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Biohydrogen Production by Modified Anaerobic Fluidized Bed Reactor (AFBR) Using Mixed Bacterial Cultures in Thermophilic Condition.

إنتاج الهيدروجين الحيوي من مجموعة بكتيريا في ظروف لا- هوائية وحرارة عالية بواسطة المفاعل اللا- هوائي المميع والمكون للحبيبات

BY

Amani Abu Rahma

Supervisors

Dr. Kamal El-Kahlout

Assistant Professor in Biotechnology

Dr.Tarek El-Bashiti

Associate Professor in Biotechnology

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List of Content

.List of tables

List of figures

List of Abbreviations

AFBR Anaerobic fluidized bed bioreactor.

AFGB Anaerobic fluidized granular bioreactor

ASBR Anaerobic sequencing batch reactor .

CAC Cylindrical activated carbon.

AC Activated carbon.

COD Chemical oxygen demand.

CSTR Continuous [flow] stirred tank reactor.

CIGSB Carrier induced granular sludge bed bioreactor.

EAMC Electrochemically assisted microbial cell

GAC Granular activated carbon.

GHG Green house gases.

HRT Hydraulic retention time.

HPR Hydrogen production rate (mmol h^{-1} L^{-1}).

HP Hydrogen productivity.

HY Hydrogen yield mol-H_2 mol-electron donor^{-1}

ICSAB Immobilized – cell- seeded anaerobic bioreactor.

MBR Membrane bioreactor.

MDGs Millennium Development Goals.

NAD^+ Nicotinamideadenine dinucleotide (oxidized form).

NADH Nicotinamideadenine dinucleotide (reduced form).

PBR Packed- bed reactor.

ppm Part per million.

ppp Pentose phosphate pathway.

PSII Photosystem II (or water-plastoquinone oxidoreductase).

ρH_2 Partial pressure of hydrogen.

SRT Sludge retention time.

CIGSB Carrier-induced granular sludge bed bioreactor.

TBR Tricking biofilter reactor.

T Temperature (k).

OLR Organic loading rate.

UASB Upflow anaerobic sludge blanket.

VFA Volatile fatty acids.

VSS Volatile suspended solids.

Abstract.

Hydrogen production represents a vital foundation for a hydrogen economy. Research, development, and demonstration, however, must continue in order to bring down the cost, increase the efficiency, and address the emissions issues associated with hydrogen production technologies. Dark fermentation using AFBR considered recently being promising and highly efficient in producing hydrogen gas in quantities exceeding even the theoretical values of 4 mol H_2 / mol glucose if certain modification in the bioreactor design and process are made.

Thermophilic fermentative biohydrogen production was studied in the anaerobic fluidized bed reactor (AFBR) operated at 65ºC with sucrose as a substrate. Theoretically, the maximum hydrogen yield (HY) is 4 mol H_2 /mol glucose when glucose is completely metabolized to acetate, H_2 and CO_2. But somehow, under most bioreactor design and operation conditions the maximum possible hydrogen yield (HY) as generally been observed not to exceed or reach 70-100% of the maximum theoretical hydrogen yield.

In this study further modification in anaerobic fluidized bed reactor namely the decrease in the total liquid volume to 3.3L, in addition to the application of external work in the form of high temperatures, high dilution rates and high rates of de-gassed effluent recycling were investigated as a means to overcome the thermodynamic constrains preventing the simultaneous achievement of high hydrogen yield (HY) and hydrogen productivity (HP) in an AFBR reactor.

Bacterial granulation was successfully induced under a thermophilic temperature of 65^0C. The bacterial granules consisted of a multispecies bacterial consortium comprised of thermophilic consortium . At a hydraulic retention time (HRT) of 1 h and effluent recycle rate of 3.6 L/ min, with V/F_{er} equal to 0.91 min, hydrogen production rate (HPR) of 7.57 L H_2 / h and hydrogen yield of 5.8 mol H_2 / mol glucose were achieved. This was greater than the yield achieved in a previous study conducted on 2012 , where the yield was 3.55 mol H_2 / mol glucose under similar experimental conditions.

Key wors: Dark fermentation.Thermophilictempreture. Modified anerobic fluidized bed reactor. Synotrophicmicrocology.

الخلاصة :

يمثل إنتاج الهيدروجين أساسا حيويا لاقتصاد الهيدروجين، ولذلك يجب أن تتواصل البحوث والعروض والتطوير من أجل خفض التكلفة، وزيادة الكفاءة، ومعالجة قضايا الانبعاثات المرتبطة بتقنيات إنتاج الهيدروجين

يعد التخمير في ظروف معتمة باستخدام المفاعل اللاهوائي المميع والمكون للحبيبات واعدا وذي كفاءة عالية في إنتاج غاز الهيدروجين بكميات تتجاوز حتى القيم النظرية التي تقول بان كل مول جلوكوز يمنح 4 مول هيدروجين وذلك إذا تم إجراء تعديل معين في تصميم المفاعل الحيوي وعملية التخمير .

