VISOKA ŠOLA ZA VARSTVO OKOLJA
ENVIRONMENTAL PROTECTION COLLEGE

MAGISTRSKO DELO
MASTER THESIS

PROIZVODNJA VODIKA IZ KOMPLEKSNE BIOMASE Z UPORABO SEVA *THERMOANAEROBACTER* AK68

HYDROGEN PRODUCTION FROM COMPLEX BIOMASS BY *THERMOANAEROBACTER* STRAIN AK68

ŽIVA VIPOTNIK

VELENJE, 2015

VISOKA ŠOLA ZA VARSTVO OKOLJA
ENVIRONMENTAL PROTECTION COLLEGE

Master thesis for 30 credit at Master of Environmental Protection and Eco-technologies

Hydrogen production from complex biomass by *Thermoanaerobacter* strain AK68

Proizvodnja vodika iz kompleksne biomase z uporabo seva *Thermoanaerobacter* AK68

ŽIVA VIPOTNIK

Supervisor: Martin Batič, Ph.D., Assistant Professor

Co-Supervisor: Jóhann Örlygsson, Ph.D., Professor

Velenje, 2015

Številka: 726-1/2015-3
Datum: 18. 6. 2015

Na podlagi Diplomskega reda izdajam naslednji

SKLEP O MAGISTRSKEM DELU

Študentka Visoke šole za varstvo okolja **Živa Vipotnik** lahko izdela magistrsko delo z naslovom v slovenskem jeziku:
Proizvodnja vodika iz kompleksne biomase z uporabo seva *Thermoanaerobacter* AK68

Naslov magistrskega dela v angleškem jeziku:
Hydrogen production from complex biomass by *Thermoanaerobacter* strain AK68

Mentor: doc. dr. Martin Batič.
Somentor: red. prof. dr. Jóhann Örlygsson.

Magistrsko delo mora biti izdelano v skladu z Diplomskim redom.

Z dnem izdaje tega sklepa preneha veljati sklep številka: 726-1/2015-2, z dne 11. 2. 2015.

Pouk o pravnem sredstvu: zoper ta sklep je dovoljena pritožba na Senat VŠVO v roku 8 delovnih dni od prejema sklepa.

Doc. dr. Boštjan Pokorny
dekan

Izjava o avtorstvu

Podpisani/a Živa Vipotnik, z vpisno številko 34122012, študentka podiplomskega študijskega programa Varstvo okolja in ekotehnologije, sem avtorica magistrskega dela z naslovom:

Hydrogen production from complex biomass by *Thermoanaerobacter* strain AK68 / Proizvodnja vodika iz kompleksne biomase z uporabo seva *Thermoanaerobacter* AK68

ki sem ga izdelal/a pod mentorstvom doc. dr. Martina Batiča in somentorstvom red. prof. dr. Jóhanna Örlygssona.

S svojim podpisom zagotavljam, da:

• je predloženo delo moje avtorsko delo, torej rezultat mojega lastnega raziskovalnega dela;

• da oddano delo ni bilo predloženo za pridobitev drugih strokovnih nazivov v Sloveniji ali tujini;

• da so dela in mnenja drugih avtorjev, ki jih uporabljam v predloženem delu, navedena oz. citirana v skladu z navodili VŠVO;

• da so vsa dela in mnenja drugih avtorjev navedena v seznamu virov, ki je sestavni element predloženega dela in je zapisan v skladu z navodili VŠVO;

• se zavedam, da je plagiatorstvo kaznivo dejanje;

• se zavedam posledic, ki jih dokazano plagiatorstvo lahko predstavlja za predloženo delo in moj status na VŠVO;

• je diplomsko delo jezikovno korektno lektorirano;

• da dovoljujem objavo diplomskega dela v elektronski obliki na spletni strani VŠVO;

• da sta tiskana in elektronska verzija oddanega dela identični.

V Velenju, dne ________________ ________________________

podpis avtorice

In the middle of every difficulty lies opportunity. Learn from yesterday, live for today, hope for tomorrow. The important thing is to not stop questioning.

(Albert Einstein)

Acknowledgments

I would like to thank my mentor Dr. Martin Batič for valuable assistance, patient guidance throughout this thesis and his helpful discussions.

I would like to thank professor Dr. Jóhann Örlygsson for not only giving me the opportunity to work in his laboratories but also being a great supervisor.

Special thanks for invaluable help to Sean M. Scully, who guided me during my work at UNAK, for advice, valuable assistance and technical support during my entire thesis.

Thanks to all my close friends who have helped me along in my studies and my family for endless support and compassion.

Abstract

Production of hydrogen as biofuel from sugars and Timothy grass hydrolysates with thermophilic bacterium was investigated in batch cultures. The thermophilic bacterial strain was isolated from a hot spring in Grensdalur, SW-Iceland. Hydrogen production from various carbon sources were investigated as well as the effects of various environmental factors including substrate concentration, effect of pH, temperature and partial pressure of hydrogen on growth and on end product formation. The effect of external electron scavenging systems on end product formation was also investigated as well as the ability to produce hydrogen from complex biomass. Various factors affect the production of biofuels from lignocellulitic biomass including initial concentration of lignocellulosic hydrolysates, acidity of pretreatment and enzymes used.

Thermoanaerobacter strain AK68, isolated from an Icelandic hot spring, was researched for hydrogen production from sugars. It is most closely related to *T. pseudoethanolicus* (99.4 % similarity) and *T. thermohydrosulfuricus* (99.3 % similarity). The strain has a temperature optimum between 65 and 70 °C and a pH optimum between pH 7.0 and 8.0. The strain AK68 degraded mostly disaccharides and hexoses examined as well as pentose xylose but not arabinose or polyssacharide starch. Various environmental variables were investigated to gain a deeper understanding of factors that are of importance for hydrogen production with the main emphasis on the fact that the strain AK68 could only partially degrade glucose at modest concentrations. Factors investigated included initial glucose concentrations, buffer concentrations, liquid-gas phase ratios, effect of various external electron acceptors, and different initial pH values.

The results for *Thermoanaerobacter* strain AK68 showed that the substrate utilization is primarily inhibited by increased partial pressure of hydrogen, as evidence by increased glucose utilization in using electron scavenging systems and manipulation of the liquid-gas phase ratio, rather than substrate or end product inhibition. The obtained 2.3 mol of hydrogen from 1 mol of glucose was the maximum hydrogen yield and represented 62.5 % of the theoretical yield in optimal conditions (the temperature between 65 and 70 °C and a pH between pH 7.0 and 8.0).

Keywords: *Thermoanaerobacter*, thermophilic bacteria, hydrogen, complex lignocellulitic biomass

Izvleček

Raziskana je bila pridelava vodika kot biogoriva iz sladkorjev in hidrolizatov kompleksne biomase s termofilno bakterijo v šaržnih kulturah. Termofilni bakterijski sev je bil izoliran iz vročega vrelca v Grensdalurju na JZ Islandije. Raziskana je bila pridelava vodika iz različnih ogljikovih virov kakor tudi učinki različnih okoljskih faktorjev kot so koncentracija substrata, pH, temperature in parcialnega tlaka vodika na rast in formiranje končnega produkta. Prav tako je bil raziskan učinek sistemov zunanjih odstranjevalcev oziroma lovilcev elektronov na nastajanje končnega produkta, kot tudi sposobnost pridelovanja vodika iz kompleksne lignocelulozne biomase. Različni faktorji vplivajo na pridelavo biogoriv iz lignocelulozne biomase, med drugim začetna koncentracija lignoceluloznih hidrolizatov, stopnja kislosti v predobdelavi in uporabljeni encimi.

Thermoanaerobacter sev AK68, izoliran iz islandskega vročega vrelca, je bil raziskan z namenom pridelave vodika iz sladkorjev. S filogenetsko analizo smo ugotovili, da je sev najbolj soroden s *T. pseudoethanolicus* (99.4 % podobnost) in *T. thermohydrosulfuricus* (99.3 % podobnost). Optimalni pogoji za rast in proizvodnjo najvišje količine vodika na 1 mol glukouze za sev AK68 so v temperaturnem območju od 65 do 70 °C in pH med 7.0 in 8.0.

Sev AK68 razgrajuje disaharidov in heksoze (C_6-sladkorje), kot tudi ksilozo, ne pa arabinoze ali polisaharidnega škroba. Raziskane so bile različne okoljske spremenljivke za boljše razumevanje faktorjev, ki pomembno vplivajo na pridelavo vodika. Glavni poudarek je na dejstvu, da lahko sev AK68 samo deloma razgradi glukozo v majhnih koncentracijah. Raziskani faktorji vsebujejo različne začetne koncentracije glukoze, koncentracije fosfatnega pufra, razmerja med tekočinsko in plinsko fazo, učinke različnih zunanjih akceptorjev elektronov in različne začetne pH vrednosti.

Rezultati za *Thermoanaerobacter* sev AK68 so pokazali, da uporabo substrata najbolj ovira povečan parcialni tlak vodika, kar dokazuje povečana poraba glukoze pri uporabi sistemov odstranjevalcev/lovilcev elektronov in spreminjanje razmerja med tekočinsko in plinsko fazo. Sev AK68 je dober proizvajalec vodika, hkrati pa proizvaja tudi etanol, acetat in ogljikov dioksid. Pridobljena 2,3 mola vodika iz 1 mola glukoze je bil največji izkoristek in predstavlja 62,5 % teoretičnega izkoristka v optimalnih pogojih.

Ključne besede: *Thermoanaerobacter*, termofilne bakterije, vodik, kompleksna lignocelulozna biomasa

Table of contents

Acknowledgments III
Abstract IV
Izvleček V
List of Figures VII
List of Tables VIII
Abbreviations IX

1. Research Objectives 1
2. Introduction 2
   2.1. Hydrogen Production 3
      2.2.1. Biological hydrogen production 3
   2.2. Biomass 8
      2.2.1. Brown macroalgae 8
      2.2.2. Terrestrial Biomass 10
      2.2.3. Biomass pretreatment 12
      2.2.4. Biohydrogen production from lignocellulosic materials using dark fermentation 14
      2.2.5. Sources of fermentation inhibitors 16
   2.3. Hydrogen producing thermophilic bacteria 17
      2.3.1. Strict anaerobes 18
      2.3.2. Factors affecting end product formation 20
3. Effect of culture conditions on hydrogen production by *Thermoanaerobacter* strain AK6825
   3.1. Introduction 25
   3.2. Materials and Methods 26
   3.3. Results 31
   3.4. Discussion 42
4. Conclusion 44
5. Povzetek 46
6. References 50
7. Appendices 62

List of Figures

Figure 1 Methods used for hydrogen production feedstock 3

Figure 2 Simplified scheme of glucose degradation to various end products by strictly anaerobic bacteria. Enzyme abbreviations: ALDH—acetaldehyde dehydrogenase; ADH—alcohol dehydrogenase; AK—acetate kinase; FNOR—ferredoxin oxidoreductase; and PTA—phosphotransacetylase 5

Figure 3 Breakdown pathway of the xylose-5-phosphate formed from the XRXDH breakdown pathway 7

Figure 4 Chemical structures of fucoidans, isolated from different members of the order *Fucales* 9

Figure 5 Ligno-cellulosic Material 11

Figure 6 Methods for pretreatment of cellulosic feedstock 14

Figure 7 A schematic diagram for biohydrogen production from cellulose 15

Figure 8 Maximum likelihood phylogenetic dendogram based on 16S rRNA gene sequences showing the position of strain AK68 within the class Clostridia. 31

Figure 9 End product formations from various substrates. 32

Figure 10 End product formations from different initial glucose concentrations. Also shown is percentage of glucose degradation. 33

Figure 11 End product formations from glucose (20 mM) at different initial pHs. 34

Figure 12 End product formations from glucose (20 mM) at different temperatures. 34

Figure 13 Effect of liquid gas ratios on end product formation. Hydrogen formation is showed as number of moles produced from one mole of glucose. 36

Figure 14 End product formation from glucose (20 mM), glucose (20 mM) and thiosulfate (40 mM) and glucose (20 mM) when cultivated in a co-culture with *Methanothermobacter* M39 37

Figure 15 End product formation in the presence of different concentrations of phosphate buffer with glucose (20 mM). 38

Figure 16 Dry weight proportion compositions of HLs samples 39

Figure 17 Results of hydrolysate experiment (acid pretreament) for strain AK68 on Timothy grass with different initial hydrolysate concentrations 40

Figure 18 Results of hydrolysate experiment (acid and enzyme pretreament) for strain AK68 on Timothy grass and brown seaweed with 5 g/L hydrolysate concentrations 40

Figure 19 End product formations in the presence of different concentrations of 5 HMF, Furfuraldehyde, Levulinic acid and Vanillin, with 20mM glucose. 41

List of Tables

Table 1 Selected hydrogen production by thermoanaerobes with different substrates 23

Table 2 Production of end products from glucose (20 mM) in different liquid-gas ratios. Values represent the mean of two replicates (± standard deviation). Hydrogen values are corrected to volume units. 35

Abbreviations

H_2	Hydrogen
CO_2	Carbon dioxide
HL	Hydrolysate
EtOH	Ethanol
ED	Entner Doudoroff pathway
EMP	Embden–Meyerhof pathway
C_5 sugars	Sugars with 5 carbon molecules (pentoses)
C_6 sugars	Sugars with 6 carbon molecules (hexoses)
ATP	Adenosine-5´-triphosphate
NADH	Nicotineamide adenine denucleotide reduced
NAD+	Nicotineamide adenine denucleotide oxidized
NAD(P)H	Nicotineamide adenine denucleotide phosphate reduced
PFOR	Pyruvate ferredoxin oxidoreductase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
PDC	Pyruvate decarboxylase
ADH	Alcohol dehydrogenase
PFR	Pyruvate-ferredoxin oxidoreductase
PDH	Pyruvate dehydrogenase complex
ADP	Adenosine di-phosphate
ρH_2	Partial pressure of hydrogen
F/M	Food-to-microorganism
HMF	Hydroxymethylfurfural
FF	Furfural
Vf	Final Volume
pK_a	Acid dissociation constant
S_2O_3	Thiosulfate
LCFA	Long chain fatty acids
VFA	Volatile fatty acids

Kazalo

Zahvala III

Abstract IV
Izvleček V
Kazalo slik VII
Kazalo tabel VI

1. Cilji raziskave 1
2. Uvod 2
 2.1. Proizvodnja vodika 3
  2.2.1. Biološka proizvodnja vodika 3
 2.2. Biomasa 8
  2.2.1. Rjave makroalge 8
  2.2.2. Rastlinska biomasa 10
  2.2.3. Obdelava biomase 12
  2.2.4. Proizvodnja biovodika s fermentacijo iz lignoceluloznega materiala 14
  2.2.5. Inhibitorji biomase 16
 2.3. Termofilni proizvajalci vodika 17
  2.3.1. Anaerobne bakterije 18
  2.3.2. Dejavniki, ki vplivajo na končni proizvod 20
3. Proizvodnja vodika z uporabo seva *Thermoanaerobacter* AK68 25
 3.1. Uvod 25
 3.2. Materiali in metode 26
 3.3. Rezultati 31
 3.4. Razprava 42
4. Sklep 44
5. Summary review (Povzetek) 46
6. Literatura 50
7. Priloge 62

Kazalo tabel

Tabela 1 Izbrane termoanaerobne bakterije, ki proizvajajo vodik iz različnih sladkorjev....... 23

Tabela 2 Končni proizvod fermentacije iz 20mM glukoze in različnimi koncentracijami tekočine/plina. Podatki so prikazani za povprečje dveh pararelnih poskusov (± standardni odklon). Kontrolni vzorci so brez glukoze 35

Kazalo slik

Slika 1	Metode za proizvodnjo vodika	3
Slika 2	Poenostavljena shema razgradnje glukoze z anaerobnimi bakterijami	5
Slika 3	razgradnja C_5 sladkorjev	7
Slika 4	Kemična struktura fukoidanov, izoliranih v različnih rjavih makroalgah, reda Fucales	9
Slika 5	Lignocelulozni material	11
Slika 6	Metode za obdelavo celulozne biomase	13
Slika 7	Diagram proizvodnje vodika iz celuloze	14
Slika 8	Filogenija, ki temelji na 16S rRNA zaporedju genov, ki kažejo položaj seva AK68 v razredu Clostridia	30
Slika 9	Končni produkt iz različnih substratov. Podatki predstavljajo povprečje dveh paralelnih poskusov	31
Slika 10	Nastanek končnega proizvoda iz različnih začetnih koncentracij glukoze. Prikazan je tudi odstotek razgradnje glukoze (n). Podatki predstavljajo povprečje dveh paralelnih poskusov	32
Slika 11	Končni proizvod z različnimi začetnimi pH vrednostmi medija in 20 mM glukoze. Razgradnja glukoze je prikazana v odstotkih (n	33
Slika 12	Končni proizvod pri različnih temperaturah in 20 mM glukoze. Razgradnja glukoze je prikazana odstotkih (n)	33
Slika 13	Učinek različnih razmerij plina in tekočine. Proizvodnja vodika je prikazana na en mol glukoze	35
Slika 14	Končni proizvod z uporabo; 20mM glukoze, 20mM glukoze in 40mM tiosulfata in 20mM glukoze inakulirane skupaj z Methanothermobacter M39.	36
Slika 15	Končni proizvod uporabe različne koncentracije fosfatnega pufra in 20mM glukoze	37
Slika 16	Sestava hidroliznih vzorcev	38
Slika 17	Rezultati hidrolizatnega eksperimenta s kislinsko obdelavo Phleum pratense in seva AK68	39
Slika 18	Rezultati hidrolize za sev AK68 s kislinsko in encimsko obdelavo Phleum pratense in rjavih makroalg	39
Slika 19	Končni proizvod fermentacije ob prisotnosti različnih koncentaracij HMF, furfurala, levulinske kisline in vanilina ter 20mM glukoze	40

1. Research Objectives

Hydrogen is considered to be the ultimate solution for clean and renewable energy due to its high energy content per unit and non-polluting characteristics as water is the only by-product after its combustion (Kim *et al.,* 2009). Biological hydrogen production by fermentation can be divided into two main categories: photosynthetic organisms that produce hydrogen using light as energy source and anaerobic bacteria that produce hydrogen via fermentation course in the dark. Lignocellulosic materials are highly attractive as substrates for hydrogen production due to its abundance, low cost, and its renewablility properties. Lignocellulosic material can be fermented directly to hydrogen by some bacteria, but the production rate is extremely slow due to the heterogeneity and crystallinity of the substrate.Thus, pretreatment is needed for the efficient conversion of biomass into energy carriers such as hydrogen (Cao *et al.,* 2014).

The research focuses on hydrogen production with the dark fermentation. The dark fermentative hydrogen production is affected by several environmental factors such as initial pH, partial pressure of hydrogen (ρH_2), substrate concentration and temperature. The partial pressure of hydrogen may especially have a significant effect on fermentation – as ρH_2 increases, some thermophilic microorganisms may switch their metabolism from acetate production to lactate or ethanol which results in a decrease in H_2 yield and production rate (Puhakka *et al.,* 2007). It has been also observed that the initial pH of cultivation affects the hydrogenase enzyme activity while substrate concentration affects the ability of hydrogen producing bacteria to produce hydrogen during dark fermentation (Reungsang and Sreela-or, 2013). Consequently the research work investigates the optimum conditions for fermentative hydrogen production by *Thermoanaerobacter* strain AK68.

The aim of this research was to isolate, characterize, and identify a thermophilic hydrogen-producing bacterium AK68 from Icelandic hot spring capable of producing hydrogen from lignocellulosic and macroalgal biomass hydrolysates. The optimum condition for hydrogen production from various sugars was also investigated.

2. Introduction

Awareness of the problems associated with the world's dependence on the non-renewable energy sources has generated a global effort to reduce dependence on fossil fuels and develop economically viable and sustainable alternative fuel sources that will significantly reduce emissions of CO_2 into the atmosphere (Kim *et al.,* 2009). Depletion of oil supplies combined with the increasing worldwide energy demand has generated an increased interest toward developing highly reneweable biofuels in the last 10 to 20 years. Another growing concern in the last 50 years is the environmental aspects of liquid fuel consumption especially regarding global warming because of increased CO_2 emissions. One of the most attractive potential portable alternative energy sources are biofuels. In addition to being renewable, biofuels can also reduce total CO_2 emissions as they are produced from stores of carbon that have not been removed from the carbon cycle (Stichnothe and Azapagic, 2009). There are several types of biofuels produced and used worldwide today. The most common are methane, ethanol (EtOH), and biodiesel but also, to a lesser extent, hydrogen (H_2), butanol, and propanol. There are several methods to produce biofuels, ranging from direct lipid extraction from fat-rich plants or animal fat (biodiesel) to the fermentation of various types of carbohydrate rich biomass (H_2, EtOH and butanol). Fermentation processes can be performed by both bacteria and yeasts (Sveinsdottir *et al.,* 2009).

Development of alternate renewable fuels with lower carbon emissions has become imperative for sustainable development and to meet the increasing demands of an increasing population (Prakasham *et al.,* 2009; Kyazze *et al.,* 2006). Hydrogen can address all the above concerns as a viable alternate energy source. Hydrogen is the most abundant and lightest element in the universe. It is found on earth in combinations with other molecules such as carbon, oxygen and nitrogen. Hydrogen (H_2) is regarded as an ideal transportation fuel for the future because it is non-pollutant, eco-friendly, efficient, and it has a high energy density (Pallavi and Anjana, 2011) and does not release any carbon dioxide during combustion. Currently, hydrogen is mainly produced by the stream reformation of methane, leading to the strong dependence of the hydrogen economy on fossil resources (Pallavi and Anjana, 2011).

Emerging techniques for the renewable hydrogen production include reformation techniques using renewable resources (Kimberly *et al.,* 2002) such as photo electrochemical hydrolysis (Deutsch *et al.,* 2008), solar thermal hydrolysis and fermenting sugar-rich feedstock (Kapdan and Kargi, 2006). Biological hydrogen production methods are more environmentally-friendly than the other aforementioned techniques as the former requires large inputs of energy or non-reneweable resources (Azbar and Levin, 2012; Wang and Wan, 2009). Among the biological hydrogen production methods, dark fermentation is often thought to be more attractive than photo-fermentation due to its high utilization efficiency of various organic wastes and feedstocks as substrate and, light-independence (Chen *et al.,* 2006). Furthermore, in dark fermentation, the hydrogen production rates are much higher compared to photo fermentation (Azbar and Levin, 2012).

2.1. Hydrogen Production

Hydrogen has been deemed as a promising alternate energy source for the future since during its combustion no carbon dioxide is produced (Masset *et al.,* 2010). It does not contribute to the greenhouse effect, producing only heat and water upon combustion and has a high energy yield of 286 kJ/mol, which is at least two times greater than that of any hydrocarbon fuel (Cai *et al.,* 2004). The various methods adopted to produce hydrogen are given in the Figure 1.

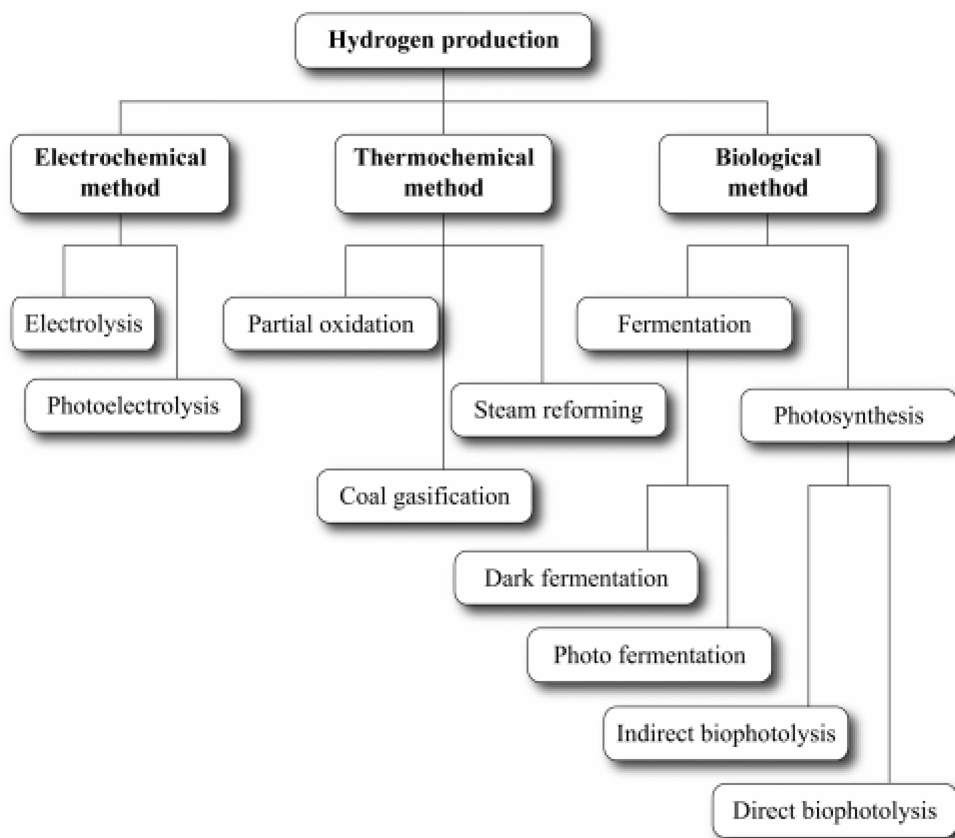


Figure 1 Methods used for hydrogen production feedstock (Saratale *et al.,* 2008)

2.2.1. Biological hydrogen production

Different microorganisms participate in biological hydrogen production. There are four mechanisms for biohydrogen production: direct biophotolysis, indirect biophotolysis, photo fermentation, and dark fermentation.

Direct Biophotolysis

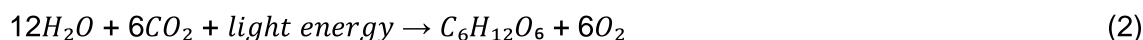
Certain bacterial-algal (green algae and cyanobacteria) systems are capable of using solar energy directly to extract electrons and protons from water resulting in evolution of hydrogen (photohydrogen) and oxygen by the following reaction (Levin *et al.,* 2004; Benemann, 1980):

$$2H_2O + light\ energy \rightarrow 2H_2 + O_2 \quad (1)$$

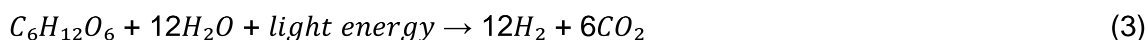
The main disadvantages of these processes are that it requires high light intensity; oxygen can be inhibitory and low photochemical efficiency (Das and Veziroglu, 2008).

Indirect Biophotolysis

Cyanobacteria (blue-green algae) can also synthesize hydrogen through photosynthesis by splitting water in a two-step process (Levin *et al.,* 2004):



$$12H_2O + 6CO_2 + light\ energy \rightarrow C_6H_{12}O_6 + 6O_2 \quad (2)$$

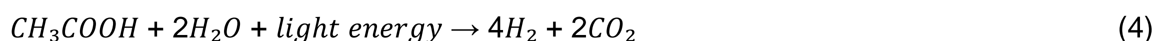


$$C_6H_{12}O_6 + 12H_2O + light\ energy \rightarrow 12H_2 + 6CO_2 \quad (3)$$

In the first step (aerobic phase), solar energy and water are used to accumulate carbohydrates through the photosynthesis process. In the second step (anaerobic phase), carbohydrates are catabolized for hydrogen production. Due to the multiple steps in indirect biophotolysis, it is less effective than direct biophotolysis (Azbar and Levin, 2012). The main disadvantage of this process is the need to remove hydrogenase enzymes to avoid the degradation of hydrogen to regenerate reduced cofactors (Das and Veziroglu, 2008).

Photo-Fermentation

Purple non-sulfur (PNS) bacteria produce hydrogen under nitrogen deficient conditions due to the presence of nitrogenase, using light energy and reduced compounds (organic acids) (Das and Veziroglu, 2008):



$$CH_3COOH + 2H_2O + light\ energy \rightarrow 4H_2 + 2CO_2 \quad (4)$$

The main disadvantages of this process are the inhibitory effect of oxygen on nitrogenase and the very low (1 % - 5 %) light conversion efficiency (Das and Veziroglu, 2008).

Anaerobic Dark Fermentation

The first step of dark fermentation is based on glycolysis, also known as Embden-Mayerhof-Parnas (EMP) pathway, which occurs in the cytosol of cells (Stryer, 1999). This pathway is initiated when one molelecule of glucose, is degraded in several steps into 2 molelecules of pyruvate. The transformation of glucose to pyruvate during glycolysis is accompanied by the formation of two molecules of Adenosine-5´-triphosphate (ATP) and two molecules of Nicotineamide adenine denucleotide reduced (NADH).

Glucose is not the only substrate that can enter into glycolysis. Simple sugars, such as fructose, galactose, or pentoses such as xylose and arabinose can be used. This, however, requires the Pentose Phosphate patheway (Figure 3). Additionally, complex sugars such as sucrose, saccharose, lactose, maltose, and cellobiose can be used as the initial substrate for glycolysis. However, the incorporation of these sugars into glycolysis pathway requires initial hydrolysis to the simple carbohydrates. Glycerol can be considred as a good substarate for glycolysis. There are also known other anaerobic pathways transforming glucose into pyruvate as e.g. Entner-Daudoroff or phosphate pentose pathway (Schlegel, 2003; Dabrock *et al.,* 1992; Vardar-Schara, 2008; Chin *et al.,* 2003; Seifert *et al.,* 2012).

