurities, because more energy is available in the crude glycerol – 25.3MJ/kg, comparing to the pure glycerol – 19MJ/kg (Sarma et al 2012). Hypothesis can be raised that for *E.aerogenes* impurities (Sodium sulfate, methanol and others) increase overall hydrogen production. Similar observation for increased H_2 concentration using crude glycerol in comparison to pure glycerol was made by Marques et al (2009) with *E.aerogenes* and Mangayil et al (2012) with consortium, mainly dominated by *Clostridium* sp. (*Clostridium sporogenes* CL3*, Clostridium subterminale* DSM 758). On contrary, as mentioned before, our study demonstrate that for pure culture fermentation with *C.sporogenes* crude glycerol impurities possibly interfere hydrogen production. Using *E.aerogenes* we demonstrated hydrogen yield: 0.4 H_2mol/mol crude glycerol with substrate concentration 6g/l. Similar results were achieved by Mangayil et al (2012) - 0.3 H_2 mol with substrate concentration 5g/l, but with microbial consortium, mainly dominated by *Clostridium* sp. They reached highest yield - 1.1 H_2 mol/mol crude glycerol (37% of the theoretical maximum) with smaller glycerol concentration 1g/l. Authors suggest that surplus of

organic compounds in higher concentrations of crude glycerol has an inhibitory effect on hydrogen production.

Our research showed unefficient substrate utilization within 96 h fermentation with concentrations of 11-17 g/l of analytical (pure) glycerol, namely, substrate utilization was not higher than 50%. Mangayil et al (2012) reported substrate utilization from 27% using concentration 5g/l of crude gycerol to 56%, using concentration 1g/l of crude glycerol. In our study with crude glycerol (conc. 6g/l) 90% substrate consumption was measured with strain *E.aerogenes.*

E.cloacae*:* Perspective hydrogen producer from analytical (pure) glycerol. Interesting, that similar dissolved hydrogen amount was detected in samples with both, pure glycerol and crude glycerol; though in the gaseous phase hydrogen appeared only in the samples with pure glycerol. Hypothesis can be proposed that there was an inhibition for hydrogen migration from liquid to gaseous phase. To ascertain whether it is regarding to dissolved hydrogen oversaturation or other factors, further investigation is needed/has to follow.

E. asburiae: This strain didn't show any hydrogen production from pure nor crude glycerol within this study.

Regarding to lactose usage for hydrogen production we showed that increased utilization of lactose was achieved by overexpression of *lacY*, and by the following selection of colonies, able to grow in the presence of elevated lactose concentrations. The selected recombinant strain 891plS showed improved growth rate and hydrogen synthesis on lactose, in comparison to the parent strain 891. *A priori* we expected that a substantial increase of lactose transport would inactivate the lactose repressor and induce the genes of the lactose operon, thus creating an effect similar to the *lacI* knock-out (Rosales-Colunga, 2010). Increased rate of lactose transport, however, must be regarded as a coin with two sides. As such, it is necessary for reaching high rates of lactose conversion; yet, a too rapid lactose transport may be deleterious for *E. coli* cell, causing stress and even cell death (Perfeito et al. 2011).

Lactose killing is a phenomenon in which 80 to 98 % of the *E. coli* cells taken from a lactose-limited chemostat die when plated on standard lactose minimal media. This unique form of cellular suicide was shown to be caused by the action of the lactose permease, the *lacY* gene product (Dykhuzein and Hartl, 1987). The occurrence of rapid

proton-dependent transport across the membrane might be the primary cause of the lactose stress phenomenon, leading to an abrupt dissipation of the transmembrane proton motive force. Inability to maintain proton motive force results in deenergization and a general stress condition of the bacterial cell. We may speculate about existence of certain 'optimum' activity of lactose permease for a lactose-converting strain: when lactose influx appears to be moderately raised, yet not reaching levels, deleterious for the cell. The reduction of plasmid content in result of selection in our experiments indicates that such fine-tuning of the lactose transporter activity might have taken place, and must be considered as one of explanations for the improved performance of strain 891plS. Clearly, this needs further transcriptomic study.

Along with the metabolomic or transcriptomic data, the variation of the macromolecular composition of microbial biomass is indicative for various stress conditions. The macromolecular composition depends on the physiological state of microbial culture, reflecting the influence of particular stress on the cellular regulatory mechanisms (Naumann, 2002, Dykhuizen and Hartl, 1987, Wang et al. 2010, Kuligowski et al. 2012). As the growth conditions in many cases affect the content of all types of biopolymers simultaneously, quantitative analysis using methods like FT-IR spectroscopy are most appropriate for they yield an integral estimate of the macromolecular composition. Here we applied FT-IR to reveal the changes of the macromolecular composition accompanying the adaptation of recombinant *E. coli* to the lactose stress conditions.