من المفترض في انتاج الهيدروجين الحيوي في المفاعل اللاهوائي المميع والمنتج للحبيبات (AFBR) عند درجة حرارة 65 درجة مئوية باستخدام السكروز في تغذية البكتيريا، أن يكون العائد الأقصى (HY) هو 4 مول هيدروجين لكل مول من الجلوكوز عندما يتم استقلاب الجلوكوز تماما الى حامض الاستيك والهيدروجين وثاني اكيد الكربون. ولكن في معظم حالات تصميم المفاعل الحيوي وظروف التشغيل كان العائد الأقصى من الهيدروجين (HY) لا يتجاوز 70–100 ٪ من العائد المفترض نظريا .

في هذه الدراسة تم اجراء مزيد من التعديلات على المفاعل اللاهوائي المميع والمكون للحبيبات وهي تحديدا خفض الحجم الكلي للمفاعل الي 3.3 لتر، بالإضافة إلى تطبيق شغل خارجي ممثلا بارتفاع درجات الحرارة، ورفع معدل التخفيف ورفع معدل إعادة تدوير السائل الخارج من المفاعل كوسيلة للتغلب على المعوقات الثيرموديناميكية التي تحول دون تحقيق عائد (HY) وانتاج عال من الهيدروجين (HP) في الوقت ذاته في مفاعل (AFBR) .

لقد تم تكوين الحبيبات البكتيرية بنجاح تحت درجة حرارة 65 مئوية. تكونت الحبيبات البكتيرية من مجموعة بكتيرية متعددة الأنواع . كان معدل انتاج الهيدروجين (HPR) ساعة واحدة عند وقت الاحتفاظ الهيدروليكي (HRT) ومعدل اعادة تدوير السائل 3.6 لتر / دقيقة، مع(V / F_{er}) يساوي 0.91 دقيقية هو 7.57 لتر هيدروجين بالساعة وعائد الهيدروجين من الجلوكوز5.8 مول هيدروجين/ مول جلوكوز وهو أعلى من عائد الهيدروجين الذي وصلت اليه دراسة سابقة اجريت عام 2012 وبلغ العائد فيها3.55 في ظروف مشابهة لهذه الدراسة.

كلمات مفتاحية: التخمير في ظروف معتمة الحرارة الثيرموفيلية، المفاعل اللاهوائي المميع والمكون للحبيبات، البيئة الدقيقة التكافلية

Chapter 1
Introduction

1.1- Overview:

Energy is at the heart of most critical economic, environmental and developmental issues facing the world today. Clean, efficient, affordable and reliable energy services are indispensable for global prosperity. Developing countries in particular need to expand access to reliable and mode energy services if they are to reduce poverty and improve the health of their citizens, while at the same time increasing productivity, enhancing competitiveness and promoting economic growth. Current energy systems are inadequate to meet the needs of the world's poor and are jeopardizing the achievement of the Millennium Development Goals (MDGs). For instance, in the absence of reliable energy services, neither health clinics nor schools can function properly **(AGECC, 2010).**

Current patterns of energy production and consumption are unsustainable and threaten the environment on both local and global scales. Emissions from the combustion of fossil fuels are major contributors to the unpredictable effects of climate change, and to urban air pollution and acidification of land and water. Reducing the carbon intensity of energy – that is, the amount of carbon emitted per unit of energy consumed – is a key objective in reaching long term climate goals. As long as the primary energy mix is biased towards fossil fuels, this would be difficult to achieve with currently available fossil fuel-based energy technologies. Given that the world economy is expected to double in size over the next twenty years, the world's consumption of energy will also increase significantly if energy supply, conversion and use continue to be inefficient. Energy system design, providing stronger incentives for reduced greenhouse gases (GHG) emissions in supply and increased end-use efficiency, will therefore be critical for reducing the risk of irreversible, catastrophic climate change **(McLamb, 2011).**

Biofuel production, if approached in a sustainable manner, can be more environmentally benign than fossil fuel technologies for several major reasons: First, biofuel production from biomass is largely carbon neutral—that is, the CO_2 produced as the fuel is combusted, is offset by the carbon absorbed as the biomass is grown. Second, bioconversion processes in general do not produce hazardous compounds, and if toxic

solvents and chemicals are avoided in the processing stages, then fewer environmental pollutants are produced Third, biomass production and microbial conversion processes can be developed and used in a more distributed manner, avoiding the need for transport of fuels via cargo ships or pipelines for long distances **(Rittmann, 2008).**

Biomass has the potential to accelerate the realization of hydrogen as a major fuel of the future. Since biomass is renewable and consumes atmospheric CO_2 during growth, it can have a small net CO_2 impact compared to fossil fuels. However, hydrogen from biomass has major challenges. There are no completed technology demonstrations. The yield of hydrogen is low from biomass since the hydrogen content in biomass is low to begin with (approximately 6% versus 25% for methane) and the energy content is low due to the 40% oxygen content of biomass **(Milne et al., 2002).**