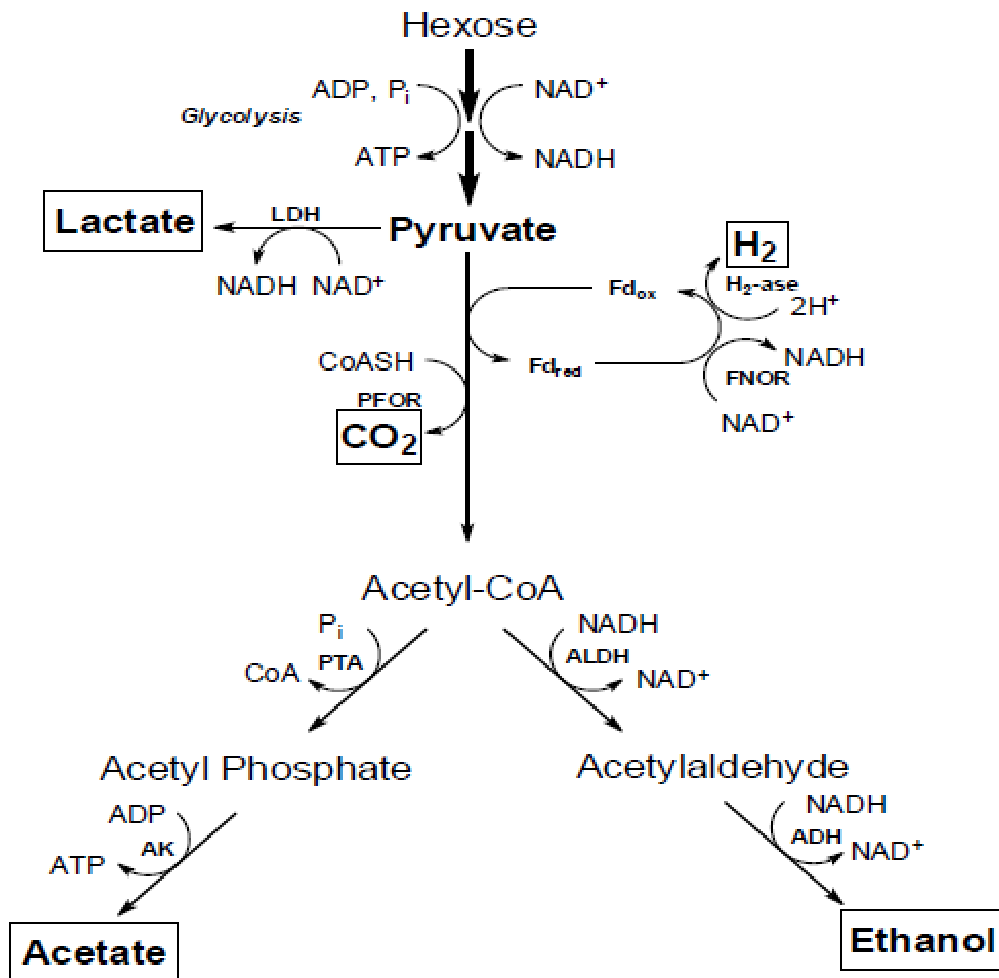
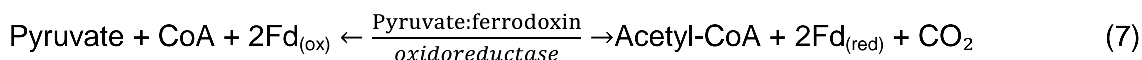
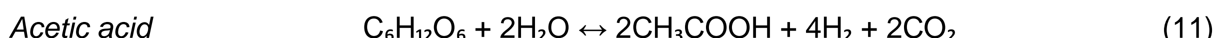


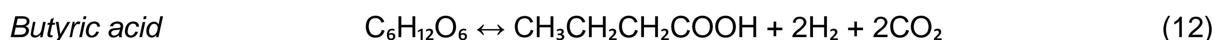
Figure 2 Simplified scheme of glucose degradation to various end products by strictly anaerobic bacteria (Scully and Orlygsson, 2015). Enzyme abbreviations: ALDH—acetaldehyde dehydrogenase; ADH—alcohol dehydrogenase; AK—acetate kinase; FNOR—ferredoxin oxidoreductase; and PTA—phosphotransacetylase

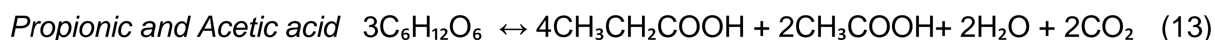
The metabolism of pyruvate is responsible for most of the hydrogen produced during anaerobic fermentation. Pyruvate decomposition is catalyzed by the enzyme pyruvate formate lyase (PFL) to produce formate (Equation 5). Formate can be further degraded by facultative anaerobic bacteria, such as enteric bacteria, in order to produce hydrogen (Equation 6) (Hallenbeck and Benemann, 2002). Strict anaerobes however, degrade pyruvate into acetyl-CoA resulting in hydrogen production is catalyzed by the enzyme pyruvate ferrodoxin oxidoreductase (PFOR) (Equation 7). Molecular hydrogen is then produced by the oxidation of the reduced ferrodoxin (Fd) by the enzyme hydrogenase (Equation 8). The acetyl-CoA generated from pyruvate can then be converted into acetylphosphate, resulting in the formation of ATP and acetate (Equation 9) (Nath and Das, 2004; Reaume, 2009).

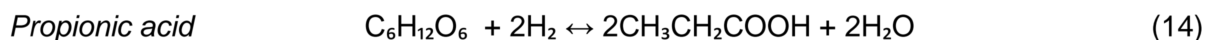


Not all pathways from pyruvate lead to hydrogen production (Equation 11 - 15). The pathways are dependant upon many different environmental conditions and the metabolic tools different microorganisms possess. For instance when sugars are degraded to ethanol (equation below) and lactate, no hydrogen is produced since end products and substrates have the same oxidation states. Below are examples of end-products of glucose degradation:









Xylose is a five carbon aldose sugar that is present in woody and non-woody biomass with a chemical formula of $C_5H_{10}O_5$. The xylose degradation pathway is denoted as the “XR-XDH” pathway. In the first step D-xylose is reduced into xylitol by the cofactors NADH or NADPH. Xylitol is further oxidized into D-xylulose, exclusively with the NAD+ cofactor. As there are a variety of cofactors needed in this pathway, which may not always be available for usage, an overproduction of xylitol may result. In the following step the D-xylulose is converted into D-xylulose-5-P where it then enters the pentose phosphate pathway (Kruger and von Schaewen, 2003).

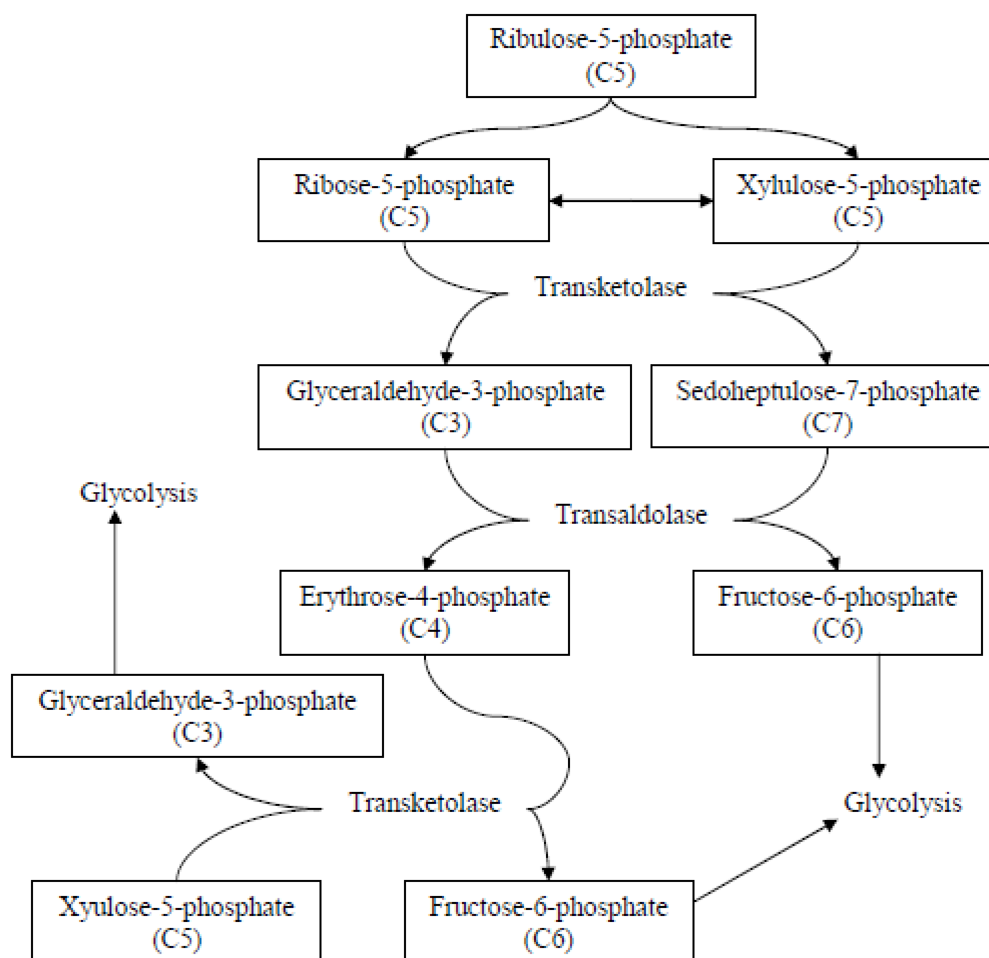
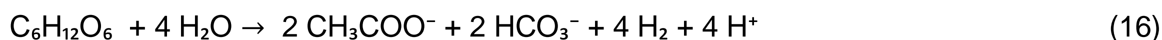


Figure 3 Breakdown pathway of the xylose-5-phosphate formed from the XRXDH breakdown pathway (Kruger and von Schaewen, 2003)

The two most common hydrogen producing routes for glucose and xylose are the acetate and butyrate pathways. Note reactions eq.15 through 18 which show that the yields of hydrogen gas are different per mol of each sugar. The glucose degradation reactions produce 4.0 and 2.0 moles of hydrogen per mol glucose according to the acetate and butyrate pathway, respectively. In case of xylose, the hydrogen yields are 3.33 and 1.67 mol of hydrogen per mol xylose for the acetate and butyrate pathways, respectively (Reaume, 2009).

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3(CH_2)_2COO^- + 2\,HCO_3^- + 2\,H_2 + 3\,H^+ \qquad (15)$$



$$C_6H_{12}O_6 + 4\,H_2O \rightarrow 2\,CH_3COO^- + 2\,HCO_3^- + 4\,H_2 + 4\,H^+ \qquad (16)$$

$$C_5H_{10}O_5 + 1.67H_2O \rightarrow 0.83CH_3(CH_2)_2COO^- + 1.67HCO_3^- + 1.67H_2 + 2.5H^+ \qquad (17)$$

$$C_5H_{10}O_5 + 3.33H_2O \rightarrow 1.67\,CH_3COO^- + 1.67HCO_3^- + 3.33\,H_2 + 3.33H^+ \qquad (18)$$

Since both the xylose and glucose pathways converge at the final stages of glycolysis, the same end products are formed. The difference between the sugars is one fewer oxygen atom, one fewer carbon atom and 2 fewer hydrogen atoms per molecule of xylose when compared to glucose therefore the different yields of hydrogen are produced.

2.2. Biomass

Biomass is an organic matter that is generated by the photosynthetic conversion of solar energy and carbon dioxide is fixed to more complex carbon compounds such as starch and cellulose. It is the only renewable energy resource that contains carbon that can be converted into solid, liquid or gaseous products that can then further be converted into heat, electricity and transport fuels. Biomass is currently the fourth largest energy source after traditional fossil fuel derived energy sources (coal, oil and natural gas). The biomass used as a feedstock for both EtOH and H_2 production can be classified as either simple or complex biomass. Simple biomass includes starch and sucrose, which is derived from corn and sugarcane, and has been used for bioethanol production for decades. Complex biomass or “plant biomass”, is composed of three different biopolymers (cellulose, hemicellulose and lignin) that are strongly bound together by non-covalent forces as well as covalent cross-linkages (Glazer and Nikado, 2007).

2.2.1. Brown macroalgae

Brown macroalgae are abundant worldwide with most of their distribution in temperate waters as varieties of Kelp (brown algae in the order *Laminariales).* The brown coloration is due to the brown pigment (fucoxanthin) and individual species vary in color from yellow to dark brown, depending on the depth. Brown macroalgae exhibit several features of an ideal feedstock that can complement the increased global demand on energy and food production. Large-scale cultivation of brown macroalgae is already being practiced in several countries, yielding over 70 million metric tons per year in 2006 (Roesijadi *et al*., 2010).

Because brown macroalgae do not contain lignin, simple biorefinery processes such as milling, leaching and extraction can separate the sugars for conversion into biofuels and renewable chemicals (Chapman, 1970). Additionally, valuable materials, such as protein meal for animal feed and potash fertilizer for crop production, can be separated to support sustainable food production. The most abundant sugars in brown macroalgae are alginate, mannitol and glucans (present as laminarin and cellulose). Conventional industrial microbes can use mannitol and hydrolysed glucans (Horn *et al*., 2000). However, the full potential of biofuel and renewable chemical production from brown macro algae cannot be realized unless alginate is co-fermented. Alginate composes 30–60 % of the total sugars in brown macroalgae (Chapman, 1970), so the inability of industrial microbes to catabolize alginate results in a substantial loss of product yield (Horn *et al*., 2000). Alginic acid and its salts, the alginates are components of the walls of brown algae. They consist of uronic acids: mannuronic acid and glucuronic acid in varying ratios. The alginates of brown algae exist both within the cell wall and in the intercellular substance. Their part in the cell wall may be as high as 40 per cent of the dry matter. They have a high affinity for divalent cations (calcium, strontium, barium, magnesium) which are important for their tendency to gel. Thus, enabling the co-fermentation of alginate and mannitol in an existing industrial microbe is a key criterion for the economic and efficient use of the sugars derived from brown macroalgae (Wargacki *et al*., 2012; Santos *et al*., 2013). In brown algae the sulphated fucan polysaccharides are common.

A number of structural variations can be found between the species. The structure is based on the C_6 carbohydrate fucose which is sulphonated and forms the polymeric backbone. Different branches of galactose and acetate side groups can be found as part of the fucan structure. Fucans are known to have therapeutic properties for blood coagulation or inflammation (Horn *et al.,* 2000).

2.2.1.1. The glycan composition of Brown seaweed

Fucoidans are heteropolysaccharides containing substantial amounts of L-fucose and sulphate ester groups. They are found in all brown seaweeds, but to a greater extent in the rocky shore seaweeds and less in the deeper water species such as *Laminaria* and *Ascophyllum*. The chemical composition of most fucoidans is complex. In addition to fucose and sulphate, fucoidans contain a range of other monosaccharides (mannose, galactose, glucose, xylose) and uronic acids, acetyl groups and protein. Furthermore, the structures of fucoidans from different brown algae vary from species to species (Figure 4). It is not clear if fucoidans are part of the structural carbohydrates, as it has been shown that they can form part of an amorphous matrix between the cells (Kloareg *et al.,* 1986). Fucoidan content, like most algal constituents, appears to be subject to seasonal variation. Crude fucoidan extracts from *Undaria pinnatifida* increased markedly from April to July (from 3 to 16 % dry weight) (Skriptsova *et al.,* 2010). A maximum fucoidan content of 7 % of dry weight was found in *Sargassum wightii* (Eluvakkal *et al.,* 2010).

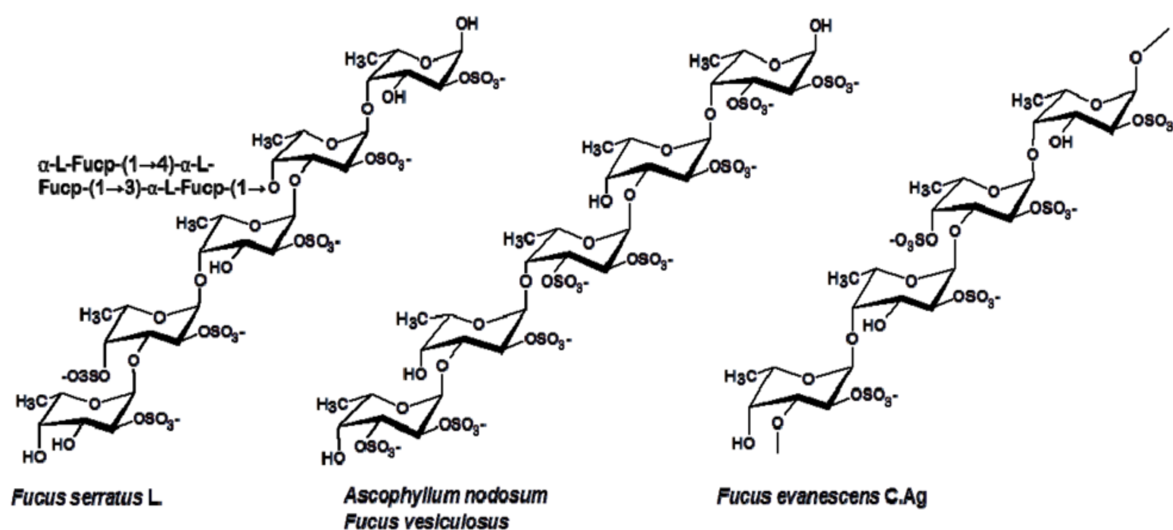


Figure 4 Chemical structures of fucoidans, isolated from different members of the order *Fucales* (Meyer *et al.,* 2011)

Alginate - a co-polymer of α-L-guluronate and its C_5 epimer β-D-mannuronate, which requires a certain pre-treatment to be utilized, but is also a possible high value co-product of a brown macroalgae bio-refinery, with use in the food industry as a water-retainer and a suspending and emulsifying agent in rubber and paint industries (Horn *et al.*, 2000). Alginates are believed to be amongst the most abundant marine biopolymers which makes them a potentially important carbohydrate target for biofuel production. The major source of alginate is found in the cell walls and the intracellular spaces of brown seaweed where it can constitute up to 40 % of the *L. hyperborea* dry weight (Horn *et al.,* 1999). Alginate is composed of mannuronic acid (M) which is β -1,4-linked and guluronic acid (G) which is α-1,4-linked in alginate.

Laminaran - A β-(1-->3) linked glucan, with some branching occurring in a β-(1-->6) fashion, which can be fermented without prior treatment provided the chosen microorganism has β-(1-->3) glucanases (Horn *et al.,* 2000). Laminarin is a storage carbohydrate of the kelps and phytoplankton (Meeuse, 1962; Painter, 1983) analogous to the starch of terrestrial plants and is stored intracellularely in vacuoles (Chiovitti *et al.,* 2004) were it can account for up to 80 % of the total carbon in diatoms and Phaeocystis (Meeuse, 1962; Alderkamp *et al.,* 2006). Laminaran structure and composition vary greatly between algae species (Chizhov *et al.,*

1998) with the laminaran backbone composed of β-1,3-linked D-glucan (Zvyagintseva *et al.,* 1999) and β-1,6-branching (Nelson and Lewis, 1974). There are two types of laminaran chains (M or G), which differ in their reducing end sugars. M chains are terminated by a non-reducing mannitol residue and have been found in species such as *Laminaria cloustoni* and *Laminaria digitata* (Goldstein *et al.,* 1959) whereas G chains end with a reducing glucose residue. Laminaran's molecular weight is approximately 5000 Da depending on the degree of polymerisation (usually 25 glucose moieties).

2.2.2. Terrestrial Biomass

Lignocellulosic material constitutes the world's largest renewable resource. There are several groups of raw materials that are differentiated by their origin, composition and structure. Forestland materials include mainly woody biomass namely, hardwoods and softwoods followed by sawdust, pruning and bark thinning residues while pasture and grassland encompass primarily agricultural residues that cover food or non-food crops and grasses such as switch grass and alfalfa (Hu *et al.,* 2008).

There are two types of woody materials that are classified into broad categories of either softwoods or hardwoods. Softwoods originate from conifers and gymnosperm trees (Hoadley, 2000) and unlike hardwoods, softwoods possess lower densities and grow faster. Gymnosperm trees, include mostly evergreen species such as pine, cedar, spruce, cypress, fir, hemlock and redwood. Hardwoods are angiosperm trees and are mostly deciduous. Unlike agricultural biomass, woody raw materials offer flexible harvesting times and avoid long latency periods of storage (Zhu and Pan, 2010). Additionally, this study reported that woody feedstock possessed more lignin than agricultural residues and less ash content. These unique characteristics of woody biomass including primarily high density and minimal ash content make woody raw material very attractive to cost-effective transportation in conjunction to its lower content in pentoses over agricultural biomass and more favorable for greater bioethanol conversion if recalcitrance is surmounted (Zhu and Pan, 2010).

Forestry wastes such as sawdust from sawmill, slashes, wood chips and branches from dead trees have also been used as bioethanol feedstocks (Perlack *et al.,* 2005). Crops residues consist of an extensive variety of types. They are mostly comprised of agricultural wastes such as corn stover, corn stalks, rice and wheat straws as well as sugarcane bagasse. Crop residues contain more hemicellulosic material than woody biomass (approximately 25-35%) (Demirbas, 2005). Aside from being an environmentally friendly process, agricultural residues help to avoid reliance on forestwoody biomass and thus reduce deforestation (non-sustainablecutting plants). Unlike trees, crop residues are characterized by a short-harvest rotation that renders them more consistently available to biofuell production (Knauf and Moniruzzaman, 2004; Kim and Dale, 2005).

Miscanthus giganteus is another fast-growing grass that is a potentially optimal candidate for biofuel production. It is native to Asia and is cultivated in Europe for combustible energy use. In addition to cellulosic feedstocks, municipal and industrial solid wastes are also a potential raw material for biofuel production. Their utilization limits environmental problems associated with the disposal of garbage household, processing papers, food-processing by-products, black liquors and pulps (Khanna, 2011). While woody biomass and agricultural residues potential was overestimated in 2005, highyielding energy crops including primarily Miscanthus have started to regain considerable interest compared to woody and agricultural residues because of their potential to cover 50-70 % of the total feedstock (Khanna, 2011). Production of biofuels form terrestrial plants leaves a large impact on the environment in general and on human beings in particular due to eutrophication, acidification and ecotoxicity. This is mostly caused by agricultural practies by the generation of waste water (Luo *et al.,* 2009).

2.2.2.1. The composition of lignocellulosic material

Lignocellulose is a class of biomass that consists of three major compounds cellulose, hemicellulose and lignin. It also includes water and a small amount of proteins and other compounds, which do not participate significantly in forming the structure of the material. Inside the lignocellulose complex, cellulose retains the crystalline fibrous structure and it appears to be the core of the complex. Hemicellulose is positioned both between the micro- and the macrofibrils of cellulose. Lignin provides a structural role of the matrix in which cellulose and hemicellu-lose is embedded. Considering that cellulose is the main material of the plant cell walls, most of the lignin is found in the interfibrous area, whereas a smaller part can also be located on the cell surface (Harmsen *et al.,* 2010).

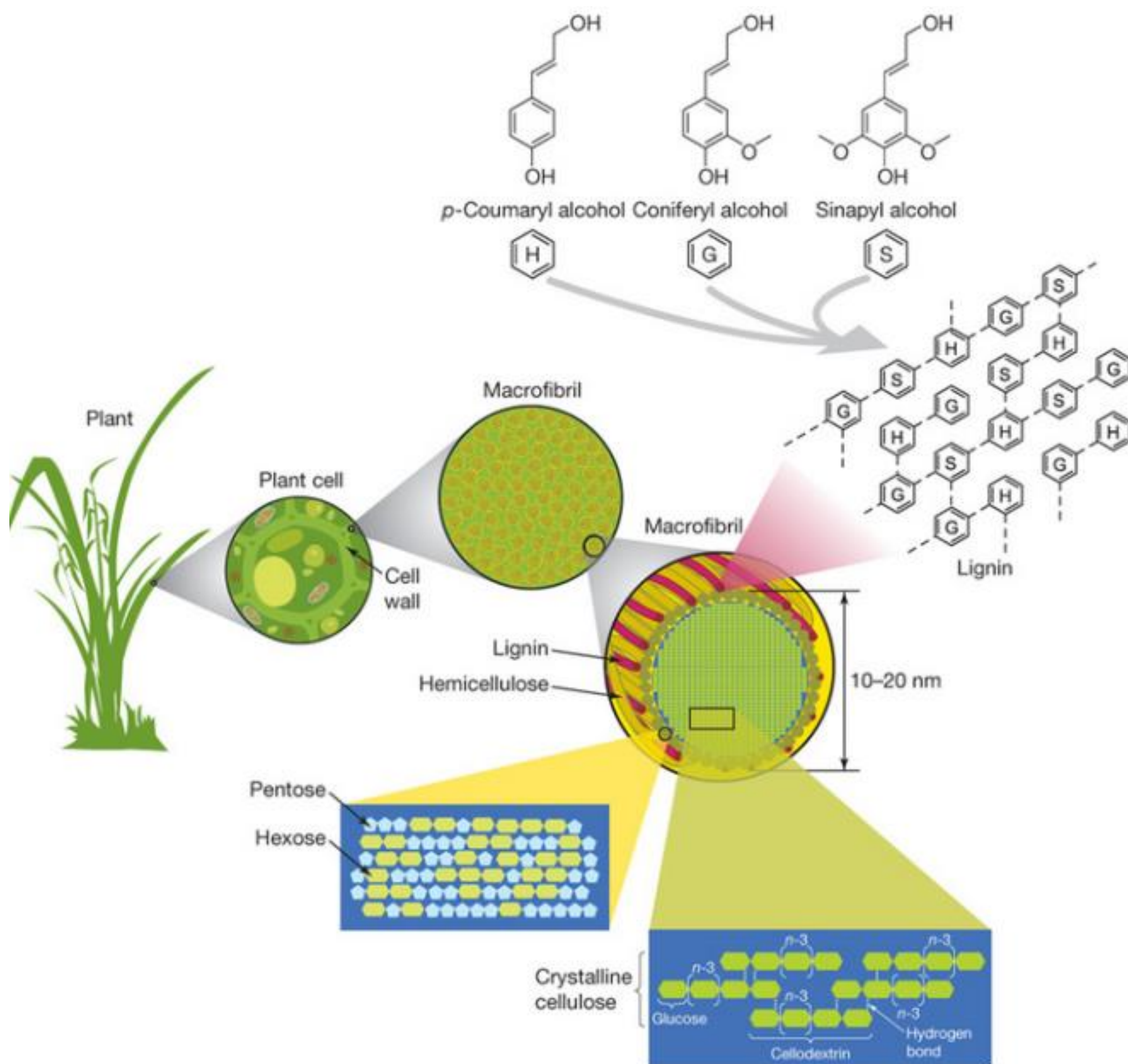


Figure 5 Ligno-cellulosic Material (Rubin, 2008)

Cellulose exists of D-glucose subunits, linked by β-1,4 *O*- glycosidic bonds. The cellulose in a plant consists of parts with a crystalline (organized) structure, and parts with a, not well-organized, amorphous structure. The cellulose strains are 'bundled' together and form so

called cellulose fibrils or cellulose bundles. These cellulose fibrils are mostly independent and weakly bound through hydrogen bonding (Laureano-Perez *et al.,* 2005; Hendriks and Zeeman, 2009).

Hemicellulose is a complex carbohydrate structure that consists of different polymers like pentoses (e.q. xylose and arabinose), hexoses (like mannose, glucose and galactose), and sugar acids. The dominant component of hemicellulose from hardwood and agricultural plants, like grasses and straw, is xylan, while this is glucomannan for softwood (Saha, 2003; Hendriks and Zeeman, 2009). Hemicellulose has a lower molecular weight than cellulose, and branches with short lateral chains that consist of different sugars, which are easy hydrolyzable polymers. Hemicellulose serves as a connection between the lignin and the cellulose fibers and gives the whole cellulose–hemicellulose–lignin network more rigidity (Laureano-Perez *et al.,* 2005). The solubility of the different hemicellulose compounds is in descending order: mannose, xylose, glucose, arabinose, and galactose. The solubility increases with increasing temperature. The solubility of higher molecular weight polymers could not be predicted, because of unknown melting points (Gray *et al.,* 2003). The solubilization of hemicellulose compounds into the water starts around 180 °C under neutral conditions. However mention that already from 150 °C parts of the hemicellulose solubalize. The solubilization of lignocellulose components not only depends on temperature, but also on other aspects like moisture content and pH. Xylan appears to be the part of lignocellulose that can be extracted the most easily. The xylan of hemicellulose can be extracted easily in an acid or alkaline environment, while glucomannan is difficult to extract in an acid environment and needs a stronger alkaline environment than xylan (Hendriks and Zeeman, 2009). Of cellulose, hemicellulose and lignin the hemicelluloses are the most thermal-chemically sensitive. During thermal–chemical pretreatment, the side groups of hemicellulose react, followed by the hemicellulose backbone (Hendriks and Zeeman, 2009).

Lignin is, after cellulose and hemicellulose, one of the most abundant polymers in nature and is present in the cellular wall. It is an amorphous heteropolymer consisting of three different phenylpropane units (*p*-coumaryl, coniferyl and sinapyl alcohol) that are held together by different kind of linkages. The main purpose of lignin is to give the plant structural support, impermeability, and resistance against microbial attack and oxidative stress. The amorphous heteropolymer is also non-water soluble and optically inactive; all this makes the degradation of lignin very tough. Lignin, just like hemicellulose, normally starts to dissolve into water around 180 °C under neutral conditions. The solubility of the lignin in acid, neutral or alkaline environments depends, however, on the composition in terms of its primary precursors (*p*-coumaryl, coniferyl, sinapyl alcohol, or combinations thereof) (Grabber, 2005; Hendriks and Zeeman, 2009).

2.2.3. Biomass pretreatment

As high water containing biomass such as macroalgae is difficult to preserve, immediate processing is usually carried out. Mechanical maceration is commonly followed by thermochemical pre-treatment processes, designed to potentially deactivate inhibitory compounds, pasteurize biomass and inactivate seaweed associated microorganisms, and/ or remove unwanted structural polymers, with the aim of improving the recovery of sugars. Plant and macroalgal tissue are naturally designed to withstand environmental challenges. For example, cell walls are complex and recalcitrant to degradation, potentially locking-in glucose-based storage products. They also contain defensive chemicals such as polyphenols that protect the plant against microbial and herbivoral attack. Polyphenols and other highly charged polymers are known to adsorb enzymes (Ximenes *et al.,* 2011), thus reducing the catalytic potential for enzymatic saccharification. As polyphenols can also make up a large proportion of seaweed (>10 %) and have shown to inhibit microbial activities,

deactivation of polyphenols using formaldehyde has already been investigated with improvements in methane production by anaerobic digestion being reported. In addition, high polyphenol containing seaweeds such as members of the *Fucales* are considered unsuitable for fermentation processes if left untreated, as polyphenols have shown to negatively impact growth of fermentative organisms such as *Saccharomyces* and *Lactobacillus spp.* (Segovia Bravo *et al.,* 2007).