The most vivid effect on the macromolecular content, characteristic for *E. coli* adaptation to elevated lactose import, appeared to be an increase of cellular polysaccharide content in the strain 891plS. The two absorption peaks at 1026 cm^{-1} (C-O-H bend) and 1154 cm^{-1} (C-O stretch) have been previously assigned to glycogen accumulation (Chiriboga et al. 2008). On the other hand, Moen *et al*. (2005) suggested that the increased carbohydrate content might be due to some osmoregulated periplasmic glucans, which appear to be important for survival of Gram-negative bacteria under various undesirable conditions. More recently, Moen *et al*. (2009) reported that *E. coli* exposed to 10 adverse conditions (sodium chloride, ethanol, glycerol, hydrochloric and acetic acid, sodium hydroxide, heat (46 ^{o}C), and cold (15 ^{o}C), as well as ethidium bromide and the disinfectant benzalkonium chloride) showed significant increase in the

two minimum peaks at 1154 and 1026 cm^{-1} in the 2nd derivative spectra. These bands have previously been associated also with the survival of *C. jejuni* under nongrowth conditions producing a polysaccharide capsule thus related to changes in the carbohydrate composition of the outer membrane (Karlyshev et al. 2001, Moen et al. 2005, Oust et al. 2006). Microscopic analysis of iodine-stained cells was therefore applied, in order to relate the spectral data to particular subcellular localization of the accumulated polysaccharide. Iodine staining was used as an indicator of intracellular glycogen accumulation. Indeed, our microscopic data showed that the strain 891plS was accumulating glycogen when grown on lactose, but we obtained no evidence of capsule formation.

Polysaccharide metabolism is highly interconnected with a wide variety of cellular processes and is adjusted to the bacterial energy and nutritional status. Different genetic studies indicate a requirement of the general stress regulator RpoS as a positive signal for glycogen biosynthesis in *E. coli* (Eydalinn et al. 2010). It has been shown that RpoS gets degraded at high ATP concentrations, but is active at low ATP, corresponding to substrate-limiting or deenergizing conditions in the cell (Peterson et al. 2012). Knowing that the elevated transmembrane lactose transport is likely to cause membrane deenergization, it is tempting to think that the polysaccharide accumulation in *E. coli* 891plS represents an enhanced manifestation of the general stress regulation by RpoS. Apparently, the underlying regulatory mechanisms, as well as the link between the increased polysaccharide content and the ability of *E. coli* cell to withstand the lactose stress remain as open problems for future study.

4.2. Hydrogen isolation possibilities using hydrogen-absorbing metals and alloys

First attempts were made for hydrogen collection from bacteria fermentation broth using metal hydrides. Various powdered metals and alloys (Pd, $LaNi_5$, AB_5, AB_2) forming hydrides were investigated to collect hydrogen directly from liquid phase. Vacuum extraction measurements were accomplished to ascertain the measured gas weight changes in thermogravimetry experiments were regarding to hydrogen and not water from broth or thermal decomposition changes of organic materials. As per $LaNi_5$,

similar behavior was seen with metal hydrides immersed in fermentation medium and hydrated metal hydride: similar weight losses occurred according to temperature changes. This could be considered as evidence that hydride-forming metals absorb H_2 not only from the gas phase but also from dissolved gas. Hydrated metal hydride weight losses with $LaNi_5$ are accordingly reported elsewhere (Broom, 2011). All materials in these experiments are known to release H_2 at low temperature: Pd at 70°C, AB_2 at 50°C, $LaNi_5$ and AB_5 at 25°C (Chen & Zhu, 2008). Weight loss of material at heating within the 30-100°C range approximate the amount of adsorbed H_2. At temperatures exceeding 150°C, organic substances from microorganisms and fermentation broth begin to decompose and can elicit additional weight losses not related to H_2 desorption. Thermal decomposition of organic materials is most active at around 200°C (Gómez et al., 2007).

In comparing weight loss, Pd and the 3 hydride alloys tested could be listed according to the amount of stored H_2 in the following order (starting with the highest amount): Pd, AB_5, AB_2 and $LaNi_5$. This could be explained by Pd's greater stability in corroding environments, as all experiments were carried out in fermentation broth and with smaller size particles. It appears that if the samples contained metals that absorbed H_2 released in fermentation, H_2 concentration in the gaseous phase at the end of the process would be lower. This was also confirmed by MS analysis. Each molecule that is produced inside the bottle increases H_2 temperature release in metal hydrides (Kleperis et al., 2001) with 1.4% being the maximum (Inspection Certificate from GfE Gesellschaft für Elektrometallurgie mbH).