Hydrogen is a very light odourless and colourless gas with very different properties from the other gaseous fuels. Burning hydrogen with air under appropriate conditions in combustion engines or gas turbines results in very low or negligible emissions. Trace hydrocarbon and carbon monoxide emissions, if any, can only come from the combustion of lubricating oil in the combustion chamber of internal combustion engines. Nitrous oxide emissions increase exponentially with the combustion temperature. They can therefore be reduced through appropriate process control. As hydrogen offers more flexibility than other fuels, a lower combustion temperature can be achieved (e.g. with a high air to fuel ratio) leading to a distinct reduction in NOx emissions compared to petroleum products and natural gas. Particulate and sulphur emissions are completely avoided but from minimal quantities of lubricant residues **(Royal Belgian, 2006).**

Hydrogen production from biological systems is called biological hydrogen or biohyhdrogen. There are numerous attractive routes to produce biohydrogen from renewable source, solar gasification, thermo- chemical gasification, pyrolysis, supercritical conversion, direct bio-photolysis, indirect biophotolysis, photo-fermentation, dark fermentation **(Karthic &Shiny, 2012).**

Dark fermentation is the fermentative conversion of organic substrate to biohydrogen. It is a complex process manifested by diverse group of bacteria by a series of biochemical reactions. Fermentative/hydrolytic microorganisms hydrolyze complex organic polymers to monomers which further converted to a mixture of lower molecular

weight organic acids and alcohols by necessary H_2 producing acidogenic bacteria **(Benemann, 1996).**

Currently, increasing efforts are being taken to improve hydrogen production by this promising approach. Although some of the research results are encouraging, there are still far way to go, with two hurdles needing to be addressed. First, bioreactor designs require improvement in several important ways. And second, low cost raw materials are needed to supply these reactors. As a corollary to both of these challenges, systems need to achieve higher levels of substrate conversion efficiency to reduce the product per-unit costs of both raw materials and processing. In the past two years, there have been many new developments in reactor optimization, raw material exploitation and two-stage hydrogen production **(Ren et al., 2011).**

The anaerobic fluidized bed reactor (AFBR) with attached biofilm has been ideally used as a biological treatment system for wastewater with high efficiency and low HRT. Although AFBRs possess favorable characteristics for the production on gaseous products like, H_2 they have been less frequently utilized for H_2 dark fermentation **(Das & Nejat, 2008).** Biohydrogen production by the various AFGB systems represents a significant technological advance **(Obazu et al., 2012).**

1.2- General objective:

This project will study dark fermentative biohydrogen production using mixed bacterial cultures in a thermophilic (65°C) and a modified fluidized bacterial granular bed bioreactor (AFBR), that facilitates maximum hydrogen production and yield.

1.3- Specific objectives:

1. The first phase of this research is to modify the AFBR design and operational strategy that facilitates maximum H_2 production and yield.
2. To develop a suitable procedure for rapid initiation, growth and development of thermophilic granules that consists of mixed hydrogen-producing microorganisms in the AFBR.
3. Investigate the effect of shortening the hydraulic retention time (HRT), increasing effluent recycle rates , total bioreactor volume on the substrate utilization, hydrogen content, hydrogen production, hydrogen yield , pH, and the distribution of soluble metabolites in the AFBR.

1.4- Significance:

Biohydrogen production is the most challenging area with respect to environmental and renewable energy problems. Palestine suffers from shortage of energy supply and sustains development resources. This research will be the first in Palestine that will produce hydrogen by dark fermentation to be used in advance for energy generation.

Chapter II

Literature review

2.1- The energy challenge

Worldwide demand for energy is growing at an alarming rate. The European "World Energy Technology and Climate Policy Outlook" (WETO) predicts an average growth rate of 1.8% per annum for the period 2000-2030 for primary energy worldwide. The increased demand is being met largely by reserves of fossil fuel that emit both greenhouse gasses and other pollutants. Those reserves are diminishing and they will become increasingly expensive. Currently, the level of CO_2 emissions per capita for developing nations is 20% of that for the major industrial nations. As developing nations industrialize، this will increase substantially **(Arvelo & Padron, 2000).**

By 2030, CO_2 emissions from developing nations could account for more than half the world CO_2 emissions. Industrialized countries should lead the development of new energy systems to offset this. Energy security is a major issue. Fossil fuel, particularly crude oil، is confined to a few areas of the world and continuity of supply is governed by political, economic and ecological factors. These factors conspire to force volatile, often high fuel prices while, at the same time, environmental policy is demanding a reduction in greenhouse gases and toxic emissions **(IPCC, 2001).**

