Domain Engineering Sciences
Rte du Rawyl 47
CH-1950 Sion 2
Phone +41 27 606 85 11
Fax +41 27 606 85 75
info@hevs.ch

www.hevs.ch

Degree Course Life Technologies

Option Biotechnology

Diploma 2010

Christel Sautaux

Quantification of biohydrogen production on defined media

Professors KURT EYER | SERGIO SCHMID

Experts: CHRISTOPH HERWIG | SIMON RITTMANN

Wien, 29.11.2010

Haute Ecole Spécialisée de Suisse occidentale
Fachhochschule Westschweiz
University of Applied Sciences Western Switzerland

Quantification of biohydrogen production on defined media

Sautaux Christel

Bachelor's Thesis
| 2010 |

Degree course
Life Technologies

Field of application
Major

Supervising professor
Sergio Schmid
Kurt Eyer
Christoph Herwig

Objectives

Objectives of this bachelor's Thesis was the quantification of biohydrogen production using *Enterobacter aerogenes* DSM 30053 with a defined medium.

Methods | Experiences | Results

In this bachelor's thesis, hydrogen production performance of facultative anaerobe *Enterobacter aerogenes* has been investigated with a defined medium. A repeted batch system was set-up for dark fermentative biohydrogen production and different concentrations of glucose, and xylose were tested for the production of biohydrogen. A continuous system for dark fermentative biohydrogen production was set-up, and production of biohydrogen was investigated among different pH and dilution rates.

It was shown that hydrogen may effectively be produced by E. aerogenes DSM 30053 by using defined medium.

The substrate of 20 g/l glucose, in batch and with a pH of 6.8 resulted in the highest yield of hydrogen (0.17 mol/C-mol). The maximum hydrogen productivity reaches 19.45 mmol/l/h with a continuous culture with glucose 20 g/l and pH 6.8. Results show a tendency to have less CO2 and more H2 production with an increasing pH, between pH 6.4 and 6.8, and a tendency to have a higher specific H2 production with a higher dilution rate.

HES-SO Valais
Route du Rawyl 47
1950 Sion

Phone 027 606 85 11
Website www.hevs.ch

☐ FSI ☒ FTV	Année académique **/** *Studienjahr* **2009/2010**	No TD / *Nr. DA* **bt/2010/61**
Mandant **/** *Auftraggeber* ☐ HES—SO Valais ☐ Industrie ☒ Etablissement partenaire **Technische Universität Wien**	Etudiant **/** *Student* **Christel Sautaux**	Lieu d'exécution / *Ausführungsort* ☐ HES—SO Valais ☐ Industrie ☒ Etablissement partenaire
Professeurs / *Dozenten* **Kurt Eyer | Sergio Schmid**	Expert / *Experte* *(données complètes)* Dr. Christoph Herwig Technische Universität Wien Karlsplatz 13 |1040 Wien	
Travail confidentiel / *vertrauliche Arbeit* ☐ oui / ja [1] ☐ non / nein		

Titre / *Titel*

Process development for enhanced fermentative biohydrogen production

Goals:

1) Find optimised process conditions for the production of biohydrogen using different substrates.
2) Establish a batch system for biohydrogen production
3) Set up a repeated batch biohydrogen production system

The process for biohydrogen production has to be established. This comprises a high sophisticated bioreactor environment with multiple measurements and controls. On-line and off-line data will be used to quantify the biological reaction. Data exploitation will comprise the calculation of rates and yields in which the biohydrogen productivity will be evaluated. The growth conditions for the microbes will be facultative and strict anaerobic.

The fermentations will be done with:

- Two different anaerobic fermentative biohydrogen producing strains (*Enterobacter aerogenes, Clostridium uliginosum*)
- Different substrates (pentoses, hexoses)
- Different bioreactor setup by using batch cultures and repeated batch cultures

Activities and Expected Results:

For efficient biohydrogen production, following factors will be studied for each strain and substrate:

- Influence of: oxidation-reduction potential (ORP), pH, N_2-sparging, agitation, temperature, initial substrate concentration
- Production of metabolic end products (i.e. acetate, formate);
- Product yield and evolution rate H_2/sugar [g/g], HER [mol H_2/g CDW/h].

[1] *Par sa signature, l'étudiant s'engage à respecter strictement le caractère confidentiel du travail de diplôme qui lui est confié et des informations mises à sa disposition; il s'engage également à appliquer formellement la directive y relative.*
Durch seine Unterschrift verpflichtet sich der Student, die Richtlinie einzuhalten sowie die Vertraulichkeit der Diplomarbeit und der dafür zur Verfügung gestellten Informationen zu wahren.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Professor Dr. Christoph Herwig that gave me the opportunity to join his group in Vienna. His enthusiasm is communicative and the word "team" has a real meaning with him.

I thank my mentor, Simon Rittmann. He had a lot of patience with me and he helped me a lot in the lab and in writing this bachelor thesis.

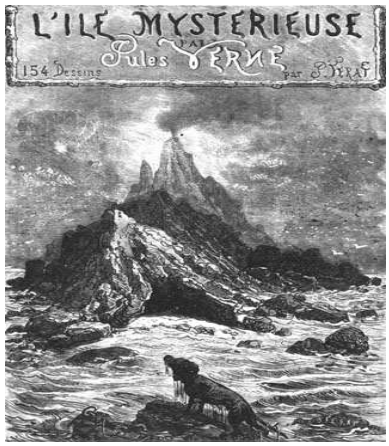
I would like to thank also my workmates Patrick Wechselberger, Oliver Spadiut, Christian Dietzsch, Andreas Posch, Arne Seifert, Mohammed Jazini, Joseph Schulze, Patrick Sagmeister, Silvia Gluderer, Birgitt Wittholm and a special thank for Katharina Denk: without her, it would not have been the same!

I also thank my professors in Sion, Sergio Schmid and Kurt Eyer that gave me the opportunity to come in Vienna and helped me to prepare my work.

Last but not least, I thank my family, and especially my mother, Claudine, for her support during my whole life. She is always present for me. MERCI!

EXCERPT OF « L'ILE MYSTERIEUSE», JULES VERNE,

… 1874 …



- *Mais que trouvera-t-on ? demanda Pencroff. L'imaginez-vous, monsieur Cyrus ?*

- *À peu près, mon ami.*

- *Et qu'est-ce qu'on brûlera à la place du charbon ?*

- *L'eau, répondit Cyrus Smith.*

- *L'eau, s'écria Pencroff, l'eau pour chauffer les bateaux à vapeur et les locomotives, l'eau pour chauffer l'eau !*

- *Oui, mais l'eau décomposée en ses éléments constitutifs, répondit Cyrus Smith, et décomposée, sans doute, par l'électricité, qui sera devenue alors une force puissante et maniable, car toutes les grandes découvertes, par une loi inexplicable, semblent concorder et se compléter au même moment. Oui, mes amis, je crois que l'eau sera un jour employée comme combustible, que l'hydrogène et l'oxygène, qui la constituent, utilisés isolément ou simultanément, fourniront une source de chaleur et de lumière inépuisables et d'une intensité que la houille ne saurait avoir. Un jour, les soutes des steamers et les tenders des locomotives, au lieu de charbon, seront chargés de ces deux gaz comprimés, qui brûleront dans les foyers avec une énorme puissance calorifique. Ainsi donc, rien à craindre. Tant que cette terre sera habitée, elle fournira aux besoins de ses habitants, et ils ne manqueront jamais ni de lumière ni de chaleur, pas plus qu'ils ne manqueront des productions des règnes végétal, minéral ou animal. Je crois donc que lorsque les gisements de houille seront épuisés, on chauffera et on se chauffera avec de l'eau. L'eau est le charbon de l'avenir.*

- *Je voudrais voir cela, dit le marin.*

- *Tu t'es levé trop tôt, Pencroff, » répondit Nab, qui n'intervint que par ces mots dans la discussion*

TABLE OF ABBREVIATIONS

Biohydrogen	biological hydrogen	
C	carbon	-
CDW	cell dry weight	-
CER	carbon dioxide evolution rate	C-mmol/l/h
CO2	carbon dioxide	-
Cubian	CuBiAn XC analyzer	-
DSM	Deutsche Sammlung von Microorganismus	
E.aerogenes	*Enterobacter aerogenes*	
glu	glucose	
h	hours	-
H2	hydrogen	-
HER	hydrogen evolution rate	mmol/l/h
HPLC	High pressure liquid chromatography	-
M	Molarity	Mol/L
min.	minutes	-
MM	Molecular mass	g/mol
N2	nitrogen	-
NaOH	Sodium hydroxide	-
OD_{600}	Optical density at 600 nm	-
PIMS	Process Information Management System	-
r_I	Volumetric rate of component I	mmol/l/h
rpm	Revolution per minute	rpm
T	Temperature	ºC
t	Time	h
V_R	Volume of the bioreactor	L
vvm	Volume air per volume media per minute	L/L/min
X	Biomass concentration	g/L
X_0	Incoming biomass concentration	g/L
$Y_{X/S}$	biomass Yield	C-mol/C-mol
μ	Specific growth rate	1/h

SUMMARY

1. INTRODUCTION

1.1 THE EMERGENCE OF THE IDEA OF A HYDROGEN ECONOMY

Energy is vital to global prosperity, yet dependence on fossil fuels as our primary energy source contributes to global climate change, environmental degradation, and health problems. More than 80% of energy consumed today is derived from burning fossil fuels [Bockris, 2002]., Since the mid-19th century, mankind has slowly been shifting utilization of primary energy sources from one form of energy to another: from solids to liquids to gases as demonstrated in the left part of the Figure 1 [Dunn, 2002].

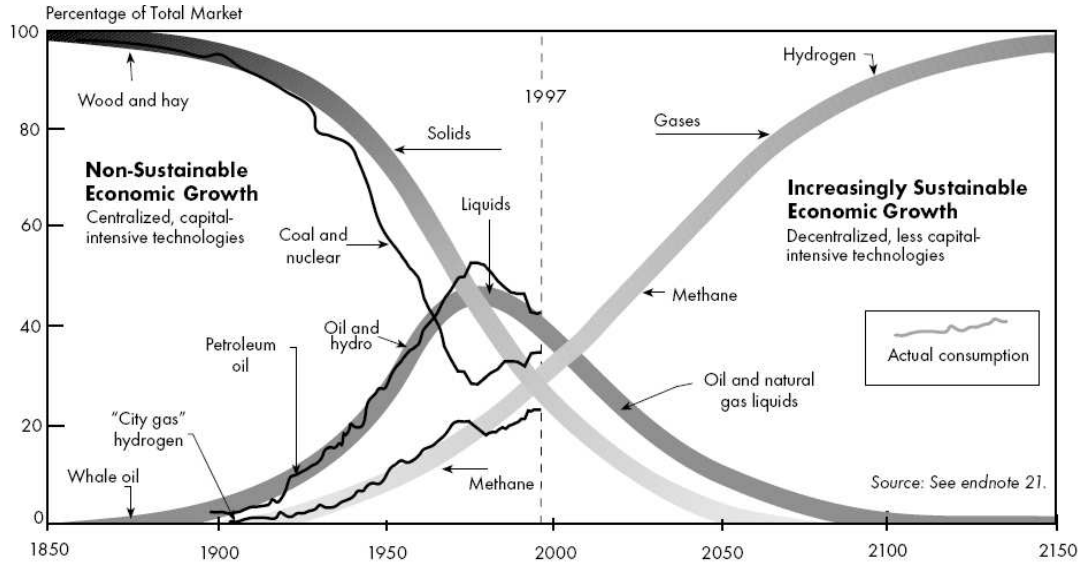


Figure 1: Global energy systems transition, 1850-2150 [Hefner, 2000]

The move from solid to liquid to gas fuels involves another sort of transition: the less visible process of “decarbonization”. From wood to coal to oil to natural gas, the ratio of hydrogen (H) to carbon (C) in the molecule of each successive source has increased. Roughly speaking, the ratio is between 1 to 3 and 1 to 10 for wood; 1 to 2 for coal; 2 to 1 for oil; and 4 to 1 for natural gas. Between 1860 and 1990, the H-C ratio rose six-fold. The trend toward ‘decarbonization’ is at the heart of understanding the evolution of the energy system [Winter, 2000].

Some scientists predict a new economy based on hydrogen, which might fundamentally change the global market, economy and society, as coal did in the 19th century and petroleum in the 20th century [Rocha, 2001]. Hydrogen is not a primary energy source, but, as shown in figure 2, hydrogen has been suggested as the energy carrier of the future, serving as a medium through which primary energy sources (such as wind or solar energy) can be stored, transmitted and utilized to fulfill our energy needs [Das, 2001].

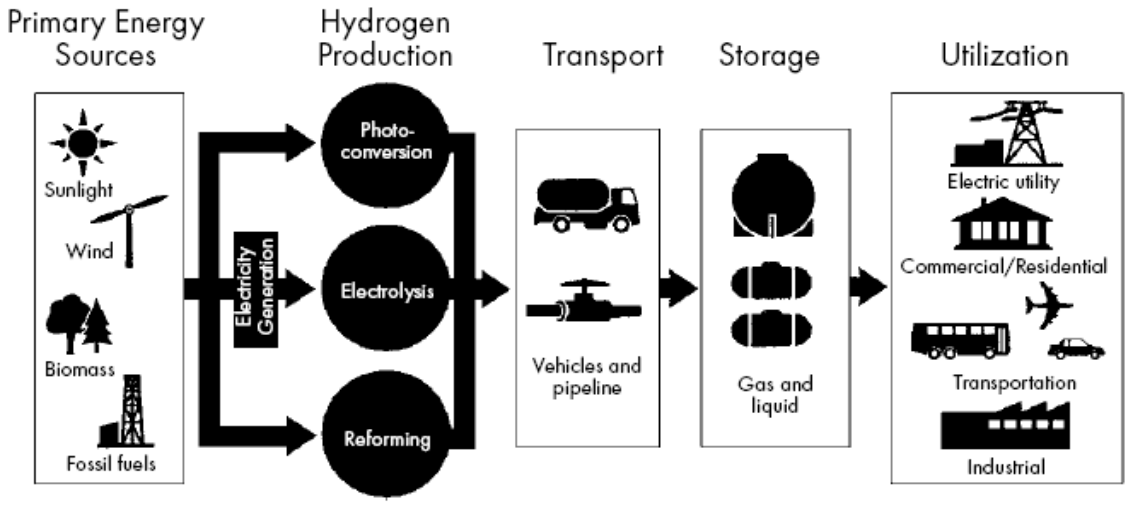


Figure 2: A hydrogen energy system: Hydrogen as energy carrier. This figure shows the current industrial production of hydrogen, which does not involve biohydrogen from dark fermentation processes. [Jensen, 2000]

Hydrogen is regarded as a clean, non-polluting fuel, because its product of combustion is H_2O, and moreover, hydrogen is harmless to mammals and the environment. This colorless, odorless and tasteless element has the highest gravimetric energy density of any known fuel and may be used in internal combustion engines or to produce electricity through fuel cells [Rittmann, 2008]. Hydrogen is everywhere, but it is hard to find on earth as a separate element. Instead, it is primarily found in combination with oxygen in water, in combination with carbon in a range of hydrocarbons, and in combination with carbon in plants, animals, and other forms of life [HTAP, 1998]. Hydrogen may be produced by a number of different processes, including electrolysis of water, thermo catalytic reformation of hydrogen-rich organic compounds, and biological processes. Currently, hydrogen is produced, almost

exclusively, by electrolysis of water or by steam reformation of methane. Biological production of hydrogen (biohydrogen), using (micro) organisms, is an exciting new area of technology development that offers the potential production of usable hydrogen from a variety of renewable resources [Levin, 2003; Rittmann, 2008]. Biohydrogen is already a part of the biocarburants as describes by the European Directive 2003/30/EC of the 8th of May 2003 that should promote the use of biofuels or renewable fuels for transport.