Our findings showed that less H_2 was detected in the gaseous phase owing to the presence of metal hydrides in all samples, except for AB_2. Vacuum extraction measurements concurred with our hypothesis that weight changes in the TG experiments were due to H_2 and not organic decomposition. Also, if some H_2 was produced by organic decomposition, CO_2 and other carbon (C) - containing gas levels would increase.

Analysis of H_2 absorption dynamics in alloys during fermentation determined that the largest amount of soak-up H_2 occurred after 18 h from the beginning of fermentation. It coincides well with the analysis of consumed glycerol – all results indicated that crude glycerol was completely expended within 24 h of fermentation. Incidentally, this may also explain the results of the first fermentation experiments (Fig.

3.26. and 3.27) with relatively small, bonded H_2 in hydride metals – because they were removed from the fermentation bottles after 72 h.

It could be hypothesized that after H_2 production stops in the fermentation process, once all of the substrate is gone, soak-up of metal hydride H_2 would gradually decreases coming out and going into the gaseous phase. Our results indicate that LC directly combined with electrospray-ionization high-resolution MS can selectively separate and detect glycerin in fermentation broth. The presence of glycerin was achieved not only from retention time compared to standard solution, but also from the exact mass of its protonated molecular ion. SEM demonstrated that bacteria attached to the material surface. Further analysis will be conducted with GC, and all alloys tested in this preliminary research (Pd, $LaNi_5$, AB_2, AB_5) will be coated in polymer activated for proton transfer. The effect of metal hydride on the amount of H_2 released in fermentation has been reported by Nishimiya et al. (International Discussion on Hydrogen Energy and Applications (IDHE) meeting, Nantes, France, 2014), who disclosed their earlier results with ZrVFe encapsulated in polymer as effective material to recover H_2 from bio-hydrogen mixtures produced by *Anabaena* in Ar atmosphere. They discovered that total H_2 amount in the gaseous phase increased 6-7 times in the presence of encapsulated alloys compared to normal H_2 production in their absence.

5. Conclusions

1. Anaerobic fermentation test-systems for on-line gas monitoring with masspectometric and microsensor measuring methods in both gaseous and liquid phase were tested and optimized.
2. In glucose fermentation with the septuple knock-out mutant *Escherichia coli* BW25113 hydrogen became oversatured in the fermentation medium - hydrogen gas accumulation in the headspace of the fermenter was facilitated by constant mixing and barbotage of the cultivation medium with inert gas.
3. Three from seven of the isolated and selected bacterial strains in Latvia are suitable for biohydrogen production research and glycerol would be a perspective substrate for hydrogen production in the fermentation process (rapidly convertible, easy accessible, large quantities of crude glycerol available).
4. *C.sporogenes* was found to be an optimal hydrogen producer with pure glycerol (reaching 7 mmol H_2/L in gaseous phase), but within this study we were not able to produce any hydrogen with the strain from crude glycerol. The findings show that crude glycerol impurities are essential inhibitor for hydrogen production with *C.sporogenes*.
5. Originally isolated *E.aerogenes* produced twice as more hydrogen from crude glycerol than from pure glycerol; presumably crude glycerol impurities increase overall energy available in the substrate. This hypothesis is supported by data gained from other authors with research on crude glycerol.
6. Increased utilization of lactose was achieved; the strain was able to grow in the presence of elevated lactose concentrations and showed improved growth and hydrogen synthesis, in comparison to parent strain *E.coli* BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE*.
7. The studies show that hydride-forming materials - Pd, AB_5, AB_2 and $LaNi_5$ - can be successfully exploited for H_2 collection from the liquid phase in crude glycerol fermentation by *E. coli* and *E. aerogenes;* H_2 accumulation from fermentation media containing AB_5 alloy powder was directly demonstrated by vacuum extraction of the alloy and MS analysis.

8. H_2 concentration in the gaseous phase after 72 h of fermentation was lower in cultivation medium loaded with hydride-forming materials than in unloaded controls, while total calculated amount of H_2 absorbed in hydrides and the gas phase exceeded the control level. The accumulation of hydrogen gas from the fermentation broth in hydrogen-forming, activated $LaNi_5$ alloy displayed similar pattern of weight loss in TG experiments, both when it was saturated with hydrogen under pressure in gas atmosphere and when it was immersed in fermentation medium, where hydrogen was produced by bacteria during glycerol fermentation.