Given the refractory nature of cellulosic and hemicellulosic biomass, it is not surprising that chemical processing techniques use mainly acids and elevated temperatures have been used for pre-treatment of terrestrial biomass (Zheng *et al.,* 2009) and seaweeds (Yazdani *et al.,* 2011; Jang *et al.,* 2012) before the application of cellulolytic enzymes. In the case of extracting soluble compounds, such as laminarin, from seaweed biomass, acid pre-treatment was reported to negatively impact ethanol production using *S. latissima* (Adams *et al,.* 2009). In addition to acids and alkalis, sodium chlorite pre-treatment has also been successfully applied as a pre-treatment method on the red seaweed *Gelidium amansii* before enzymatic saccharification were glucose yields improved from 5 to 70 % for pre-treated samples (Wi *et al.,* 2009). Pre-treatment is among the most costly steps in biochemical conversion of biomass, accounting for up to 40 % of the total processing cost (Zhang *et al.,* 2009). Mechanical size reduction is unavoidable and has both economic and energy costs unless fragmented waste or by-products are used as the starting material. Diverse techniques have been explored and described for the pretreatment of size-reduced biomass materials, with the aim of producing substrates that can be more rapidly and effectively hydrolysed by either chemical or enzymatic means to yield mixtures of fermentable sugars. Chemical, physical, and thermochemical methods are used to increase the surface area and open up the biomas, and thus create conditions for enzymatic attack (Figure 6). Therefore, pre-treatment using thermal, physical, chemical, biological or a combination of these is crucial in the conversion of biomass to a suitable fermentation substrate.

Lignocellulosic material must be pretreated prior to fermentation to hydrogen in order to remove lignin and hemicelluloses, reduce the cellulose crystallinity and increase the surface area of the material to enhance the release of sugars (Xia and Sheng, 2004). Physico-chemical pre-treatment of lignocellulosic material, such as the application of acid, alkaline or oxidative conditions at ambient or elevated temperatures, yields a mixture of pentoses and hexoses (Ren *et al.,* 2011). Efficient microbial fermentation of hexoses and pentoses is, therefore, the key step for hydrogen production from plant biomass. However, combined fermentation of mixtures of hexoses and pentoses is often prevented due to catabolic repression; in the presence of glucose, pentoses might be converted to a lesser extent thereby decreasing overall fermentation yields (Abreu *et al.,* 2010). Moreover, efficient hydrogen production from sugars is dependent on the different possible fermentation pathways.

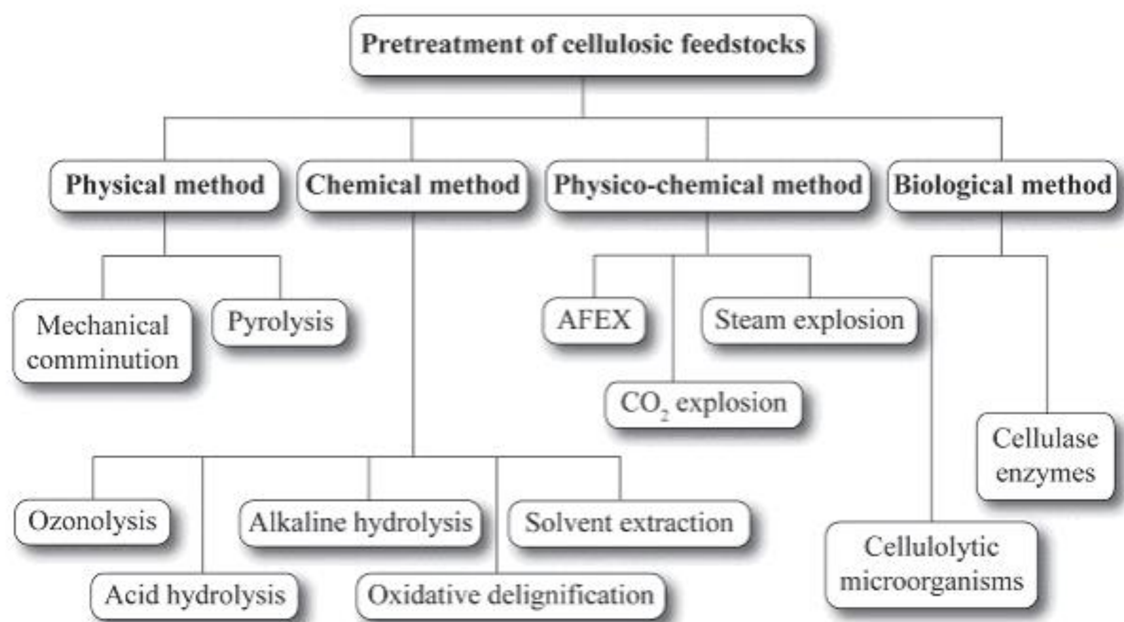


Figure 6 Methods for pretreatment of cellulosic feedstock (Saratale *et al.,* 2008)

2.2.4. Biohydrogen production from lignocellulosic materials using dark fermentation

Bio-conversion of biomass to produce H_2 has been demonstrated utilizing anaerobic fermentation of some well-defined compounds in liquid. However, the limitation was the pretreatment process involved to degrade cellulose to simple sugars (Mosier *et al.,* 2005). Main source of H_2 producing during a biological fermentative process is carbohydrate, either as oligosaccharide or as its polymeric form (cellulose, hemicellulose and starch). For effective H_2 yield directly from cellulose materials using dark fermentation require pretreatment process (delignification and hydrolysis) to dissolve organic matter from a lignocellulose complex and makes process expensive. Microbial (efficient celluloytic microorganisms) and enzymatic (cellulase complex) pretreatment have potential to convert cellulosic biomass into fermentable sugars and to make process cost effective. Biohydrogen production from cellulosic feedstock under dark anaerobic fermentation could be achieved by either a direct process in which cellulose is simultaneously hydrolyzed and converted into H_2 gas in a single stage or by two-stage process where cellulose hydrolysis and biohydrogen production are carried out seperatley.

Hydrogen production from lignocellulosic materials would be a potential process with the help of pretreatment on materials and cellulolytic microorganisms. Figure 7 shows a schematic diagram of possible hydrogen production route from lignocellulosic materials. Utilization of cellulose degrading bacteria would be an alternative way to degrade cellulose.

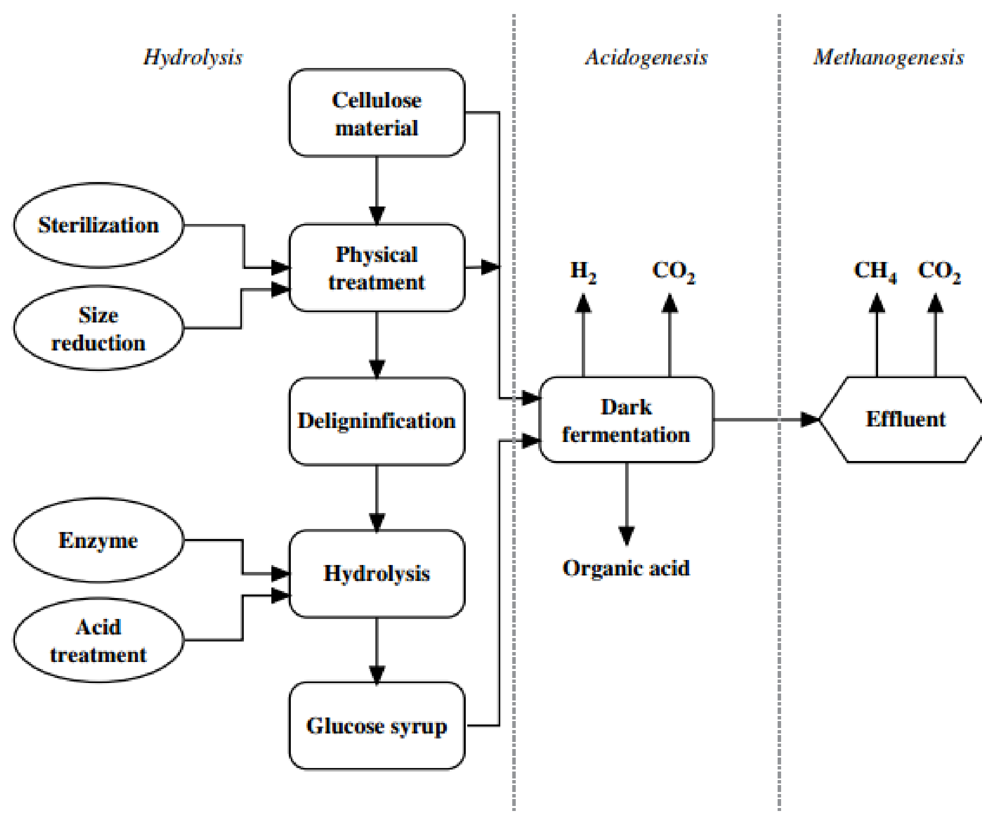


Figure 7 A schematic diagram for biohydrogen production from cellulose (Chong *et al.,* 2009)

Taguchi *et al.,* (1995) used a single bacterial strain to hydrolyze cellulose to sugars which were further degraded to produce hydrogen. . During 81 h stationary culture, the bacterium consumed 0.92 mmol glucose/h and produced 4.1 mmol H_2/h (Chong *et al.,* 2009). Cellulose degrading hydrogen producer, *Clostridium thermocellum* was studied for hydrogen production using cellulose-based medium (Levin *et al.,* 2006). They reported that delignified wood fibers were good substrates with the yield of 1.6 mol H_2/mol glucose. For efficient hydrolysis of cellulosic materials, the bacterial cell has to adhere to the cellulose (Chong *et al.,* 2009). Hydrogen production using heat treated sludge as inoculum was studied by Lay, (2001). Increased concentration of cellulose from 12.5 g/L to 50 g/L resultedin a lower hydrogen yields, from 2.18 mmol/g cellulose to 0.42 mmol/g, respectively. Higher temperature gave higher conversion of cellulose to hydrogen, which is from 43 ml H_2/g cellulose at 37 °C to 69 ml H_2/g cellulose at 55 °C. The maximum hydrogen yield obtained was 102 ml H_2/g cellulose at pH 6.5 and 55 °C. Theoretically, 1 g of cellulose yields 567 ml H_2 (Liu *et al.,* 2003). Wang *et al.,* (2008) had demonstrated a co-culture of *Clostridium acetobutylicum* X9 and *E. ethanoligenens harbinense* B49 in microcrystalline cellulose for hydrogen production. *E. harbinense* can produce hydrogen efficiently from monosaccharides but not cellulose. The bioaugmentation of both strains improved hydrogen production from microcrystalline cellulose from 755 ml H_2/L-medium to 1810 ml H_2/L-medium. The relationship of reducing sugar in cellulose hydrolysate was reported by Lo *et al.,* (2008). Lignocellulosic materials were initially degraded by *Cellulomonas sp.* rich sludge, and the reducing sugar-rich hydrolysate was readily used by pure *Clostridium sp.* for hydrogen production. With an initial reducing sugar (RS) concentration of 0.8 g/L, the hydrogen production and yield were approximately 23.8 ml H_2/L and 1.21 mmol H_2/g RS (1.09 mmol H_2/g cellulose), respectively. Acidification of lignocellulosic waste materials using diluted HCl successfully increased hydrogen production from 10 to 136-fold compared to hydrogen

production from raw waste (Fan *et al.*, 2006; Fan *et al.,* 2006; Zhang *et al.,* 2007; Chong *et al.,* 2009).

2.2.5. Sources of fermentation inhibitors

Pretreatment of lignocellulosic biomass may lead to the production of products that are inhibitory for the microbes used in the fermentation process. The level of toxicity depends in part on fermentation variables including cell physiological conditions, dissolved oxygen concentration and pH of the medium. In addition, the fermenting organisms may, to some extent, be resistant to inhibitors or may become gradually adapted to their presence. However, the optimal approach is to prevent the formation of inhibitors as much as possible through the pretreatment process conditions or other measures. The inhibitory effect of these compounds is higher when they are present together due to a synergistic effect (Mussatto and Roberto, 2004).

Sugar degradation products; subsequent to hemicellulose hydrolysis, pentose sugar monomers may dehydrate to the inhibitor furfural. Similarly hexose sugars (e.g. glucose) may degrade to the toxic hydroxymethyl-furfural (HMF). Furfural and HMF affect cell growth and respiration. HMF is considered less toxic than furfural and its concentration in (hemi) cellulose hydrolysates is usually low. It is clear that extensive degradation of (hemi) cellulose is responsible for the formation of the latter inhibitor compounds. Kinetic studies have shown that the production of furfural strongly increases with temperature and reaction time. Temperatures higher than 160 °C and residence time of acid pre-treatment longer than 4 hours have been reported to be sufficient for furfural or HMF to form, with their formation being more significant at higher temperature or longer residence times (McKillip and Collin, 2002).

Lignin degradation products; a variety of compounds (e.g. aromatic, polyaromatic, phenols, and aldehydes) may be released from the lignin fraction. Phenolic compounds have a considerable inhibitory effect and are more toxic (even at low concentrations) than furfural and HMF. Low molecular weight phenolics are the most toxic. Phenolic compounds cause partition and loss of integrity of cell membranes of the fermenting microorganisms, thus reducing cell growth and sugar assimilation. The main factors influencing formation are process temperature and residence time. At temperatures lower than 180 °C lignin degradation is negligible, if no strong acid or alkaline conditions are present (Harmsen *et al.,* 2010).

Acetic acid; Acetic acid is derived from the acetyl groups in hemicellulose. At low pH in the fermentation medium the acetic acid (pK_a = 4.75) is in the undissociated form, is liposoluble and diffuses into the cells. In the cell (pH = 7.4) the acid dissociates causing a lowering of cell pH that inhibits cell activity. The toxicity varies according to the fermentation conditions. Since the formation of acetic acid is inherent to hemi-cellulose hydrolysis, its formation cannot be prevented. However, a higher fermentation pH can reduce this effect or the acid can be neutralized before fermentation (Harmsen *et al.,* 2010).

Inhibitory extractives; Extractives are derived from the lignocellulose structure and include acidic resins, tanninic and terpene acids. These extractives are less toxic than lignin breakdown products or acetic acid (Harmsen *et al.,* 2010).

Heavy metal ions; Heavy metal ions (e.g. Fe, Cr, Ni, and Cu) may originate from corrosion of process equipment. Their toxicity may inhibit enzymes in the fermenting organisms metabolism (Harmsen *et al.,* 2010).

2.3. Hydrogen producing thermophilic bacteria

Thermophilic Anaerobes

Microorganisms can be divided into four groups based on their optimum temperature for growth: psycrophiles, mesophiles, thermophiles, and hyperthermophiles. Natural environments for anaerobic thermophiles range from terrestrial volcanic sites (including solfatara fields) with temperatures slightly above ambient temperature, to submarine hydrothermal systems (sediments, submarine volcanoes, fumaroles and vents) with temperatures exceeding 300 °C, subterranean sites such as oil reservoirs, and solar heated surface soils with temperatures up to 65 °C. There are also human-made hot environments such as compost piles (usually around 60 – 70 °C but as high as 100 °C) slag heaps, industrial processes and water heaters (Oshima and Moriya, 2008). Besides natural thermal environments, thermophilic anaerobes are also found within anthropogenically heated environments, including coal refuse piles and compost heaps, and nuclear power plant effluent channels which contain not only spore-forming species, but also vegetative and active cells including *Bacteria* and *Archaea*. Many environments are also temporarily hot, adaptation to which may be the reason some thermophiles are very fast-growing. Among the geothermally heated habitats are the alkaline, mainly carbonate-containing hot springs around a neutral pH, and acidic areas including some mud-holes. Most of the acidic high-temperature habitats contain elemental sulfur and metal sulfides and most isolates from these areas metabolize sulfur by either anaerobic respiration or fermentation (Canganella and Wiegel, 2014).

Major fermentation products formed by glycolytic thermophilic anaerobes include acetate, butyrate, lactate, ethanol, CO_2, and H_2 and to a lesser degree propionate, propanol and butanol. Traces of various branched-chain fatty acids from amino acid degradation are also detected since many glycolytic anaerobic thermophiles require yeast extract for growth and some even for metabolic activity (Canganella and Wiegel, 2014).

The main interest in anaerobic thermophiles during the last decades has mainly been on two issues dealing with basic and applied research: 1) the discovery of many novel hyperthermophilic *Archaea* (of which many can grow at 100 °C and above and a few even up to 121 °C), has attracted a great interest among the scientific community; 2) the realization that anaerobic thermophilic microorganisms can serve as excellent sources for thermostable biocatalysts was the driving force for implementing basic and applied research on thermophiles. Due to the stress of living at such extreme temperatures, anaerobic thermophiles have evolved a variety of mechanisms that allow them to survive at temperatures other organisms cannot thrive at. These traits include unique membrane lipid composition, thermostable membrane proteins, and higher turnover rates for various protein enzymes. One of the most important attributes to the maintenance of homeostasis within the organism is that of the plasma membrane surrounding the organism. Aside from having to stabilize the plasma membrane at high temperatures, anaerobic thermophiles must also stabilize their proteins, DNA, RNA, and ATP (Canganella and Wiegel, 2014).

Thermophilic bacteria in soil seldom live at their optimum temperature. However they adapt to conditions in the environment and grow faster when temperature becomes favorable. Thus, hot springs are favorable habitats for thermophilic bacteria because of the stability of that particular environment and they can grow close to their optimum temperature. Thermophilic bacteria are organisms that can live grow optimally at temperatures higher temperatures, higher than 45 °C. These bacteria are classified depending on what temperature they live at and on their optimum temperature for growth:

- Thermophilic bacteria, live at 45 - 68 °C with optimum around 60 °C
- Extreme thermophilic bacteria, live at 65 – 95 °C with optimum around 88 °C

- Hyperthermophiles such as Pyrophilic archaea, live at even higher temperatures for example *Pyrolobusfumarii* with optimum temperature at 106 °C and can survive up to 115 °C. These bacteria do not grow below 60 °C.

Strictly anaerobic bacteria grow only in the absence of oxygen (O_2) and its presence can in some cases be often fatal for them. These bacteria are called obligate anaerobes. Facultative anaerobes grow with or without oxygen. The solubility of oxygen is very poor at high temperatures and the amount of oxidizing gases that are formed in hot-springs forcesand most thermophilic bacteria to live as strict anaerobes or microaerophiles. Other species of bacteria that also use anaerobic metabolism besides thermophilic bacteria are acidophiles, halophiles and alkalophiles. These are all species that live at extreme conditions. Obligate anaerobic bacteria are mostly found in areas where oxygen is in low concentrations and are often responsible for oxidizing organic material which can then be used by aerobic bacteria. This kind of collaboration interactions can often be found with bacterial communities that live in these extreme conditions. There are even bacteria that can exchange hydrogen molecules, where one strain cannot utilize some reduced organic compounds unless there is another strain that uses the hydrogen that is produced.

2.3.1. Strict anaerobes

The hyperthermophile *Pyrococcus furiosus*, an archae, produces H_2, organic acids and CO_2 from carbohydrates. From characterization studies of utilized substrates and produced products many extreme- and hyperthermophiles are known to produce hydrogen from carbohydrates. Cellulolytic thermophiles producing hydrogen such as species of *Anaerocellum, Caldicellulosiruptor, Clostridium, Dictyoglomus, Fervidobacterium, Spirocheta, Thermotoga, Thermoanaerobacterium and Thermoanaerobacter,* range from moderately thermophilic to hyperextremophiles. Schröder *et al.*, (1994) reported on batch fermentations at 80 ºC with *Thermotoga maritima*. A H_2 yield on glucose of 4 mmol/mol was obtained which is equal to the maximal theoretical value. However, glucose consumption was low (1.6 mM) and low cell densities (1.4 x 108 per mL) were reached. Maximal hydrogen production rates of approximately 10 mmol/L.h were measured. Similar stoichiometries as for *T. maritima* were obtained for two moderate thermophiles, *Acetothermus paucivorans* and *Acetomicrobium flavidum*, grown at 60 ºC. Recently, results on growth and hydrogen production by two other extreme thermophiles during sugar fermentation have been published (van Niel *et al.,* 2002). In cultures of *Caldicellulosiruptor* saccharolyticus grown on sucrose at 70 ºC and *Thermotoga elfii* grown on glucose at 65 ºC stoichiometries of 3.3 mole H_2 per mole hexose were obtained which is 83 % of the theoretical maximum. Maximal hydrogen production rates of 8.4 and 2.7 mmol/L.h, respectively, were measured. These results show that higher hydrogen yields on hexose can be reached by extreme and hyper-themophiles compared to mesophilic facultative and strict anaerobes (de Vrije and Claassen, 2003).

Clostridium

The genus *Clostridium* belongs to the family *Clostridiaceae*, order *Clostridiales*, class *Clostridia* and *phylum Firmicutes*. The genus *Clostridium* is a very large genus and contains over 200 validly described species. Phylogenetic analysis of *Clostridium* bacteria has shown big distances in relations within the genus and in fact it is high time that these bacteria be reclassified (Collins *et al.*, 1994). These bacteria are spore forming and often present in environments which are rich in plant decaying material. It is thus not surprising that many species are capable of polymer hydrolyzation and this is one of the main reasons for extensive research on biofuel production from complex biomass by these bacteria (Canganella and Wiegel, 1993; Carreira and Ljungdahl, 1993). Several cellulose-degrading

enzymes form a structure called cellulosome, located and embedded on the external surface of the cell membrane (Demain *et al.,* 2005).

Caldicellulosiruptor

Caldicellulosiruptor species belong to a group of extremely thermophilic obligate anaerobes, which possess a natural ability to produce hydrogen from a wide range of mono-, di-, and oligo-saccharides and raw materials (Pawar *et al.,* 2013; Ivanova *et al.,* 2009; Pawar *et al.,* 2015). In addition to this, various other beneficial metabolic features enable the genus *Caldicellulosiruptor* as one of the best, yet not ideal, groups of bacteria with the natural ability to produce H_2 (Pawar and van Niel, 2013). Within this genus, *Caldicellulosiruptor saccharolyticus* and *Caldicellulosiruptor owensensis* are two of the best-studied species, both known to produce H_2 near the theoretical maximum of 4 mol · mol^{-1} (Zeidan and van Niel, 2013; de Vrije *et al.,* 2007).

Thermoanaerobacter

Bacteria within this genus were originally classified within the genus *Clostridium* because of close phylogenetic relationship and physiological properties. These bacteria use the classical EMP pathway for sugar degradation and produce EtOH, acetate and lactate as major end products (Lee *et al.,* 1993; Sveinsdottir *et al.*, 2009). Most species have broad substrate range and can degrade both pentoses and hexoses. The genus consists of 24 species (subspecies included) originating from various environments like hot springs and oil fields. Most species produce EtOH and H_2 as well as lactate, and in some cases alanine as end products (Sveinsdottir *et al.*, 2009).

Rumen bacteria

Other strict anaerobic bacteria producing hydrogen are rumen bacteria. *Ruminococcus albus* has long been known to produce H_2 together with other products like acetate, ethanol, formate and CO_2 from carbohydrates. In a continuous culture a H_2 yield of 2.4 mol/mol glucose was reported by Innotti *et al.*, (1973). Since then production of H_2 by *R. albus* was not studied further (de Vrije and Claassen, 2003).

Methanogens

Methanogens are characterized by the presence of hydrogenase, which is usually involved in the oxidation of H_2 coupled to CH_4 production and CO_2 reduction. Methanogens are prokaryotic microorganisms that produce methane as an endproduct of their metabolism. They are strictly anaerobic archaea belonging to the taxon *Euryarchaeota*. Methanogens occupy a wide variety of anaerobic environments, even extreme habitats characterized by high temperature, salinity, and extreme pH (Liu and Whitman, 2008).

Methanogens share primarily two common physiological characteristics, namely growing strictly anaerobically and producing methane as the exclusive final product of energy metabolism (Garcia, 1990). In contrast to their significantly similar energy metabolism, methanogens inhabit extremely diverse environments, including freshwater and marine sediments, the digestive and intestinal tracts of animals and anaerobic waste digesters. So far, 28 genera of methanogens have been described. The majority of rod-shaped methanogens are affiliated to the order *Methanobacteriales*, which consists of three mesophilic genera (*Methanobacterium, Methanobrevibacter* and *Methanosphaera*) and two thermophilic or hyperthermophilic genera (*Methanothermobacter* and *Methanothermus*). The anaerobic digester is a compatible surrounding for the growth of mesophilic methanogens and *Methanobacterium* strains constitute the main microbial flora, which play an important

role in the anaerobic degradation of organic compounds as the terminal metabolic groups (Ma *et al.,* 2005).

2.3.2. Factors affecting end product formation

Yields of end products are affected by many factors which include substrate type, environmental conditions, and microbial and engineering design factors. Reduced substrates rich in electrons and that are easily degradable will generate greater higher hydrogen yields compared to those which are poor electron donors and are more oxidized substrates. Temperature and pH are factors affecting enzymatic activities which in turn have effect on the end product profile. At higher temperatures hydrogen yield is greater than that obtained from low temperatures. The operating pH also affects - under low pH conditions, the production of reduced products is more favorable such as butyrate than the more oxidized products such as acetate (Yu *et al.,* 2002). An important environmental factor concerning the utilization of thermophilic bacteria for scale up production of ethanol and hydrogen is the substrate loading. It is a well-known fact that thermophilic bacteria do not tolerate high substrate loadings compared to yeasts (Lacis and Lawford, 1992; Sommer *et al.,* 2004). The manipulation of the electrons produced during oxidation of carbon substrates is of importance concerning energy gain and deeper understanding of the physiology of the strains. Environmental factors like high initial substrate concentration and the partial pressure of hydrogen can play an important role in both ethanol and hydrogen production (Sommer *et al.,* 2004).

The effects of several major factors including inoculum, substrate, reactor configuration, temperature, pH, nitrogen, phosphorus and metal ions, on hydrogen production by fermentation have been studied (Yang and Shen, 2006; Wang and Wan, 2009; Kim *et al.,* 2012). In addition, if some hydrogen-consuming bacteria like methanogens, sulfate reducing bacteria and homoacetogens existed in microflora, the hydrogen yield would be reduced because of their ability of using H_2 to gain energy (Guo *et al.,* 2010). Therefore, it is vital to enhance the metabolic activity of hydrogen-producing bacteria and meanwhile inhibit the ability of hydrogen-consuming bacteria for improving the efficiency of fermentation hydrogen production. Yeast extract contains peptide, amino acid, vitamins and trace elements, and these nutrients are important for growth and metabolism of the microorganisms (Bibi *et al.,* 2012). In addition, some vitamins and trace elements are cofactors of certain enzymes in microbial metabolic processes.

Nutritients; Nutrients such as nitrogen (peptone and yeast extract), phosphorus, vitamins and mineral salts were shown to be essential for the synthesis of macro- and micro-molecules in the cells (Li and Fang, 2007). Bacteria require an electron donor, a carbon source, salts, vitamins and trace elements to grow. Nutritients such as nitrogen which is used for DNA and protein synthesis and phosphorus is another important nutrient which is used for energy storage and in DNA synthesis. Certain heavy metals, trace elements at low levels are also important for the metabolism of all cells. Metals such as magnesium, sodium, zinc, and iron are important cofactors for enzyme function (Lin and Lay, 2005). Other metals, such as zinc, sodium, iron, and magnesium have been shown to have toxic effects (Li and Fang, 2007). An important component of operating a successful biological reactor is to provide the proper amount of necessary nutrients. Too much macro and micronutrients can have a negative effect on the biological processes. That is why the reactors must be properly maintained so that the bacteria have ideal operating conditions in order to produce the maximum amount (Fang *et al.,* 2004).

pH; The pH is another important factor affecting the production of hydrogen. It has effects on the hydrolytic and catabolic bacterial pathways (Lay, 2001). Low pH is inhibitory to methanogenic activity in anaerobic biological processes (Li and Fang, 2007). Also, the

activity of hydrogenase, an iron-containing enzyme, is inhibited by low pH (Dabrock *et al.,* 1992). Therefore, controlling the pH is necessary due its effects on hydrogenase enzymes and enzymes mediating many metabolic pathways (Chen *et al.,* 2002). The pH is a major factor affecting the control of methanogenic pathways in which hydrogen is consumed (Lay *et al.,* 2001). Ren and coworkers found out that application of pH out of the optimum range decreased the level of adenosine triphosphate (ATP) in the cell which leads to the inhibition of the hydrogen producing bacteria's growth. In mixed culture, the optimum pH for H_2 production varied widely, and it may be due to the complex nature of the prevailing bacterial populations and substrates. Therefore, discovering an effective initial pH and also ability to control pH of the medium to be within a favorable range will be crucial (Ren *et al.,* 2011).

Temperature; The effect of temperature has a significant impact on the end production. Research has shown that in mesophilic and thermophilic reactor systems the rate of hydrogen production is higher than in psychropilic systems. For every increase of 10 °C, the enzymatic activity within a range of 20 °C to 60 °C is expected to double (Li and Fang, 2007). There is a tradeoff in an increase in temperature and the increased rate of hydrogen production due to the many engineering and biological factors. When a bacteria's cell membrane is warmer it behaves more fluid-like. The phospholipids become more fluid and flexible hence, there is easier diffusion of molecules by passive and active transport processes allowing more compounds to pass through (Cirne *et al.,* 2007). Biological hydrogen production can be affected by variation in the environmental temperature. Hydrogen producing bacteria are mostly categorized in two main groups: Mesophilic mesophilic and thermophilic bacteria whose favorable ranges of temperature are 30 – 40 and 45 – 55 °C, respectively. Sudden increase in temperature impedes biohydrogen production adversely. Nevertheless, a sudden fall in temperature decreases the hydrogen concentration rapidly, although it can be alleviated following the adaptation of the microorganisms to the new culture environment. Moreover, in some cases when the temperature shifted considerably lower than the safe ranges, the bacteria needed more ferrous ion to activate the hydrogenase so that it could oxidize reduced ferredoxin to produce more hydrogen. As a matter of fact, there is no exact optimum temperature in biohydrogen production. The optimum temperature for producing hydrogen via dark fermentation depends on the type of H_2 producers and also the carbon source used.

Substrate Source; Carbohydrates, such as sugars, cellulose and starch are commonly used as electron donors for hydrogen-producing fermentations as they are relatively inexpensive and readily available. An optimization of the substrate to biomass concentration or microorganism (F/M) ratio is needed to ensure proper operational efficiency of the process (Lay, 2001). Research by van Ginkel *et al.*, (2001) suggests that higher substrate concentrations can have an inhibitory effect due to the increased acid production which leads to lower pH values and increased hydrogen partial pressures (Li and Fang, 2007).