1.2 BIOHYDROGEN PRODUCTION

There are several biological hydrogen production processes that can be classified as follows:

- Biophotolysis of water (direct or indirect)
- Photofermentation
- Bioelectrohydrogenesis
- Fermentative hydrogen production (this is the process used in the present work and this point will be discussed with more attention in chapter 2)

1.2.1 Biophotolysis of water

A production of hydrogen by **direct biophotolysis** is a biological process that uses solar energy, as well as the photosynthetic system of algae or cyanobacteria to convert water in chemical energy [Suzuki, 1982]. The principal advantage of direct photolysis is the production of hydrogen only with water and solar energy, as follows:

$$2H_2O + \text{light energy} \rightarrow 2H_2 + O_2 \qquad \text{Eq. 1}$$

The principal disadvantages of this process are the requirement for a high intensity of light, which is very expensive in a technologic point of view, and the oxygen sensitivity of the several enzymes participating in photo biohydrogen production [Nath, 2004].

In **indirect biophotolysis,** problems of the oxygen sensitivity in the hydrogen evolving process are potentially circumvented by separating temporally and/or spatially oxygen evolution and hydrogen evolution [Hallenbeck, 2002]. In this process, CO_2 is continually fixed and relaxed, and used for the transport of electrons between the reaction that produce O_2 (scission of water), and the reactions that are O_2-sensitive (with hydrogenase).

In indirect biophotolysis, H_2 is produced as follows [Das, 2008]:

$$12H_2O + 6CO_2 + \text{light energy} \rightarrow C_6H_{12}O_6 + 6O_2 \qquad \text{Eq. 2}$$

$$C_6H_{12}O_6 + 12H_2O + \text{light energy} \rightarrow 12H_2 + 6CO_2 \qquad \text{Eq. 3}$$

Cyanobacteria are essentially used for that type of biophotolysis.

1.2.2 Photofermentation

Purple non-sulfur bacteria evolve molecular hydrogen catalyzed by the enzyme nitrogenase under nitrogen-deficient conditions using light energy and reduced compounds (organic acids) as follows [Levin, 2004]:

$$C_6H_{12}O_6 + 12\ H_2O + \text{light energy} \rightarrow 12\ H_2 + 6\ CO_2 \qquad \text{Eq. 4}$$

The major benefits of photofermentation are given below [Das, 2001]:

- A large energy spectrum can be used by these bacteria
- A lot of different organic wastes can be used as substrate. This could be use in association with wastewater treatment.
- High theoretical conversion yield
- This process takes place under anaerobic conditions and can be combined with a dark fermentation that gives acetic acid as final product

The major problem with this approach is the scale-up. It is necessary to expose the microbes to a very big surface to obtain a sufficient solar energy. In addition, the solar energy conversion efficiency is very low.

1.2.3 Bioelectrohydrogenesis

Bioelectrohydrogenesis uses microorganisms that are electrochemically active for hydrogen production. In the presence of a moderate voltage, organic compounds are used as energy and carbon source and hydrogen is evolved as a by-product. These reactions take place inside an electrochemical cell through coupled anode/cathode reactions. The density of the current on the surface of the electrodes is low and low volumetric production of hydrogen is obtained. Actually, this volumetric production is lower than the production obtained in dark- and/or photofermentations [Hallenbeck, 2009].

1.2.4 Hybrid system using photosynthetic and fermentative bacteria

Hybrid systems consist of non-photosynthetic and photosynthetic bacteria and can enhance the hydrogen yield. A variety of carbohydrates may be degraded by bacteria that produce hydrogen using dark fermentation. Other resulting products from dark fermentation could be sources for photosynthetic bacteria to produce hydrogen, as shown in Figure 3.

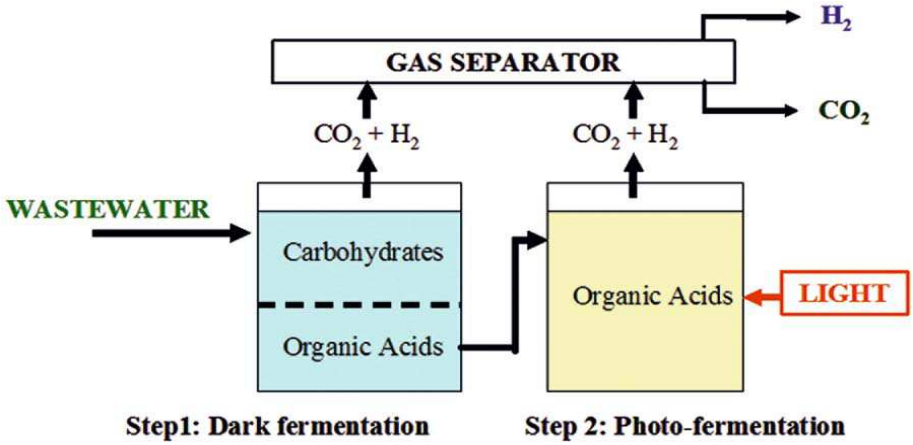


Figure 3: Hybrid system using photosynthetic and fermentative bacteria. [Tao, 2007]

The concomitant production of hydrogen from different substrates by dark and photo fermentation, not only reduces the light energy demand of photosynthetic bacteria, but also increases overall hydrogen production yield [Das, 2001; Mogidell, 2007].

2. THEORITICAL PART

2.1 DARK FERMENTATION

Dark fermentative biohydrogen production is ubiquitously occurring in several microbial species. This phenomenon occurs under anaerobic condition (for detailed information on microbes capable of dark fermentative biohydrogen production, please refer to the relevant literature [Rittmann, 2008; Nath 2004]). When bacteria grow on organic substrates (heterotrophic growth), these substrates are degraded by oxidation to provide building blocks and energy for growth and metabolism. This oxidation generates electrons which need to be disposed off to maintain electrical neutrality. In anaerobic environments, hydrogen acts as electron acceptor [Nath, 2004]. The evolution rate of hydrogen is higher from fermentative processes, than using other applications (for example biophotolysis or photofermentation), but, in comparison, the hydrogen yield (mol H_2/mol substrate) is lower [Nandi, 2001].

2.1.1 Advantages and limitations of hydrogen production by dark fermentation

Biohydrogen production by dark fermentation offers several advantages in comparison to other processes:

- H_2 can be produced all day long without the requirement of a light source
- It is an anaerobic process; there is no O_2 limitation problem
- Some fermentatively growing bacteria comprise a very high hydrogen evolution rate. For instance Ito et al., 2005, obtained a volumetric H_2 production of 80 mmol H_2/l.h by using an *Enterobacter* sp. [Ito, 2004]
- A variety of carbon sources (biomass, pure substance, various wastes) may be used as substrates for biohydrogen production (please refer to figure 4) [Das, 2004; Das, 2001]

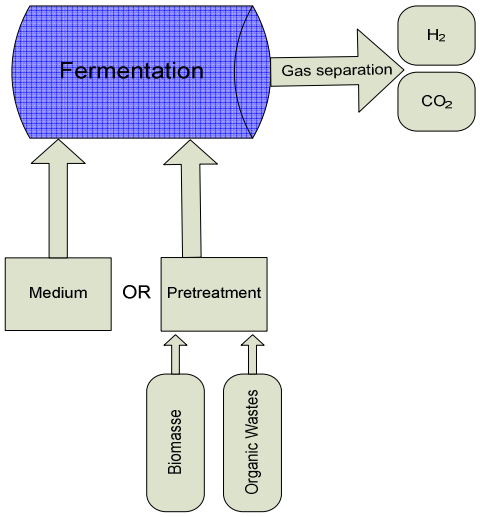


Figure 4: Schematic process of biohydrogen production via dark fermentation.

Pure substrates used for biohydrogen production are for example: glucose, xylose, cellulose, sucrose or maltose. Following wastewaters were, for example, investigated for feasibility of biohydrogen production: sugary wastewater, bean curd manufacturing waste or rice bran [Hallenbeck, 2002]. Moreover, metabolites produced by dark fermentation, for example, acetate, lactate, glutamate, may for instance be used in a second stage, by photofermentative microbes, also leading to an enhanced overall H_2 evolution [Madigan, 2001; Modigell, 2007].

The major limitations in the process using dark fermentation are the following [Nath, 2004]:

- Low achievable yields of H_2, in comparison to hydrogen produced by electrolysis
- If H_2 yields increase, H_2 fermentation becomes thermodynamically unfavourable, because end-product inhibition occurs [Hallenbeck, 2002]
- Product gas mixture contains CO_2 which has to be separated

Various approaches exist to overcome these limitations, for example:

- Metabolic shift of biochemical pathways to arrest the formation of alcohol and acids [Mahyudin, 1997; Kumar, 2001]
- Maintaining low partial pressure of H_2 and CO_2 to keep biohydrogen fermentation thermodynamically favourable, e.g. by inert gas sparging [Nath, 2004].

2.1.2 Fermentative biohydrogen production pathways

As shown in Figure 5, there are three representative pathways for the production of biohydrogen by fermentative bacteria [Tanisho, 1997]

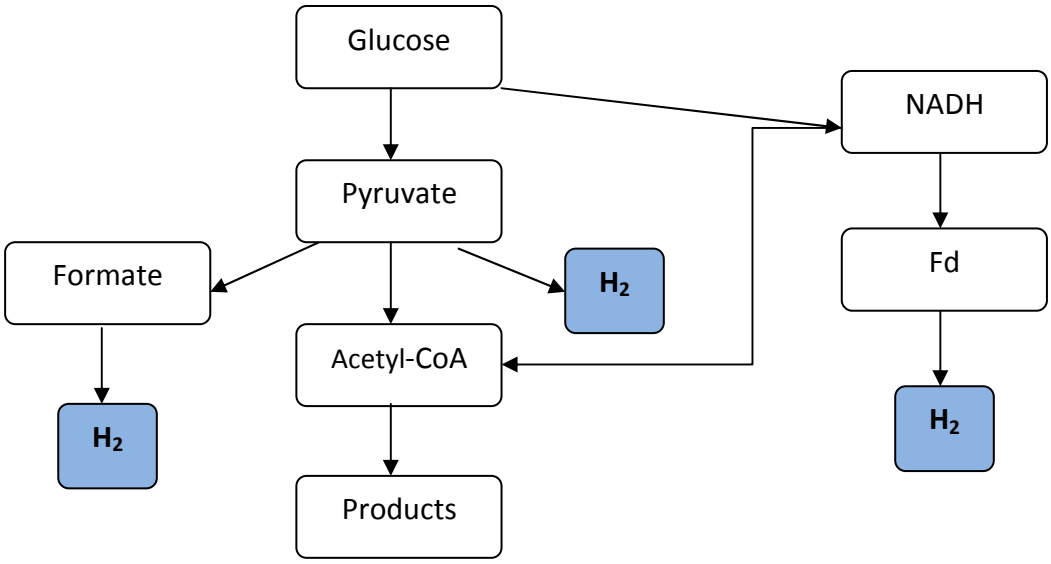


Figure 5: Representative pathways of fermentative hydrogen evolution.

The main pathway of glycolysis, that converts glucose into pyruvate, can be expressed as follows:

$$C_6H_{12}O_6 + 2NAD^+ \rightarrow 2CH_3COCOOH + 2NADH + 2H^+ \quad \text{Eq. 5}$$

NADH pathway:

The evolution of hydrogen through NADH pathway is driven by the necessity for reoxidizing the residual NADH of metabolic reactions as follows:

$$NADH + H^+ \rightarrow NAD^+ + H_2 \quad \text{Eq. 6}$$

Pyruvate pathway:

The pathway of pyruvate decomposition through acetyl-CoA produces formate as follows, by using the enzyme PFL (pyruvate/formate lyase):

$$CH_3COCOOH + HCoA \rightarrow CH_3CO\ CoA + HCOOH \qquad \text{Eq. 7}$$

In the next step, hydrogen is obtained by decomposition of formate (HCOOH) as follows:

$$HCOOH \rightarrow H_2 + CO_2 \qquad \text{Eq. 8}$$

Under most conditions during "enteric-type" (performed by species belonging to the order *Enterobacteriales*) fermentations, the degradation of formate is incomplete, because formate is only broken down to H_2 and CO_2 under acetic conditions and when intracellular formate concentrations are high.

The acetyl CoA gives rise to metabolites, such as acetate and ethanol, which is then excreted:

$$CH_3\ CO\ CoA + H_2O \rightarrow CH_3\ COOH + H\ CoA \qquad \text{Eq. 9}$$

$$CH_3\ CO\ CoA + 2NADH + 2H^+ \rightarrow CH3CH2OH + H\ CoA + 2NAD^+ \qquad \text{Eq. 10}$$

The oxidation of acetyl-CoA, as described in the equation 6, results in the reduction of ferredoxine (Fd). The reduced form of Fd(red) is oxidized by hydrogenase that regenerates Fd(ox) and transmittes electrons for the reduction of H_2. The complete reaction can be written as follows [Nath, 2004]:

$$Pyruvate + CoA + 2Fd(ox) \rightarrow Acetyl\text{-}CoA + 2Fd(red) + CO_2 \qquad \text{Eq. 11}$$

$$2H^+ + Fd(red) \rightarrow H_2 + Fd(ox) \qquad \text{Eq. 12}$$

If CO_2 is available, pyruvate can lead to the production of formate and succinate as follows:

$$CH_3COCOOH + CO_2 + NADH + H^+ \rightarrow 2HCOOH + H_2O + NAD^+ \qquad \text{Eq. 13}$$

$$CH_3COCOOH + CO_2 + 2NADH + 2H^+ \rightarrow 2CH_3COOH + H_2O + 2NAD^+ \qquad \text{Eq. 14}$$

From the above equations, it is understood that the production of succinate and formate should be decreased by some means to obtain high yield of hydrogen. Thus, if CO_2 is removed compulsorily form the culture liquid, the production of formate and succinate may be reduced. This will result in an increased hydrogen production, due to more availability of NADH [Das, 2001; Tanisho 1997].

The maximum stoichiometric hydrogen yield from the "enteric-type" fermentation might be 2 mole H_2 per mole of glucose, but in practice only half of the theoretical amount is observed [Hallenbeck, 2005]. Several reasons can explain this lower amount of H_2, for example the degradation of glucose that may involve different pathways without producing hydrogen, the partial consumption of glucose for construction of cellular biomass or the intracellular consumption of hydrogen for the synthesis of other metabolites [Woodward, 2000; Rittmann, 2008].

2.2 CHOICE OF A MICRO-ORGANISM FOR BIOHYDROGEN PRODUCTION

Microbial species from all domains of life are able to perform biohydrogen production. Prokaryotes that possess the ability to perform hydrogen production include strict anaerobes (clostridia, methylotrophs, rumen bacteria, archaea), facultative anaerobes (*Escherichia coli, Enterobacter* spp., *Citrobacter* spp.) and aerobes (*Alcaligenes* spp., *Bacillus* spp.) [Nandi, 1998].