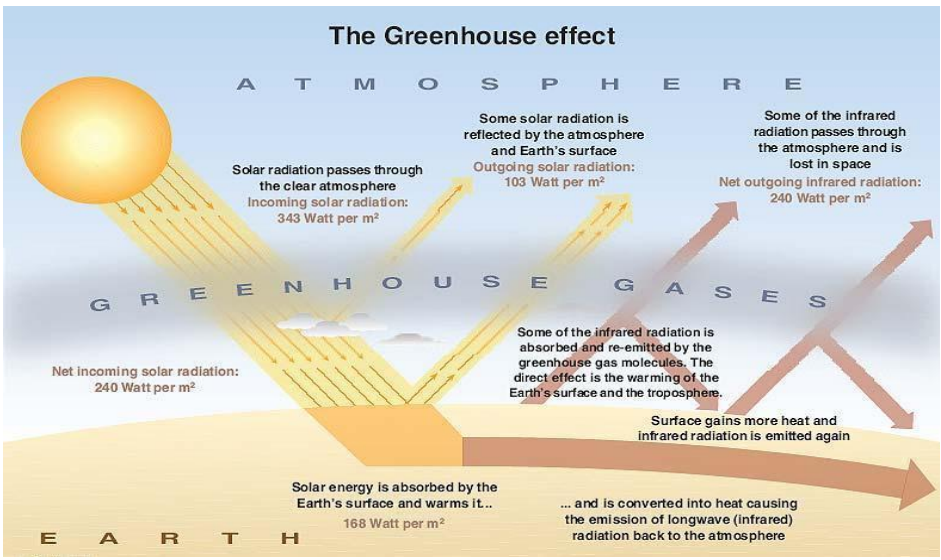


Figure 2.1- A schematic illustration of the greenhouse effect (Source Okanagan University college in Canada, Department of Geography; University of Oxford, School of Geography; United states Protection Agency (EPA) Washington, Intergovernmental Panel on Climate Change (IPCC) 1996).

A coherent energy strategy is required, addressing both energy supply and demand, taking account of the whole energy lifecycle including fuel production, transmission and distribution, and energy conversion, and the impact on energy equipment manufacturers and the end-users of energy systems. In the short term, the aim should be to achieve higher energy efficiency and increased supply from local energy sources, in particular renewables. In the long term, a hydrogen-based economy will have an impact on all these sectors. In view of technological developments, vehicle and component manufacturers، transport providers, the energy industry, and even householders are seriously looking at alternative energy sources and fuels and more efficient and cleaner technologies – especially hydrogen and hydrogen-powered fuel cells **(Arvelo& Padron, 2005).**

Renewable energy deriving from solar, wind, and biomass sources has great potential for growth to meet our future energy needs. Fuels such as ethanol, methane, and hydrogen are characterized as **biofuels** because they can be produced by the activity of biological organisms. Which of these fuels will play a major role in our future? The answer is not clear, as factors such as land availability, future technical innovation, environmental policy regulating greenhouse gas emissions, governmental subsidies for fossil fuel extraction/ processing, implementation of net metering, and public support for alternative fuels will all affect the outcome. A critical point is that as research and development continue to improve the efficiency of biofuel production processes, economic feasibility will continue to improve **(Drapcho et al., 2008).**

2.2- Hydrogen as energy carrier.

Hydrogen is a very light odourless and colourless gas with very different properties from the other gaseous fuels. Water is made of 11.2 % hydrogen by weight. Gaseous hydrogen density is 0.09 kg/m^3 (air is 14.4 times as dense and methane 8 times). Hydrogen boils at -253° C. Hydrogen has the highest energy to weight ratio of all fuels. 1 kg of hydrogen contains the same amount of energy as 2.1 kg of natural gas or 2.8 kg of gasoline. The energy to volume ratio amounts for the liquid to about 1/4 of crude oil, and for the gas to about 1/3 of natural gas. Hydrogen burns in air at volume concentrations from 4 % to 74.5 % (methane burns at 5.3 to 15 % and propane at 2.1 to 9.5 % volume concentrations). The highest flame temperature of hydrogen of 2318 °C is reached at 29 % volume concentration in air, whereas hydrogen in an oxygen atmosphere can reach temperatures up to 3000 °C (the highest temperature reached in air for methane is 2148 °C

and for propane 2385 °C). The minimum required ignition energy required for a stoechiometric fuel/oxygen mixture is 0.02 mJ for hydrogen, 0.29 mJ for methane and 0.26 mJ for propane. Since even the energy of a static electric discharge from the arching of a spark is sufficient to ignite natural gas, the lower value for hydrogen ignition (only one tenth) is therefore not a practical disadvantage. The temperatures for spontaneous ignition of hydrogen, methane and propane in air are 585 °C, 540 °C and 487 °C respectively **(Pritchard & Rattigan, 2010).**

The explosive concentrations in air for hydrogen and methane lie (detonation limits) between 18.3 to 59 % and 6.3 to 14 % respectively. The explosive range for hydrogen is clearly much greater, whereas methane is already explosive at a much lower concentration. The 0.61 cm^3/s diffusion coefficient of hydrogen is 4 times that of methane. Hydrogen therefore mixes with air considerably faster than methane or petrol vapors. From a safety point of view, it is advantageous in the open air but presents a disadvantage in badly ventilated indoors. Since both hydrogen and natural gas are lighter than air they raise quickly, hydrogen being much the faster. Propane and petrol vapor on the contrary are heavier than air and lay on the ground, leading to accumulation and presenting a greater hazard of major explosions **(Swain & Swain, 1992).**