Heat treatment; In spore forming bacteria the original cell replicates its genetic material, which is surrounded by a tough coating. The outer cell then is destroyed releasing the spore which is now well protected against a variety of environmental conditions, including temperature extremes, radiation, and an absence of nutrients. There was no reported optimal value for heat treatment in terms of temperature or duration of heating. However, the most common treatment for an anaerobic mixed community inoculum is at 100 °C for 15 min (Li and Fang, 2007). Oh *et al.,* (2003) found that some homoacetogenic bacteria may survive heat treatment and eventually use hydrogen for the production of acetate.

Partial pressure of hydrogen; higher partial pressure (ρH_2) of hydrogen leads to icreased ethanol production and less acetate production from glucose fermentations (Brynjarsdottir *et al.,* 2012; Fardeau *et al.,* 1996). Hydrogen itself can become inhibitory to its own production as it has been shown that a hydrogen partial pressure of 2.0 kPa prevented growth and butyrate consumption as compared to pressures as low as 0.1 kPa. The hydrogen generated by fermentative bacteria is rapidly consumed by hydrogen-utilizing organisms, including

methanogens and sulfate-reducers (Cord-Ruwisch *et al.,* 1988). Thus, hydrogen accumulation does not occur allowing for a complete catabolism of glucose to end products. The elevated hydrogen partial pressure is a problem that has to be dealt with in order to reduce the levels of hydrogen in solution. Therefore, researchers have developed methods to maximize the yield of hydrogen. One approach is to release the dissolved hydrogen by sparging the culture with an inert. The second is to use a specialized membrane which is permeable only to hydrogen. Due to the relatively small size of the hydrogen molecule, it can pass through a small pore size where other products or reactants are retained in the gas phase (Li and Fang, 2007).

Electric Current; hydrogen-producing bacteria could be separated from methane gas producing bacteria in a sludge sample by electric current. Using an application of low-voltage (3.0 – 4.5 V) electric current, cellulose containing landfill sludge and municipal sewage sludge were shown to accumulate hydrogen without the accumulation of methane (Roychowdhury, 2000).

Product Inhibition; during hydrogen fermentation, acetate and butyrate productions are always accompanied with hydrogen production. However, these products could result in the feedback of product inhibition to the microbes' activities. Therefore, product inhibition is always the critical factor leading to a worse performance scenario in biological hydrogen reactions. An investigation reported that the IC_{50} values, which the butyric concentration cause 50 % inhibition at bioactivity of hydrogen producing bacteria, were estimated as 19.39 and 20.78 g/L with respect to hydrogen production rate and hydrogen yield (Zheng and Yu, 2005).

Long Chain Fatty Acids Inhibition; Long chain fatty acids (LCFA's) are compounds with hydrophilic and hydrophobic groups. They are attached to a glycerol back bone by ester bonds to form glycerides. The glycerides can exist in the mono- di- and tri- forms. LCFAs are linear carbon compounds classified into saturated and unsaturated fatty acids. Saturated fatty acids are carbon chains with only single bonds between the carbons whereas unsaturated fatty acids are carbon chains with one or more double bonds between the carbons. The double bond causes branching of the fatty acid which makes it more of a liquid at room temperature whereas saturated fattyacids tend to be solid at room temperature. LCFAs act by several mechanisms to inhibit a variety of microorganisms. They cause the death of bacteria by surrounding the cell in a lipid layer (Alves *et al.,* 2001). This lipid layer causes the bacteria to float and is washed out in the effluent. LCFAs also impose mass transfer limitations across the cell membrane (Cirne *et al.,* 2007) needed for energy (Reaume, 2009).

Inocolum; the microbial populations are very crucial as they are responsible for degradation of organic compounds to hydrogen and other end-products. Numerous microorganisms have been identified as hydrogen producers, and strictly anaerobic bacteria, mesophilic or thermophilic, are the most common class of bacteria that produce hydrogen (Vertes *et al.,* 2009). Pure bacterial isolates have also been studied as mono-cultures or co cultures. Utilizing complex material requires a wide range of hydrolytic and catabolic activities, which is where mixed microbial populations are useful and more advantageous than pure cultures. Additionally, pure cultures are substrate specific, whereas, mixed cultures have a broader source of feedstock (Wang and Wan, 2009). During harsh conditions, hydrogen-producing bacteria have a better chance of survival than hydrogen-consuming bacteria. Hydrogen producing bacteria can form protective spores in restrictive environments such as high temperature, extreme acidity and alkalinity, but hydrogen consuming bacteria are not able to withstand such extreme conditions (Zhu and Beland, 2006).

Table 1 Selected hydrogen production by thermoanaerobes with different substrates

Inocolum	Substrate	Reactor	T (°C)	Yield (mol H_2/mol C_6)	References
Thermoanaerobacterium thermosaccharolyticum W16	Glucose	Batch	60	2,42	Ren *et al.,*2008
Thermoanaerobacterium thermosaccharolyticum W16	Corn stover	Batch	60	2,2	Ren *et al.,* 2010
Caldicellulosiruptor saccharolyticus	Glucose	Batch	70	3,4	Mars *et al.,*2010
Thermotoga elfi	Glucose	Batch	65	3,33	van Niel *et al.,*2002
Clostridium paraputrificum M-21	Glucose	Batch	45	1,1	Evvyernie *et al.,* 2001
Clostridium butyricum and *Enterobacter aerogenes HO-39*	Sweet potato starch residue	Batch	37	2,7	Yokoi *et al.,* 2002
Rhodopseudomonas palustris P4	Glucose	Batch	37	2,76	Oh *et al.,* 2002
Caldicellulosiruptor owensis	Glucose	Continuous	70	3,8	Zeidan and van Niel, 2010
Caldicellulosiruptor owensis	Xylose	Continuous	70	2,7	

Table 1 (Cont) Selected hydrogen production by thermoanaerobes with different substrate

Inocolum	Substrate	Reactor	T (°C)	Yield (mol H_2/mol C_6)	References
Caldicellulosiruptor saccharolyticus	Glucose	Continuous	70	3	Willquist *et al,*. 2009
Caldicellulosiruptor saccharolyticus	Wheat straw	Batch	70	3,8	Ivanova *et al.,* 2009
Caldicellulosiruptor saccharolyticus	Sugarcane bagasse	Batch	70	2,3	
Caldicellulosiruptor saccharolyticus	maize leaves	Batch	70	3,67	
Cattle manure	Glucose	Batch	55	0,35	Cheong and Hansen, 2007
Anaerobic mixed cultures	Glucose	Expanded granular sludge bed reactor	70	0,75	Abreu *et al.,* 2012
Thermophilic waste activated sludge	Starch (10g/L)	CSTR HRT 24 hr	55	2,32	Akutse *et al.,* 2008
Thermophilic digested cattle manure				1,17	
Thermophilic acidified potato				2,02	
Clostridium thermocellum and *Thermoanaerobacterium thermosaccharolyticum*	Micro-crystalline cellulose (5 g/L)	Batch	60	1,8	Liu *et al.,* 2008

3. Effect of culture conditions on hydrogen production by *Thermoanaerobacter* strain AK68

3.1. Introduction

Thermoanaerobacter species are of interest for the production of biofuels including biohydrogen due to their tolerance towards harsh environments such as pH and temperature extremes, short generation times, and their broad substrate spectrum (Wiegel and Ljungdhal, 1981; Taylor *et al.,* 2009; Scully and Orlygsson, 2015). Species within the genus have been noted to be both good hydrogen and ethanol producers from various sugars and carbohydrates from complex biomass (Wiegel and Ljungdhal, 1981; Soboh *et al.,* 2004; Lacis and Lawford, 1988; Lacis and Lawford, 1989; Carriera *et al.,* 1982; Fardeau *et al.,* 1996; Jessen and Orlygsson, 2012). *Thermoanaerobacter ethanolicus* has, for example, been reported to produce close to the theoretical yield of ethanol, 1.95 mol ethanol/mol glucose (Carriera *et al.,* 1982) which is 97.5 % of the theoretical yield. Alternately, *T. tengcongensis* produces 4 moles of H_2 per mole of glucose during continuous gas sparging during fermentation (Soboh *et al.,* 2004) which is the theoretical yield of hydrogen from a hexose (Jones, 2008). Recent investigations on *Thermoanaerobacter* species have focused on their ability to produce high-value fine chemicals such as branched-chain alcohols (Scully and Orlygsson, 2014).

Most thermophiles use the Emden-Meyerhof pathway for sugar degradation to pyruvate and further to various end products including ethanol, acetate, lactate, butyrate, alanine, carbon dioxide, and hydrogen (Taylor *et al.,* 2009; Scully and Orlygsson, 2015; Wagner and Wiegel, 2008). It is well known that various environmental factors such as pH, initial substrate concentration, product inhibition, partial pressure of hydrogen (pH_2), and the presence of alternative external electron acceptors are of great importance for determining the end product formation patterns. For instance, pyruvate can be used as an electron sink resulting in the production of reduced end products such as lactate and ethanol or pyruvate can be oxidized leading to production of acetate and butyrate. Several studies on *Thermoanaerobacter* have shown that some strains can be extremely sensitive towards very low initial substrate concentrations, such as *Thermoanaerobacter* strain GHL15 which could only degrade and 11 mM of initial 20 mM glucose concentrations in batch cultures (Brynjarsdottir *et al.,* 2013). Similar inhibitions have been observed with *Thermoanaerobacter ethanolicus* (Lacis and Lawford, 1988), *Thermoanaerobacterium* sp. AK17 (Almarsdottir *et al.,* 2012), and more. It was not clear from the aforementioned studies whether this inhibition is due to direct substrate loadings or by end product inhibition either due to accumulation of hydrogen or acids that lower the pH in batch cultures. It is known that using different liquid – gas ratios in closed batch systems is of great importance regarding substrate utilization (Jessen and Orlygsson, 2012; Brynjarsdottir *et al.,* 2012) suggesting that hydrogen accumulation may be to responsible for this inhibition. Also, it is well known that hydrogen accumulation or hydrogen scavenging has profound effects on the types of end products formed in *Thermoanaerobacter* species (Wiegel and Ljungdahl, 1981; Soboh *et al.,* 2004; Brynjarsdottir *et al.,* 2012).

The present study investigates the role of culture conditions on the production of hydrogen by *Thermoanaerobacter* strain AK68. This was done by altering culture conditions, including temperature, pH, liquid-gas phase ratio, the presence of an electron scavenging system, buffer concentrations, and initial substrate concentration. Particular emphasis was placed on the role of the partial pressure of hydrogen or by scavenging hydrogen chemically (addition of thiosulfate) or biologically (co-culture with hydrogenotrophic methanogen). Finally, the capacity of the strain to produce hydrogen from more complex biomass (brown macroalgae and Timothy grass) was investigated.

3.2. Materials and Methods

Culture Media

The medium (per liter), hereafter referred to as BM medium, consisted of NH_4Cl 0.3 g, NaCl 0.3 g, $CaCl_2$ 0.11 g, $MgCl_2 \times 6H_2O$ 0.1 g, yeast extract 2.0 g, resazurin 1 mg, trace element solution 1 mL, vitamin solution 1 mL, and $NaHCO_3$ 0.8 g. Carbon and energy sources were 20 mM or in the case of polymers, 3 g l^{-1}. Phosphate buffers were also used where 1 M stock solutions of NaH_2PO_4 and Na_2HPO_4 were made and added to the media to give a buffer capacity of 50 mM. The vitamin solution was prepared according to DSM 141. The trace element solution consists of (g/L) $FeCl_2 \times 4H_2O$, 2.0, EDTA, 0.5, $CuCl_2$, 0.03, H_3BO_4, $ZnCl_2$, $MnCl_2 \times 4H_2O$, $(NH_4)_6Mo_7O_{24} \times 4H_2O$, $AlCl_3$, $CoCl_2 \times 6H_2O$, $NiCl_2$, all 0.05 mg, and 1 mL of concentrated HCl. The medium was prepared by adding the phosphate buffer, yeast extract, and resazurin to distilled water, which was then boiled for 5 – 10 min and cooled while flushing with nitrogen. After pouring the medium into serum bottles they were closed and the gas phase was sparged with nitrogen. Finally the medium was autoclaved for 60 minutes. All other components of the medium were added separately through filter (0.45 µm) sterilized solutions. All experiments were conducted in duplicate at 65 °C and at pH of 7.0; the strain was cultivated for 5 days in duplicate without shaking.

Inoculation with culture - the serum bottles were inoculated with volume that is 2 % (v/v) of the final serum bottle volume, unless otherwise noted.

Isolation of strains

Thermoanaerobacter strain AK68, originally named as J4, was isolated from a hot spring (64.6 °C/pH 8.0) from Grensdalur, SW-Iceland (Jessen, 2013). Samples were collected and transported in serum bottles to laboratory. Five mL of the sample was inoculated into 45 mL BM medium containing 20 mM of glucose in 117.5 mL serum bottle and incubated at 65 °C (pH 7.0). Positive growth was confirmed by hydrogen production and was re-enriched three times into a glucose containing medium. From the final enrichment series, a tenfold dilution was made and from the most diluted sample showing positive growth, end point dilutions series were performed in glucose containing medium with agar (3 % w/v). Pure colonies were picked from the most diluted positive sample and re-inoculated to liquid medium containing 20 mM of glucose.

The hydrogenotrophic methanogen used, *Methanothermobacter* M39 was isolated in BM medium but with H_2/CO_2 (80/20 % v/v) in the gas phase instead of nitrogen as described by Brynjarsdottir *et al.,* (2012).

Phylogenetic analysis

The 16S rRNA from *Thermoanaerobacter* strain AK68 was partially sequenced (1081 nt) by 16S rRNA as described by Vesteinsdottir *et al.,* (2011). The sequence was compared to sequences in the NCBI (National Center for Biotechnology Information) database using the nucleotide-nucleotide BLAST tool (Altschul *et al.,* 1997). The most similar sequences obtained were aligned with sequencing results in MEGA6 (Tamura *et al.,* 2013) and the maximum likelihood method based on the Tamura-Nei model was used to generate a phylogenetic tree (Tamura and Nei, 1993). The hydrogenotrophic methanogen, strain M39 was analyzed for 16S rDNA by conventional methods performed by DSMZ. Genomic DNA extraction was carried out using MasterPure™ Gram Positive DNA Purification Kits from Epicentre®Biotechnologies, Germany according to the manufacturer's instructions. PCR

mediated amplification of the 16S rDNA and purification of the PCR product was carried out. Purified PCR products were sequenced using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) as described in the manufacturer's protocol. Sequence reactions were electrophoresed using the 3500 xL Genetic Analyzer from Applied Biosystems. The resulting sequence data were put in the alignment editor (Maidak *et al.,* 1999), aligned manually and compared with representative 16S rRNA gene sequences of organism belonging to Archaea (Maidak *et al.,* 1999).

Determination of optimum growth conditions

Cell concentration was determined by measuring absorbance at 600 nm by a Perkin Elmer Lambda-25 UV-Vis spectrophotometer. Maximum (specific) growth rate (μ_{max}) for each growth experiment done as previously reported (Sveinsdottir *et al.,* 2009). The temperature optimum (T_{opt}) was determined by cultivating the strain at pH 7.0 at temperatures ranging from 45 °C to 80 °C. The strain's optimum pH (pH_{opt}) for growth was determined by incubating the strain at 65 °C and at a pH from 3.0 to 9.0 in 0.5 pH unit increments.

Substrate utilization

The ability of *Thermoanaerobacter* strain AK68 to utilize different substrates was tested using the BM medium supplemented with various filter sterilized substrates, except for xylan, starch, and cellulose, which were autoclaved with the medium. Substrate concentration was 20 mM or, in case of polymers, 2 g/L. Experiments were done in 25 mL serum bottles containing a final volume of 12.5 mL (liquid-gas phase of 1:1). The substrates tested were: xylose, ribose, arabinose, glucose, fructose, galactose, mannose, L-rhamnose, L-fucose, sucrose, lactose, sorbitol, lactate, formate, succinate, malate, pyruvate, oxalate, crotonate, tartaric acid, mannitol, glycerol, inositol, starch, cellulose, xylan, pectin, casamino acids, yeast extract, beef extract, peptone, alanine, aspartate, glycine, glutamate, *N*-acetyl-D-glucosamine, glucosamine, serine, theronine, histidine, and cysteine. Positive growth was indicated by growth greater than that of control bottles and hydrogen production; where positive growth was detected, end products were analysed.

Effect of liquid-gas (L-G) volume ratio and initial glucose concentration on end-product formation

The influence of partial hydrogen pressure (pH_2) on end product formation was investigated with different ratios of liquid and gas phases when grown on 20 mM glucose. The liquid phase varied from 2 to 90 mL in serum bottles with a total volume of 117.5 mL; thus, the L-G volume ratio varied from 0.017 to 3.27. After incubation, the glucose content and the end products formed (hydrogen, acetate, and ethanol) were analysed.

Effect of different initial pH for glucose degradation and end product formation

The strain was cultivated at different initial pH levels on 20 mM of glucose with increments of 0.5 pH units. The experiment was done in 25 mL serum bottles containing a final volume of 12.5 mL (L-G of 1:1). After 5 day cultivation, glucose, hydrogen, acetate and ethanol were analysed.

Effect of electron scavenging systems on glucose degradation and end product formation

Strain AK68 was co-cultivated either with thiosulfate (40 mM) or in a co-culture with *Methanothermobacter* M39 in one set of experiments. The methanogen was pre-cultivated in BM medium with a gas phase consisting of H_2/CO_2 (80/20 v/v) for one week in 118 mL bottles containing a final volume of 20 mL (L-G of 0.2). Thereafter, the culture bottles were flushed with nitrogen before glucose (20 mM) and strain AK68 (2 % v/v) were added. The co-culture was incubated for five days and samples were taken for analysis of both volatiles and gases.

Hydrolysate experiment

In this experiment four different types of Hydrolysates (HL); Timothy grass (*Phleum pratense*) and *Fucus vesiculosus*, *Ascophyllum nodosum* and *Laminaria digitata* were used. Whatman paper was used as control.

Timothy grass (*Phleum pratense*) was collected in the summer of 2012, *Fucus vesiculosus* and *Ascophyllum nodosum* were collected from the intertidal zone of Eyjafjordur (Iceland) in the summer of 2013 and *Laminaria digitata* was obtained from Thorverk (Iceland). Collected algae samples were dried at 45-50 °C for 48 hours. Dried biomass and Whatman paper (cellulose) were milled in a Waring blender and passed through a 1 mm sieve. Biomass samples were analyzed for fat by soxhlet extraction with diethyl ether, protein content using the Kjeldahl method, water content by oven drying at 105 °C for 1 hour, and ash content according to the published NREL Laboratory Analytical Procedures (Laurens, 2013).

The first step of the HL preparation procedure was to cut the Whatman paper to very small pieces with a Waring blender. For the hydrolysate 25 g/L and 100 g/L of the biomass (Timothy grass and WP) was put into 1000ml bottles, with 5,15 mL of 97 % H_2SO_4 and 800 mL of dH_2O. Biomass was suspended in slightly less than desired final volume (i.e. 800mL for 1000 mL Vf) and autoclaved for 60 minutes at 121 °C. After cooling down bottles were adjusted to pH 5.0 with HCl and NaOH and 15 ml of Novozyme 188 and 15 ml of Cellulast were added to each bottle and filled to desired volume 1000mL. It was incubated for 68 hours at 45 °C during shaking (120 RPM). Thereafter the material was centrifuged at 4700 rpm for 20 min and the pH adjusted to 7.0.

For each algae hydrolysate, 0.5 grams of the biomass was put into four 100 ml bottles. For each hydrolysate four different pretreatments were performed; Acid, Acid + Enzymes, Enzymes and No pretreatment.

For acid and enzyme pretreatment it was used 0.1 ml of 97 % (v/v) H_2SO_4 and 1ml of enzymes Cellurest and 1 ml of Novozymes 188, and filling the bottle up to 100 ml with distilled water. The content of each bottle was mixed for a few seconds with a blender, and then autoclaved for 60 minutes.

After cooling down, the acidity of each bottle was adjusted to pH 7.0. The final concentration of each HL's was 5 g/L. All biomass was first filtering through a filter paper with a vacuum pump, then two nylon filters (53 μm and 5 μm) and the last filtering the HL into closed and sterile bottles with syringe filter (0.45 μm).The 24.5 ml bottles were used and the total liquid volume of each culture was 12.2 ml. Inoculation with culture - the serum bottles were

inoculated with volume that is 2 % (v/v) of the final serum bottle volume.This corresponds to a L/G ratio of 0.69. The incubation time was 5 days at 65 °C.

Effect of inhibitory compounds

To investigate the effect on inhibitory compound 24.5 ml bottles were used. Four inhibitory compounds (2-furfuraldehyde, 5-hydroxymethyl-2-furfuraldehyde, Levulinic acid, Vanillin) were investigated at concentrations from 10 to 80 mM in the prescence of 20 mM glucose. The total liquid volume of each culture was 12.2 ml and the incubation time of the cultures was 5 days at 65 °C.

Analytical methods

Hydrogen and methane were analyzed using a Perkin Elmer gas chromatograph equipped with thermo conductivity detector. Nitrogen was used as carrier gas at a rate of 3 mL/min, with another 10 ml/min as make-up gas in the detectors. The separation was performed on a Supelco 1010 Carboxen CC Plot Capillary Column. The oven temperature was 80 °C and the injector and detector temperatures were 200 °C. Volatile fatty acids and alcohols were analyzed by gas chromatograph (Perkin Elmer Clarus 580) using a FID detector with 30 m DB-FFAP capillary column (Agilent Industries Inc, Palo Alto, CA, US). Samples (1 mL) were centrifuged for 5 min at 13000 rpm. The supernatants were acidified with 25 % formic acid and crotonic acid was used as the internal standard. Optical density was then measured with UV-Vis spectrophotometer (Perkin Elmer, Lambda 25) at 600 nm with the pathlength of 1 cm. The final pH of the cultures was measured by a Thermo Scientific 4 star pH meter. Glucose was analysed by using the anthrone method (Laurentin and Edwards 2003). Biomass samples were analyzed for fat by soxhlet extraction with diethyl ether, protein content using the Kjeldahl method, water content by oven drying at 105 °C for 1 hour, and ash content according to the published NREL Laboratory Analytical Procedures.For the experiments performed on grass and algae, high performance liquid chromatography (HPLC) with a refractive index detector was used. Sugars and lactate were quantified by HPLC using a Shimadzu equpped with an LC20-AD pump, RID-10A Refractive index detector (40 °C) and an HPX-87H (BioRad, State) at ambient temperature with a 20 µL injection volume. 5 mM H_2SO_4 was used as a mobile phase at a flow rate of 1.2 mL per min.

Statistical methods

Obtained data in the experimental work were statistically processed with Microsoft Excel programme. The measure of average, standard deviation and standard curves were calculated, eq. 20 through 22 (Strode and Brokaw, 2013);

Measures of Average: calculating the sample average by summing all the data points:

$$\bar{x} = \frac{\sum x_i}{n} \qquad (20)$$

Standard Deviation; calculate the average of the deviation between each measurement in the sample and the sample mean (x). The formula (eq.21) for calculating the sample standard deviation:

$$s = \sqrt{\frac{\sum (x_i - \overline{x})^2}{(n-1)}} \tag{21}$$

Standard Curves; measuring correlations between sets of data. The correlation coefficient (r) provides a measure of how related two variables are, and it is expressed as a value between +1 and −1. The closer the value is to 0, the weaker the correlation. Calculating r involves determining the sample mean of the predictor variable ($\overline{x}$) and its standard deviation (sx), the sample mean of the response variable ($\overline{y}$) and its standard deviation (sy), and the number of pairs (*X*, *Y*) of individuals in the sample (n) and the coefficient of determination, uses the square of r.

$$r = \frac{\sum_{i=1}^{n}\left(\frac{x_i - \overline{x}}{s_x}\right)\left(\frac{y_i - \overline{y}}{s_y}\right)}{n-1} \tag{22}$$

3.3. Results

Phylogeny and phenotypic characterization

One basis of the 16S rRNA gene, *Thermoanaerobacter* strain AK68 is most closely related to *Thermoanaerobacter thermohydrosulfuricus* (99.7 %), *Thermoanaerobacter brockii* (98.6 %), and *Thermoanaerobacter pseudoethanolicus* (98.2 %) as shown in Fig. 8.

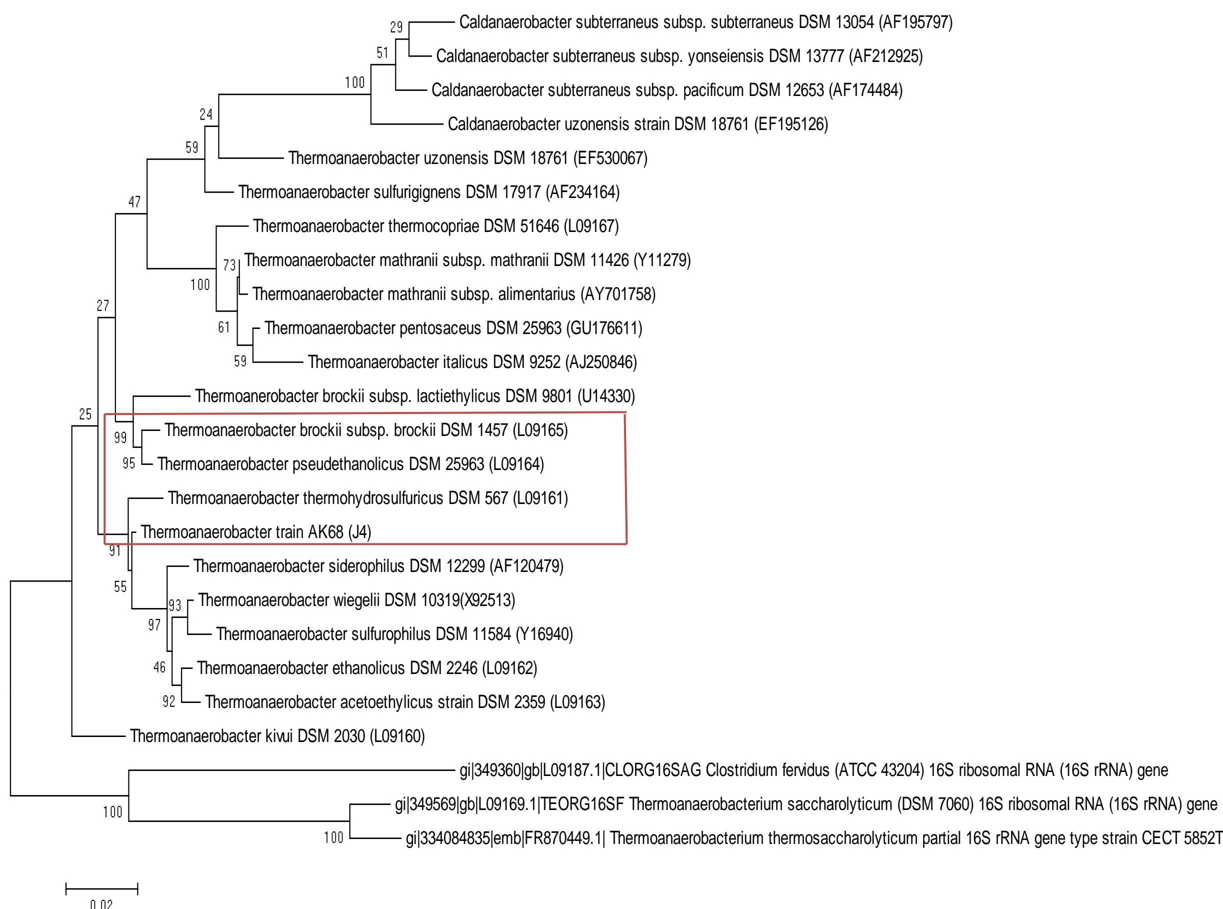


Figure 8 Maximum likelihood phylogenetic dendogram based on 16S rRNA gene sequences showing the position of strain AK68 within the class Clostridia. Bootstrap values (> 50%) based on 1000 replicates are shown at branch nodes. Bar represents 1 substitution per 100 nucleotides.

The strain shows a broad substrate spectrum degrading xylose, various hexoses (D-glucose, D-mannose, D-galactose, fructose), disaccharides (maltose, cellobiose, sucrose, lactose, and trehalose), trisaccharide (raffinose), xylan, pyruvate, and serine (Fig. 9). The main end products formed by strain AK68 from tested substrates were acetate and hydrogen; ethanol was only produced in small quantities (< 4 mM) except for xylose (17.5 mM) and trehalose (8.0 mM). Acetate and hydrogen concentrations were typically between 10 to 20 mM but highest values were observed on glucose where acetate and hydrogen were 20.6 and 27.4 mmol/L, respectively. The pH at the end fermentation on various carbon substrates was between pH 6.3 to 7.1.

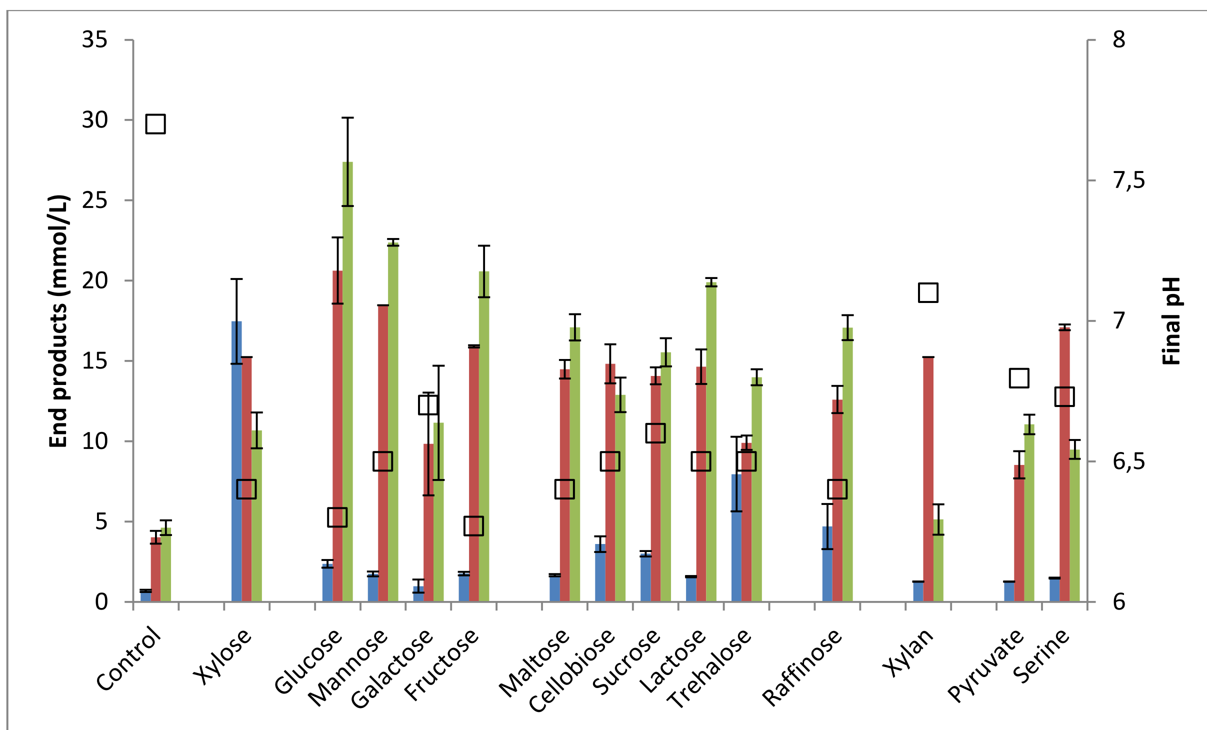


Figure 9 End product formations from various substrates. Data represent the average of two replicate experiments. Standard deviations are shown as error bars. Columns from left to right: ethanol, acetate and hydrogen. Final pH (□).