A facultative organism may be a better choice for hydrogen production than a strict anaerob, because they are less sensitive towards oxygen and may restore hydrogen production after accidental oxygen contact with the system, by rapidly depleting oxygen in the broth [Oh, 2002; Rittmann, 2008]. *E. aerogenes*, which is used in the present work, is a gram-negative, rod-shaped and facultative anaerobic bacterium.

3. OBJECTIVES

The objectives of this work were the following:

- Establishment of a batch system for dark fermentative biohydrogen production
- Set up a repeated batch system for dark fermentative biohydrogen production
- Set up a continuous system for dark fermentative biohydrogen production
- Quantification of biohydrogen production using *Enterobacter aerogenes* DSM 30053 with a defined medium
- Comparison of product yields, rates and specific growth rates between using defined medium and complex medium, whereas results from dark fermentations using complex medium are obtained from literature
- Comparison of yields, rates and specific growth rates between:
    - different initial concentrations of glucose
    - glucose and xylose
    - different pH

The process for biohydrogen production had to be established. This comprised a sophisticated bioreactor environment with online, in-line and offline measurements and controls. Online and offline data were used to quantify the biological reactions. Data exploitation comprised the calculation of rates and yields for describing biohydrogen productivity.

4. MATERIAL AND METHODS

4.1 MICROORGANISM AND CULTURE CONDITION

4.1.1 Microorganism :

The facultative-anaerobic bacterial strain *Enterobacter aerogenes* DSM 30053 was used in the present work for all cultivations. Cryocultures were stored at -80°C in 2 ml cryo vials (VWR, Bohemia, NY, USA) in a final concentration of glycerol of 12,5% (v/v).

4.1.2 Preculture preparation:

For preculture preparation, a cryovial of *Enterobacter aerogenes* DSM 30053 was removed from -80°C and 10µl of bacterial suspension were transferred to an Erlenmeyer-flask containing medium by using an inoculation loop, which was extensively decontaminated by flaming using a Bunsen burner. The procedure was performed twice in a laminar flow chamber (Hermasafe, Thermo, Germany). Moreover, a negative control was also established by applying the same procedure as mentioned above, but without using suspension for inoculation. Afterwards all Erlenmeyer-flasks were incubated at 30°C on a rotary shaker with 170 rpm (Multitron, Infors, Switzerland) for 12 hours.

4.1.3 Cultivation conditions for fermentation using 1L Applikon bioreactor :

The cultivation conditions were as follows:

- Temperature: 30 °C
- N_2 flow: 0,1 l/min
- Agitator speed: Lag phase: 400 rpm

  Exponential phase: 800 rpm

4.1.4 Medium Preparation

The composition of the medium is given in Table 1. Medium was prepared according to Delisa et al., 1999 with minor modifications. (Addition of PPG against foam, changes of hydratations of some products because of availability in the lab) For preparation of 1L of medium, a stock solution of $MgSO_4$ (62 g L^{-1}) and trace elements (0,25 g $CoCl_2 \cdot 6H_2O$ L^{-1}; 1.5 g $MnCl_2 \cdot 4H_2O$ L^{-1}; 0.12 g $CuCL_2 \cdot 2H_2O$ L^{-1}; 0,3 g H_3BO_3 L^{-1}; 0,25 g $Na_2MoO_4 \cdot 2H_2O$ L^{-1}; 1,3 g $Zn(CH_3COO)_2$ $2H_2O$ L^{-1}; 10 g Fe^{III}citrate L^{-1}) were mixed in 100 ml of distilled water and sterilized for 20 min at 121°C.

KH_2PO_4 (13,3 g/l), $(NH_4)_2HPO_4$ (4 g/l), PPG (100 µl) and citric acid (1,7 g/l) were mixed in 770 ml of distilled water and sterilized in the bioreactor for 20 min at 121°C. Stock solutions of glucose (400 g/l), xylose (200 g/l) and EDTA (0.84 g/l) were sterilized separately. Thiamine (0.45 g/l) was sterilized by filtration, by using 0.2 µm sterile filters (ReZist, Whatman, Dassel, Germany). The pH of 6.8 was adjusted by using 2 M (80g/l) NaOH solution, which was autoclaved before application.

Table 1: Medium composition

Component	Batch Medium (per L)
Glucose/Xylose	5 - 40 g
KH_2PO_4	13.3 g
$(NH_4)_2HPO_4$	4.0 g
$MgSO_4 \cdot H_2O$	0.6 g
Citric acid	1.7 g
EDTA	8.4 mg
$CoCl_2 \cdot 6H_2O$	2.5 mg
$MnCl_2 \cdot 4H_2O$	15.0 mg
$CuCL_2 \cdot 2H_2O$	1.2 mg
H_3BO_3	3.0 mg
$Na_2MoO_4 \cdot 2H_2O$	2.5 mg
$Zn(CH_3COO)_2 \cdot 2H_2O$	13.0 mg
Fe^{III}citrate	100.0 mg
Thiamine · HCl	4.5 mg
PPG	100 µl

4.1.5 Microbiological quality assurance:

For investigation of culture pureness, strain *Enterobacter aerogenes* DSM 30053 was routinely streaked out on two agar plates prepared as indicated in appendix by using an thoroughly flamed inoculation loop, and withdrawal of approximately 10 µl of culture from each the Erlenmeyer flask. Incubation of cultures was performed in an incubation chamber (HT Infors AG, Bottmingen, Switzerland) over night. Afterwards single colonies were individually picked, streaked out on an object carrier with cover slip and investigated by using an inverse microscope (IX2-SLP, Olympus, Japan) with a total magnification of 1000. Moreover, fermentation culture was also investigated by using this procedure. Colony shape and coloring was not taken into account for colony pureness.

4.2 EXPERIMENTAL PROCEDURE

4.2.1 The bioreactor

A 1 L bioreactor (Applikon, The Netherlands), was used for all the fermentations. As shown in Figure 6, the reactor is equipped with an internal stirrer, a sparger for air/nitrogen and several ports, which could be used for sampling, feeding, or for base supply. The remaining ports were closed by applying a septum in order to be able to inoculate, and for injection of additional solutions, when required.

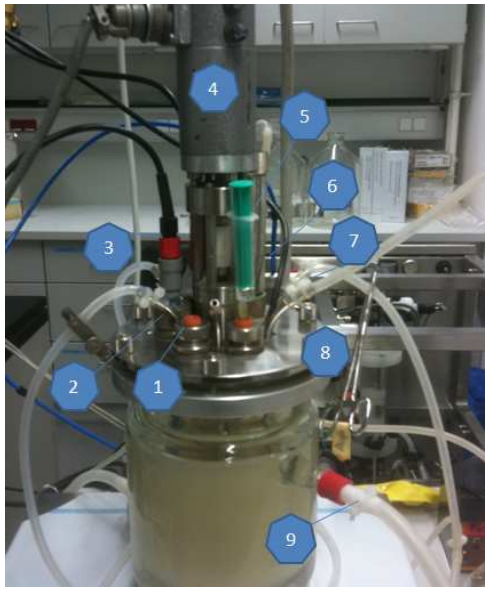


Figure 6: Bioreactor used for the fermentations: 1. Septum; 2. Base income; 3. pH probe; 4. Motor; 5. Syringe for injection; 6. Condenser; 7. Output for probes; 8. Air/nitrogen inlet; 9. Water outlet for the double vessel.

4.2.2 Bioreactor Set-up

First, the bioreactor was cleaned, and equipped with all probes, septa and tubes needed for fermentation. In order to keep the sterility inside the bioreactor, a 0,45 µl sterile filter was used on all ingas tubes. This kind of sterile filter was also used on the outgas tube, to assure that no bacteria were able to contaminate the environment. For autoclavation, every tube was closed by a horse clamp.

The bioreactor-condenser was connected to an off-gas outlet tube leading to an empty pressure-resistant bottle (Schott, Mainz, Germany) (used as before passing to the O_2/CO_2 gas analyzer system (DasGip, Bluesens, Germany) and the H_2 gas analyzer system (Bluesens, Germany). Before autoclavation, the pH probe was calibrated applying a two point calibration using calibration buffers of pH 7 and pH 4 (Hamilton Duracel Buffer, Bonaduz, Switzerland). The redox probe was calibrated also using a two point calibration, with redox calibration buffer of 220 mV and 180 mV (Mettler Toledo, Greifensee, Switzerland).

Finally, some elements of the medium, as explained in part 1.1.3, were transferred and autoclaved with the bioreactor for 20 minutes at 121°C. The autoclave used was an industrial autoclave (Zirbus, Germany).

After autoclaving and cooling of the bioreactor, the entire system was connected to the required analysis and detection devices. Parameters were adjusted to cultivation conditions.

The pH of the cultures was controlled by the automatic addition of NaOH (1M – 3M). The amount of NaOH solution added was determined gravimetrically by placing the NaOH containing pressure-resistant bottle on a balance (Sartorius, Germany).

The technical N_2 (Messer, Austria) inlet flow was maintained constant at 0.1 l/min by using a mass flow controller (4800-Series, Brooks Instruments, Hatfield, USA)

4.3 ANALYTICAL METHODS

4.3.1 Determination of biomass concentration

During all experiments, samples were taken in regular intervals, and biomass quantification was carried out in four individual tubes in parallel. To determine cell dry weights (CDW), 10 ml of culture medium was added to pre-weighted glass tubes, centrifuged (4000 rpm at 4°C for 20 min, centrifuge Sigma 3K30, rotor 11156). 2 ml of supernatant were removed carefully by aspiration and transferred to 2 ml of Eppendorf-tubes and concomitantly frozen at -20°C. Biomass pellets were resuspended in 5 ml of 4°C water using a Vortex (Genie2, VWR, Bohemia, NY, USA) for resuspension of cells. Biomass containing tubes were centrifuged at 4000 rpm for 10 minutes and the supernatant was discarded. The cell pellet washing procedure was performed twice. Following the second washing procedure the biomass containing glass tubes were transferred to a drying oven and stored inside for 72 hours on 105°C. Before being weighted on an analytical balance, dried biomass containing tubes were kept in a desiccator in order to prevent rehydration [SOP001, BioVT, TUWien].

4.3.2 Determination of the optical density (OD)

The optical density (OD) of each sample was measured at λ 600 nm in a spectrophotometer (HITACHI U-1100, Japan). Distilled water was used as a reference. When necessary, the samples were diluted with distilled water in order to measure within the linear range (ranging from $OD_{(600nm)}$ 0.2 to 0.8) [SOP001, BioVT, TUWien].

4.3.3 Gas Analyzer

Bioreactor off-gas was passed through two gas analyzers (GA-4 DasGip, Bluesens, Jühlich, Germany for CO_2 and Blusens,Jühlich, Germany for H_2). Data were recorded by using the Process and Information Management System Lucullus (Biospectra, Schlieren, Switzerland). For calibration of gas detection devices the protocols were followed precisely (please refer to relevant manuals). CO_2 analyser was calibrated between 0.04 % (with air) and 7 % (with check gas). H_2 analyser was calibrated between 0 % (nitrogen) and 50 % (mixture 50% N_2 / 50% H_2).

4.3.4 Quantification of substrates and metabolites

Acetate

Acetate acid produced during the fermentation by *Enterobacter aerogenes* DSM 30053 was measured by using an enzymatic assay for spectrophotometric detection using CuBiAn XC (Innovatis, Germany)(Figure 7). For detection of acetate in supernatant of fermentation samples, 1 ml of was taken and centrifuged in a centrifuge (Eppendorf 5804R, Germany) at 4°C and 10000 rpm, to make sure that no cellular debris would interfere in spectrophotometric measurement.

Figure 7: Picture of the CuBiAn XC analyzer. It is a compact version of a benchtop random access biochemistry analyzer (Innovatis, Bielefeld, Germany). It is applied for enzymatic and photometric assays.

Ethanol, Glucose, Lactate

HPLC analysis was performed in order to quantify the ethanol, glucose and lactate concentrations. All measurements were performed on an Agilent 1100 HPLC device (Agilent Technologies Incorporation, Santa Clara, USA) with DAD (Diode Array Detection) and RID (Refractive Index Detection) detectors and a Supelcogel column C-610H (Sigma-Aldrich Corporation, St Louis, MO, USA) 0.1% H_3PO_4 was used as the mobile phase at a flow rate of 0,3 mL/min and a temperature of 41°C.

4.4 DATA EXPLOITATION

4.4.1 Batch culture fermentation

Mass flow controllers were justified on gasflow rates in normliters per minute, but our gas was used at standard termperatures, so adaption of flow rates was necessary. This was performed by using the equation given below:

$$H_2(cor)[\%] = H_2\,[\%] - (0{,}107 \cdot CO_2\,offgas\,[\%]) \qquad \text{Eq. 15}$$

The rates were calculated as follows:

Please note that these calculations are valid in case of batch mode and constant working volumes.

$$r_s\left[\frac{C-mmol}{l\cdot h}\right] = \frac{\Delta glu\,[\frac{g}{l}]}{\Delta t\,[h]} \cdot \frac{1000}{MMs\,[\frac{g}{C-mol}]} \qquad \text{Eq. 16}$$

$$r_x\left[\frac{C-mmol}{l\cdot h}\right] = \frac{\Delta biomasse\,[\frac{g}{l}]}{\Delta t\,[h]} \cdot \frac{1000}{MMx\,[\frac{g}{C-mol}]} \qquad \text{Eq. 17}$$

$$r_{base}\left[\frac{mmol}{l\cdot h}\right] = \frac{\Delta base[g]}{\Delta t\,[h]} \cdot \frac{1000}{V_R \cdot MMbase\,[\frac{g}{mol}]} \qquad \text{Eq. 18}$$

$$r_{Hac}\left[\frac{C-mmol}{l\cdot h}\right] = \frac{\Delta Hac[g/l]}{\Delta t\,[h]} \cdot \frac{1000}{MMhac\,[\frac{g}{C-mol}]} \qquad \text{Eq. 19}$$

$$r_{EtOH}\left[\frac{C-mmol}{l\cdot h}\right] = \frac{\Delta EtOH[g/l]}{\Delta t\,[h]} \cdot \frac{1000}{MMetoh[\frac{g}{C-mol}]} \qquad \text{Eq. 20}$$

$$r_{inert}\,[-] = \frac{100}{(100-CO_2-H_2-H_2O)} \qquad \text{Eq. 21}$$

Carbon dioxide evolution rate (CER) and Hydrogen evolution rate (HER) were calculated as follow:

$$CER\left[\frac{C-mmol}{l\cdot h}\right] = \frac{Air_{IN}\left[\frac{l}{min}\right]\cdot 60\left[\frac{min}{h}\right]\cdot CO_2[\%]\cdot r_{inert}[-]\cdot 1000\,[\frac{mmol}{mol}]}{22{,}41\left[\frac{l}{min}\right]\cdot 100\cdot V_R\,[l]} \qquad \text{Eq. 22}$$

$$HER\left[\frac{mmol}{l\cdot h}\right] = \frac{Air_{IN}\left[\frac{l}{min}\right]\cdot 60\left[\frac{min}{h}\right]\cdot H_2[\%]\cdot r_{inert}[-]\cdot 1000\,[\frac{mmol}{mol}]}{22{,}41\left[\frac{l}{min}\right]\cdot 100\cdot V_R\,[l]} \qquad \text{Eq. 23}$$