9. TG data ranked the tested hydride-forming materials by their H_2 sorption capacity in the fermentation broth in the following order (highest to lowest): Pd, AB_5, AB_2 and $LaNi_5$.

10. The presence of powdered Pd or hydride-forming alloys in cultivation medium did not impede glycerol consumption: it was fully degraded during 24 h of fermentation. Concomitantly, the highest amount of absorbed H_2 was measured by the weight loss of hydride-forming materials in TG experiments after 18 h of glycerol fermentation.

6. Defense thesis

1) Hydrogen that is formed in liquid media during bacterial fermentation only partly escapes in gaseous phase, but part of it stays dissolved and is oversaturating in the liquid phase thereby limiting further hydrogen production. Hydrogen concentration in the gaseous phase can be increased by constant fermentation broth liquid mixing and/or barbotage with inert gas. Technically inovative test-systems are necesary for on-line gas monitoring anaerobic fermentation process realization.

2) Hydride-forming metal or alloy presence in the fermentation broth absorbs dissolved hydrogen to prevent inhibation of hydrogease enzyme. Depending from fermentation broth, hydride-forming material and surface composition, synergy development is possible between microorganisms and metal hydride, resulting in improved efficiency of hydrogen production process.

3) In Latvia naturaly isolated facultative anaerobe *Enterobacter aerogenes* is an optimal hydrogen producer from industrial waste product - biodiesel production byproduct - crude glycerol, that has been manufactured in Latvia. Impurities of the crude glycerol increase the overall hydrogen yield using specifically this bacteria strain.

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APPENDIX

Appendix 1

H_2 production measurements in liquid phase and the final production of H_2 of selected anaerobic bacteria strains using both crude and analytical glycerol as substrates.

Substrate	Genus taxonomy	H_2 concentration in liquid phase mmol/L	Total H_2 concentration (gaseous and liquid phase) mmol/l
Crude glycerol	*E. aerogenes I*	1,474	5,230
	Cl. sporogenes I	0,018	0,018
	E. cloacea I	1,377	1,377
	E. asburiae I	0,034	0,034
	E. aerogenes II	3,618	13,257
	Cl. sporogenes II	0,028	0,068
	E. cloacea II	0,028	0,029
	E. asburiae II	0,024	0,025
	E. aerogenes III	1,859	6,386
	Cl. Sporogenes III	0,033	0,072
	E. cloacea III	3,657	3,657
	E. asburiae III	0,011	0,011
Pure glycerol	*E. aerogenes I*	1,171	6,481
	Cl. sporogenes I	0,028	0,029
	E. cloacea I	0,065	0,065
	E. asburiae I	0,050	0,050
	E. aerogenes II	1,511	5,002
	Cl. sporogenes II	1,315	8,458
	E. cloacea II	1,707	9,542
	E. asburiae II	0,036	0,036

Appendix 2

Table 1. pH of media with *E. aerogenes* without crude glycerol (B), with crude glycerol concentration 3 g/ L (B + 3 g/ L), 6 g/ L (B + 6 g/ L), 12 g/ L (B + 12 g/ L) and crude glycerol solution in water with concentration of 6 g/ L (6 g/ L) at the beginning of the cultivation and after 24 h of cultivation.

Medium	pH measuring time - beginning (B) of the cultivation and after (A) 24 h of cultivation.	pH value				AverPGe
		1. sample	2. sample	3. sample	4. sample	
B	B	5,82	5,70	5,81	5,83	5,79 ± 0,06
	A	5,34	5,26	5,33	5,24	5,29 ± 0,05
B+3g/l	B	6,11	6,04	5,92	6,07	6,04 ± 0,08
	A	5,20	5,10	5,10	5,13	5,13 ± 0,05
B+6g/l	B	6,39	6,36	6,35	6,27	6,34 ± 0,05
	A	4,69	4,78	4,94	4,73	4,79 ± 0,11
B+12g/l	B	6,73	6,80	6,90	6,86	6,82 ± 0,07
	A	4,58	4,52	4,56	4,53	4,55 ± 0,03
6g/l	B	7,00	7,63	7,64	7,69	7,49 ± 0,33
	A	6,40	6,43	6,43	6,45	6,43 ± 0,02

Table 2. Colony forming units (kvv · 10^6/ mL medium) of *E. aerogenes* in media with metal hydride AB_5 alloys (B +6g/l+ AB_5) and without it (B+6g/l).

Type of medium	Colony formit units kvv · 10^7/ mL			AverPGe
	1. sample	2. sample	3. sample	
B+6g/l+AB5	15,0	20,2	20,8	18,7 ± 3,2
B+6g/l	14,7	10,6	8,0	11,1 ± 3,4