Historically the main reasons for promoting hydrogen as an energy carrier are its outstanding properties for environmental protection. Burning hydrogen with air under appropriate conditions in combustion engines or gas turbines results in very low or negligible emissions. Trace hydrocarbon and carbon monoxide emissions, if any, can only come from the combustion of lubricating oil in the combustion chamber of internal combustion engines. Nitrous oxide emissions increase exponentially with the combustion temperature. They can therefore be reduced through appropriate process control. As hydrogen offers more flexibility than other fuels, a lower combustion temperature can be achieved (e.g. with a high air to fuel ratio) leading to a distinct reduction in NOx emissions compared to petroleum products and natural gas. Particulate and sulphur emissions are completely avoided but from minimal quantities of lubricant residues **(Momirlana & Veziroglub, 2005).**

The use of hydrogen for propulsion in low temperature fuel cells (PEMFC) completely eliminates all polluting emissions. The single by-product resulting from the generation of electricity from hydrogen and air is demineralized water. The use of

hydrogen in fuel cells at higher temperature (MCFC and SOFC) causes up to 100 times fewer emissions than conventional power stations. Let us remember however that hydrogen originates from a primary source. If it is obtained from methane, methanol or a fossil fuel, the reforming process itself will results in carbon dioxide emissions**.** This carbon dioxide from the reforming process is highly concentrated, therefore making it much cheaper to recover than from diluted exhaust gases of gas turbines. Hydrogen shows therefore an economical advantage, should the capture and storage of carbon dioxide become a practical reality**.** Several production processes drastically reduce - or even avoid emissions, especially of carbon dioxide (CO_2) - in the whole fuel cycle. This is the case for the most diverse renewable energies. Hydrogen has some advantageous properties which are at least as important as its outstanding environmental characteristics. They are listed below and put in balance with their drawbacks **(Royal Belgian, 2006).**

Main advantages:

- Uncoupling of primary energy sources and utilization.
- Hydrogen is a gas, thus easier to store than electricity.
- Hydrogen can be obtained from any primary energy source, including renewable.
- Decentralized production is possible. Hydrogen is viewed as capable of providing services where electricity is not available, in particular as a fuel for vehicles and energy storage in remote areas.
- Very efficient when used in fuel cells.
- Very good experience of hydrogen as a chemical reactant (ammonia, methanol, oil refining).
- Very good safety records (for a specific range of applications however) **(Royal Belgian, 2006).**

Main Drawbacks:

- Poor overall energy efficiency when produced from electricity made with fossil fuels.
- Very low density and poor specific volume energy density.
- Need for high pressures and very low temperatures if stored in the liquid phase.
- Specific safety problems and poor public acceptance (Hindenburg syndrome, Apollo Challenger space shuttle).
- No existing infrastructures for transport, distribution and storage.
- Rather high cost (up to now) **(Royal Belgian, 2006).**

Table 2.2: Basic Properties of Hydrogen, Methane, and Propane

Gas Properties:	Hydrogen	Methane	Propane
Chemical Formula	H2	CH4	C3H8
Molecular Weight	2.016	16.04	44.097
Gas Density (KgLm3) @ STP	0.0808	0.643	1.767
Diffusivity (m2Lsec) × 105	6.11	1.60	1.00
Combustiom Properties:			
Stoichiometric Fuel Volume ranction %	29.5%	9.48%	4.03%
Lower Heating Value (MJ/m3)	9.9	32.6	81.2
Lower Heating Value (MJ/kg)	118.8	50.0	46.35
Adiabatic Flame Temperature (K)	2380	2226	2267
Flammability Limits (Volume %)			
Lean limit:	4%	5.3%	2.2%
Rich Limit:	75%	15%	9.5%
Max. Flame Velocity (m/sec)	3.06	0.39	0.45
Min. Lgmition Temperature (K)1	845	905	766
Min. Lgnition Energy (10-5 J)1	2.0	33	30.5
Storage Condiions:			
Tank Type	Cylimder	Cylimder	Barbecue
Volume (liters)	49	49	21
Pressure (psi)2	34 MPa	17 MPa	1.6 MPa
Phase	Gas	Gas	Gas
Mass (kg)	1.35	5.36	0.61

2.3- Energy and air pollution profile in Palestine.

The main sources of air pollution in Palestine are the various means of transportation, the smoke rising from the chimneys of factories, the heavy dust from quarries, the burning of solid wastes, and the effects of water treatment projects. The Israeli industries in the West Bank, Gaza Strip, and inside the part of Palestine occupied in 1948 are the biggest cause of atmospheric pollution in Palestine. Many Israeli sawmills pollute the air across the West Bank with large quantities of greenhouse gases. A 2009 study prepared by George Karzam of the Ma'an Development Centre predicts that the

greenhouse gases emitted from the territories occupied in 1948 will increase by 40 percent by the year 2020 **(Karaeen, 2012).**