The yields were calculated as follows:

$$Y_{H_2/CO_2}[\frac{mol}{mol}] = \frac{HER\,[\frac{mmol}{l \cdot h}]}{CER[\frac{mmol}{l \cdot h}]} \quad \textbf{Eq. 24}$$

$$Y_{H_2/s}\,[\frac{mol}{C-mol}] = \frac{HER\,[\frac{mmol}{l \cdot h}]}{r_S[\frac{C-mmol}{l \cdot h}]} \quad \textbf{Eq. 25}$$

$$Y_{Base/x}[\frac{mol}{C-mol}] = \frac{r_{base}\,[\frac{mmol}{l \cdot h}]}{r_x\,[\frac{C-mmol}{l \cdot h}]} \quad \textbf{Eq. 26}$$

$$Y_{x/s}[\frac{C-mol}{C-mol}] = \frac{r_x\,[\frac{C-mmol}{l \cdot h}]}{r_S[\frac{C-mmol}{l \cdot h}]} \quad \textbf{Eq. 27}$$

The carbon recovery was calculated as follows:

$$C - balance = \frac{CER + r_x + r_{Hac} + r_{EtOH}}{r_S} \quad \textbf{Eq. 28}$$

4.4.2 Continuous fermentations

The law of the conservation of the mass can be expressed for a dynamic mass balance where biological reactions, such as the following ones, may occur:

$$\begin{pmatrix}\text{Rate of}\\ \text{accumulation}\\ \text{of mass}\\ \text{in the system}\end{pmatrix} = \begin{pmatrix}\text{Rate of}\\ \text{mass}\\ \text{flow}\\ \text{in}\end{pmatrix} - \begin{pmatrix}\text{Rate of}\\ \text{mass}\\ \text{flow}\\ \text{out}\end{pmatrix} \pm \begin{pmatrix}\text{Rate of}\\ \text{production or consumption}\\ \text{of the component}\\ \text{by reaction}\end{pmatrix} \quad \text{Eq. 29}$$

$$\frac{d(c\cdot V)}{dt} = F_{in}\cdot c_{in} - F_{out}\cdot c_{out} + r\cdot V \quad \text{Eq. 30}$$

Assumptions:

1) *Rate of accumulation = 0*
2) *C_{out}(substrate) = 0*

That gives the following rates:

$$r_s\left[\frac{C-mmol}{l\cdot h}\right] = \frac{c_{in}\cdot F_{in}}{V_R} = \frac{Feedrate\left[\frac{g}{l}\right]\cdot c\left[\frac{g}{l}\right]}{V_R\,[l]}\frac{1000\left[\frac{mmol}{mol}\right]}{30\left[\frac{g}{C-mol}\right]}\cdot\frac{1}{1000\left[\frac{g}{l}\right]} \quad \text{Eq. 31}$$

$$PHI_{base}\left[\frac{g}{h}\right] = -\frac{\Delta base[g]}{\Delta t\,[h]} \quad \text{Eq. 32}$$

$$r_x\left[\frac{C-mmol}{l\cdot h}\right] = \left(\frac{\left(PHI_{Base}\left[\frac{g}{h}\right]+Feedrate\left[\frac{g}{h}\right]\right)\cdot c_x\left[\frac{g}{l}\right]}{V_R\,[l]\cdot MWx\left[\frac{g}{C-mol}\right]}\right)\left(\frac{1000\left[\frac{mmol}{mol}\right]}{1000\left[\frac{g}{l}\right]}\right) \quad \text{Eq. 33}$$

$$r_{Hac}\left[\frac{C-mmol}{l\cdot h}\right] = \left(\frac{\left(PHI_{Base}\left[\frac{g}{h}\right]+Feedrate\left[\frac{g}{h}\right]\right)\cdot c_{Hac}\left[\frac{g}{l}\right]}{V_R\,[l]\cdot MW_{Hac}\left[\frac{g}{C-mol}\right]}\right)\left(\frac{1000\left[\frac{mmol}{mol}\right]}{1000\left[\frac{g}{l}\right]}\right) \quad \text{Eq. 34}$$

$$r_{EtOH}\left[\frac{C-mmol}{l\cdot h}\right] = \left(\frac{\left(PHI_{Base}\left[\frac{g}{h}\right]+Feedrate\left[\frac{g}{h}\right]\right)\cdot c_{EtOH}\left[\frac{g}{l}\right]}{V_R\,[l]\cdot MW_{EtOH}\left[\frac{g}{C-mol}\right]}\right)\left(\frac{1000\left[\frac{mmol}{mol}\right]}{1000\left[\frac{g}{l}\right]}\right) \quad \text{Eq. 35}$$

$$r_{base}\left[\frac{mmol}{l\cdot h}\right] = \left(\frac{\left(PHI_{Base}\left[\frac{g}{h}\right]\right)}{V_R\,[l]\cdot MW_{Base}\left[\frac{g}{mol}\right]}\right)\cdot\left(1000\ \left[\frac{mmol}{mol}\right]\right) \quad \text{Eq. 36}$$

$$r_{inert}\,[-] = \frac{100}{(100-CO_2-H_2-H_2O)} \quad \text{Eq. 37}$$

Carbon dioxide evolution rate (CER), Hydrogen evolution rate (HER), and all the yield were calculated in the same way than in the calculations of the batch cultures.

The carbon recovery was calculated as follows:

$$C-balance = \frac{r_x+r_{Hac}+r_{EtOH}+CER}{r_S} \quad \text{Eq. 38}$$

5. RESULTS

Many publications report the ability of microbial strains to produce biohydrogen. However, hydrogen production is always accomplished by using complex medium. In this work, hydrogen production by *Enterobacter aerogenes* DSM 30053 is performed using a defined medium (please refer to chapter 4.1.4). Quantification of experimental data and comparison with results obtained from literature using complex medium is performed. Biohydrogen production of rates, yields, specific productivity, and volumetric productivity are expressed in different units by researchers, which does not make it easy to quickly compare different results between different articles. In the following part of this bachelor thesis, all results obtained during the current work are reported with the units that were given in chapter 4, in order to allow comparability towards publications from the other working groups.

The results can be divided into three different parts. The first part demonstrates the suitability of *E. aerogenes* DSM 30053 using defined medium to produce hydrogen in batch mode. In the second part results from repetitive batch fermentation are shown. In the third part results from continuous fermentations are presented.

5.1 SUITABILITY OF THE DEFINED MEDIUM TO PRODUCE HYDROGEN WITH ENTEROBACTER AEROGENES DSM 30053

In the present work a defined medium, as described by Delisa et al., 1999, has been used for all fermentations. A batch fermentation of *E. aerogenes* DSM 30053 glucose (20 g/l) as substrate is shown in Figure 8.

As shown in Figure 8, hydrogen may effectively be produced by *E. aerogenes* DSM 30053 by using defined medium described in part 4.1.4. This figure shows that more CO_2 than H_2 was produced, especially at the end the exponential phase of the batch. Production of acetate and ethanol were also measured.

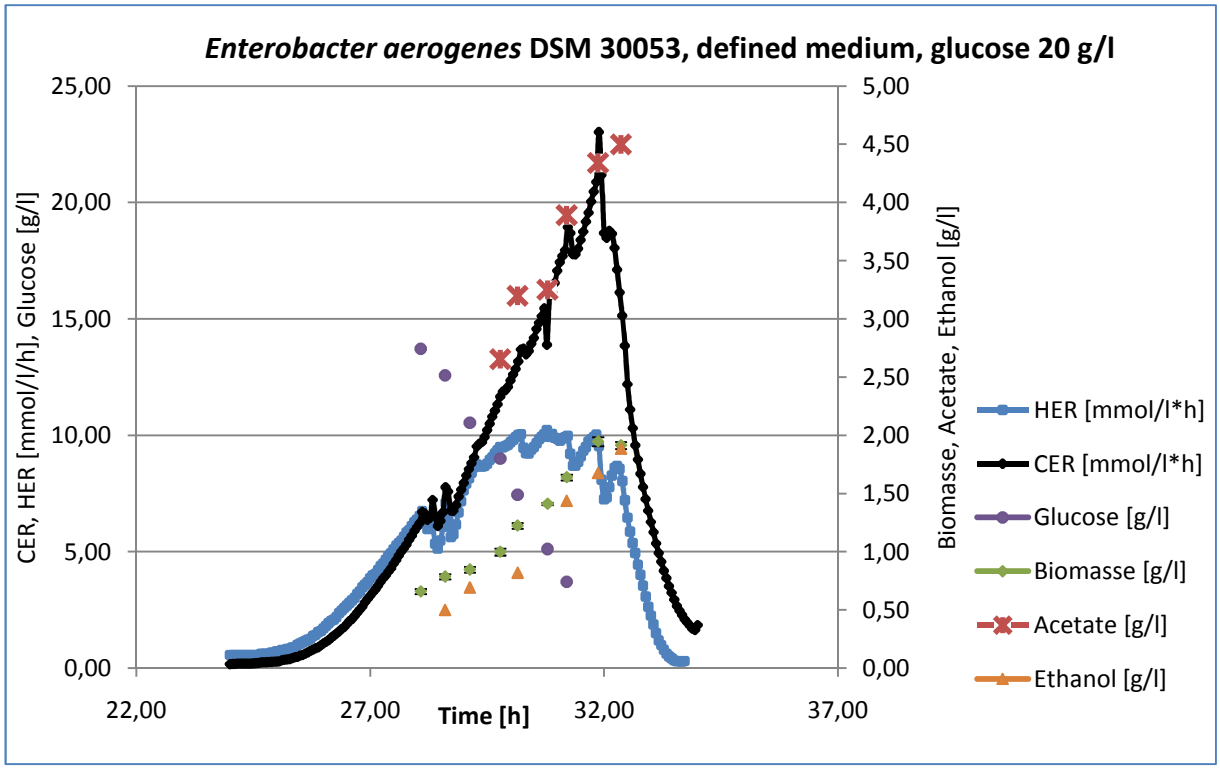


Figure 8: Batch process with *E. aerogenes* DSM 30053, using glucose 20 g/l as substrate

Analysis of elementary composition of *E. aerogenes* DSM 30053 biomass was performed at the Universität Wien, Fakultät für Chemie, Mikroanalytisches Labor, Vienna, Austria (please refer to table 1). This sample was taken from a continuous culture fermenting glucose (20 g/l). This biomass composition has been used for calculation of the molecular weight.

Table 2: Elementar analysis of biomass:

Element	C	H	N	O	Ashes	Biomass
[%]	46,72	7,07	12,76	28,55	3	12,65 g/mol
[C-mol]	1,00	0,15	0,27	0,61	0,10	**25,78 g/C-mol**

5.2 HYDROGEN PRODUCTION BY *ENTEROBACTER AEROGENES* DSM 30053 IN BATCH MODE:

5.2.1 Effect of initial glucose concentration:

The effect of different glucose concentrations on biohydrogen production using *E. aerogenes* DSM 30053 was studied. The signals obtained by online and in-line measurements were recorded by the PIMS (process and information management system) Lucullus. Different repetitive batch experiments are shown in Figure 9.

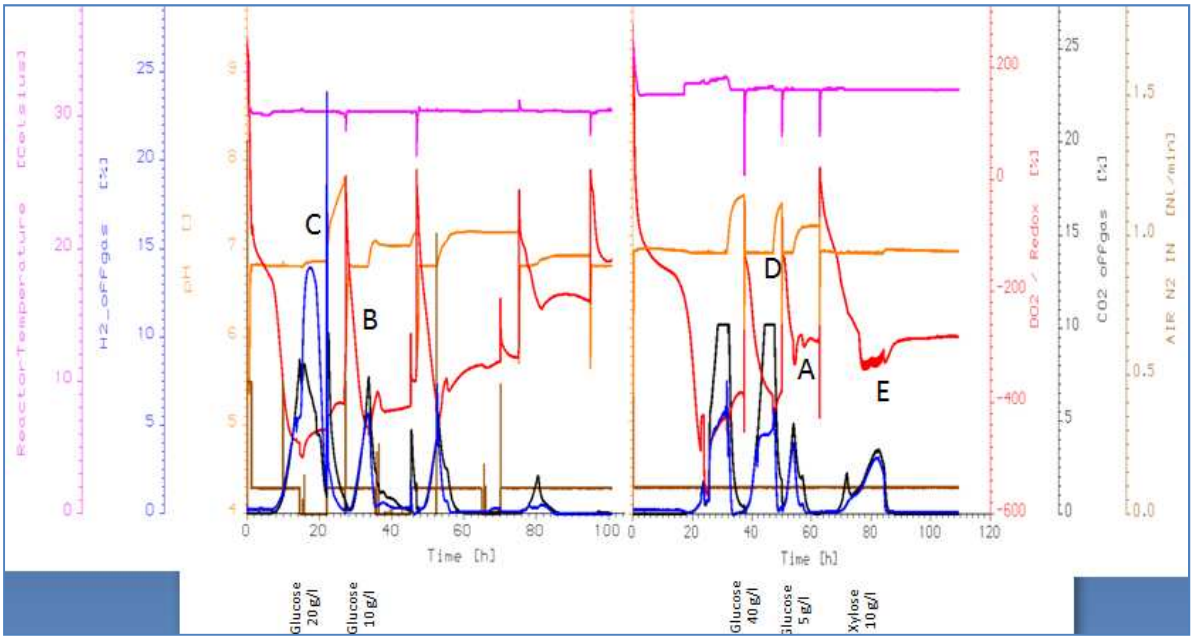


Figure 9: Signals from PIMS, for two series of repeated batches using different initial glucose concentrations or xylose. Graph coloring: blue: H_2 offgas [%], black: CO_2 offgas [%], red: Redox [mV], brown: N_2 ingas flow [l/min], orange: pH [-], purple: temperature [°C]. Peaks: A: glucose 5 g/l, B: glucose 10 g/l, C: glucose 20 g/l, D: glucose 40 g/l, E: xylose 10 g/l.

Online and in-line data obtained from distinct and marked peaks from Figure 9 are presented separately in Figure 10, as well as showing offline data obtained by HPLC and enzymatic measurements by using CuBiAn (glucose, ethanol and acetate) and biomass concentration.