Additionally, the strain was found to be proteolytic, degrading yeast extract, peptone, casamino acids and beef extract (not shown in Fig. 9). The strain did not degrade ribose, arabinose, L-fucose, L-rhamnose, mannitol, sorbitol, lactate, formate, succinate, malate, oxalate, crotonate, tartaric acid, mannitol, glycerol, inositol, starch, cellulose, pectin, alanine, aspartate, glycine, glutamate, *N*-acetyl-D-glucosamine, glucosamine, theronine, histidine, and cysteine.

Temperature and pH optimum

Thermoanaerobacter strain AK68 grows optimally at 65 °C (range 50.0 to 70.0 °C) between pH 7.0 and 8.0 (range 4.0 to 10.0). Generation time under optimal conditions was 2.5h.

Effect of initial glucose concentration

To investigate the effect substrate and/or end product formation the strain was cultivated with different initial glucose (5 to 100 mM). Strain AK68 degraded most of the glucose at 5 and 10 mM concentrations to mainly acetate and hydrogen (Fig. 10).

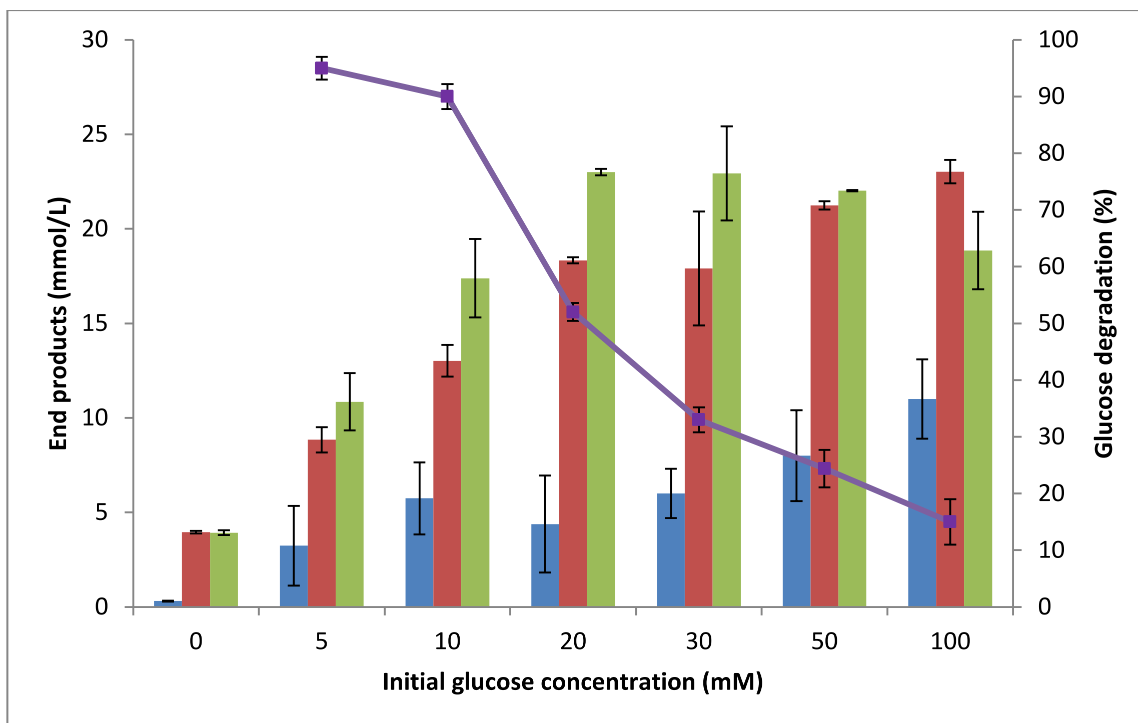


Figure 10 End product formations from different initial glucose concentrations. Also shown is percentage of glucose degradation (■). Data represent the average of two replicate experiments. Standard deviations are shown as error bars. Columns from left to right: ethanol, acetate and hydrogen.

The carbon balances for 5 and 10 mM glucose concentrations were 90.4 and 87.5 %, respectively. At a concentration of 20 mM glucose, only 48.0 % of the glucose was degraded, mainly to acetate and hydrogen. Above 20 mM concentrations, less and less portion of the glucose was degraded and ethanol and acetate were produced in similar concentrations as observed at 20 mM initial glucose concentrations. The carbon balances for 30, 50, and 100 mM were 94.0, 98.1 and 88.1 %, respectively.

Effect of initial pH values

To investigate the effects of initial pH on end product formation, the strain was cultivated pH values between 3 and 11 in the prescence of 20 mM of glucose. The pH optimum is between pH 7.0 and 8.0 (see earlier) and at these pH values (pH 7.0, 7.5, and 8.0), highest concentrations of end products were indeed observed (Fig. 11).

The strain grew between pH 4 and 9.5 although performance diminishes below pH 7 and above pH 8. As observed earlier, the glucose was only partially degraded under all conditions; the highst hydrogen/acetate yields were obtained between pH 7 and 8 with 38 to 53 % glucose utalization. Lower hydrogen yields were obtained below pH 7 and above pH 8 with glucose consumption reflecting end product formation.

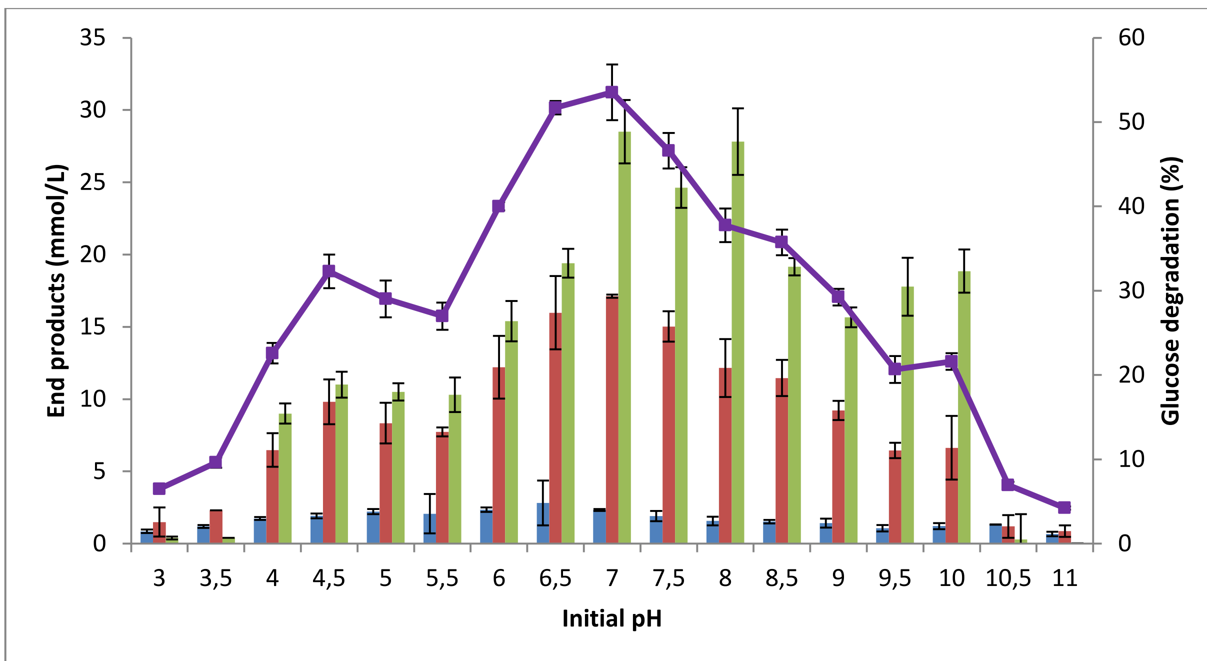


Figure 11 End product formations from glucose (20 mM) at different initial pHs. Data represent the average of two replicate experiments. Standard deviations are shown as error bars. Columns from left to right: ethanol, acetate and hydrogen. Glucose degradation in percent (■).

Effect of temperature

End product formation was determined at temperatures ranging from 40 to 80 °C. The strain produced the most acetate and hydrogen between 65 and 75 °C; acetate concentrations were between 14.7 to 21.2 mM with hydrogen concentrations from 25.5 to 27.5 mmol/L (Fig. 12).

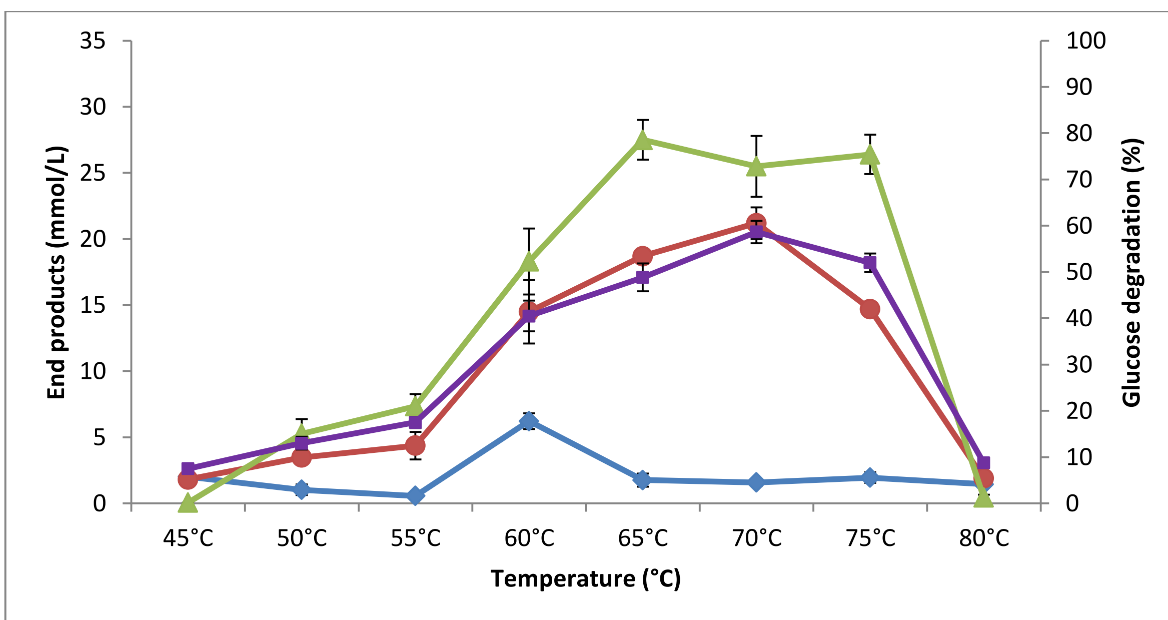


Figure 12 End product formations from glucose (20 mM) at different temperatures. Data represent the average of two replicate experiments. Standard deviations are shown as error bars. Ethanol (♦), Acetate (●), Hydrogen (▲). Glucose degradation in percent (■).

Between 50 to 60 % of the glucose were degraded from these three temperatures. At 45, 50, and 80 °C very little end products were produced and less than 10 % of the glucose was degraded. Very little ethanol was produced under all conditions, except for 60 °C were 6.2 mM of ethanol were analysed in the cultures.

Effect of partial pressure of hydrogen

The effects of the partial pressure of hydrogen by strain AK68 was done by cultivating the strain on glucose (20 mM) in 117.5 mL serum bottles but with different liquid-gas (L-G) phases (lowest 0.02; highest 3.27). Table 2 shows end product formation for strain AK68 after 5 day cultivation.

Table 2 Production of end products from glucose (20 mM) in different liquid-gas ratios. Values represent the mean of two replicates (± standard deviation). Hydrogen values are corrected to volume units. Controls are without glucose.

	Concentration (mmol L^{-1})						
Liquid – gas phase	Ethanol	Acetate	Hydrogen	Ethanol/ Acetate Ratio	Glucose degraded (%)	Final pH	Carbon balances (%)
0.02 (Control)	0.5 ± 0.0	8.8 ± 0.5	20.7 ± 0.5	ND	NA	7.6	NA
0.02	3.4 ± 0.2	35.4 ± 0.6	45.4 ± 1.3	0.10	80.1	6.5	88.0
0.04 (Control)	0.7 ± 0.1	7.4 ± 0.4	13.4 ± 3.7	ND	NA	7.3	NA
0.04	3.4 ± 0.1	30.0 ± 3.2	37.9 ± 0.5	0.11	78.0	6.3	81.9
0.09 (Control)	0.5 ± 0.0	5.7 ± 0.3	7.7 ± 1.3	ND	NA	7.3	NA
0.09	3.33 ± 0.5	30.1 ± 0.6	40.2 ± 3.3	1.07	80.0	6.2	84.8
0.34 (Control)	0.5 ± 0.0	4.3 ± 0.1	6.7 ± 0.2	ND	NA	7.2	NA
0.34	4.7 ± 0.5	23.7 ± 2.0	36.3± 1.4	0.20	59.9	6.0	95.9
1.06 (Control)	0.6 ± 0.1	3.8 ± 0.2	4.6 ± 0.1	ND	NA	7.2	NA
1.06	7.4 ± 1.1	15.7 ± 1.6	20.7 ± 1.0	0.47	50.1	6.2	93.3
3.34 (Control)	2.1 ± 0.2	4.9 ± 0.0	4.0 ± 0.2	ND	NA	7.3	NA
3.34	28.7 ± 2.5	5.5 ± 0.5	6.4 ± 0.3	5.21	77.1	6.5	99.3

The ethanol concentrations are relatively stable (between 3.4 to 4.5 mM) for all the L-G ratios used, with the exception of L/G ratio of 3.27 where increased dramatically (to 28.7 mM) and acetate lowers to similar extent (Table 2). Acetate and hydrogen formation steadily decrease from low to high liquid-gas rations. Acetate decrease from 35.4 to 5.5 mM and hydrogen from 45.4 to 6.4 mmol/L. Thus the ethanol/acetate ratio ranged from 0.1 (lowest L-G ratio) to 5.2 (highest L-G ratio). The highest hydrogen yields were observed at the lowest liquid-gas ratio, or 2.3 mol H_2/mol glucose degraded (Fig. 13).

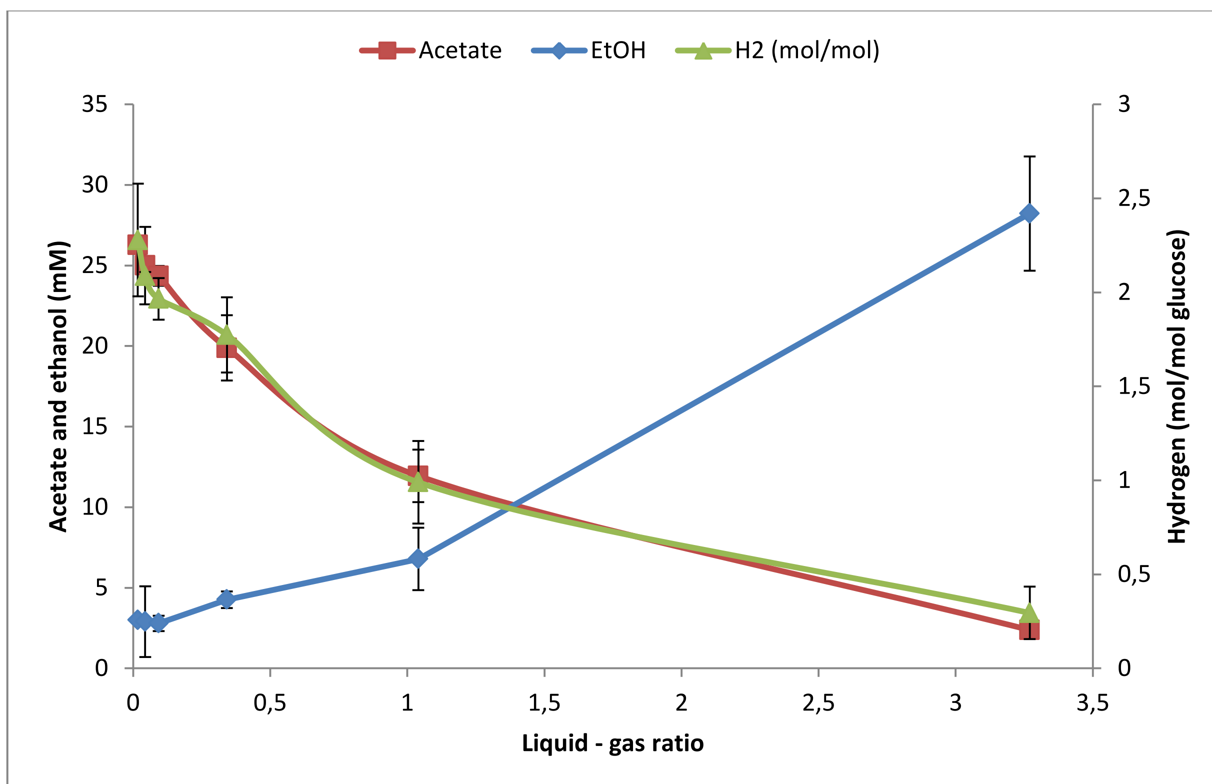


Figure 13 Effect of liquid gas ratios on end product formation. Hydrogen formation is showed as number of moles produced from one mole of glucose. Data represent the average of two replicate experiments. Standard deviations are shown as error bars. Ethanol (♦), Acetate (■), Hydrogen (▲)

Effect of electron scavenging systems

To further get insight into the relevance of the importance of hydrogen as end product the strain was cultivated on glucose (20 mM) in the presence of thiosulfate and in a co-culture of hydrogenotrophic methanogen as well as without any electron scavenging systems. When glucose was degraded in the presence of thiosulfate, almost 16 mM (80 %) of the glucose was degraded and higher amounts of acetate were compared as compared with glucose fermentation without thiosulfate addition of which only 48 % of the initial glucose was consumed (Fig. 14).

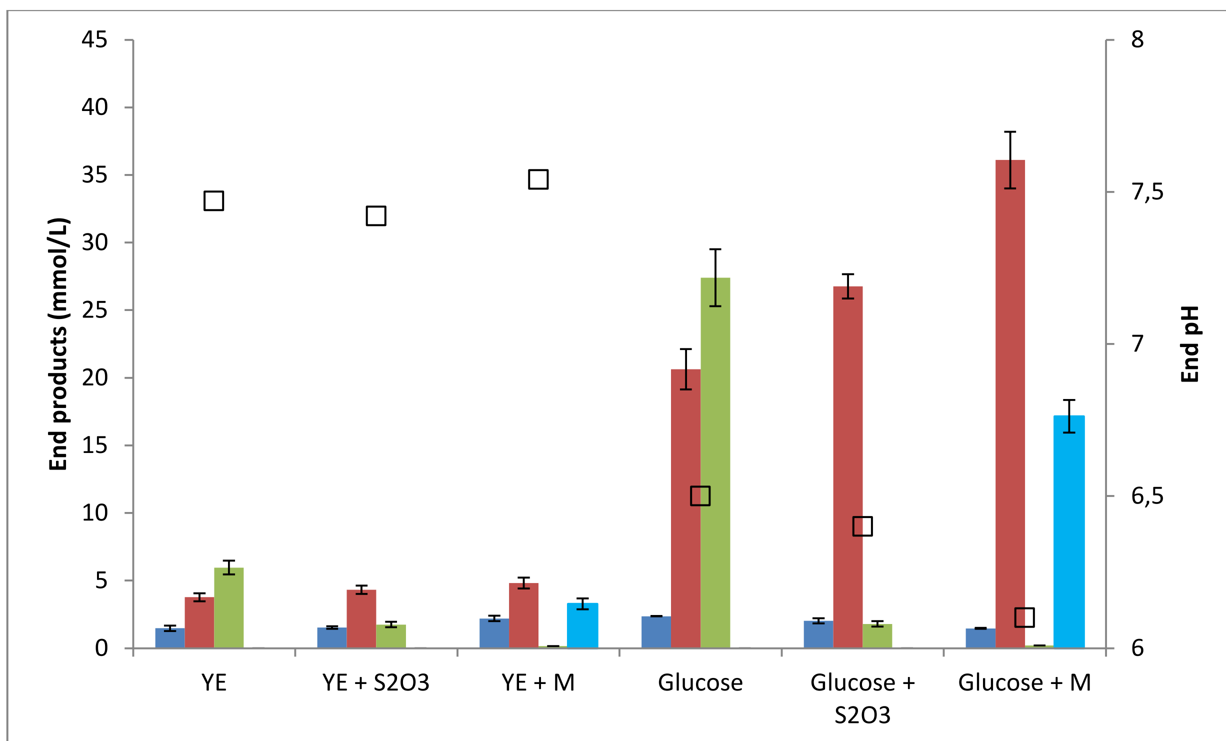


Figure 14 End product formation from glucose (20 mM), glucose (20 mM) and thiosulfate (40 mM) and glucose (20 mM) when cultivated in a co-culture with *Methanothermobacter* M39. Columns from left to right: ethanol, acetate, hydrogen, methane and final pH (□).

Hydrogen concentrations in the presence of thiosulfate at end of fermentation were very low (1.8 mmol/L). Hydrogen sulphide and sulphur were not analysed in the experimental bottles. When strain AK68 was co-cultivated in the presence of *Methanothermobacter* M39, almost complete degradation of glucose was observed and more acetate was produced together with methane. The stoichiometry for glucose degradation under methanogenic condition (controls subtracted) were: 1.00 Glucose → 1.60 Acetate + 0.72 CH_4. The maximum yields of hydrogen from one mole of glucose are 4 moles, occurring when acetate is the only volatile end product. Since hydrogenotrophic methanogens use 4 mole of hydrogen to produce one mole of methane, the theoretical methane yields for co-culture of strain AK68 and *Methanothermobacter* M39 are 0.80 moles which is slightly higher than obtained yields (0.72 moles). Ethanol production under these condition were slightly lower as compared with controls and are thus not in the equation above. Interestingly, final pH is lowest when strain AK68 was cultivated on glucose only (6.34) and highest in the co-culture (6.96) showing that increased acetate production do not necessary result in lower final pH.

Effect of phosphate buffer concentration

The effects of different initial phospate buffer concentration are shown in Figure 15. The concentration of phosphate in the medium used is normally 50 mM. At both 50 and 75 mM the strains behaves as earlier described; producing low amounts of ethanol and almost 20 mM of acetate and hydrogen amounts were always higher than acetate concentrations.

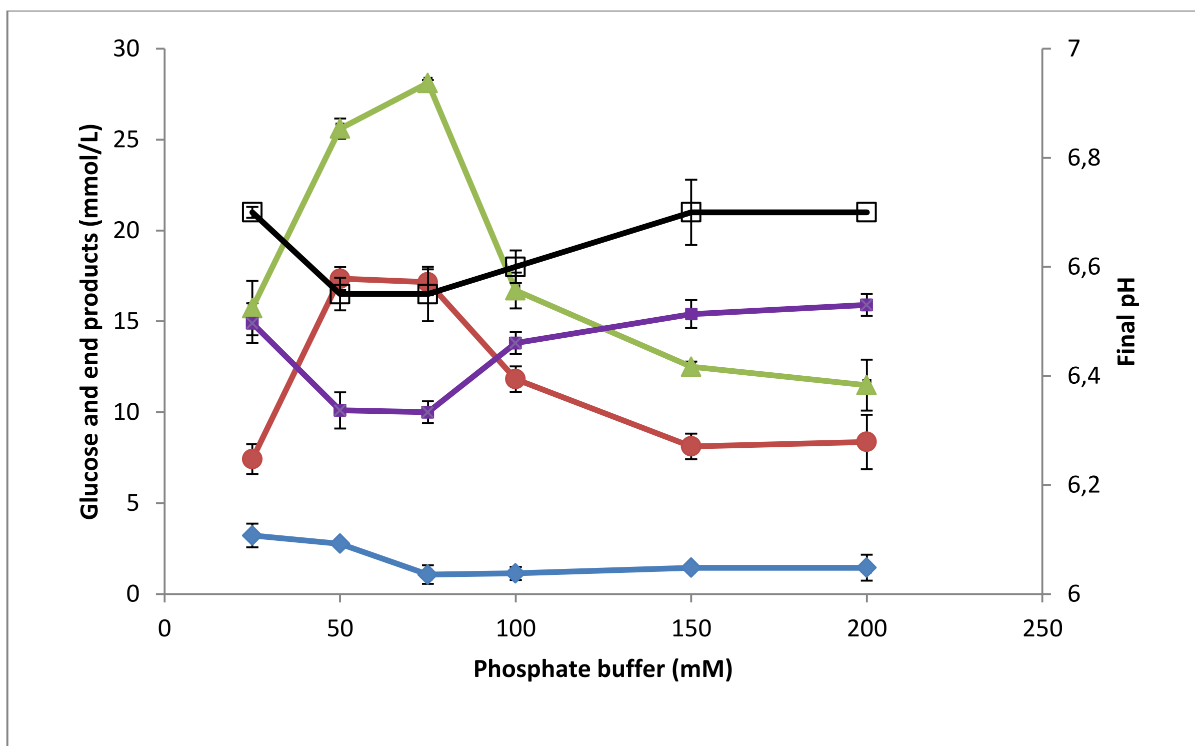


Figure 15 End product formation in the presence of different concentrations of phosphate buffer with glucose (20 mM). Glucose concentration after fermentation (■), Ethanol (♦), Acetate (●), Hydrogen (▲), final pH (□).

Decreasing the buffer concentrartion to 25 mM and increasing it above 75 mM resulted in lower end product formation and lower portion of glucose was degraded. At 50 and 75 mM phosphate concentrations, only about 50 % of the glucose was degraded, resulting in a production of about 17 mM of acetate and between 25 to 28 mmol/L of hydrogen. Lowest pH values observed at the end of cultivation were observed at 50 mM and 75 mM buffer concentrations.

Hydrolysate experiment with Timothy grass and brown macroalgae

Chemcial composition of brown macroalgae and Timothy grass

There is a great variation in chemical composition between the three algae species (Fig 16). About 85 % dry weight compostion in *Laminaria* represent carbohydrate, 12 % proteins and 11 % ash. Dry weight composition in *Fucus sp*. were 52 % ash, 24 % carbohydrate and 16 % of protein. The proportion composition in *Ascophyllum* and *Timothy grass* are very similar, with slight different.

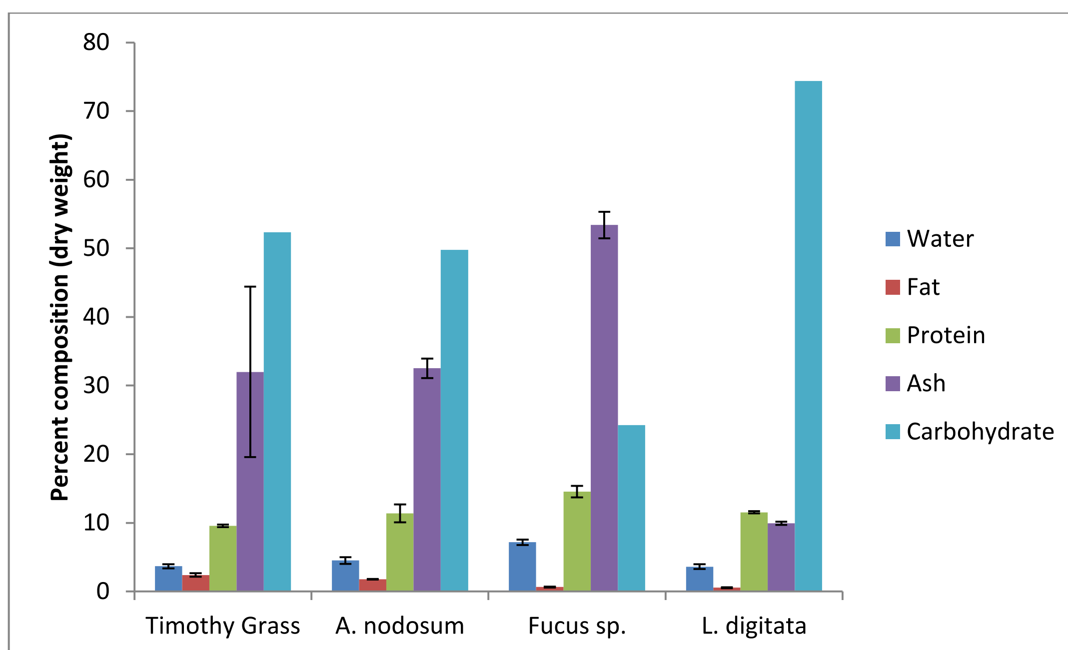


Figure 16 Dry weight proportion compositions of HLs samples

Hydrogen production from brown macroalgae and Timothy grass

Figure 17 shows end product formation from Whatman paper and grass HLs. The main end products of fermentation were acetate and hydrogen. But increased the substrate loadings, did not increase end product formation. The strain produced between 12.0 and 14.2 mM acetate in different concentration of Whatman paper HL and 10.9 and 18.1 mmol/L hydrogen whereas values for the grass HL were 9.0 to 12.5 and 10.8 to 12.1 mmol/L. This levelling of of end product formation also is also reflected in the fact that less and less of the available sugars were degraded at increased substrate loadings. In the lowest concentration of Whatman paper used, only 54 % of the glucose was degraded and this decreased to 31.7 % and 19.0 % at 5.0 and 10.0 g/L, respectively. For grass HL, especially at 5 and 10 g/L, also the majority of sugars were not degraded.