In the Gaza strip, the issue of air pollution is attributed to the density of motor vehicles, estimated at about 60,000, and especially to the number of old vehicles. Also, air pollution is caused by the gases and smog emitted from Israeli factories, especially from coal-operated power stations, and transferred to Gaza Strip by the wind. These factories are located in Isdude (Ashdod) and Al-Majdal (Ashqelon) inside the part of Palestine occupied in 1948**.** If we were to look at the effects the unjust siege of the Gaza Strip has had on the environment, we would find painful facts about air pollution. Toxic gases, including sulphur dioxide and carbon monoxide, which harm the respiratory system, are released into the air as a result of the use of the large numbers of people who run home generators to compensate for the shortage in electricity caused by Gaza's inability to run its power plant full-time due to the acute shortage of fuel. It is estimated that there are about 100,000 of these generators in use and that they consume about 500,000 litres of fuel per day. Therefore, the environment in Gaza Strip requires a more thoughtful and comprehensive policy of planning, awareness, and conservation **(Karaeen, 2012).**

Palestine is a developing nation, its access to considerable amounts of energy is essential to achieve economic growth and development. While most of Palestine has access to electricity there are many challenges facing Palestine, arising mainly from its energy dependence. Its energy is not provided through domestic means but rather provided through Israel which controls the quantity and quality of energy imported. With complete dependency on Israel for its energy needs, Palestine is put in a vulnerable position given its complex political and security situation. Such a threat has given rise to the importance of using renewable energy such as solar, wind, geothermal and biomass. Renewable energy can offer Palestine many benefits which will not only revolve around the reduction of conventional energy consumption and reducing harmful emissions, but most importantly will help achieve sustainable development for a Palestine that has little natural resources**.** Biomass, if utilized properly, could become one of Palestine's major energy resources. Currently, biomass energy constitutes approximately 15% of Palestinian energy supply; it is used mainly for heating purposes. Palestine is known, historically, for its agriculture and trading. Agriculture is still a predominant economic activity. As a result, Palestine has a strong potential for biomass energy. People living in rural areas may benefit from

producing biomass energy in various forms, including wood, crop residues and biogas. Presently, no crops are grown in Palestine specifically for use as fuel **(Abu Hamed et al., 2011).**

2.4- Hydrogen Production.

H_2 can be generated in four ways: (1) electrochemical processes; (2) thermochemical processes; (3) photochemical process, photocatalytic process, or photo electrochemical process and (4) Fermentative hydrogen production. The first three processes have the disadvantages in that they do not reduce waste, do not produce energy, but consume it through the use of electricity derived from fossil fuel combustion. On the other hand, fermentative H_2 production produces energy and reduces waste **(Han& Shin, 2004)**. Fermentative H_2 production can be classified into two categories: anaerobic fermentation and photosynthesis **(Kotsopoulos et al., 2006).** The efficiency of photosynthetic H_2 production is low and cannot be operated in the absence of light. Furthermore, photosynthetic H_2 production rates are relatively low, from 0.07 to 0.16 mmol of H_2 (L/h) **(Levin et al., 2004).** In contrast, fermentative H_2 can produce H_2 all day without light, using various kinds of substrates such as organic wastes, and has higher H_2 production rate reaching 120 mmol of H_2/(L/h), simple control requirements, lower operating costs and higher feasibility for industrialization **(Li &Fang, 2007).** Thus, fermentative H_2 production is more feasible and widely used. It is of great significance to produce H_2 from organic wastes by fermentative H_2 production, because it plays the dual role of waste reduction and energy production **(Xing et al., 2008).**

2.5-Biological production technologies.

Even though photosynthetic hydrogen production is a theoretically perfect process with transforming solar energy into hydrogen by photosynthetic bacteria, applying it to practice is difficult due to the low utilization efficiency of light and difficulties in designing the reactors for hydrogen production. However, fermentative hydrogen production has the advantages of rapid hydrogen production rate and simple operation. Moreover, it can use various organic wastes as substrate for fermentative
hydrogen production. Thus, compared with the photosynthetic hydrogen production, fermentative hydrogen production is more feasible and thus widely used. In addition, it is of great significance to produce hydrogen from organic wastes by fermentative hydrogen

production, because it can not only treat organic wastes, but also produce very clean energy. Therefore fermentative hydrogen production has been received increasing attention in recent years **(Karthic & Shing, 2012).**

2.5.1-Photofermentation.

The photo-decomposition of organic compounds by phototrophic bacteria has shown great potential as a biohydrogen production system. The purple non-sulphur bacteria are photofermenters and produce hydrogen by absorbing light and fermenting reduced compounds such as organic acids. These anaerobic photoheterotrophic bacteria do not possess PSII and as a result do not produce O_2, thus there is no inhibition in hydrogen production. Furthermore, they are able to utilize a variety of organic and inorganic substrates as electron donors (as opposed to water, by photoautotrophs), and a number of studies corroborate this in different experimental environments, including batch processes continuous cultures as well as immobilized whole cell systems with various solid support matrices The photofermentative process essentially involves solar energy being captured and utilized to produce ATP and release electrons via reverse electron flow, which reduces ferredoxin, and together with ATP, drive hydrogen evolution via proton reduction carried out by nitrogenase **(Tizzone, 2010).**

2.5.2- Hydrogen dark fermentation.