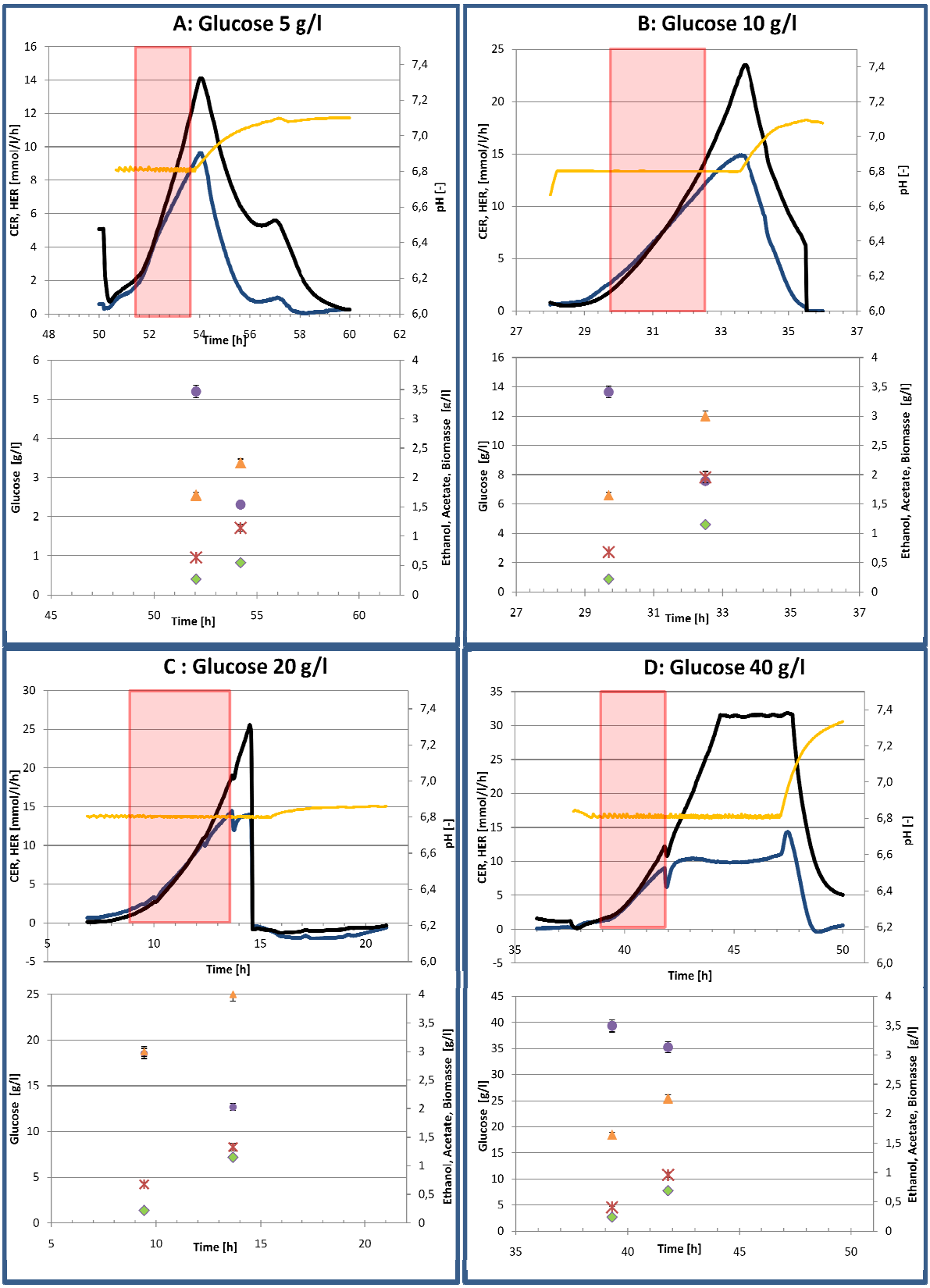


Figure 10: Repeated batch process with *E. aerogenes* DSM 30053, using different glucose concentrations as substrate. Purple: glucose [g/l], orange: ethanol [g/l], red: acetate [g/l], green: biomass [g/l], blue: HER, black CER, yellow: pH. A: Glucose 5 g/l, B: Glucose 10g/l, C: Glucose 20 g/l, D: Glucose 40 g/l.

As shown in the upper graphs of figure 10 as well as in Figure 9, at the end of each exponential phase, the pH increased, as a result of acid utilization by *E. aerogenes* DSM 30053 until new substrate is applied.

Batches A and B are very similar, with only a difference of height and area.

In the batch C, H_2% offgas peak is higher than CO_2 % offgas. During this batch C, N_2 sparging stopped. The big H_2 peak (batch C) can be explained by an accumulation of hydrogen in the bioreactor, and a modification of gas composition occurred, which may be due to the lack of N_2.

In the batch D, the CO_2 offgas could not be measured in total, because of saturation of the CO_2 analyzer. The shape of the H_2 peak, showing a low ascending slope, may be due to a high concentration of CO_2 offgas that may result in a decrease of hydrogen production by CO_2 inhibition [Tanisho, 1997].

Batch E, a fermentation using xylose as substrate, will be discussed in chapter 5.2.2.

Product yields, hydrogen evolution rate and maximum specific growth rate have been calculated using the formula given in chapter 4.4.1 Results for the repeted batches using glucose are shown in the table 3.

Figure 11 shows comparisons of hydrogen yield. These results show that these four experiments with different initial substrate concentration all resulted in biohydrogen production. Among the different concentrations of glucose, the batch with 20 g/l of glucose showed the maximum yield H_2/CO_2 with 0.93 mol H_2/mol CO_2. This substrate concentration also showed the best yield H_2/Glu with 0.17 mol H_2/mol glu. If, in both cases, results show no significant differences, however, these results show a tendency for the 20 g/l concentration to be the best one for the yields of H_2.

Table 3: Comparison of the hydrogen production performance on different concentrations of glucose

Parameters	Units	Glucose 5 g/l	Glucose 10 g/l	Glucose 20 g/l	Glucose 40 g/l
Y H_2/CO_2	mol/C-mol	0,77	0,86	0,93	0,86
Y CO_2/glu	C-mol/C-mol	0,21	0,11	0,18	0,11
Y H_2/glu	mol/C-mol	0,16	0,09	0,17	0,09
Y Hac/glu	C-mol/C-mol	0,18	0,14	0,11	0,14
Y x/glu	C-mol/C-mol	0,11	0,13	0,18	0,13
Y EtOH/glu	C-mol/C-mol	0,25	0,20	0,22	0,20
Y base/glu	mol/C-mol	3,48	2,89	3,63	2,89
qH_2	mmol/g/h	25,23	11,55	8,49	11,40
H_2 productivity	mmol/l/h	7,06	11,89	7,9	5,13
µ	1/h	0,49	0,51	0,54	0,61
Carbon recovery	%	74	89	71	57
Batch duration	h	8	6,5	8	13

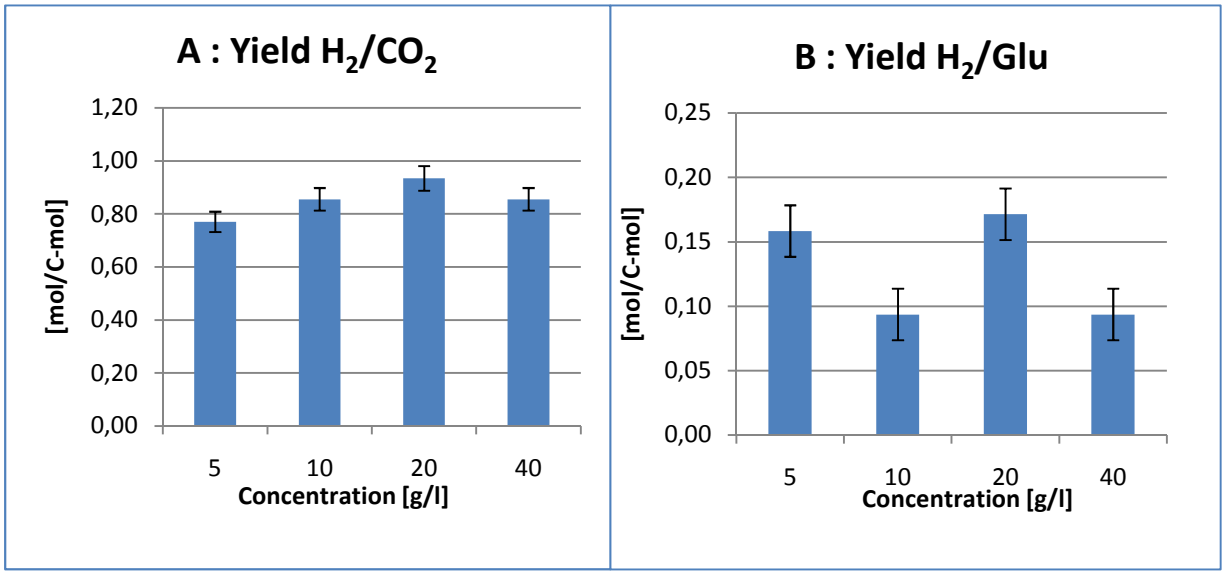


Figure 11: Comparison of yields of H_2. A: Yield H_2/CO_2 ; B: Yield H_2/Glu

Yields of the other products as shown in figure 12, are depicting that 20 g/l initial glucose concentration results in enhanced yield of biomass $Y_{X/Glu}$ and in a low yield of $Y_{Hac/glu}$. Yields $Y_{CO2/glu}$ and $Y_{EtOH/glu}$ seem to decrease among the augmentation of glucose concentrations. Specific H_2 production rate (q_{H2}) shows the best result (25,23 mmol H_2/gDW*h) for the initial substrate concentration of 5 g/l of glucose (see table 3).

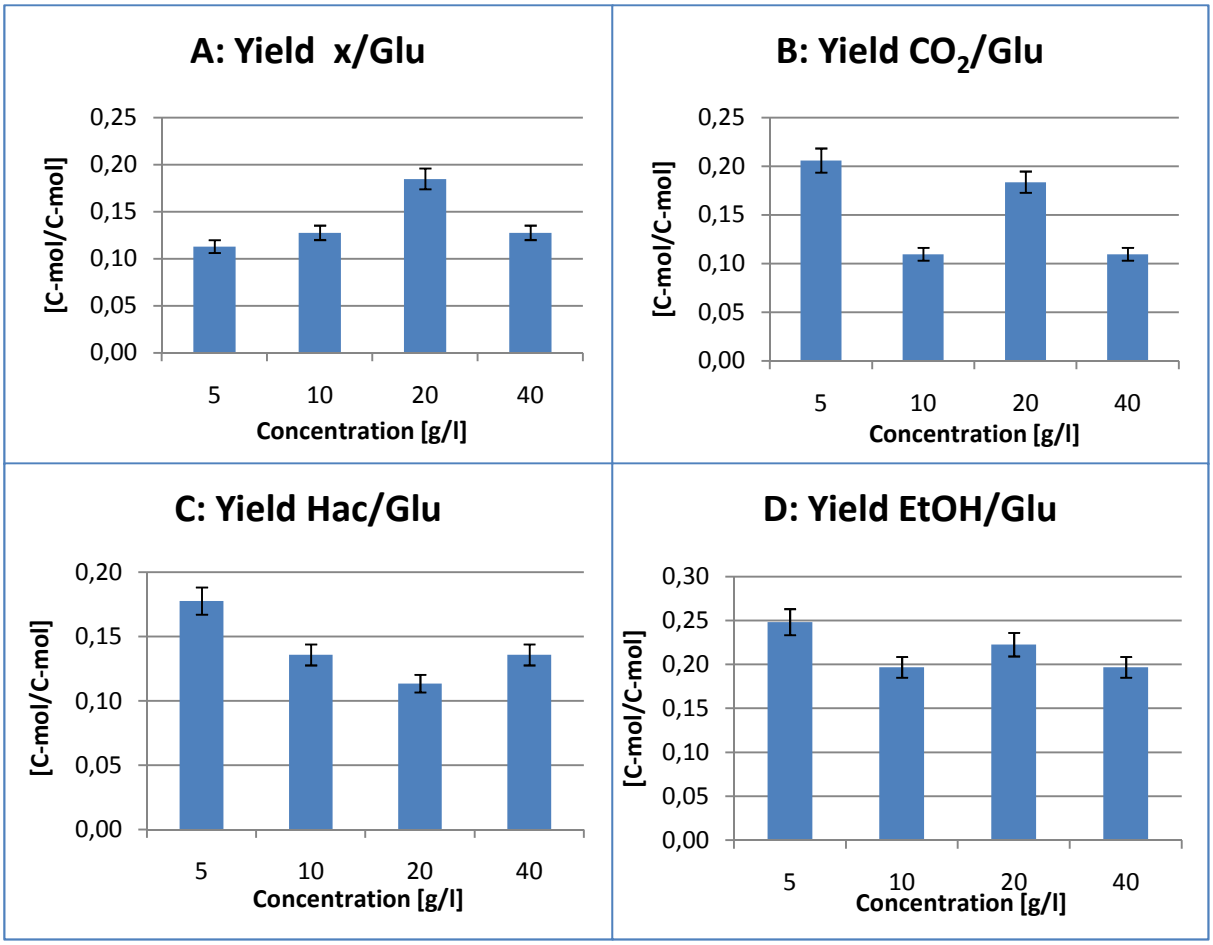


Figure 12-Comparison of yields for different products as a function of the initial substrate concentration. A: Yield x/Glu ; B: Yield CO_2/Glu ;C: Yield Hac/Glu; D: Yield EtOH/Glu

In these batches, C-balances were not closing (see table 3). Hydrogen, carbon dioxide, acetate, ethanol and lactate were identified as fermentation products. Lactate was qualitatively identified. Chromatograms (figure 13) showed peaks for products of glucose fermentation by *E. aerogenes* DSM 30053 that have not been identified yet.

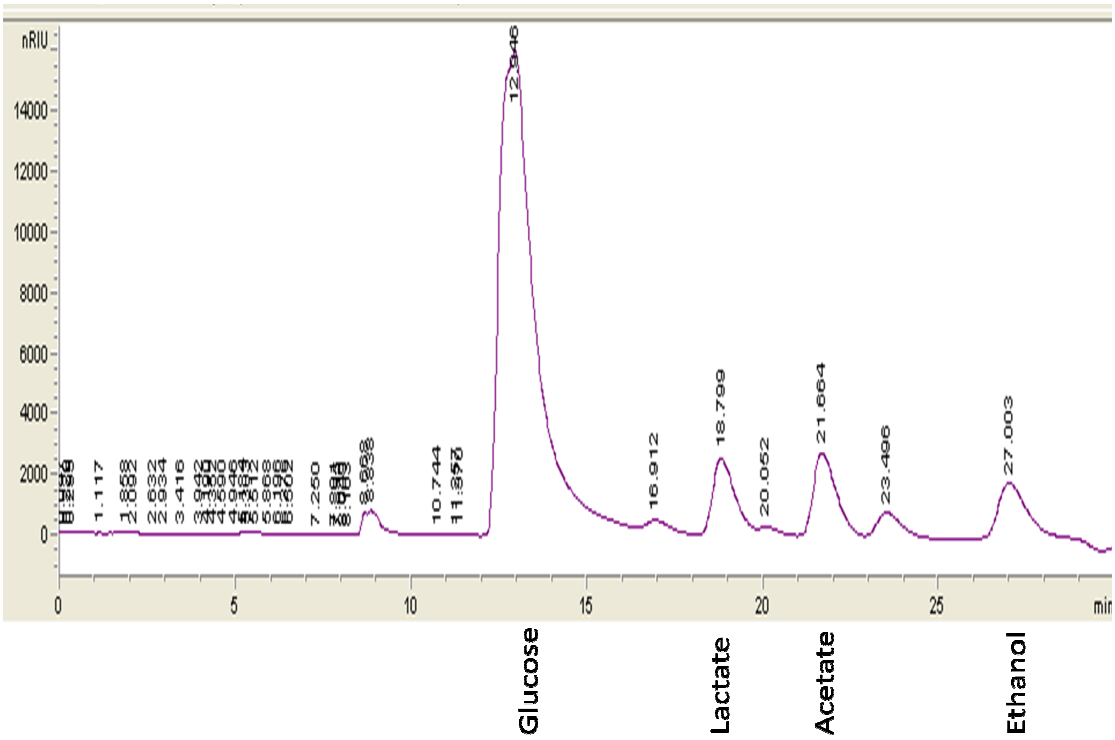


Figure 13: Chromatogram of supernatant derived from a repeated batch on glucose with an initial glucose concentration of 10 g/l. Glucose and ethanol were measured with HPLC, Acetate was measured with CuBiAn, Lactate was identified with HPLC.