Maximum hydrogen yields from cellulose were obtained on 2.5 g/L Whatman paper, 6.4 mmol H_2/g cellulose. Much lower yields were obtained on the higher substrate loadings. When calculated on basis of glucose equivalents degraded these yields increase to 12.5 mmol H_2/glucose. Also, the lowest yields of hydrogen on grass were on the lowest substrate loadings, or 3.0 mmol H_2/g grass HL.

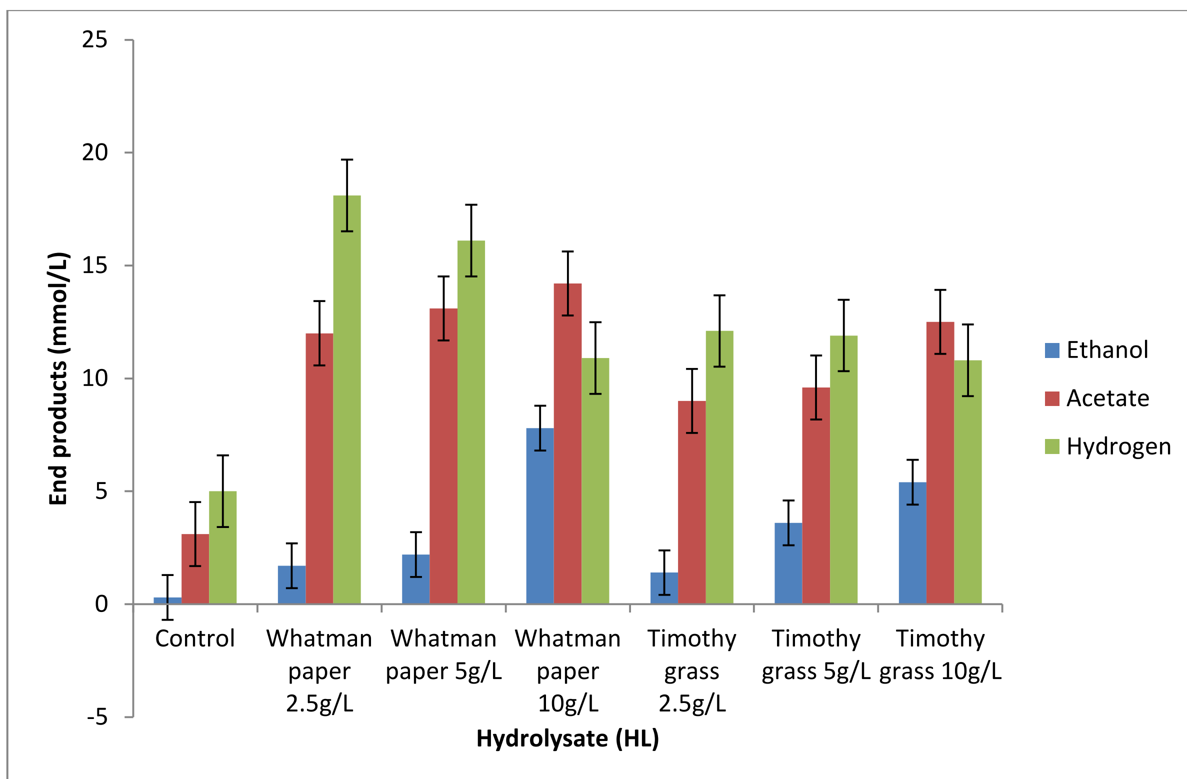


Figure 17 Results of hydrolysate experiment (acid pretreament) for strain AK68 on Timothy grass with different initial hydrolysate concentrations

Figure 18 shows results of hydrolysate experiment with acid and enzyme pretreatment (5 g/L HL concentration), the control are without glucose, only yeast extract. The main volatile end product by Timothy Grass and *Ascophyllum nodosum* is acetate. Fermentation of the AK68 on grass results in 9.1 mM acetate, 5.3 mmol/L H_2, 2.2 mM EtOH and 3.8 mM acetate 0.9mmol/L H_2 and 1,9 mM EtOH by *Ascophyllum.*

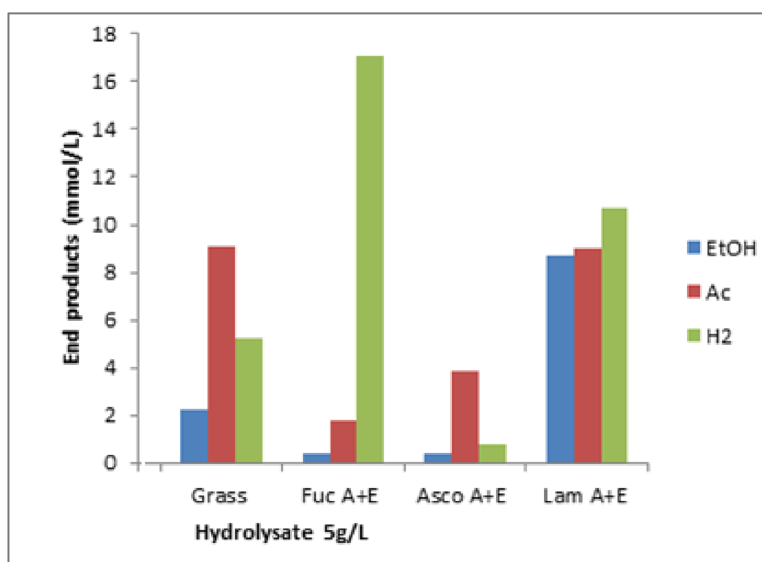


Figure 18 Results of hydrolysate experiment (acid and enzyme pretreament) for strain AK68 on Timothy grass and brown seaweed with 5 g/L hydrolysate concentrations; A, acid pretreatment; E, enzymatic pretreatment

When Laminaria was used as a substrate, strain AK68 produced 8.6 mM of acetate, 1.2 mmol/L of H_2, and 8.4 mM of EtOH.
During growth on *Fucus* HL, especially when pretreated with acid and enzyme the majority of sugars were not degraded. The sugars were mostly degraded with the acid-enzyme pretreatment and without pretreatment, also the initial concentration of xylose were the lowest. On the other hand to get most of sugars degraded by *Ascophyllum* there need to be acid-enzyme pretreatment, otherwise the majority of sugars were not degraded.

Effect of inhibitory compounds

Figure 19 shows end product formation in presence of 5HMF, FF, Levulinic acid and Vanillin. By gradually increase the initial concentrations of 5HMF and furfural less and less amounts of end products are formed ant 40 mM concentration, the strain is completley inhibited and no end products formed.
The end product formation in presence of Levulinic acid - as previously increased concentrations of the acid lead to gradually less and less amounts of end products formed, and complete inhibition occurs at 80 mM initial concentration.
Finally, when using vanillin, simila spectra was observed as for 5 HMF and furfural; a complete inhibition of end products at 40 mM initial vanillin concentrations.

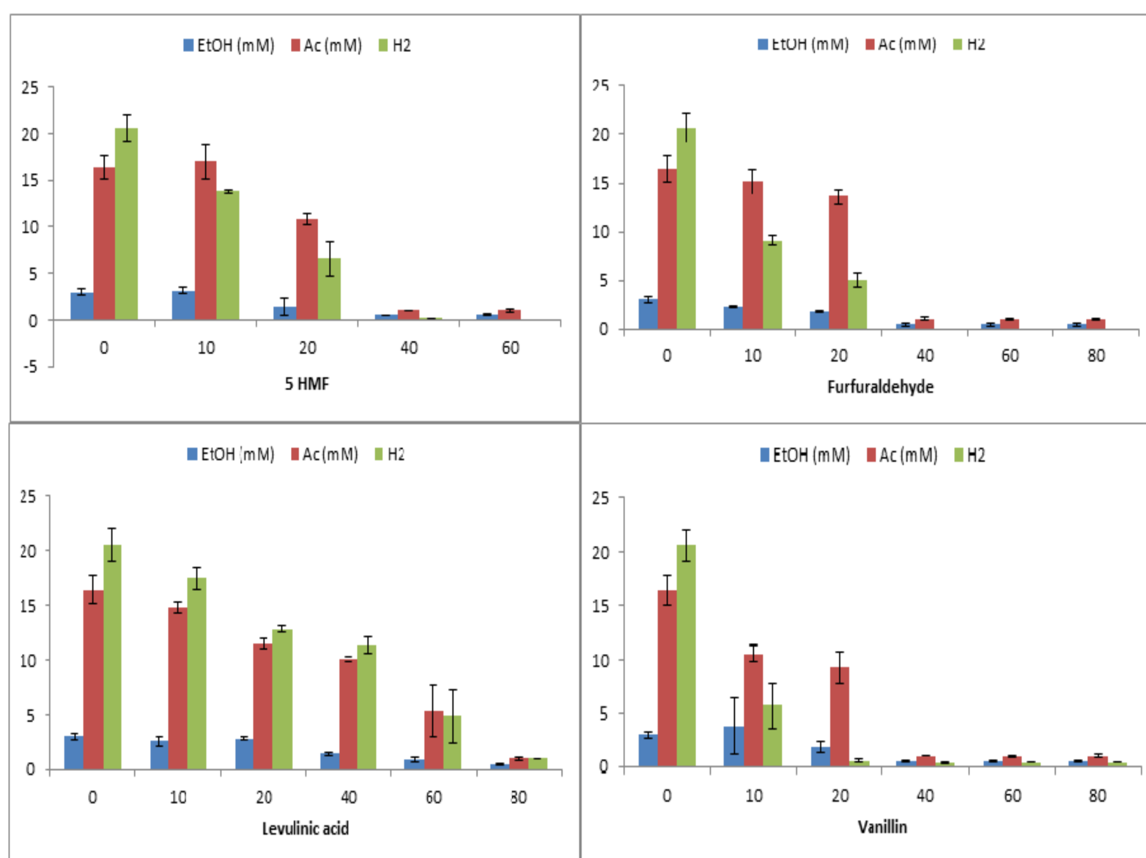


Figure 19 End product formations in the presence of different concentrations of 5 HMF, Furfuraldehyde, Levulinic acid and Vanillin, with 20mM glucose.

3.4. Discussion

Most species within the genera of *Thermoanaerobacter* and *Thermoanaerobacterium* that have been investigated for the production of ethanol and hydrogen show very low tolerance toward initial substrate concentrations (Scully and Orlygsson, 2015; Almarsdottir *et al.,* 2012; Brynjarsdottir *et al.,* 2012). Most often these experiments have been performed in batch culture conditions and the inhibition observed may be contributed to indirect effects such as pH drop in the medium or accumulation of hydrogen but not caused by substrate inhibition. Thus, it is not clear from the various data available whether it is a direct inhibition (substrate) or indirect inhibition (pH, pH_2) that is the main inhibitory factor. The main aim of present investigation was towards solving this question for strain AK68, a moderate good hydrogen producing bacterium.

Strain AK68 is most closely related to *T. pseudoethanolicus* (99.4 %) and *T. thermohydrosulfuricus* (99.3 % similarity) based upon ribotyping (Fig. 8). The temperature and pH optimal range are very close to these two species (Lee et al., 1993; Onyenwoke et al., 2007). The substrate spectra for strain AK68 is also similar to *T. pseudoethanolicus* and *T. thermohydrosulfuricus,* degrading common hexoses, xylose, disaccharides, and xylan (Fig. 9) although the dominant end product for AK68 is acetate and hydrogen but for *T. thermohydrosulfuricus* and *T. pseudoethanolicus* ethanol (Zeikus *et al.,* 1980). Interestingly, strain AK68 does not degrade starch and arabinose, unlike many other species of *Thermoanaerobacter* including those to which AK68 is most closely related. Strain AK68 produces the most hydrogen and acetate at 70 °C with a noticeable drop in hydrogen production and substrate utilization above 75 °C (Fig. 12). Additionally, hydrogen production by strain AK68 was highest between pH 7 and 8 with a decrease in substrate utilization and hydrogen production at lower and higher pH values (Fig. 11).

From the data presented, it is clear that strain AK68 is extremely sensitive to very low initial glucose concentrations in batch culture. At 20 mM (3.6 g/L) concentrations it only degrades about 50 % of glucose and further increase in initial glucose concentrations does not increase end product formation; rather less and less portion of the glucose is degraded (Fig. 10). This is in line with other studies on thermoanaerobes where incomplete substrate utilization is observed at concentrations between 10 to 30 mM (Almarsdottir *et al.,* 2012; Brynjarsdottir *et al.,* 2012). When the strain was cultivated at different buffer concentrations, lower end products were formed at very low and high concentrations (Fig. 14). This indicates that the buffer concentration of the BM medium has sufficient buffer capacity to deal with acid formation during glucose fermentation. The lower amounts of end products observed at high phosphate concentrations are likely due to high osmolarity under these conditions. Interestingly, the cultivation of AK68 at high liquid-gas phase ratios (3.27) lead to the formation of large amounts of ethanol with acetate as a minor end product whereas cultivation at low L-G (0.02) yielded acetate/hydrogen as the dominant products. This further underscores the importance in pH_2 not only in terms of inhibition, but also in terms of directing end product formation patterns.

Additionally, cultivating strain AK68 in the presence of excess thiosulfate resulted in approximately 80 % of substrate degradation and almost 100% when co-cultivated with *Methanothermobacter* strain M39 (Fig. 13). Both these experiments were performed in 50 mM buffer concentrations leading to the conclusion that it is indeed not substrate inhibition or the lowering of the pH that causes incomplete glucose utilization but more likely to be the partial pressure of hydrogen. In most experiments performed, the hydrogen concentrations were between 15 to 25 mmol/L at end of fermentation. Hydrogen has been reported as a strong growth inhibitor for *Caldicellulosiruptor saccharolyticus* (Donnison *et al.,* 1989; van Niel *et al.,* 2003) and elevated pH_2 causes a switch in end product formation; high liquid gas phase ratios lead to increased ethanol and lactate formation with lower acetate and hydrogen yields. It has been found that the initiation of lactate formation with *C. saccharolyticum* coincided with an increase in both internal $NADH/NAD^+$ ratio as well as the pH_2 of the

system. These changes in end product formation may be explained by the thermodynamics behind the reactions involved. Under standard conditions, acetate formation is less thermodynamically favourable than ethanol production ($\Delta G°'$ = -142.6 kJ/reaction and -231 kJ/reaction, respectively). Acetate formation from sugars are however heavily dependent on hydrogen concentrations in the system but not for ethanol production (Bielen *et al.,* 2013). Thus, complete oxidation of sugars to acetate and hydrogen becomes more favourable when pH_2 is kept very low in the system. With *Thermoanaerobacter* strain AK68, the data indicates that it is similarly sensitive to increased hydrogen concentration as evidenced by a shift in end production formation under high L-G ratios and increased substrate consumption under electron scavenging conditions.

The brown algae represent an especially efficient and resilient class of biomass relative to lingo biomass.The order *Laminariales* and *Fucales* of the brown algae (division *Phaeophyta*) are the most important groups of algae to the field of biosorption because of the abundance of their cell wall matrix polysaccharides and extracellular polymers. The chemical composition of brown algae varies considerably between species, throughout the year and between habitats. Brown seaweeds exposed to seasonal changes usually accumulate mannitol and laminaran in the light season (spring to autumn), and consume these carbohydrates during growth in the dark season (Haug and Jensen, 1954; Horn, 2000). Hanssen and coworkers reported carbohydrate content of *Laminaria spp.* were highest in the fall, while chemical composition of *A.nodsium* shows no seasonal variation (Hanssen *et al.,* 1987). The performance of each pretreatment method depends on the type and composition of the biomass used. Furthermore, the microorganisms used for biofuel production have different tolerance to the various types of inhibiting compounds which originate to the biomass pretreatment, but the types and amounts of these compounds depend strongly on the pretreatment method used (Galbe and Zacchi, 2007).

Furfural, HMF and acetic acid are the degradation products that are often present at easily detectable concentrations and are often used as a measure of the inhibiting effect of the pre-treatment liquid. However these compounds are probably not the most inhibitory compounds and it is, of course, the sum of all inhibitors present that determines biofuel productivity and yield. It is also possible that the presence of different inhibitory compounds may give rise to synergistic inhibitory effects (Martin and Johnsson, 2003; Liu *et al.,* 2004). Furfural, HMF, levulinic acid, vanillin and acetic acid are common substances that originate during pretreatment of biomass. These compounds are well known as strong inhibitors for microorganisms. A study of Almarsdottir and coworkers on *Thermoanaerobacterium AK17* during growth on glucose with different initial concetrations of 5 HMF and fufural showed that the strain was completely inhibited at 2.8 mM (2 g/L) and 31.7 mM (4 g/L) concentrations, respectively (Almarsdottir *et al.,* 2012). To our knowledge this is the highest tolerance of a thermoanarobe on these compounds known.

Jessen (2013) investigated in his thesis different pretreatment methods for grass HL. His data shows the highest H_2 production when grass is pretreatment with acid. For different initial hydrolysate concentration he got similar results as presented in this study. Additionally, the focus of Jessen investigation was towards the effect of the partial pressure of hydrogen on hydrogen yields from grass hydrolystates. Not surprisingly, lowering of the liquid gas ratio, lead to increased yields of hydrogen; L-G ratio of 0.1, 1.0 and 3.46 lead to 2.3, 0.64 and 0.38 mol H_2/mol glucose equivalent.

4. Conclusion

The main challenge of converting lignocellulosic biomass into biofuels such as hydrogen, ethanol and methane is the degradation of its chemical constituents – cellulose, hemicellulose and lignin – into more simple types of carbohydrates in order for fermentation occur. Thermophilic anaerobes that have been isolated from Icelandic hot springs thrive in hot and hostile environments of the geothermal areas. Their heat tolerance is of great advantage for the conversion process from complex biomass to ethanol, hydrogen and methane, as it gives the possibility of raising the process temperature, and increasing the rate and thermodynamic favorability of the reaction of each fermentational stage. Moreover, thermophilic anaerobes have broad spectra of substrate degradation including various hexoses, pentoses, disaccharides, and polymeric substrates, which is a great advantage when degrading complex biomass source such as waste and crop residues. Factors such as initial glucose concentration, enzyme concentration, partial pressure of hydrogen, inhibitory effects of furan derivatives, and tolerance to end-products such as ethanol are amongst the crucial factors that have been shown to have important effects on the yield of lignocellulosic degradation of thermophilic anaerobes. One extremely important factor is the different types and amounts of inhibitory compounds produced during the pretreatment and the effect very depent on the complex biomass operating conditions and composition. All pretreatments have their disadvantages and advantages, and need to be optimized.

There are also other feedstocks that should be considered for biohydrogen production. Algae biofuels have the potential to become a renewable, cost-effective alternative for fossil fuels with reduced impact on the environment. Algae hold tremendous potential to provide a non-food, high-yield, non-arable land use source of renewable fuels like hydrogen, biodiesel, bioethanol etc.

Algae also produce proteins, isoprenoids and polysaccharides. Some strains of algae ferment sugars to produce alcohols, under the right growing conditions. Their biomass can be processed to different sorts of chemicals and polymers (Polysaccharides, enzymes, pigments and minerals), biofuels (e.g. biodiesel, alkanes and alcohols), food and animal feed as well as bioactive compounds (antibiotics, antioxidant and metabolites) through down-processing technology such as transesterification, pyrolysis and continuous catalysis using microspheres.

Fucoidans present in brown seaweeds are usually very complex nonregular polysaccharides, which may be even mixtures of molecules of different structural types. Algae is regarded as important raw material of biomass because using the algae that causes marine pollution problems through excessive breeding, economic effects as well as environmentally sound effects are expected. Marcoalgae are considered ideal substrates for anaerobic fermentation due to their high content of easily degradable polysaccharides such as alginate, laminarian and the sugar alcohol mannitol.

The present study included the production of biohydrogen by thermophilic bacteria strain AK68, isolated from Grensdalur (SW-Iceland). The strain has broad substrate spectra and tolerates a wide range of tempertures and pH. However, the strain is inhibited by relatively modest substrate loadings. This inhibition is most likely linked with hydrogen accumulation rather than pH drop or substrate inhibition as was shown byadding thiosulfate as an external electron acceptor or by co-culture with a hydrogenotrophic methanogen. Manipulation of the liquid-gas phase ratio also changes the end product spectra to predominately ethanol. Strain was examined regarding their ability to degrade various carbon substrates present in complex biomass and produce acetate and hydrogen. The strain showed a strong negative correlation between L/G ratios and production of both acetate and hydrogen, as observed before. Strain AK68 was inhibited by increased hydrolysate concentrations, that are in compliance with results on initial concentration of glucose. The strain was able to grow on

hydrolysates from different biomass. Even that strain showed negative correlation between L/G ratio, over 14 mmol/L of hydrogen were observed for all the biomass types used, except *Ascophyllum nodosum* where main product was acetate. *Thermoanaerobacter* strain AK68 is a good hydrogen producer producing 2.3 mol hydrogen from one mole of glucose which is 62.5 % of the theoretical yield under optimum conditions.

5. Povzetek

V nalogi je predstavljena alternativa trenutno uporabljenega vodika. Predstavlja ga biokemijska produkcija vodika, pri čemer vodik nastaja kot metabolni produkt, mikrobiološke razgradnje biomase. Biovodik je obnovljivo biogorivo, proizvedeno iz obnovljivih surovin s kemijskimi, termokemijskimi, biološkimi ter biokemijskimi procesi. Danes je veliko raziskav usmerjenih ravno k proizvodnji vodika iz obnovljivih surovin. Biovodik lahko učinkovito tekmuje s fosilnim gorivom, saj nastaja v procesu anaerobne mikrobne razgradnje z nizkoogljično bilanco, ki je čista in okolju prijazna metoda. Poleg tega pri zgorevanju vodika nastaja voda in ne toplogredni plini kot pri fosilnih gorivih. Pri sobni temperaturi in pri normalnem (atmosferskem) tlaku je vodik brezbarven, brez vonja in nestrupen plin, ki je lažji od zraka ter helija. Gori z bledim modrim, skoraj nevidnim plamenom. Na Zemlji je pogost, ampak ne v elementarnem stanju, temveč kot del drugih spojin (npr. vode). Vodik ima najvišjo sežigno energijo na enoto mase od vseh navadno uporabljenih materialov, kar je približno trikrat več kot pri kemičnih gorivih, kot so tekoči ogljikovodiki. Pri gorenju vodika nastaja vodna para, ki je okolju in ljudem neškodljiva, za razliko od ogljikovega dioksida (CO_2), žveplovega dioksida (SO_2) ter drugih toplogrednih plinov, ki nastajajo pri izgorevanju fosilnih goriv. Nadalje je vodik zdravju neškodljiv za razliko od propana, ki draži pljuča in druge organe. Prav tako ne vsebuje težkih kovin za razliko od bencina ali nafte. Ima približno dvakrat večjo kurilno vrednost na enoto mase od ostalih tradicionalnih fosilnih goriv.

Obstajajo trije tipi mikroorganizmov, ki so sposobni proizvodnje vodika: - cianobakterije, - anaerobne bakterije ter - aerobne fermentativne bakterije. Cianobakterije v procesu fotosinteze vodo neposredno razcepijo na vodik in kisik s pomočjo svetlobe. Fotosintetske bakterije uporabljajo organske substrate, npr. organske kisline. V procesu anaerobne razgradnje bakterije kot edini vir elektronov in energije uporabljajo organske snovi in jih pretvarjajo v vodik. Biovodik lahko proizvedemo tudi s pomočjo termofilnih bakterij s spremljanjem in prilagajanjem temperature, pH vrednosti, zadrževalnega časa v reaktorju ter ostalih dejavnikov. Izraz biovodik se nanaša na nefosilno ter biorazgradljivo gorivo, proizvedeno iz rastlin, živali in mikroorganizmov. Biovodik je opredeljen kot vodik, proizveden na biološki način, zlasti s pomočjo bakterij, kot biogorivo iz odpadnih organskih snovi. Najbolj znane industrijske metode za pridobivanje vodika vključujejo t.i. parno preoblikovanje zemeljskega plina, uplinjanje premoga ter cepljenje vode z elektriko, najpogosteje iz fosilnih goriv. Ti energetsko intenzivni procesi okolje obremenjujejo z velikimi količinami sproščenega ogljikovega dioksida in drugih toplogrednih plinov, ki se pojavljajo kot stranski produkt produkcije vodika. Poleg toplotnega proizvajanja vodika z uplinjanjem biomase lahko vodik proizvajamo tudi biološko – s pomočjo mikroorganizmov.

Proizvodnja vodika s pomočjo mikroorganizmov ima velik potencial kot neomejen, poceni in obnovljiv vir čiste energije. Prav zaradi tega dejstva je pritegnila svetovno pozornost. Študije v proizvodnji biovodika so se osredotočile predvsem na biofotokatalizo vode z uporabo alg in cianobakterij, svetlobni razkroj organskih snovi s pomočjo fotosintetskih bakterij ter fermentacijo v temi iz organskih odpadkov s pomočjo anaerobnih mikroorganizmov. Anaerobna, imenovana tudi temna, fermentacija se je izkazala za najbolj ugodno, saj lahko s to metodo proizvedemo večje količine vodika kot z ostalimi metodami. Temna fermentacija je katabolizem: anaerobne bakterije pretvarjajo sladkorje in beljakovine v karboksilne kisline, vodik, ogljikov dioksid in druge snovi. Temna fermentacija je biološko-kemijska reakcija, ki poteka v striktno anaerobnih pogojih, v strogi odsotnosti kisika in v prisotnosti zadostne količine vode/medija. Je proces, pri katerem določeni organizmi razgrajujejo različne organske snovi, pri čemer nastaja t.i. biovodik – mešanica ogljikovega dioksida, vodika ter ostalih maščobnih kislin. Za proizvodnjo vodika so v glavnem odgovorne različne skupine bakterij, najpogosteje rodov *Enterobacter, Clostridium* in *Bacillus.* Metabolni proces anaerobne razgradnje se vrši v štirih stopnjah (hidroliza, acidogeneza, acetogeneza ter metanogeneza), pri čemer je potrebno omeniti, da metanogeneza v proizvodnji biovodika ni prisotna. Proizvodnja biovodika s fermentacijo se lahko izvede preko širokega spektra

mikroorganizmov, ki pa imajo zelo različne potrebe po substratu, pH vrednosti in temperaturi. Ti parametri so ključni za napoved rasti mikroorganizmov, pomembno vlogo pa imajo tudi pri metabolni poti mikroorganizmov. Proizvodnjo biovodika lahko dosežemo bodisi z mešanimi acidogenimi mikrobiološkimi kulturami, ki bodisi izhajajo iz naravnega okolja ali s čistimi kulturami. Za trajnostno in izboljšano produkcijo biovodika je potrebno zavreti rast potrošnikov vodika – metanogenih organizmov in sulfatnih reducentov v mešani kulturi. Z različnimi metodami (toplotna obdelava, kontrola pH vrednosti in hidravličnega zadrževalnega časa, oblika in zasnova reaktorja) lahko selektivno zaviramo rast neželenih organizmov ter spodbujamo rast organizmov, ki so sposobni proizvodnje biovodika, npr. bakterije iz rodu; *Anaerocellum, Caldicellulosiruptor, Clostridium, Dictyoglomus, Fervidobacterium, Spirocheta, Thermotoga, Thermoanaerobacterium* in *Thermoanaerobacter.*

Biomasa je vsa masa rastlinskega in živalskega izvora v naravi. Je celotna biocenoza na določeno površino ali prostornino v določenem času. Biomasa je tudi skupno ime za odmrl organski material, ki je predvsem rastlinskega izvora in iz katerega si lahko človek naredi gorivo ali iz njega pridobiva energijo. Vse to je tudi surovina, ki jo lahko obdelamo z kemičnimi, fizikalnimi ali biološkimi procesi, da bi iz nje pridobili vse sestavine, ki jih ima biomasa. Pod biomaso spadajo obnovljivi odpadki, hitrorastoče rastline, morske rastline in alge, prehrambne rastline, sladkorni trs, industrijski organski odpadki. Zaradi obnovljivosti je ta vrsta surovine privlačna za trg. Z obnovljivostjo poskrbimo za trajnostno in dolgoročno rabo.

Biomasa se uporablja kot surovina za proizvodnjo etanola in vodika, sestavljena je lahko kot; enostavni sladkorji (škrob in saharoza, ki je pridobljena iz koruze in sladkornega trsa) ali pa kot kompleska biomasa oz. rastlinska biomasa, ki je sestavljena iz treh različnih polimerov (celuloze, hemiceluloze in lignina) ki jih skupaj vežejo nekovalentne, kot tudi križne kovalentne vezi. Lignocelulozna biomasa je v glavnem zgrajena iz celuloze in neceluloznih strukturnih polisaharidov, ki se s procesom hidrolize pretvorijo v topne sladkorje, ki lahko naprej fermentirajo v tekoča biogoriva. Ena večjih omejitev je encimska razgradnja kompleksne matrice lignoceluloznih polimerov, ki tvorijo celično steno. Znotraj celične stene se namreč nahajajo heksoze in pentoze, ki so najbolj pomembni sladkorji za tvorbo biogoriv. Lignin je brezbarven, trden heterogeni polimer, ki nastaja v rastlinah iz fenolnih spojin, torej ga sestavljajo aromatski obroči. Celuloza je polisaharid, sestavljen iz ß-D-glukoze, ki so med seboj povezane z ß-1,4-glikozidno vezjo. Je najpomenbnejša in tudi najbolj razširjena sestavina rastlinskih celičnih sten. Celuloza je brezbarvna snov, netopna v vodi in organskih topilih, vendar razgradljiva v kislinah. Hidroliza pri višjih temperaturah s koncentriranimi kislinami poteče do posameznih glukoznih enot, iz katerih se lahko pripravlja različne kemikalije s kemijskim ali biološkim procesom. Hemiceluloza so polisaharidi različne sestave, ki so topne v razredčenih kislinah in bazah. Hemiceluloze so zamreženi polimeri, zgrajeni iz monosaharidnih enot, poleg glukoze pa vsebujejo še manozo in galaktozo in pontozi ksilozo in arabinozo, med seboj pa so povezane z ß-1,4-glikozno vezjo.