Dark fermentation is the fermentative conversion of organic substrate to biohydrogen. It is a complex process manifested by diverse group of bacteria by a series of biochemical reactions. Fermentative/hydrolytic microorganisms hydrolyze complex organic polymers to monomers which further converted to a mixture of lower molecular weight organic acids and alcohols by necessary H_2 producing acidogenic bacteria**.** A wide range of Carbohydrates-rich substrates which can be used for the generation of hydrogen, includes feedstock from energy crops (sugar beet, grasses, including lignocelluloses fractions), solid waste (food waste, organic fraction of municipal solidwaste), and industrial wastewaters (food industries, pulp and paper industry). Due to global environment and energy security concerns, a non-polluting inexpensive feedstock must be used for hydrogen generation. Utilization of wastes to generate H_2 energy could reduce the production cost, making H_2 gas more available and cheaper **(Levin et al., 2007).**

Majority of H_2 dark fermentation studies have been conducted with model compounds, mainly with glucose or sucrose. Glucose is the monomeric unit of the most abundant biopolymers, cellulose and starch while sucrose is a major component in some crops and food industry wastes (sugar industry, brewing etc.). Hydrogen production from xylose, a sugar constituent of hemicellulose, has been demonstrated. In a similar manner, H_2 production from lactose, a main carbohydrate constituent of diary wastes, has been demonstrated. Further, H_2 production from the sugar polymers, starch and cellulose, has been reported. In addition, H_2 fermentation from chitin and N-acetyl-D-glucosamine, the monomer of chitin, has been demosnbstrated by *Clostridium paraputrificum M-21***.** Hydrogen production has been demonstrated from several wastes and potential energy crop materials. Pilot-scale H_2 production has been demonstrated from molasses, spent grains, citric acid production waste water, office paper slurry and food waste. Depending on the feedstock and microorganisms used, the material may require pretreatment with processes, such as mechanical cutting or crushing, cid, enzymatic hydrolysis, or sterilization. The treated feedstock may need to be diluted and supplied with nutrients and buffers prior to feeding to the reactors **(koshinen, 2008).**

The process by which H_2 is formed involves a complex interaction of various microorganisms and takes place in basically four separate phases namely: hydrolysis (phase 1), acidogenesis (phase 2), acetogenesis (phase 3) and methanogenesis (phase 4) **(Masilela, 2011).**

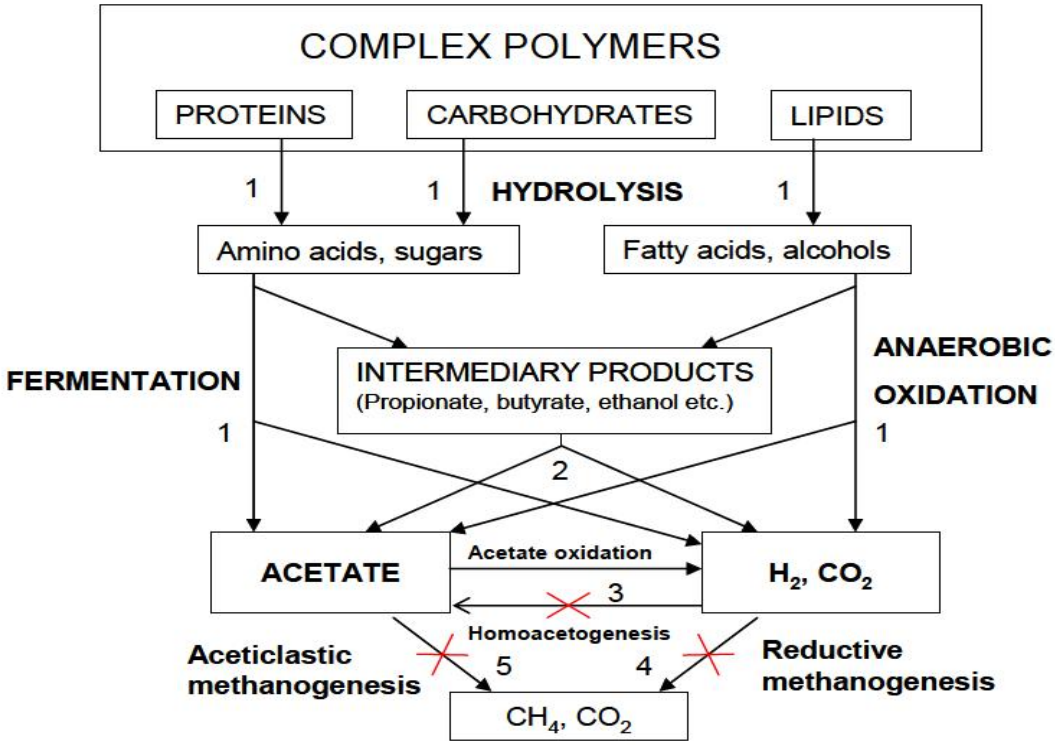


Figure 2.5.2: Different stages of anaerobic digestion of organic matter and the microbial groups involved.1, Fermentative bacteria; 2, hydrogen-producing acetogenic bacteria; 3, hydrogenconsuming acetogenic bacteria; 4, carbon dioxide-reducing methanogens; 5, Aceticlastcic methanogens .The crosses represent hydrogen consuming reactions, methanogenesis and homoacetogenesis, which are undesirable in H_2 producing reactors **(Valdez-Vazquez& Poggi-Varaldo, 2009).**