The sensitivity and the linear response range were checked, therefore a calibration was established.

According to the Figure 14, the linear relation between the area and the concentration was maintained in the whole measurement range of ethanol. No peak was detected with a 0 g/l concentration of ethanol; the calibration was forced to zero.

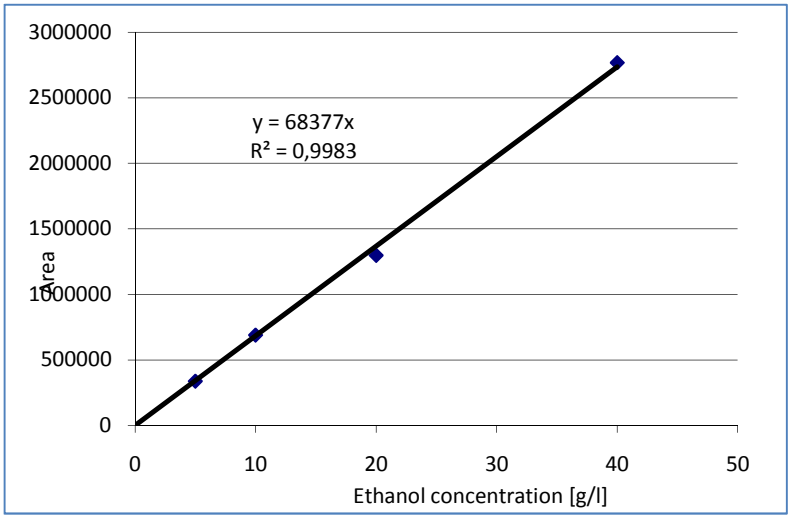


Figure 14: Ethanol calibration with aqueous standards. Concentration values: 0; 5; 10; 20; 40 g/l.

According to the Figure 15, the linear relation between the area and the concentration was maintained in the whole measurement range of glucose. No peak was detected with a 0 g/l concentration of glucose; the calibration was forced to zero.

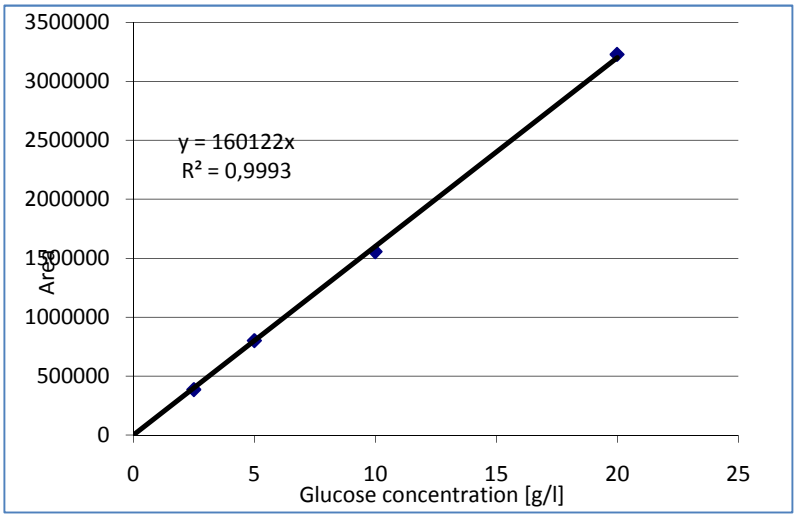


Figure 15: Glucose calibration with aqueous standards. Concentration values: 0; 2,5; 5; 10; 20 [g/l].

5.2.2 Comparison yields and physiological response of *E. aerogenes* DSM 30053 towards an initial glucose concentration and initial xylose concentration

The effect of a different substrate on biohydrogen production using *E. aerogenes* DSM 30053 was studied. Online and in-line data obtained from distinct and marked peaks are presented separately in figure 16 as well as showing offline data obtained by HPLC and enzymatic measurements by using CuBiAn (glucose, ethanol and acetate) and biomass concentration. A main difference between these two batches was the duration of the batches: The duration of the batch with glucose 10 g/l was 6,5 h. The duration of the batch with xylose 10 g/l was 16 h.

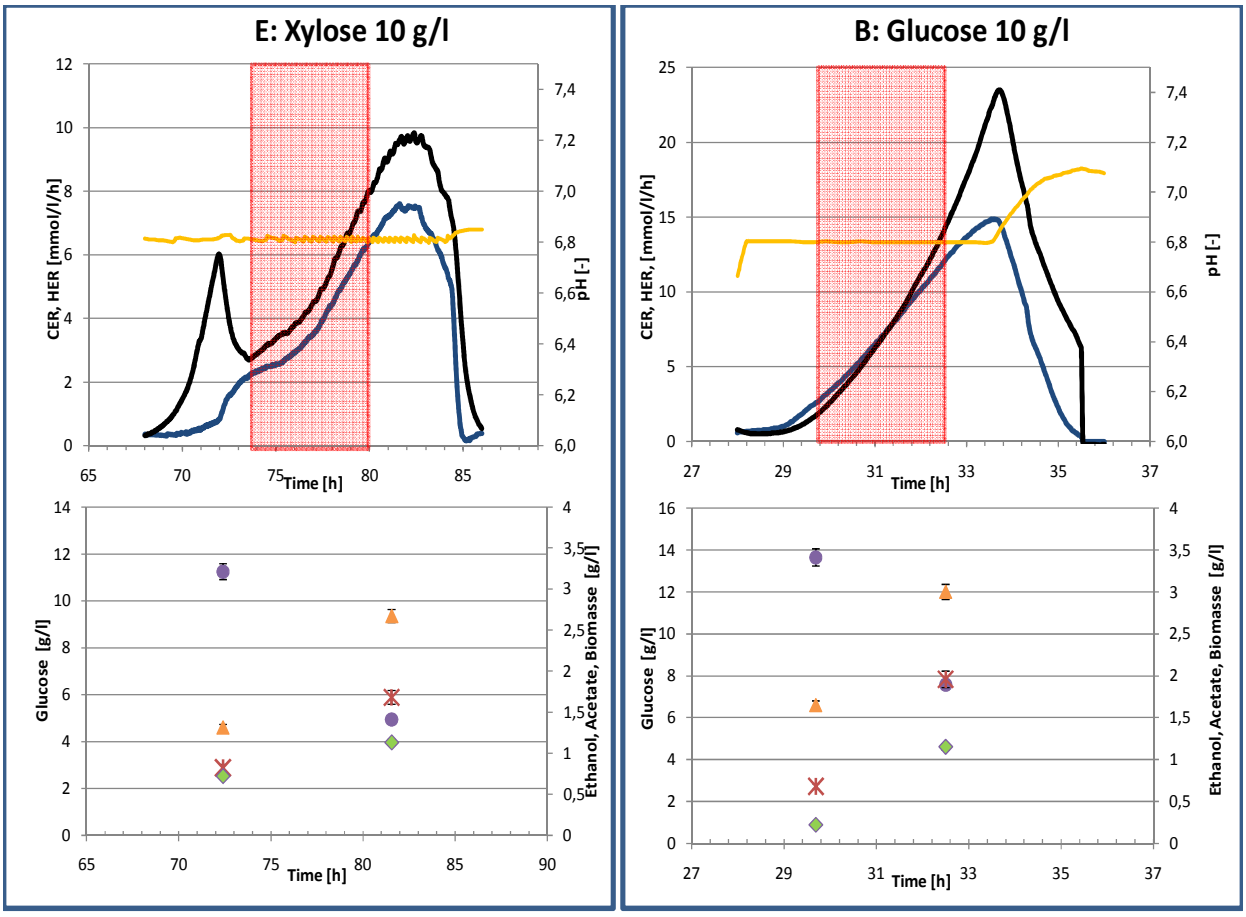


Figure 16: Repeated batch process with *E.aerogenes* DSM 30053, using two different substrates. The upper graphs show CER, HER and pH data. The down graphs show glucose, ethanol, acetate and biomass concentrations.

Peaks: E: Xylose 10 g/l, B: Glucose 10g/l. Purple: glucose [g/l], orange: ethanol [g/l], red: acetate [g/l], green: biomass [g/l], blue: HER, black CER, yellow: pH.

Product yields, hydrogen evolution rate and maximum specific growth rate for the repeated batches using glucose 10 g/l and xylose (10 g/l) are shown in the Table 4. As a result of the long duration of the batch with xylose, these results show that the batch with 10 g/l glucose gave better results for qH_2, with 11,55 mmol/l/h and for H_2 productivity, with 11,89 mmol/l/h. Also μ is more than two times higher with the batch using glucose, with 0,51 h^{-1}.

All results shown in table 4 were calculated from data obtained from graphs of the red window shown in figure 16.

Table 4: Comparison of the hydrogen production performance on two different substrates.

Parameters	Units	Xylose 10 g/l	Glucose 10 g/l
Y H_2/CO_2	mol/C-mol	0,78	0,86
Y CO_2/glu	C-mol/C-mol	0,23	0,11
Y H_2/glu	mol/C-mol	0,18	0,09
Y Hac/glu	C-mol/C-mol	0,14	0,14
Y x/glu	C-mol/C-mol	0,08	0,13
Y EtOH/glu	C-mol/C-mol	0,28	0,20
Y base/glu	mol/C-mol	2,21	2,89
qH_2	mmol/g/h	9,96	11,55
H_2 productivity	mmol/l/h	4,08	11,89
μ	1/h	0,2	0,51
Carbon recovery	%	72	89
Batch duration	h	6,5	16

Among the different substrates, the batch with 10 g/l of glucose showed the maximum yield Y H_2/CO_2 with 0,86 mol H_2/ mol CO_2. However, the batch with 10g/l xylose showed the best yield Y H_2/s with 0,18 mol H_2/ mol substrate (see figure 17).

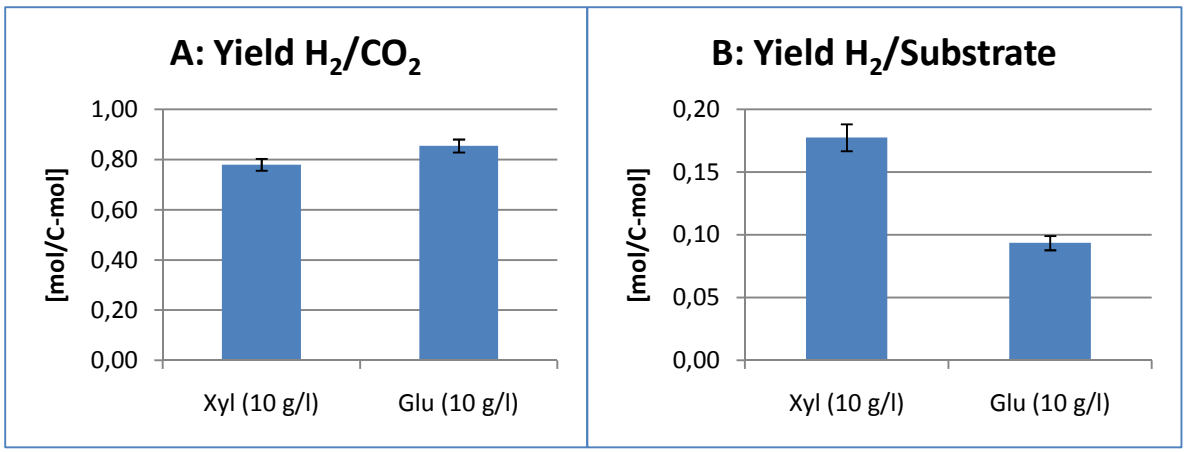


Figure 17: Comparison of the yields of hydrogen, among two differents substrates: glucose (10g/l) and xylose (10g/l). A: Yield H_2/CO_2 ; B: Yield H_2/Substrate.

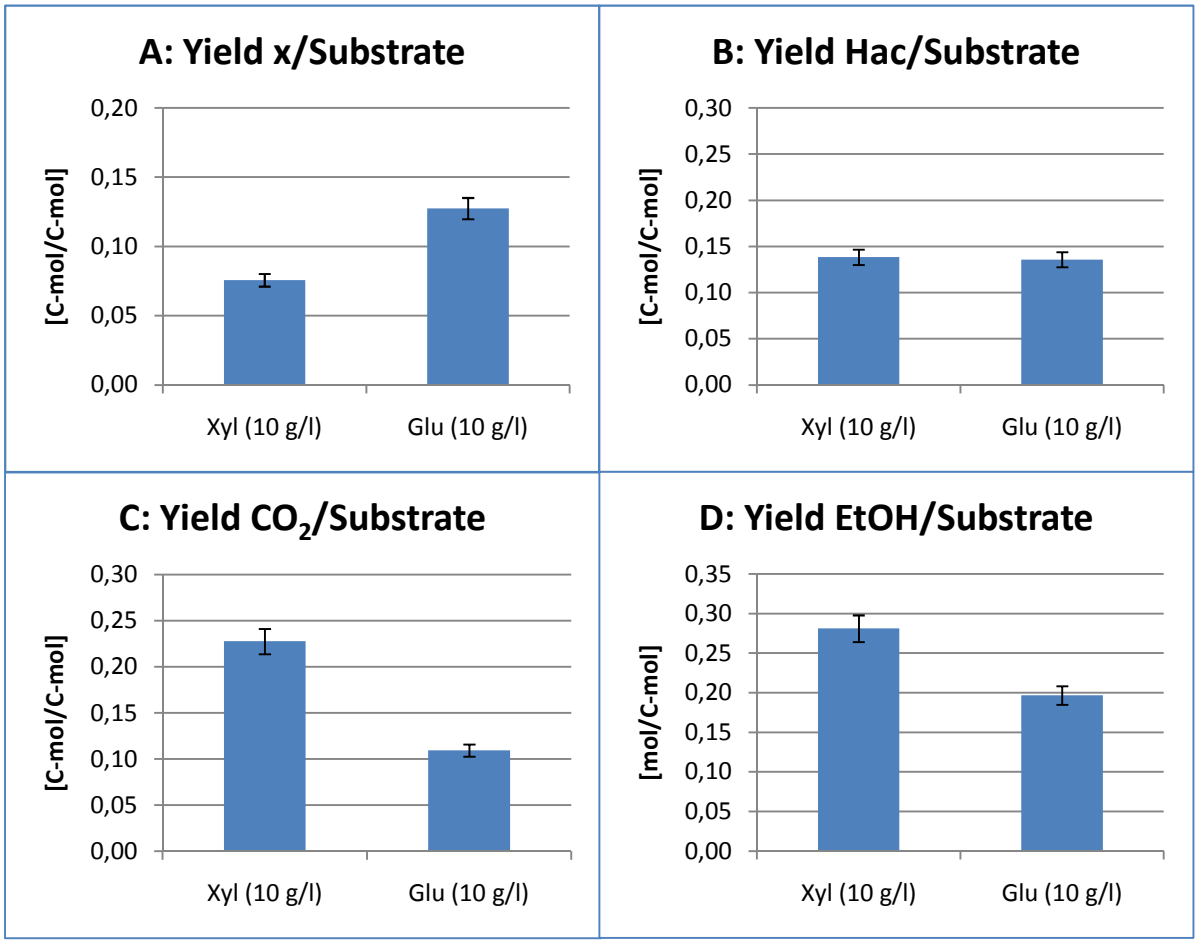


Figure 18: Comparison of yields for different products as a function of the different substrates.