Hidroliza je reakcija z ioni ali molekulami vode, pomeni cepljenje kemijske vezi ob prisotnosti vode. Hidroliza predstavlja stopnjo v razgradnji snovi. V hidrolitske reakcije vstopajo nekateri ioni soli, estri in številne druge organske spojine. Pri hidrolizi biomase nastajajo produkti, ki vsebujejo različne koncentracije organskih kislin, furfurala, aldehidov, ketonov in sladkorjev. Eden izmed izzivov predstavlja premagati vpliv, ki ga imajo inhibitorne komponente, ki nastajajo v pretvorbi iz lignoceluloznega materiala v biogoriva. Glavni komponenti za pretvorbo v biogoriva sta polisaharida celuloza in hemiceluloza, ki razpadeta na enostavne sladkorje, ti pa predstavljajo substrata fermentativnim mikroorganizmom. Da pridemo do enostavnih sladkorjev iz biomase se uporablja več postopkov, kot so: hidroliza z razredčeno kislino, hidroliza z bazo, encimska hidroliza, parna eksplozija, mokra oksidacija. Splošni problem pri vsakem postopku pa je nastanek inhibitornih komponent iz različnih stranskih produktov med pretvorbami, v glavnem pri dehidraciji sladkorjev in razpadu ligninskih

fragmentov. Inhibitorne komponente, ki nastajajo pri razgradnji predstavljajo največjo viro za rast mikrobov in fermentacijo. Iz hemiceluloze se sprostijo pentoze in nekatere heksoze, z dehidracijo teh pa se tvorita furfural in 5-hidriksimetilfurfural, ki sta znana kot največja inhibitorja rasti mikrobov. Oba aldehida dalje razpadeta v levulinsko in metanojsko kislino. Etanojska in druge organske kisline se sprostijo iz hemiceluloze. Mnoge aldehidne in fenolne spojine pa se sprostijo iz lignina.

Eksperiment:

Raziskava se je osredotočila na pridobivanje vodika iz različnih sladkorjev s hidrolizo kompleksne biomase s pomočjo termofilnih bakterij, ki so bile izolirane na gejzirnih območjih Grensdalur-ja, na JZ Islandije.

Sestava medija na liter: NaH_2PO_4 2.34 g, Na_2HPO_4 3.33 g 2.3 g, NH_4Cl 2.2 g, NaCl 3.0 g, $CaCl_2$ 8.8 g, $MgCl_2$ x $6H_2O$ 0.8 g, kvasni ekstrakt 2.0 g, resazurin 1 mg, trace elementi 1 ml, vitaminski pripravki 1 ml in $NaHCO_3$ 0.8 g. 20mM energijskega vira oz, 3 g l^{-1} v primeru polimerov. Sestava trace elementa na liter: $FeCl_2$ x 4 H_2O 2.0g, EDTA 0.5 g, $CuCl_2$ 0.03 g, H_3BO_3, $ZnCl_2$, $MnCl_2$ x 4 H_2O, $(NH_4)Mo_7O_{24}$, $AlCl_3$, $CoCl_2$ x 6 H_2O, $NiCl_2$, all 0.05 g, Na_2S x 9 H_2O 0.3 g in 1 mL koncentriranega HCl. Vitaminski preparati so bili pripravljeni po DSM141. Gojišča so bila pripravljena po Örlygsson-u in Baldursson-u, 2007. Vse komponente v medij so bile dodane ločeno skozi filter (0.45 µm). Vsi eksperimenti so bili opravljeni v paralelkah pri 65 °C in pri pH 7.0, trajali so 5 dni.

Analitične metode: Vodik in metan sta bila analizirana z uporabo Perkin Elmer plinskega kromatografa, opremljenega z detektorjem toplotne prevodnosti. VFA in alkohole smo analizirali s plinsko kromatografijo (Perkin Elmer Clarus 580) s pomočjo FID detektorja z 30 m DB-FFAP kapilarno kolono (Agilent Industries Inc., Palo Alto, CA, ZDA). Optična gostota je bila merjena z UV-Vis spektrometrom pri 600 nm (Perkin Elmer, Lambda 25). pH vrednost kultur je bil merjen z Thermo Scientific 4 star pH meter, glukoza je bila analizirana po anhronovi metodi (Laurentin and Edwards 2003). Vzorci biomase so bili analizirani z visokotlačno tekočinsko kromatografijo (HPLC – Shimadzu z LC20-AD črpalko, RID-10A detektorja indeksa refrakcije (40 °C) in HPX-87H (BioRad, State). HPLC metoda je bila uporaljena za določanje koncentracije sladkorjev, ocetne kisline, bioetanola in vodika, spojin prisotnih v gojiščih z biomaso. Prad analizo smo vzorce centrifugirali 5 min pri 13000 vrt./min. Dobljeni supernantant - 20 µL smo injicirali v kromatograf, kot topilo se je uporabljal 5 mM H_2SO_4 s pretokom 1.2 mL/min.

Analizirana je bila proizvodnja vodika glede na različne sladkorje, prav tako tudi vplivanje okoljskih faktorjev, kot so substrati, pH, temperatura, delni pritisk vodika na rast in končni proizvod. Različni dejavniki vplivajo tudi na proizvodnjo biogoriv iz lignocelulozne biomase, vključno z začetno koncentracijo lignoceluloznih hidrolizatov in različno obdelavo. Kot potencialni sev za proizvodnjo vodika je bil analiziran sev AK68.

Zaključki:

Analiza 16S rRNA je pokazala, da sev pripada rodou *Thermoanaerobacter* in je blizu *T. thermohydrosulfuricum* (99.2 %), *T. brockii* (97.9 %), *T. kivui* (94.8 %), and *T. ethanolicus* (89.0 %). Optimalni pogoji za rast in tvorbo H_2 za sev AK68 so pri temperaturi 65 °C in pH med 7.0 in 8.0. Sev je dober proizvajalec vodika, hkrati pa proizvaja tudi etanol, acetat in ogljikov dioksid. Kot je vidno iz opravljenih eksperimentov je AK68 zelo občutljiv na zelo nizke začetne koncentracije glukoze. Pri 20mM (3,6 g/L) koncentracije se razgradi le okoli 50 % glukoze, zaradi česar nadaljne povečevanje začetni koncentracije glukoze ni smiselno, saj ne povečuje končnega produkta - vodika. AK68 je zelo občutljiv na razmerje plina/tekočine,

saj je pri nizki L-G (0.02) glavni proizvod vodik in acetat, medtem ko pri visokem L-G razmerju (3.27) kot glavni proizvod dobimo etanol. Prav tako z dodajanjem pH_2 sev postalne etanogen, s čimer lahko sev usmerjamo glede na to kateri končni produkt želimo. Sev *Thermoanaerobacter* AK68 je dober proizvajalec vodika, s proizvodnjo 2,3 mol vodika iz enega mola glukoze, kar je 62,5 % teoretične vrednosti v optimalnih pogojih.

Glavni izziv pri pretvorbi kompleksne lignocelulozne biomase v biogoriva, kot so vodik, etanol in metan je pretvorba kemičnih sestavin – celuloze in hemiceluloze v več vrst enostavnih sladkorjev, kot tudi ločevanje in odstranjevanje lignina, da je primeren za fermentacijo. Termofilni anaerobi izolirani na Islandskih vročih vrelcih uspevajo v geotermalnih razmerah, njihova toplotna odpornost pa predstavlja veliko prednost pri fermentaciji s kompleksno biomaso, predvsem zaradi rasti in tvorbe bioplinov pri temperaturah na 60 °C in imajo hkrati širok spekter razgradnje substratov in različnih sladkorjev.

Kot biomaso bi bilo potrebno upoštevati tudi druge vire surovin - enega takšnega predstavljajo makroalge, ki imajo velik potencial, da postanejo učinkovit obnovljivi viri, saj so storškovna učinkovita alternativa, ki ne zasedajo obdelovalnih površin namenjenih pridelavi hrane ter imajo visoko vrednost možnosti izkoriščanja za pridobitev vodika, etanola, metana z minimalnim vplivom na okolje.

6. References

Abreu, A.A., Alves, J.I., Pereira, M.A., Karakashev, D., Alves, M.M., Angelidaki, I. (2010). Engineered heat treated methanogenic granules: a promising biotechnological approach for extreme thermophilic biohydrogen production. *Bioresour Technol.* doi: 10.1016/j.biortech.2010.07.070.

Abreu, A., Karakashev, D., Angelidaki, I., Sousa, D., Alves, M. (2012). Biohydrogen production from arabinose and glucose using extreme thermophilic anaerobic mixed cultures. *Biotechnology for Biofuels*, 5(6).

Adams, J., Gallagher, J.A., Donnison, I.S. (2009). Fermentation study on *Saccharina latissima* for bioethanol production considering variable pre-treatments. *Journal of Applied Phycology* 21(5), 569-574.

Akutse, Y., Li, Y.Y., Tandukar, M., Kubota, K., Harada, H. (2008). Effects of seed sludge on fermentative characteristics and microbial community structures in thermophilic hydrogen fermentations of starch. *Int J Hydrogen Energy.* 33:6541- 6548.

Alderkamp, A.C., Sintes, E., Herndl, G.J. (2006). Abundance and activity of major groups of prokaryotic plankton in the coastal North Sea during spring and summer. *Aquat Microb Ecol,* 45, 237–246.

Almarsdottir, A.R., Sigurbjornsdottir, M.A., Orlygsson, J. (2012). Effects of various factors on ethanol yields from lignocellulosic biomass by *Thermoanaerobacterium* AK17. *Biotechnol Bioeng;* 109;686-6694.

Altschul, S., Madden, T., Schaffer, A., Zhang, J., Zhang, Z., Miller, W., Lipman, D. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389-3402.

Alves, M.M., Mota Vieira, J.A., Alvares Pereira, R.M., Pereira, M.A., Mota, M. (2001). Effects of lipids and oleic acid on biomass development in anaerobic fixed-bed reactors. Part I: Biofilm growth and activity. *Water Research*, 35-1, 255-263.

Azbar, N., Levin, D. (2012) State of the art and progress in production of biohydrogen. *Bentham Science Publishers.*

Benemann, J.R. (1980). Bioengineering aspects of biophotolysis. *Enzyme Microbiol Technology* 2, 103-111.

Bibi, R., Arshad, M., Asghar, H.N. (2012). Optimization of factors for accelerated biodegradation of reactive black-5 azo dye. *Int. J. Agric.Biol.,* 14, 353–359

Bielen, A.A.M., Verhaart, M.R.A., van der Oost, J., Kengen, S.W.M. (2013). Biohydrogen production by the thermophilic bacterium Caldicellulosiruptor saccharolyticus: Current status and perspectives. *Life*,3, 52-85.

Brynjarsdottir, H., Scully, M.S., Orlygsson, J. (2013). Production of biohydrogen from sugars and lignocellulosic biomass using Thermoanaerobacter GHL15. *Int J Hydrogen Energ.* 38, 14467-14475.

Brynjarsdottir, H., Wawiernia, B., Orlygsson, J. (2012). Ethanol production from sugars and complex biomass by *Thermoanaerobacter* AK5: The effect of electron-scavenging systems on end-product formation. *Energy Fuel*; 7, 4568-4574.

Cai, M., Liu, J., Wei, Y. (2004). Enhanced biohydrogen production from sewage sludge with alkaline pretreatment. *Environ. Sci. Technol.* 38, 3195-3202.

Canganella, F.; Wiegel, J. (1993). The Potential of Thermophilic *Clostridia* in Biotechnology. In *The Clostridia and Biotechnology*; Woods, D.R., Ed.; *Butterworths Publishers: Stoneham*, MA, USA, pp. 391–429.

Canganella, F., Wiegel, J. (2014). Anaerobic Thermophiles. *Life,* 4(1), 77-104. doi:10.3390/life4010077

Cao, G., Guo, W., Wang, A., Zhu, Y., Liu, B., Xu, J., Ren, N. (2010). Biological hydrogen production from corn stover by moderately thermophile *Thermoanaerobacterium thermosaccharolyticum W16. International Journal of Hydrogen Energy*, 35, 2708-2712.

Cao, G., Zhao, L., Wang, A., Zhen, Y., Ren, N. (2014). Single-step bioconversion of lignocellulose to hydrogen using novel moderately thermophilic bacteria. *Biotechnology for Biofuels,* 7, 82. doi:10.1186/1754-6834-7-82.

Carreira, L.H.; Ljungdahl, L.G. (1993). Production of ethanol from biomass using anaerobic thermophilic bacteria. In *Liquid Fuel Developments*; Wise, D.L., Ed.; CRC Press: Boca Raton, FL, USA, pp. 1–28.

Carriera, L.H., Ljungdahl, L.G., Bryant, F., Szulcynski, M., Wiegel, J. (1982). Control of product formation with Thermoanaerobacter ethanolicus: enzymology and physiology. In: Proc. 4th International Symposium on Genetics of Industrial Microorganisms. pp. 351-355. Kodansha Ltd., Tokyo, Japan.

Chapman, V. J. (1970). *Seaweeds and Their Uses,* Chapman & Hall. London, 334.

Chen, C.C., Lin, C.Y., Lin, M.C. (2002). Acid-base enrichment enhances anaerobic hydrogen production process. *Appl. Microbiol. Biotechnol.* 58, 224-228.

Chen, X., Sun, Y., Xiu, Z., Li, X., Zhang D. (2006). Stoichiometric analysis of biological hydrogen production by fermentative bacteria. *Int J Hydrogen Energy.* 31, 539-549.

Cheong, D.Y., Hansen, C.L., (2007). Feasibility of hydrogen production in thermophilic fermentation by natural anaerobes. *Bioresour Technol. 98*: 2229- 2239.

Chiovitti, A., Molino, P., Crawford, S.A., Teng, R., Spurck, T., Wetherbee, R. (2004). The glucans extracted with warm water from diatoms are mainly derived from intracellular chrysolaminaran and not extracellular polysaccharides. *European Journal of phycology,* 39, 117-128.

Chin, H.L.; Chen, C.P., Chou, C.P. (2003). Fed batch operation using *Clostridium acetobutylicum* suspension culture as biocatalyst for enhancing hydrogen production, *Biotechnology Progress*, vol.19, pp.383-388.

Chizhov, A.O., Dell, A., Morris, H.r., Reason, A.J., Haslam, S.M., McDowell, R.A., Chizov, O.S., Usov, A.i. (1998). Structural analysis of laminarans by MALDI and FAB mass spectrometry. *Carbohydr. Res.,* 310, 203-210.

Chong, M.L., Sabaratnam, V., Shirai, Y., Hassan, M.A. (2009). Biohydrogen production from biomass and industrial wastes by dark fermentation. *International Journal of Hydrogen Energy* 8:34, pp.3277–3287.

Cirne, D.G., Paloumet, X., Bjornsson, L., Alves, M.M., Mattiasson, B. (2007). Anaerobic digestion of lipid-rich waste-Effects of lipid concentration. *Renewable Energy*, 32, 59-75.

Collins, M.D., Lawson, P.A., Willems, A., Cordoba, J.J., Fernandezgarayzabal, J., Garcia, P. (1994). The phylogeny of the genus *Clostridium* - proposal of 5 new genera and 11 new species combinations. *International Journal of Systematic Bacteriology*, 44, 812-826.

Cord-Ruwisch, R., Seitz, H., Conrad, R. (1988). The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor. *Archives of Microbiology*, 149, 350-357.

Dabrock, B., Bahl, H., Gottschalk, G. (1992). Parameters affecting solvent production by *Clostridium pasteurianum*, *Applied and Environmental Microbiology*, vol.58, pp.1233-1239.

Das, D., Veziroglu, T.N. (2008). Advances in biological hydrogen production processes. International *Journal of Hydrogen Energy.* 33, 6046-6057.

Demain, A.L., Newcomb, M., Wu, J.H.D. (2005). Cellulase, Clostridia, and Ethanol. *Microbiology and Molecular Biology Reviews*, 69, 124-154.

Demirbas A. (2005) Bioethanol from cellulosic materials: a renewable motor fuel from biomass. *Energy Source,* 27, 327e37.

Deutsch, T.G., Head, J.L., Turner, J.A. (2008). Photoelectrochemical characterization and durability analysis of GaInPN epilayers. *J. Electrochem. Soc.* 155, B903– B907.

Donnison, A.M., Brockelsby, C.M., Morgan, H.W., Daniel, R.M. (1989). The degradation of lignocellulosics by extremely thermophilic microorganisms. *Biotechnol Bioeng,* 33, 1495-1499.

Eluvakkal., T., Sivakumar, S.r., Arunkumar, K. (2010). Fucoidan in some Indian brown seaweeds found in along the Coast Gulf of Mannar. *International Journal of botany*, 6 (2), 176-181.

Evvyernie, D., Morimoto, K., Karita, S., Kimura, T., Sakka, K., Ohmiya, K. (2001). Conversion of Chitinous waste to hydrogen gas by *Clostridium paraputrificum M-21*. *J Biosci Bioengineering* 91, 339-343.

Fan, Y.T., Zhang, G.S., Guo, X.Y., Xing, Y., Fan, M.H. (2006). Biohydrogen production from beer lees biomass by cow dung compost. *Biomass Bioenergy,* 30, 493–6.

Fan, Y.T., Zhang, Y.H., Zhang, S.F., Hou, H.W., Ren, B.Z. (2006). Efficient conversion of wheat straw wastes into biohydrogen gas by cow dung compost. *Biores Technol.,* 97, 500–5.

Fang, H.H.P., Liu, H. and Zhang, T. (2004). Bio-hydrogen production from wastewater. *Water Sci. Technol.* 4(1), 77–85.

Fardeau, M.L., Faudon, C., Cayol, J.L., Magot, M., Patel, B.K.C., Ollivier, B. (1996). Effect of thiosulphate as electron acceptor on glucose and xylose oxidation by *Thermoanaerobacter finnii* and a *Thermoanaerobacter* sp isolated from oil field water. *Res Microbiol.,* 147, 159-165.

Galbe, M., Zacchi, G. (2007). Pretreatment of lignocellulosic materials for efficient bioethanol production. *Advances in Biochemical Engineering/Biotechnology,* 108, 41-65.

Garcia, J.L. (1990). Taxonomy and ecology of methanogens. *FEMS Microbiol Rev* 87, 297–308.

Ginkel van, S., Sung, S. and Lay, J. (2001). Biohydrogen production as a function of pH and substrate concentration. *Environ. Sci. Technol.,* 35, 4726-4730.

Glazer, A.N., Nikaido, H. (2007). *Microbial Biotechnology: Fundamentals of Applied Microbiology*, Second Edition. New York. W.H. Freeman and Company.

Goldstein, I.J., Smith, F., Unrau, A.M. (1959). Constitution of laminarian. *Chem. Ind. (London),* 124-125.

Grabber, J.H. (2005). How do lignin composition, structure, and cross-linking affect degradability? A review of cell wall model studies. *Crop Sci.* 45, 820–831.

Gray, M.C., Converse, A.O., Wyman, C.E., (2003). Sugar monomer and oligomer solubility. Data and predictions for application to biomass hydrolysis. *Appl. Biochem. Biotechnol.,* 179–193.

Guo, P., Zhu, W., Wang, H., Lu, Y., Wang, X., Zheng, D., Cui, Z. (2010). Functional characteristics and diversity of a novel lignocelluloses degrading composite microbial system with high xylanase activity. *Journal of Microbiology and Biotechnology*, 20(2), 254-264.

Guo, X.M., Trably E., Latrille, E., Carrère, H., Steyer, J. (2010). Hydrogen production from agricultural waste by dark fermentation: a review. *Int.J. Hydrogen Energy*, 35, 10660−10673.

Hallenbeck, P.C., Benemann, J.R. (2002). Biological hydrogen production; fundamentals and limiting processes. *Int. J. Hydrogen Energy.*, 27, 185-1193.

Hanssen, J.F., Indergaard, M., Østgaard, K., Bævre, O.A., Pedersen, T.A., Jensen, A. (1987). Anaerobic digestion of *Laminaria spp.* and *Aschophyllum nodosum* and application of end products. *Biomass,* 14, 1-13.

Harmsen, P.F.H., Huijgen, W.J.J., Bernudez lopez, L.M., Bakker, R.C.C. (2010). *Literature Review of Physical and Chemical Pretreatment process for Lignocellulosic Biomass.* BioSynergy project. Food & Biobased Research Centre, Wageningen University, The Netherlands.

Haug, A., Jensen, A. (1954). Seasonal variation in the chemical composition of Alaria esculenta, *Laminaria saccharina*, *Laminaria hyperbora* and *Laminaria digitata* from Northern Norway. *Rep. Norw. Inst. Seaweed Res.* No. 4. 1-14.

Hendriks, A.T.W.M., Zeeman, G. (2009). Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource Technology*, 100, 10–18.

Hoadley, R.B. (2000). *Understanding wood: a craftsman’s guide to wood technology.* 2nd ed. Newtown, CT: Taunton Press.

Horn, S.J., Moen, E., Ostgaard, K., 1999, Direct determination of alginate content in brown algae by near infra-red (NIR) spectroscopy. *Journal of Applied Phycology,* 11(1), 9-13.

Horn, S. J., Aasen, M., Řstgaard, K. (2000). Ethanol production from seaweed extract. *J. Ind. Microbiol. Biotechnol.,* 25, 249–254.

Hu, G., Heitmann, J.A., Rojas, O.J. (2008). Feedstock pretreatments strategies for producing ethanol from wood, bark, and forest residues. *BioResources*, 3, 270e94

Innotti, E.L., Kafkawitz, D., Wolin, M.J., Bryant, M.P. (1973). Glucose fermentation products of *Ruminococcus albus* grown in continuous culture with *Vibrio succinogenes:* changes caused by interspecies transfer of H_2. *J Bacteriol.,* 114, 1231-1240.

Ivanova, G., Rakhely, G., Kovacs, K.L. (2009). Thermophilic biohydrogen production from energy plants by *Caldicellulosiruptor saccharolyticus* and comparison with related studies. *Int J Hydrogen Energy*, 34, 3659-3670.

Jang, S. S., Shirai, Y., Uchida, M., Wakisaka, M. (2012). Production of mono sugar from acid hydrolysis of seaweed. *African Journal of Biotechnology,* 11(8), 1953-1963. doi: 10.5897/AJB10.1681

Jessen, J.E. (2013). *Biofuel production from lignocellulosic biomass by thermophilic bacteria.* University of Akureyri.

Jessen, J.A., Orlygsson, J. (2012). Production of ethanol from sugars and lignocellulosic biomass by *Thermoanaerobacter* J1 isolated from a hot spring in Iceland. *J Biomed Biotechnol*, doi:10.1155/2012/186982.

Jones, P.R. (2008). Improving fermentative biomass-derived H_2 production by engineering microbial metabolism. *Int J Hydrogen Energ,* 33, 5122-5130.

Kapdan, I.K., Kargi, F. (2006). Biohydrogen production from waste materials. *Enzyme Microb Technol.*, 38, 569–582.

Khanna, M. (2011). A billion tons of biomass a viable goal but at high price. *Am J Agric Econ.*

Kim, D.H., Kim, M.S. (2012). Thermophilic fermentative hydrogen production from various carbon sources by anaerobic mixed cultures. *Int J Hydrogen Energy*. 37, 2021-27.

Kim, D.H., Kim, S.H., Shin, H.S. (2009). Sodium inhibition of fermentative hydrogen production. International *Journal of Hydrogen Energy,* vol. 34, no. 8, p. 3295-3304.

Kim, S., Dale, B.E. (2005). Global potential bioethanol production from wasted crops and crops residues. *Biomass Bioenerg.,* 29, 361e75.

Kimberly, M., Stefan, C., Richard, F., Yves, P., Marc, R., Esteban, C. (2002). Fluidizable Catalysts for Producing Hydrogen by Steam Reforming Biomass Pyrolysis Liquids. In: N.R.E. Labtorary (Ed.) U.S. DOE Hydrogen Program Review, Golden.

Kloareg, B., Demarty, M., Mabeau, S. (1986). Polyanionic characteristics of purified sulphated homofucans from brown algae. *Int. J. Biol. Macromol.*, 8, 380-386.

Knauf, M., Moniruzzaman, M. (2004). Lignocellulosic biomass processing. *Persp Int Sugar J.,* 106, 147e50.

Kruger, N. J., von Schaewen, A. (2003). The oxidative pentose phosphate pathway: structure and organization. *Curr. Opin. Plant Biol.* 6(3), 236–246.

Kyazze, G., Martinez-Perez, N., Dinsdale, R., Premier, G.C., Hawkes, F.R., Guwy, A.J., Hawkes, D.L. (2006). Influence of substrate concentration on the stability and yield of continuous biohydrogen production. *Biotechnol Bioeng.*, 93, 971-979

Lacis, L.S., Lawford, H.G. (1988). Ethanol production from xylose by *Thermoanaerobacter ethanolicus* in batch and continuous culture. *Arch Microbiol.,* 150, 48-55.

Lacis, L.S., Lawford, H.G. (1989). Analysis of the variation in ethanol yield from glucose or xylose with continuously grown Thermoanaerobacter ethanolicus. *Appl Biochem Biotechn.,* 20-21,479-490.

Lacis, L., Lawford, H. (1992). Strain selection in carbon-limited chemostats affects reproductibility of Termoanaerobacter-ethanolicus fermentations. *Applied and Environmental Microbiology*, 58(2), 761- 764.

Laureano-Perez, L., Teymouri, F., Alizadeh, H., Dale, B.E., (2005). Understanding factors that limit enzymatic hydrolysis of biomass. *Appl. Biochem. Biotechnol.,* 1081–1099.

Laurens, L.M.L. (2013). Summative Mass Analysis of Algal Biomass – Integration of Analytical Procedures. Laboratory Analytical Procedure (LAP). NREL.

Laurentin, A., Edwards, C.A. (2003). A microtiter modification of the anthrone-sulfuric acid colorimetric assay for glucose-based carbohydrates. *Anal Biochem.,* 315, 143-145.

Lay, J.J. (2001). Biohydrogen generation by mesophilic anaerobic fermentation of microcrystalline cellulose. *Biotechnol. Bioeng.,* 74, 280-287.

Lee, Y.E., Jain, M.K., Lee, C., Lowe, S.E., Zeikus, J.G. (1993). Taxonomic distinction of saccharolytic thermophilic anaerobes: Description of *Thermoanaerobacterium xylanolyticum* gen. nov., sp. nov., and *Thermoanaerobacterium saccharolyticum* gen. nov., sp. nov.; reclassification of *Thermoanaerobium brockii*, *Clostridium thermosulfurogenes*, and *Clostridium thermohydrosulfuricum* E100-69 as *Thermoanaerobacter brockii* comb. nov., *Thermoanaerobacterium thermosulfurigenes* comb. nov., and *Thermoanaerobacter thermohydrosulfuricus* comb. nov., respectively; and transfer of *Clostridium thermohydrosulfuricum* 39E to *Thermoanaerobacter ethanolicus*. *International Journal of Systematic Bacteriology,* 43, 41-51.

Levin, D.B., Islam, R., Cicek, N., Sparling, R. (2006). Hydrogen production by *Clostridium* thermocellum 27405 from cellulosic biomass substrates. *Int J Hydrogen Energy.,* 31,1496–503.

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

Li, C., Fang, H.H.P. (2007). Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. *Crit Rev Environ Sci Technol.*, 37, 1-39.

Lin, C.Y. and Lay, C.H. (2005). A nutrient formulation for fermentative hydrogen production using anaerobic sewage sludge microflora, *Int. J. Hydrogen Energy,* 30(3), 285–292.

Liu, H., Zhang, T., Fang, H.P.P. (2003). Thermophilic H_2 production from cellulose containing wastewater. *Biotechnol Lett.,* 25 365–9.

Liu, Y., Whitman, W.B. (2008). Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. *Ann NY Acad Sci,* 1125,171–189.

Liu, Y., Yu, P., Song, X., Qu, Y. (2008). Hydrogen production from cellulose by co-culture of *Clostridium thermocellum* JN4 and *Thermoanaerobacterium thermosaccharolyticum* GD17. *Int J Hydrogen Energy*, 33, 2927-2933.

Liu, Z.L., Slininger, P.J., Dien, B.S., Berhow, M.A., Kurtzman, C.P., Gorsich, S.W. (2004). Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethylfuran. *J Ind Microbiol Biotechnol,* 31, 345–352.

Lo, Y.C., Bai, M.D., Chen, W.M., Chang, J.S. (2008). Cellulosic hydrogen production with a sequencing bacterial hydrolysis and dark fermentation strategy. *Biores Technol.,* 99, 8299–308.

Luo, W., Proschold, T., Bock, C., Krienitz, L. (2009). Generic concept in *Chlorella*-related coccoid green algae (Chlorophyta, Trebouxiophyceae). *Plant Biology*, Volume 12, Issue 3, pages 545–553.

Ma, K., Liu, X., Dong, X. (2005). *Methanobacterium beijingense* sp. nov., a novel methanogen isolated from anaerobic digesters. *International Journal of Systematic and Evolutionary Microbiology* 55, 325–329. doi 10.1099/ijs.0.63254-0

Maidak, B.L., Cole, J.R., Parker, C.T., Garrity, G.M., Larsen, N., Li, B., Lilburn, T.G., McCaughey, M.J., Olsen, G.J., Overbeek, R., Pramanik, S., Schmidt, T.M., Tiedje, J.M., Woese, C.R. (1999). A new version of the RPD (Ribosomal Database Project). *Nucleic Acid Research*, 27,171-173.

Mars, A.E., Veuskens, T., Budde, M.A.W., van Doeveren, F.N.M., Lips, S.J., Bakker, R.R., de Vrije, T., Claassen, A.M., (2010). Biohydrogen production from untreated and hydrolyzed steam peels by extreme thermophilies *Caldicellulosiruptor saccharoluticus* and *Thermotoga neopolitana*. *Int J Hydrogen Energy*, 35. 7730-7737.

Martin, C., Jonsson, L.J. (2003). Comparison of the resistance of industrial and laboratory strains of *Saccharomyces* and *Zygosaccharomyces* to lignocellulose-derived fermentation inhibitors. *Enzyme Microbial Technol,* 32, 386–395.

Masset, J., Hiligsmann, S., Hamilton, C., Beckers, L., Franck, F., Thonart, P. (2010). Effect of pH on glucose and starch fermentation in batch and sequencedbatch mode with a recently isolated strain of hydrogen-producing *Clostridium butyricum CWBI1009*. *Int J Hydrogen Energy.* 35, 3371-3378.

McKillip, W.J., Collin, G. (2002). *Ullmann's Encyclopedia of Industrial Chemistry,* Sixth edition, Weinheim, Germany, Wiley-VCH.

Meeuse, B. J. D. (1962). Storage products. In Lewin, R. A. (ed), *Physiology and Biochemistry of Algae. Academic Press*, New York, pp. 289–291.

Meyer, A., S., Ale, M., T., Mikkelsen, J., D. (2011). Review of Structure-Function Relations and Extraction Methods for Fucose-Containing Sulfated Polysaccharides from Brown Seaweeds. *Marine Drugs*, 9(10), 2106-2130. doi:10.3390/md9102106

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M. (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology,* 96, 673–86.

Mussatto, S.I., Roberto, I.C. (2004). Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: A review. *Bioresource Technology,* 93(1), 1-10.

National Renewable Energy Laboratory. (2011). [online]. Available at: www.nrel.gov/biomass/analytical_procedures.html

Nath, K. and Das, D. (2004). Improvement of fermentative hydrogen production: Various approaches. *Appl. Microbiol. Biotechnol.*, 65, 520-529.

Nelson, T.E., Lewis, B.A. (1974). Separation and characterization of the soluble and insoluble components of insoluble laminarian. *Carbohydrate Research,* 33(1), 63-74.

Niel van, E.W.J., Budde, M.A.W., de Haas, G.G., van der Val, F.J., Claassen, P.A.M., Stams, A.J.M., (2002). Distinctive properties of high hydrogen producing extreme thermophiles, *Caldicellulosiruptor saccharaolyticus* and *Thermotoga elfii. Int J Hydrogen Energy,* 27, 1391-1398.

Niel van, E.W.J., Claassen, P.A., Stams, A.J.M. (2003). Substrate and product inhibition of hydrogen production by the extreme thermophile, *Caldicellulosiruptor saccharolyticus. Biotechnol Bioeng.,* 81, 255-262.

Oh, S., Van Ginkel, S., Logan, B.E. (2003). The relative effectiveness of pH control and heat treatment for enhancing biohydrogen gas production. *Environ. Sci. Technol.,* 37, 5186-5190.

Oh, Y.K., Seol, E.H., Lee, E.Y., Park, S., (2002). Fermentative hydrogen production by a new chemoheterotrophic bacterium *Rhodopseudomonas palustris* P4. *Int J Hydrogen Energy*, 27, 1373-1379.

Onyenwoke, R.U., Kevbrin, V.V., Lysenko, A.M., Wiegel, J. (2007). *Thermoanaerobacter pseudoethanolicus* sp. nov., a thermophilic heterotrophic anaerobe from Yellowstone National Park. *International Journal of Systematic and Evolutionary Microbiology*, 57, 2191-2193.

Orlygsson, J., Baldursson, S.R.B. (2007). Phylogenetic and physiological studies of four hydrogen-producing thermoanaerobes from Icelandic geothermal areas. *Icelandic Agricult Sci,* 20, 93-106.

Oshima, T., Moriya, T. (2008). A Preliminary Analysis of Microbial and Biochemical Properties of High-Temperature Compost. Annals of the New York Academy of Sciences Volume 1125, Incredible Anaerobes From Physiology to Genomics to Fuels, 338–344. doi:10.1196/annals.1419.012

Painter, T.J. (1983). Algal polysaccharides. In Aspinall OG (ed.) *The Polysaccharides,* Vol. 2. Academic Press, New York, 195-285.

Pallavi, S., Anjana, P. (2011). An evaluative report and challenges for fermentative biohydrogen production. *International Journal of Hydrogen Energy* 36, 7460-7478.

Pawar, S.S., Nkemka, V.N., Zeidan, A.A., Murto, M., van Niel, E.W.J. (2013). Biohydrogen production from wheat straw hydrolysate using *Caldicellulosiruptor saccharolyticus* followed by biogas production in a two-step uncoupled process. *Int J Hydrog Energy*, 38, 9121-30.

Pawar, S.S., van Niel, E.W.J. (2013). Thermophilic biohydrogen production: how far are we? *Appl Microbiol Biotechnol.,* 97(18), 7999–8009. doi: 10.1007/s00253-013-5141-1

Pawar, S.S., Vongkumpeang, T., Grey, C., van Niel, E.W.J. (2015). Biofilm formation by designed co-cultures of Caldicellulosiruptor species as a means to improve hydrogen productivity. *Biotechnology for Biofuels,* 8, 19. doi:10.1186/s13068-015-0201-7

Perlack, R.D., Wright, L., Turhollow, L.A., Graham, R.L., Stokes, B., Erbach, D.C. (2005). Biomass as feedstock for a bioenergy and bioproducts industry: the technical feasibility of a

billion-ton annual supply. Oak Ridge National Laboratory Report ORNL/TM-2005/66. Oak Ridge, TN: US Dept. of Energy.

Prakasham, R.S., Brahmaiah, P., Sathish, T., Rao, K.R.S.S., (2009). Fermentative biohydrogen production by mixed anaerobic consortia: Impact of glucose to xylose ratio. *Int. J. Hydrogen Energy,* 34 (23), 9354-9361.

Puhakka., J.A., Koskinen, P.E.P., Lay, C.H., Steinar, R.B., Tolvanen, K.E.S., Kaksonen, A.H., Orlygsson., J. Lin, C.Y. (2007). Bioprospecting Thermophilic Microorganisms from Icelandic Hot Springs for Hydrogen and Ethanol Production. *Energy & Fuels* 01/2008; 22(1). doi:10.1021/ef700275w

Rainey, F.A., Ward-Rainey, N., Krooppenstedt, R.M., Stackebrand, E. (1996). The genus Nocardiopis represents a phylogenetically coherent taxon and a distinct actinomycete lineage; proposal of Nocardiopsscae fam. nov. *Int J Syst Bacterol.*, 46, 1088-1092.

Reaume, S.(2009). *Fermentation of Glucose and Xylose to Hydrogen in the presence of Long Chain Fatty Acid.* University of Windsor.

Ren, N., Cao, G., Guo, W., Wang, A., Zhu, Y., Liu, B., Xu, J. (2010). Biological hydrogen production from corn stover by moderately thermophile *Thermoanaerobacterium thermosaccharolyticum* W16. *International Journal of Hydrogen Energy*, 35, 2708-2712

Ren, N., Cao, G., Wang, A., Lee, D., Guo, W., Zhu, Y. (2008). Dark fermentation of xylose and glucose mix using isolated *Thermoanaerobacterium thermosaccharolyticum W16. International Journal of Hydrogen energy*, 33(21), 6124-6132.

Ren, N., Guo, W., Liu, B., Cao, G., Ding, J. (2011). Biological hydrogen production by dark fermentation: challenges and prospects towards scaled-up production. *Curr Opin Biotechnol,* 22, 365-370. doi:10.1016/j.copbio.2011.04.022

Reungsang, A., Sreela-or, C. (2013). Bio-Hydrogen Production from Pineapple Waste Extract by Anaerobic Mixed Cultures. *Energies*, 6, 2175-2190. doi:10.3390/en6042175

Roesijadi, G., Jones, S. B., Snowden-Swan, L. J., Zhu, Y. (2010). PNNL-19944: Macroalgae as a Biomass Feedstock: A Preliminary Analysis (Pacific Northwest National Laboratory)

Roychowdhury, S. (2000). *Process for production of hydrogen from anaerobically decomposed organic materials.* U.S. Patent. US 006090266A.

Rubin, E.M. (2008). Genomics of cellulosic biofuels. *Nature* 454, 841-845. doi:10.1038/nature07190

Saha, B.C. (2003). Hemicellulose bioconversion*. J. Ind. Microbiol. Biotechnol.,* 30, 279– 291.

Santos, C. N., Regitsky, D. D., Yoshikuni, Y. (2013). Implementation of stable and complex biological systems through recombinase-assisted genome engineering. *Nature Comm.,* 4, 2503.

Saratale, G.D., Chen S.D., Lo, Y.C., Sartale, R.G., Chang, J.S. (2008). Outlook of biohydrogen production from lignocellilosic feedstock using dark fermentation – a review. *Journal of Scientific & Industrial Research.* Vol.67, pp 962-979

Schlegel, G. (2003). PWN, Warszawa

Schröder, C., Selig, M., Schonheit, P. (1994). Glucose fermentation to acetate, CO_2 and H_2 in the anaerobic hyperthermophilic eubacterium *Thermotoga maritima*: involvement of the Embden-Meyerhof pathway. *Arch. Microbiol.* 161, 460-470.

Scully SM, Orlygsson J. (2014). Branched-chain alcohol formation from branched-chain amino acids by *Thermoanerobacter brockii* and *Thermoanerobacter yonseiensis*. *Anaerobe*, 30, 82-84.

Scully, S.M., Orlygsson, J. (2015). Recent Advances in Second Generation Ethanol Production by *Thermophilic* Bacteria. *Energies*, *8*, 1-30, doi:10.3390/en8010001

Segovia Bravo, K. A., Lopez, F.N.A., garcia, P.G., Quintana, D., Fernandez, A.G. (2007). Treatment of green table olive solutions with ozone. Effect on their polyphenol content and on *Lactobacillus pentosus* and *Saccharomyces cerevisiae* growth. *International Journal of Food Microbiology,* 114(1), 60-68.

Seifert, K.,Thiel, M., Wicher, E.,Włodarczak,M., Łaniecki, M. (2012). *Microbiological Methods of Hydrogen Generation.* Faculty of Chemistry, A. Mickiewicz University, Poznań, Poland.

Skriptsova, A.V., Shevchenko, N.M., Zvyangintseva, T.N., Imbs, T.I. (2010). Monthly changes in the content and monosaccharide composition of fucoidan from *Undaria pinnatifida* (*Laminariales, Phaeophyta*). *Journal of Applied Phycology,* Volume 22, Issue 1, pp 79-86.

Soboh, B., Linder, D., Hedderich, R.A. (2004). A multisubunit membrane-bound (NiFe) hydrogenase and an NADH-dependent Fe-only hydrogenase in the fermenting bacterium *Thermoanerobacter tengcongensis*. *Microbiology*, 150, 2451-63.

Sommer, P., Georgieva, T., Ahring, B.K. (2004). Potential for using thermophilic anaerobic bacteria for bioethanol production from hemicellulose. *Biochemical Society Transactions* 32, 283-289.

Stichnothe, H., Azapagic, A. (2009). Bioethanol from waste: Life cycle estimation of the greenhouse gas saving potential. *Resources, Conservation and Recycling,* 53(11), 624-630.

Strode, P., Brokaw, A., (2013). Using BioInteractive Resources to Teach Mathematics and Statistics in Biology. Available at: http://www.hhmi.org/biointeractive/teacher-guide-math-and-statistics.

Stryer, L. (1999). Biochemistry PWN, Warszawa

Sveinsdottir, M., Baldursson, S., Orlygsson, J. (2009). Ethanol production from monosugars and lignocellulosic biomass by thermophilic bacteria isolated from Icelandic hot springs. *Icelandic Agricultural Sciences*, 22, 45-58.

Taguchi, F., Mizukami, N., Yamada, K., Hasegawa, K., Saito-Taki, T. (1995). Direct conversion of cellulosic materials to hydrogen by *Clostridiumsp.* strain no. 2. *EnzyMicrob Technol.,* 17, 147–50.

Tamura, K., Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol.,* 10, 512-526.

Tamura, K., Stecher, G., Peterson, A., Kumar, S. (2013). MEGA6: Molecular evolutionary genetic analysis. Version 6.0. *Mol Biol Evol.,* 30, 2725-2729.

Taylor, M.P., Eley, K.L., Martin, S., Tuffin, M.I., Burton, S.G., Cowan, D.A. (2009). *Thermophilic ethanologenesis*: future prospects for second-generation bioethanol production. *Trends Biotechnol.*, 27, 398-405.

Vardar-Schara, G.; Maeda, T.; Wood, T.K. (2008). Metabolically engineered bacteria for producing hydrogen via fermentation, *Microbial Biotechnology*, vol.1, pp. 107-125.

Vertes, A., Qureshi, N., Blaschek, H., Yukawa, H. (2009). Biomass to biofuels: Strategies for global industries. John Wiley and Sons, Ltd.

Vesteinsdottir, H., Reynisdottir, D., Orlygsson, J. (2011). *Thiomonas islandica* sp. nov., a moderately thermophilic, hydrogen- and sulfur-oxidizing betaproteobacterium isolated from hot spring. *Int J Syst Evol Micr.*, 61, 132-137.

Wagner, I.D., Wiegel, J. (2008). Diversity of thermophilic anaerobes. In: Incredible anaerobes: From physiology to genomic fuels. Ann. N. Y. *Acad. Sci.,* 1125, 1-43.

Wang, A., Ren, N., Shi, Y., Lee, D.J. (2008). Bioaugmented hydrogen production from microcrystalline cellulose using co-culture – *Clostridium acetobutyricum X9* and *Ethanoligenens harbinense B49. Int J Hydrogen Energy*, 33, 912–7.

Wang, J., Wan, W., (2009). Factors influencing fermentative hydrogen production: A review. *Int J Hydrogen Energy*, 34, 799-811.

Wargacki, A. J., Loenard, E., Win, M.N., Regitsky, D.D., Santos, C.N.S., Kim, P.B., Cooper, S.R., Raisner, R.M., Herman, A., Sivitz, A.B., Lakshmanaswamy, A., Kashiyama, Y., Baker, D., Yoshikuni, Y. (2012). An engineered microbial platformfor direct biofuel production from brown macroalgae. *Science* 335, 308–313. doi: 10.1126/science.1214547

Wi, S. G., Kim, H. J., Mahadevan, S. A., Yang, D.J., Bae, H. J. 2009. The potential value of the seaweed Ceylon moss (Gelidium amansii) as an alternative bioenergy resource. *Bioresour.Technol.* 100, 6658–6660.

Wiegel, J., Ljungdahl, L.G. (1981). "*Thermoanaerobacter ethanolicus* gen. nov., spec. nov., a new, extreme thermophilic, anaerobic bacterium". *Arch Microbiol.,* 128, 343-348.

Willquist, K., Claassen, P.A.M., van Niel, E.W.J. (2009). Evaluation of the influence of CO_2 on hydrogen production by *Caldicellulosiruptor saccharolyticus. Int J Hydrogen Energy,* 34, 4718–4726.

Vrije de, G., Mars, A., Budde, M., Lai, M., Dijkema, C., de Waard, P., Claassen, P. (2007). Glycolytic pathway and hydrogen yield studies of the extreme thermophile Caldicellulosiruptor saccharolyticus. *Applied Microbiology and Biotechnology,* 74(6), 1358-1367.

Vrije de, T., Claassen, P.A.M. (2003). Dark hydrogen fermentation.Bio-methane & Bio-hydrogen. Status and perspectives of biological methane and hydrogen production, 103-104.

Xia, L.M., Sheng, X.L: (2004). High yield cellulase production by *Trichoderma* reesei ZU-02 on corncob residues. *Bioresour Technol.*, 91, 259-262.

Ximenes, E., Kim, Y., Mosier, N., Dien, B., Ladisch, M. (2011). Deactivation of cellulases by phenols. *Enzyme Microb Technol.*, 48, 54–60.

Yang, H., Shen, J. (2006). Effect of ferrous iron concentration on anaerobic bio−hydrogen production from soluble starch. *Int. J.Hydrogen Energy,* 31, 2137−2146

Yazdani, P., Karimi, K. Taherzadeh, M.J. (2011). *Improvement of enzymatic hydrolysis of a marine macro-alga by dilute acid hydrolysis pretreatment.* World Renewable Energy Congress, Linkoeping, Sweden, 186-191.

Yokoi, H., Maki, R., Hirose, J., Hayashi, S. (2002). Microbial production of hydrogen from starch-manufacturing wastes. *Biomass Bioenergy*, 22, 389-395.

Yu, H., Zhu, Z., Hu, W., Zhang, H., (2002). Hydrogen production from rice winery wastewater in an upflow anaerobic reactor by using mixed anaerobic cultures. *Int. J. Hydrogen Energy* 27, 1359-1365.

Zeidan, A.A. van Niel, W.J. (2010). A quantitative analysis of hydrogen production efficiency of the extreme thermophile *Caldicellulosiruptor owensensis* OLT. *International Journal of Hydrogen Energy,* 35(3), 1128-1137.

Zeikus, J.G., Ben-Bassat, A., Hegge, P.W. (1980). Microbiology of *methanogenesis* in thermal, volcanic environments. *J Bacteriol*, 141, 432-440.

Zhang, M.L., Fan, Y.T., Xing, Y., Pan, C.M., Zhang, G.S., Lay, J.J. (2007). Enhanced biohydrogen production from corn stalk wastes with acidification pretreatment by mixed anaerobic culture. *Biomass Bioenergy*, 31, 250–4.

Zhang, P.Y.H., Berson, E., Sarkanen, S., Dale, B.E. (2009). Pretreatment and Biomass Recalcitrance: Fundamentals and Progress. *Appl. Biochem Biotechnol,* 153, 80-83. doi10.1007/s12010-009-8610-3

Zheng, Y., Pan, Z., Zhang, R. (2009). Overview of biomass pretreatment for cellulosic ethanol production. *Int J Agric & Biol Eng.,* 2(3), 51-68. doi: 10.3965/j.issn.1934-6344.2009.03.051-068

Zheng, X.J., Yu, H.Q. (2005). Inhibition effects of butyrate on biological hydrogen production with mixed anaerobic cultures. *J. Environ. Management,* 74, 65-70.

Zhu, H., Beland, M., (2006). Evaluation of alternative methods of preparing hydrogen producing seeds from digested wastewater sludge. *Int J Hydrogen Energy,* 31, 1980-1988.

Zhu, J.Y., Pan, H.J. (2010). Woody biomass treatment for cellulosic ethanol production: technology and energy consumption evaluation. *Bioresour Technol.*

Zvyagintseva, T.N., Shevchenko, N.M., Popivnich, I.B., Isakov, V.V., Scobun, A.S., Sundukova, E.V. and Elyakova, L.A. (1999). A new procedure for the separation of the water-soluble polysaccharides from brown seaweeds. *Carbohydrate Research* 322, 32–39.

7. Appendices

Appendix A: Hydrogen Calibration Curve

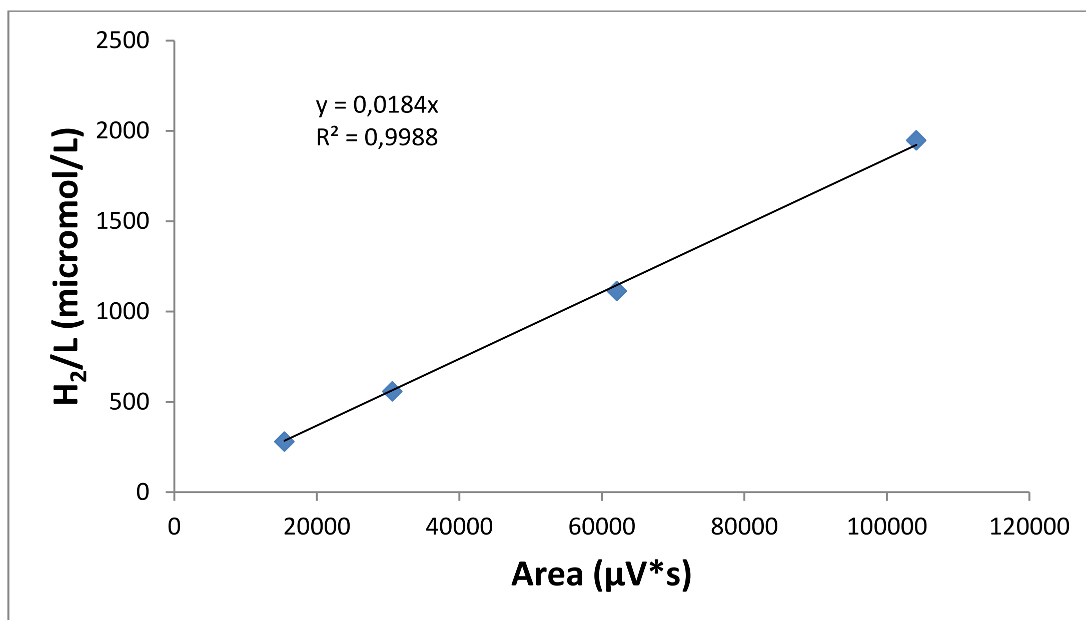


Figure A.1 Hydrogen calibration curve (mmol/L)

Appendix B: Volatile Fatty Acid Calibration Curves

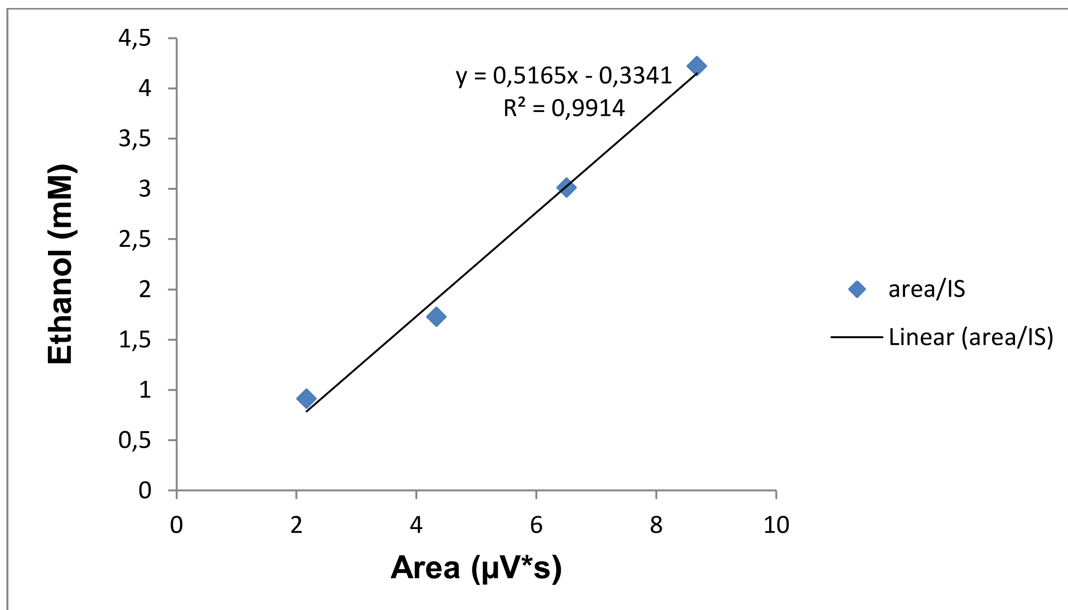


Figure B.1 Ethanol Volatile Fatty Acid Calibration Curve

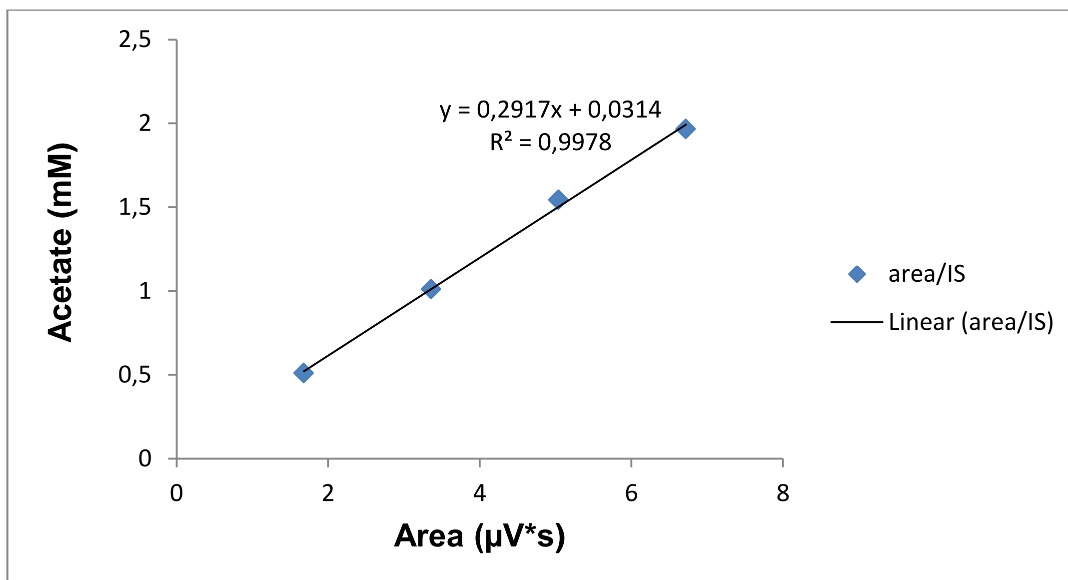


Figure B.2 Acetate Volatile Fatty Acid Calibration Curve

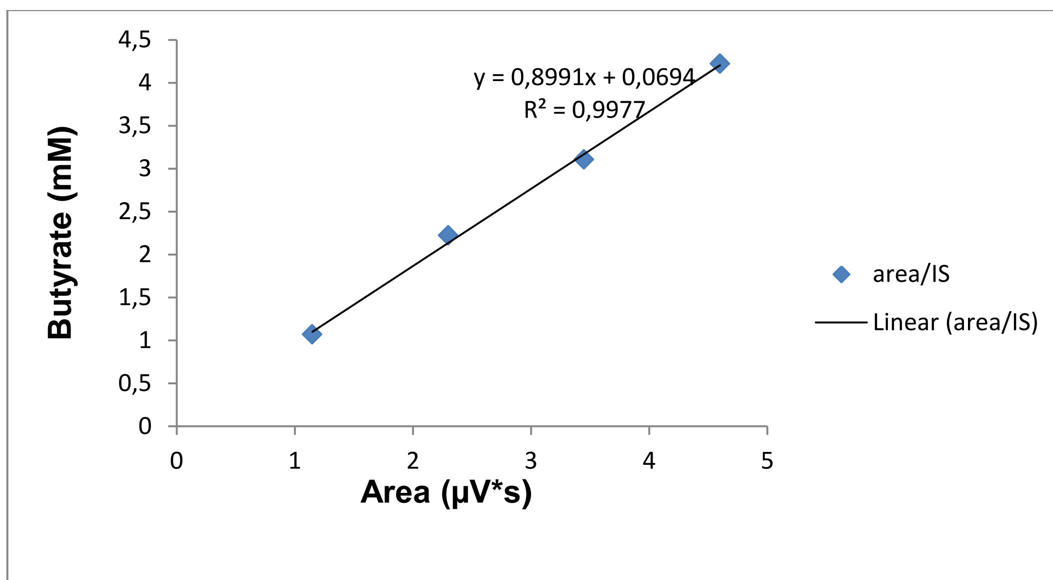


Figure B.3 Butyrate Volatile Fatty Acid Calibration Curve

Appendix C: Sugar Calibration Curves

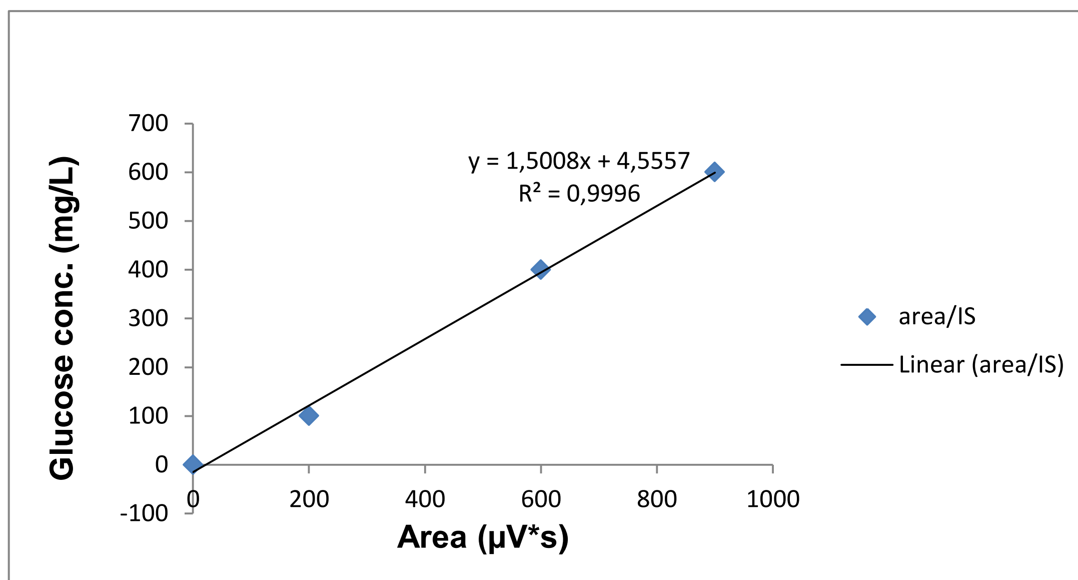


Figure C.1 Glucose Calibration Curve

Appendix D: Publication

The part of work done in this master's thesis was also submitted (17 April 2015) for publication in "International Journal of Hydrogen Energy" under the title:

Effect of culture conditions on hydrogen production by *Thermoanaerobacter* strain AK68

Ziva Vipotnik[1], Jan Eric Jessen[2], Sean Michael Scully[2], and Johann Orlygsson[2]*

ABSTRACT

Thermoanaerobacter strain AK68, isolated from an Icelandic hot spring, was investigated for hydrogen production from sugars. It is most closely related to T. pseudoethanolicus (99.4% similarity) and T. thermohydrosulfuricus (99.3% similarity). The strain has a temperature optimum between 65 and 70°C and a pH optimum between pH 7.0 and 8.0. The strain degraded most hexoses and disaccharides examined as well as xylose but not arabinose or starch. Various environmental variables were investigated to gain a deeper understanding the factors that are of importance for hydrogen production with the main emphasis on the fact that the strain could only partially degrade glucose at modest concentrations. Factors investigated included initial glucose concentration, buffer concentration, liquid-gas phase ratio, effect of various external electron acceptors, and different initial pH values. The main conclusion is that substrate utilization is mainly primarily inhibited by increased partial pressure of hydrogen, as evidenced by increased utilization of glucose using electron scavenging systems and manipulation of the liquid-gas phase ratio, rather than substrate or end product inhibition.

[1]Environmental Protection College, 3320 Velenje, Slovenia

[2]Faculty of Natural Resource Sciences, University of Akureyri, Borgir, Nordurslod 2, IS-600 Akureyri, Iceland. Tel: +354 4608511. Fax: + 354 4609889.

E-mail address: jorlygs@unak.is*

Acknowledgements

This work was sponsored by RANNÍS, (Technology Development Fund, projects 081303408 (BioEthanol) and RAN091016-2376 (BioFuel), The Nordic Energy Fund (NER; project BioH2, 06-Hydr-C13), The Research Fund of the University of Akureyri and the KEA fund.