5.3 HYDROGEN PRODUCTION IN CONTINUOUS CULTURE

5.3.1 Effect of pH

The effect of different pH on biohydrogen production using *E. aerogenes* DSM 30053 in continuous culture was studied using defined medium described in chapter 4. Product yields, hydrogen evolution rate and maximum specific growth rate have been calculated using the formulas given in chapter 4.4.2. Results for the continuous culture process using glucose are shown in the Table 5.

Table 5: Comparison of the hydrogen production performance on continuous culture.

Parameters	Units	pH 6,4	pH 6,6	pH 6,7	pH 6,8	pH 6,8
YH2/CO2	mol/mol	0,37	0,48	0,82	0,70	0,61
Y H2/glu	mol/C-mol		0,10	0,17	0,14	0,12
Y CO2/glu	mol/C-mol		0,19	0,20	0,20	0,20
Ybase/biomass	mol/C-mol		15,75	62,86	15,51	27,31
Yeth/s	C-mol/C-mol		0,16	0,15	0,20	0,15
YHAc/s	C-mol/C-mol		0,21	0,29	0,23	0,25
Y x/s	C-mol/C-mol		0,13	0,13	0,15	0,14
Y base/x+Hac	C-mol/C-mol		6,19	18,95	6,33	9,64
Y base/Hac	C-mol/C-mol		10,20	27,13	11,93	14,90
qCO2	mmol/(g*h)	15,67	15,23	7,10	6,74	14,44
qH2	mmol/(g*h)	5,73	7,30	5,79	4,73	8,85
H2 productivity	mmol/l/h	15,62	16,15	10,60	11,92	19,45
C-Balance	%		70	78	78	73
DoR balance	%		63	074	76	68
D	1/h	0,25	0,25	0,10	0,13	0,25

As in the case of repeted batches, the C-balances were not completed. This will be discussed in the chapter 6.

Effect of pH was studied with a dilution rate D of 0,25 h^{-1}. As shown in Figure 19, the best specific H2 production, 8,85 mmol/g/h was obtained with a pH of 6,8. The lower value of specific CO2 production, 14,44 mmol/g/h, was also obtained with a pH of 6,8. These results also show a tendency to have less CO2 and more H2 production with an increasing pH.

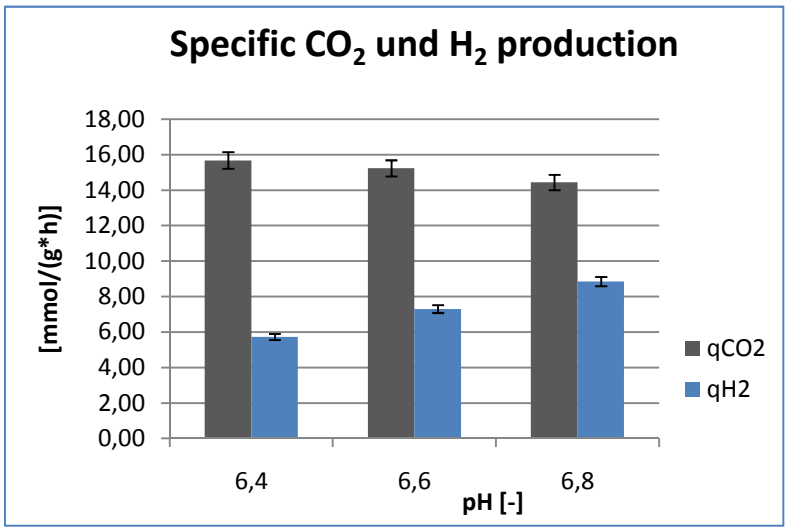


Figure 19: Specific CO_2 und H_2 production at different pH, with a dilution rate D=0,251/h

As a consequence of the evolution of specific H_2 and CO_2 production in function of pH, the yield Y H_2/CO_2 increased at the different pH tested. The best yield, 0,61 mol/mol was obtained with pH 6,8 (see figure 20 A). Figures 20 A and B show an increasing of the yield Y H_2/CO_2 and Y H_2/Glu among the augmentation of pH.

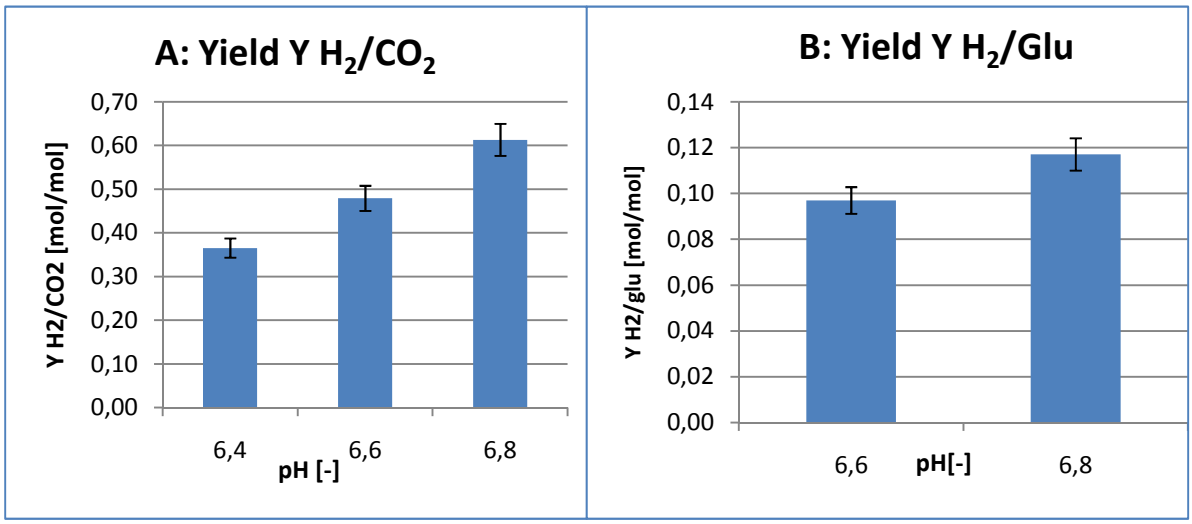


Figure 20: A: Yields Y H_2/CO_2; B: Yield Y H_2/glu at different pH.

Yields of the other products, as shown in figure 22, had a tendency to have a lower yield Y Hac/substrate with a lower pH, but this figure does not show a significant evolution of the yield Y eth/substrate and the yield $Y_{x/s}$ at the different pH.

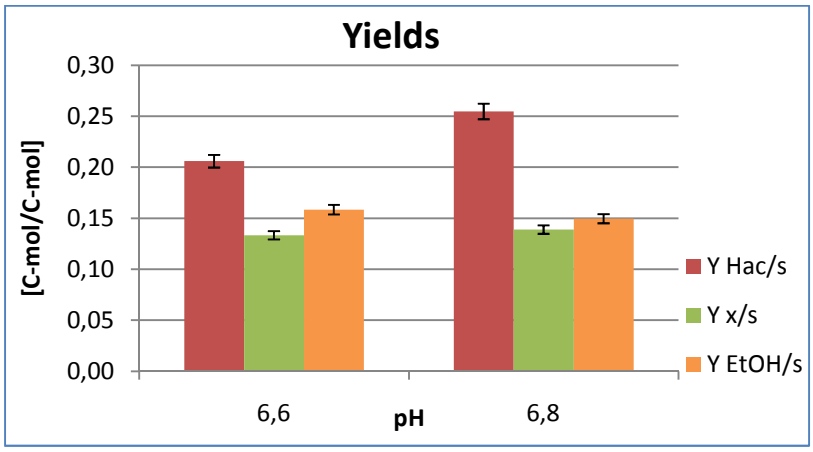


Figure 21: Comparison of yields for different products as a function of the pH

5.3.2 Effect of dilution rate

The effect of different dilution rate on biohydrogen production using *E. aerogenes* DSM 30053 in continuous culture process with a defined medium could also be studied with a pH 6,8. As shown in figure 23, the best specific H_2 production, 8,85 mmol/g/h was obtained with a dilution rate of 0,25 h^{-1}. These results show a higher specific H_2 production with a higher dilution rate.

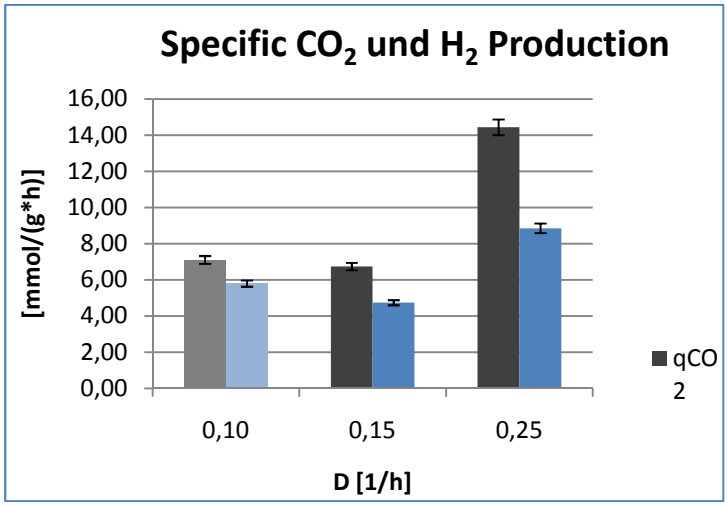


Figure 22: Specific CO_2 und H_2 production among different dilution rates with pH=6,8 (light-coloured: pH 6,7)

Figure 24 shows that yields Y H_2/CO_2 and Y H_2/glu lower among increasing of the dilution rate. The best yield Y H_2/CO_2, (0,82 mol/mol), and the best yield Y H_2/glu (0,17 mol/c-mol) were obtained with a dilution rate of 0,10 (with pH= 6,7).

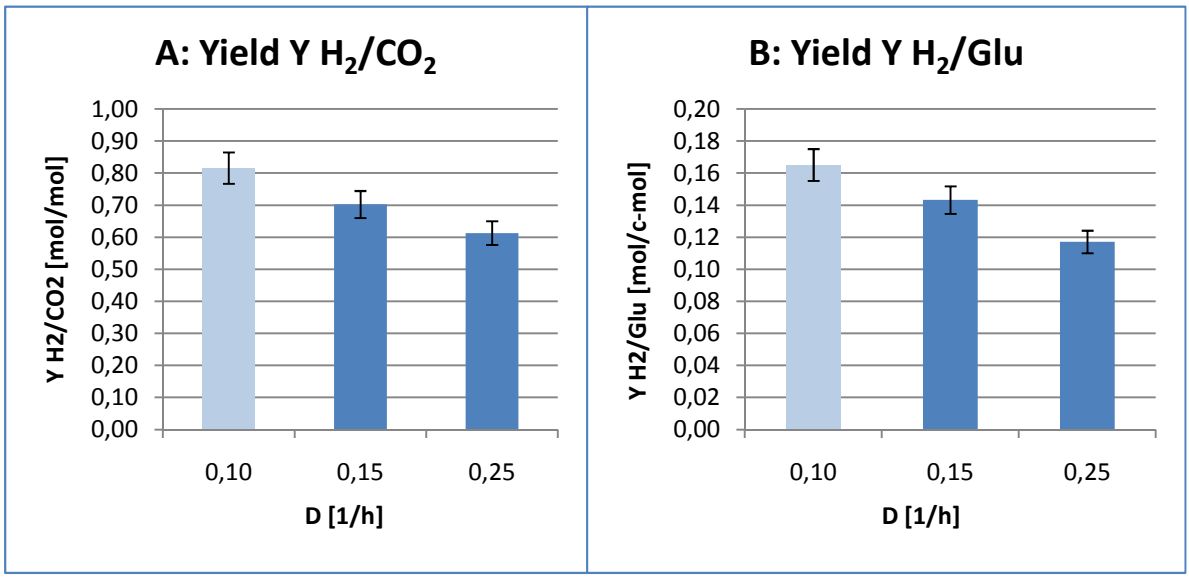


Figure 23: Yields Y H2/CO2 and Y H2/glu at different dilution rate with pH=6,8 (light-coloured: pH 6,7)

Yields of the other products, as shown in Figure 24, do not show a tendency towards dilution rates.

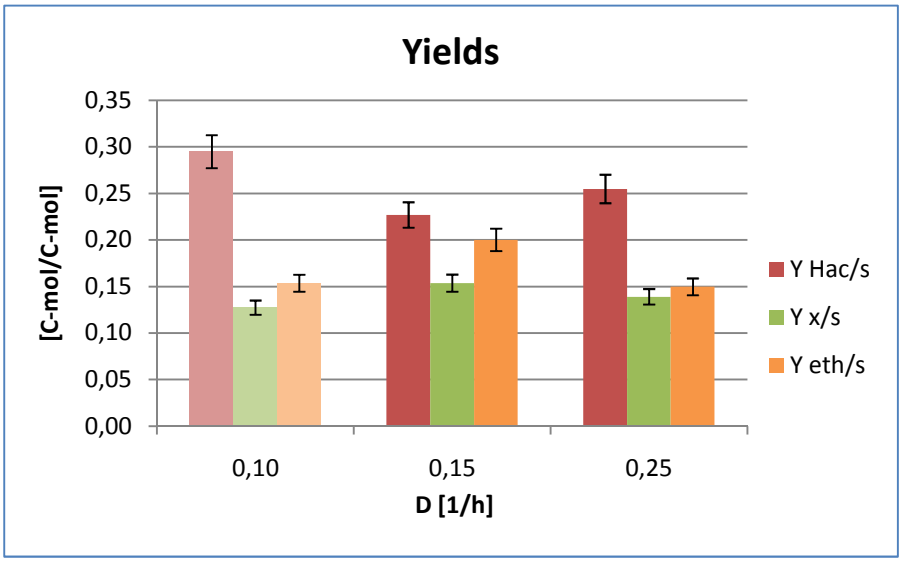


Figure 24: Comparison of yields for different products as a function of the dilution rate, with pH=6,8 (light-coloured: pH 6,7).

6. DISCUSSION

6.1 SUITABILITY OF THE DEFINED MEDIUM TO PRODUCE HYDROGEN WITH E AEROGENES DSM 30053

Figure 8 shows that the defined medium used is suitable for hydrogen production with *E. aerogenes* DSM 30053. Our work clearly differs from work conducted by Ren et.al, 2009 and Kumar et Das, 2001 [Ren, 2009; Kumar, 2001] because in our experiments defined medium was used lacking for instance of yeast extract and peptone as these substances were used in the cited works.

6.2 HYDROGEN PRODUCTION BY *ENTEROBACTER AEROGENES* DSM 30053 IN BATCH MODE:

6.2.1 Effect of initial glucose concentration:

As it was not possible to find similar studies on *Enterobacter aerogenes* DSM 30053 in the literature, the comparison will be done with studies on other strains of *Enterobacter aerogenes* strains, under similar culture conditions using complex media.

Palazzi et al., 2002, studied *Enterobacter aerogenes* NCIMB 10102 using complex medium, with glucose in function of the glucose concentration (30 g/l; 40 g/l, and 50g/l) [Palazzi, 2002]. The pH value was 5.5. No information about yields could be found about these results. The following specific H_2 production rates were obtained:

Table 6: Results obtained by Pallazi 2002 with different glucose concentrations

[glucose] [g/l]	H_2 production rate [mmol H_2/g DW.h]
30	3,2
30	3,9
40	8,3
40	9,8
50	14,5
50	15,8

In these results, a clear increase of specific H_2 production rate concomitant to the increase in initial glucose concentration in the medium is observed. It is not the case in the results of the present study (see table 3), which shows a decrease of the H_2 production rate from 5g/l until 20 g/l. In the present work, different initial concentrations of glucose were lower as in the experiments of Palazzi et al. [Palazzi, 2002]. For that reason, a very little deviation in the measure of the glucose, especially in the case of the 5g/l concentration, could have given a different result. The different results could also be due to the different medium used.

In figure 10, peak D does not have the same shape as the other observed peaks. This peak shows the batch experiment with the highest glucose concentration (40 g/l). In this culture high concentrations of CO_2 were produced, and not removed from the bioreactor. Some studies show that the concentration of CO_2 affects the rate of synthesis and final yield of H_2 [Levin, 2004; Das, 2001]. It was showed that a CO_2 removal can increase the production of H_2 [Tanisho, 1997]. It can be done for example with a CO_2 absorber with aqueous NaOH used to absorb the by-product CO_2 gas [Ren 2009]. As this was not done in this experiment, it can explain why the yields Y H_2/glu, and Y H_2/CO_2 were less high with this 40 g/l glucose concentration than with the other concentrations, unlike in other studies [Palazzi, 2002].

As it was mentioned in the result parts, the C-balances were not closing completely. This may be because some metabolites were not detected. In one of the chromatograms from a batch with a glucose 10 g/l concentration (figure 13), with calibration lines above (figures 14-15), glucose, ethanol, lactate, and acetate were measured and/or identified with HPLC or CuBiAN. Ito et al., 2004, identified H_2, CO_2, lactate, acetate, formate, succinate and 2,3-Butanediol as fermentation end-products from glucose with complex medium in batch culture with *Enterobacter aerogenes* HU-101 [Ito, 2004]. Nakashimada et al.,2002, identified H_2, CO_2, ethanol, lactate, acetate, acetoin and butanediol when *Enterobacter aerogenes* HU-101 was cultivated on glucose as substrate in complex medium [Nakashimada, 2002]. It is possible that such products were also present in the experiments conducted in the present work but have not been identified. Identifying these products with analytical devices could help to close the C-balance. Due to the missing components, the yield coefficients respective to substrates need to be carefully interpreted, when comparing the experiments among each other.

6.2.2 Comparison yields and physiological response of Enterobacter aerogenes DSM 30053 towards an initial glucose concentration (10 g/l,) and initial xylose concentration (10 g/l)

To the best of knowledge, fermentative hydrogen production from xylose by using facultative anaerobe has been reported just once, with complex medium [Ren, 2009]. It can be seen from figure 16 that hydrogen can be produced effectively by *E. aerogenes* from xylose in defined medium, as described in chapter 5. Yield Y H_2/xyl of 0,18 mol/C-mol was obtained in the xylose concentration of 10 g/l. This is comparable to the reported value from the cultivation with a complex medium for the yield Y H_2/xyl of 0,22 mol/C-mol [Ren, 2009]. It is also comparable to the reported value from the hydrogen-producing enrichment culture such as *Clostridium* sp. Strain No.2 [Taguchi, 1994].

H_2 productivity of 4,08 mmol/l/h was obtained in the xylose concentration of 10 g/l. This is lower than the reported value for the H_2 productivity (12 mmol/l/h), that was obtained with a complex medium, and with CO2 removal. [Ren, 2009].

It can be seen from figure 17 that the yield Y H_2/substrate is two times better with xylose than with glucose, with a same concentration (10 g/l). In the same time, figure 18-C shows that the Yield CO_2/substrate is also two times higher with xylose than with glucose. Table 4 shows that H_2 productivity is fast three times higher for the hydrogen production with medium containing glucose. This comparable to the reported value from the cultivation with a complex medium that gave better H_2 productivity from hexoses than from pentoses [Ren, 2009].

6.3 HYDROGEN PRODUCTION IN CONTINUOUS CULTURE

In Table 7 results are presented, which were obtained from literature with glucose concentration of 20g/l and at different pH conditions (between 6.3 and 6.8) from several *Enterobacter aerogenes* strains:

Table 7: Results from literature on biohydrogen production with *E. aerogenes*, with glucose

pH	Strain	H_2 yield	H_2 productivity	Reference
[-]		[mol H_2/c-mol]	mmol H_2/l/h	
6.8	A-1	0,14		[Rachman, 1998]
6.8	AY-2	0,20		[Rachman, 1998]
6.8	HU-101	0,33		[Rachman, 1998]
6,8	HU-101	0,09		[Rachman, 1998]
6,5	E.82005	0,18	16,6	[Tanischo, 1987]
6,3	AY-2	0,25		[Nakashimada, 2002]
6,3	E.82005	0,14		[Tanischo, 1987]
6,3	E.82005	0,12		[Tanischo, 1987]
6,3	HU-101	0,13		[Rachman, 1998]

As shown in Table 7, there is often only the H_2 yield for these experiences trying different pH conditions. These results were obtained with different strains, and different type of cultures. It is difficult to compare with results obtained in the table 5 in chapter 5. The yields H_2/glu given in the table 5 with a defined medium in continuous culture are comparable to the yields given in table 7 with complex media. However, it is difficult to see a tendency among the pH in the results from the literature between 6.4 and 6.8.

Any literature about production of biohydrogen with *E.aerogenes* in function of the dilution rate could not be found. But reported results using *E.cloacae* showed that H_2 production rate was increased among the dilution rate, and that the yield Y H_2/glu was decreasing among the dilution rate [Kumar, 2001]. The same tendency can be observed in figure 23 and 24 B. However, it is difficult to confirm a tendency, because only a few different dilution rates were tested with the same pH value.

7. CONCLUSION AND PERSPECTIVES

From the discussion and results above, it can be concluded that hydrogen may be produced by *E. aerogenes* DSM 30053 by using defined medium.

The substrate of 20 g/l glucose, in batch and with a pH of 6.8 resulted in the highest yield of hydrogen (0.17 mol / C-mol). The maximum hydrogen productivity reaches 19.45 mmol/l/h with a continuous culture with glucose 20 g/l and pH 6.8. Results show a tendency to have less CO2 and more H2 production with an increasing pH, between pH 6.4 and 6.8, and a tendency to have a higher specific H_2 production with a higher dilution rate. However, due to the missing components, the yield coefficients respective to substrates need to be carefully interpreted, when comparing the experiments among each other.

Some more experiments could be done with *E.aerogenes* on defined medium with different substrates, like arabinose, mannose, rhamnose or galactose, to compare the H_2 production rates and yields with results from the literature. Some other defined media could also be tried. At length, it could be interesting to try to produce biohydrogen on defined medium with other anaerobe or other facultative anaerobe strains.

8. BIBLIOGRAPHY

Bockris JO'M (2002). The origin of ideas on a hydrogen economy and its solution to the decay of the environment. *Int J Hydrogen Energy*. Vol.27, pp 731-40

Cammack R (1999). Hydrogenase sophistication. *Nature* Vol.397, pp 214-215

Das D, Veziroglu TN (2001). Hydrogen production by biological processes: a survey of literature. *International Journal of Hydrogen Energy.* Vol. 26, pp 13-28

Das D, Khanna and Veziroglu TN (2008). Recent developments in biological hydrogen production processes. *Chemical Industry & Chemical Engineering Quarterly .* Vol. 14 (2) pp 57-67

Dunn S (2002). Hydrogen futures: toward a sustainable energy system. *Int J Hydrogen Energy*. Vol.27, pp 235-264

Hallenbeck PC, Benemann JR (2002).Biological hydrogen production; fundamentals and limiting processes. *International Journal of Hydrogen Energy.* Vol. 27, pp 1185-1193

Hallenbeck PC (2005). Fundamentals of the fermentative production of hydrogen. *Water Science and Technology* Vol.52(1-2), pp 21-29

Hallenbeck PC, Ghosh and Monika (2009). Microbiological and engineering aspects of biohydrogen production. *Indian J Microbiol* Vol. 49, pp 48-59

Hefner RA (2000). The Age of Energy Gases, "Where is Energy Going? *The Industrial Physicist*, pp 16–19

Hydrogen Technical Advisory Panel (HTAP) (1998). Fuel choice for fuel cell vehicules. *National Renewable Energy Laboratory*

Ito T, Nakashimada Y, Senba K, Matsui T, Nishio, N, (2005). Hydrogen and ethanol production from glycerol-containing wastes discharged after biodiesel manufacturing process. *Journal of Bioscience and Bioengineering*, Vol 100(3), pp 260-265

Jensen, MW, Ross, M (2000). The ultimate challenge: developing an infrastructure for fuel cell vehicles. *Environment 2000,* Vol 42(7), pp 10-22

Kumar N, Das D (2001). Redirection of biochemical pathways for the enhancement of H_2 production by Enterobacter cloacae. Biotechnol Lett Vol.23 pp 537-541

Levin, Pitt, Love (2004). Biohydrogen production: prospects and limitations to practical application*. Int J Hydrogen Energy.* Vol.29, pp 173-185

Li C, Fang HP (2007). Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. *Critical Reviews in Environmental Science and Technology*. Vol 37, pp 1-39

Madigan MT, Martinko JM, Parker J (2001). Brock Mikrobiologie. Heidelberg/Berlin, Spektrum Akademischer Verlag.

Mahyudin AR, Furutani Y, Nakashimada Y, Kakizono T, Nishio (1997). Enhanced hydrogen production in altered mixed acid fermentation of glucose by Enterobacter aerogenes. J Ferm Bioeng Vol 83(4), pp 358-363

Melis T (2002). Green alga production: process, challenges, and prospects. *Int J Hydrogen Energy,* Vol 27, pp 1217-1228

Modigell M, Schumacher M, Claassen P.A.M (2007). Hyvolution - Entwicklung eines zweistufigen Bioprocesses zur Produktion von Wasserstoff aus Biomass; Chemie-Ingenieur-Technik, Vol 79(5) pp 637-641

Nakashimada Y, Rachman MA, Kakizono T, Nishio N, (2002). Hydrogen production of *Enterobacter aerogenes* altered by extracellular and intracellular redox states. *International Journal of Hydrogen Energy* Vol 27 pp 1399-1405

Nandi R, Sengupta S (1998). Microbial Production of Hydrogen: An Overview. *Critical Reviews in Microbiology.* Vol. 24(1), pp 61 - 84

Nandi R, Dey S, Sengupta S (2001). Thiosulfate improves yield of hydrogen production from glucose by the immobilized formate hydrogenlyase system of Escherichia coli. *Biotechnology and Bioengineering* 75(4), pp 492-494

Nath K, Das D (2004). Byohydrogen production as a potential energy resource - Present state-of-art. *Joural of Scientific & Industrial Research* Vol 63 pp 729-738

Nath K, Das D (2004). Improvment of fermentative hydrogen production: various approaches. *Appl Microbiol Biotechnol.* Vol. 65, pp 520-529

Oh Y-K, Seol E-H, Yeol Lee E, Park S (2002). Fermentative hydrogen production by a new chemolithotrophic bacterium Rhodopseudomonas palustris P4. *International Journal of Hydrogen Energy* Vol. 27, pp 1373-1379

Ren Y, et al. (2009). Hydrogen production from the monomeric sugars hydrolyzed from hemicellulose by *Enterobacter aerogenes*. *Renewable Energy.* Vol. 34, pp 2774-2779

Rachman M. A., Furutani Y., Nakashimada Y., Kakizono T., Nishio N. (1998). Enhanced hydrogen production in altered mixed acid fermentation of glucose by *Enterobacter aerogenes*. *Journal of Fermentation and Bioengineering* Vol. 83(4) pp 358–363

Rocha JS, Barbosa MJ, Wijffels RH (2001). Hydrogen production by photosynthetic bacteria: culture media, yields and efficiencies. Biohydrogen II, *An approach to Environmentally Acceptable Technology.* J. Miyake, Matsunaga, T., Pietro, A. S.,. Oxford, UK, Pergamon/Elsevier: pp 3-32

Rittmann S (2008). Overview on Fermentative Microorganisms Capable of Biohydrogen Production - a Putative Comprehensive Survey of Literature.

Suzuki Int. J (1982). *International Jouranl of Hydrogen Energy.* Vol. 7, pp 227-230

Tanisho S, Suzuki Y, Wakao N, (1987). Fermentative hydrogen evolution by*Enterobacter aerogenes* strain E.82005. *International Journal of Hydrogen Energy* Vol 12(9) pp 623-627

Tanisho S, Kuromoto M, Kadokura N (1997). Effect of CO_2removal on hydrogen production by fermentation. *International Jouranl of Hydrogen Energy.* Vol. 23 (7), pp 559-563

Tao Yongzhen, Chen Yang, Wu Yongqiang, He Yanling, Zhou Zhihua (2007). High hydrogen yield from a two-step process of dark- and photo-fermentation of sucrose" *International Journal of Hydrogen Energy*, Vol 32 (2), pp 200–206

Winter C-J (2000). On energies of change, the hydrogen solution. *Gerling Akademie Verlag*, pp 67-82

Woodward J, Orr M, Cordray K, Greenbaum E, (2000). Enzymatic production of biohydrogen. *Nature* Vol. 405, pp 1014-1015

Woodward J, Mattingly SM, Danson M, Hough D, Ward N, Adams M (2000). In vitro hydrogen production by glucose dehydrogenase and hydrogenase. Nat Biotechnol Vol 14, pp 872-874

9. APPENDIX

9.1 STOCK SOLUTION

Table A-1 : Trace elements stock (100-fold).

Chemical substance	used amount
$MgSO_4$ H_2O	6.2 g
$CoCl_2 \cdot 6\ H_2O$	0.025 g
$MnCl_2 \cdot 4\ H_2O$	0.150 g
$CuCl_2 \cdot 2\ H_2O$	0.012 g
H_3BO_3	0.030 g
$NaMoO_4 \cdot 2\ H_2O$	0.025 g
$Zn(CH_3COO)_2 \cdot 2\ H_2O$	0.130 g
Fe(III) citrate	1.0 g
E-H_2O add to 100 mL	

Table 2: EDTA stock solution (100-fold). *

Chemical substance	used amount
EDTA	0.084 g
E-H_2O add to 100 mL	

Table A-3: Vitamin stock (100 fold)*

Chemical substance	used amount
Thiamine HCl	0,025 g
E-H_2O add to 50 mL	

*** Filtered sterilizied**

Table A-4:. Nutrient agar

Chemical substance	used amount
Peptone	5 g
Meat extract	5 g
Agar	15 g
E-H_2O add to 1000 mL, pH adjust to pH 7 with NaOH 1M	