2.5.2.1- Hydrolysis:

Hydrolysis is the first step in anaerobic process whereby complex organic compounds (e.g. carbohydrates, proteins and lipids) are split into simpler components or simple monomers. These monomers which are the products of external hydrolytic reactions can be taken up across cell membranes and used as substrates for catabolism and anabolism. The breakdown of large biopolymers into the constituent monomers are catalysed by extracellular hydrolytic enzymes (cellulase, protease, lipase) released by facultative or obligate anaerobic bacteria **(Gavrilescu, 2002).**

2.5.2.2- Acidogenesis:

Acidogenesis, also called fermentation is a process by which soluble molecules are used as carbon and energy sources by fermentative bacteria and converted into volatile fatty acids (VFAs), alcohols, and biogas. Acidogenesis is very important in anaerobic digestion as it is a step where H_2 is produced. H_2 comes from the mechanism of

dehydrogenation of pyruvate by ferredoxin and NADH reductase enzymes and also from the conversion of formic acid by formate dehydrogenase. H_2 is one of the substrates from which methane (CH_4) is formed. For acidogenesis to take place, some conditions such as nature of the culture, temperature, pH and H_2 partial pressure must be controlled to direct the process to the formation of expected end products **(Gavrilescu, 2002).**

There are mainly four fermentation types in the anaerobic acidogenesis of organic matters (e.g. glucose), namely acetic acid fermentation, propionic acid type fermentation, butyric acid type fermentation and ethanol type fermentation. Most of microbial communities exhibit acetic acid fermentation with acetate acid as a major product **(Chan& Holtzaphe, 2003).**

2.5.2.3-Acetogenesis:

Acetogenesis is part of the fermentation process where more reduced compounds such as aromatic compounds, long VFAs and alcohols are converted to acetic acid and H_2. VFAs such as acetate, propionate, butyrate, are major intermediate products in acidogenesis and acetogenesis stages of anaerobic biochemical degradation. The stability of over-all biochemical reactions relied on the degradation of VFA by anaerobes to the final gaseous products. Butyrate degradation differs from that of acetate as it includes acetogenesis step in the biochemical reactions, shown in the following reaction,

$$\text{Butyrate} + 2\,H_2O \rightarrow 2\,\text{acetate} + 2\,H_2 + H^+$$

Conversion of butyrate to acetate is not thermodynamically favorable unless the acetate and hydrogen produced by the acetogens can be readily removed by acetotrophic and hydrogenotrophic bacteria, respectively. The conversion of acetate to hydrogen according to this reaction:

$$CH_3COOH + 2\,H_2O \rightarrow 4\,H_2 + CO_2 \qquad \Delta G^o = +\,104.6\ \text{kJ}$$

Is thermodynamically unfavorable at moderate temperatures (ΔG =+ 104.6 kJ mol-1) and is strongly determined by the hydrogen partial pressure. For acetate oxidation to hydrogen the H_2 partial pressure must be kept very low by H_2 removal **(Claassen et al., 1999).**

2.5.2.4-Methanogenesis:

This process involves methanogenic bacteria which convert H_2 and acetate and CO_2 produced by the fermentation step to methane (CH_4). Methanogenesis is the final stage of the anaerobic digestion. Two groups of methanogenic microorganisms are involved: aceticlastic methanogenesis, and hydrogenotrophic methanogenesis which involve hydrogen oxidation to methane:

Aceticlastic methanogenesis

$$\text{Acetate} + H_2O \rightarrow CH_4 + HCO_3^- \qquad \Delta G^o = -31 \text{ kJ/mol methane}$$

Hydrogenotrophic methanogenesis

$$4\,H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 2\,H_2O \qquad \Delta G^o = -135.6 \text{ kJ/mol methane}$$

By using the mass balance the complete oxidation of glucose substrate to H_2, CO_2 and by products form, can be used to estimate the net hydrogen yield by each type of bacteria **(Masilela, 2011).**

2.6-Biochemical pathways of hydrogen fermentation:

Because fermentation involves no respiratory electron acceptor, energy conservation occurs only through substrate-level phosphorylation. Still, the microorganisms must generate reducing power in the form of intracellular electron carriers (e.g., $NADH_2$). Figure (2.6) shows where ATP and $NADH_2$ are generated in the reactions that ferment glucose to the usual products; here, $NADH_2$ is short for NADH + H+. $NADH_2$ and ATP are coproduced during the initial glycolysis step (reaction 1). ATP also is synthesized in formation of acetate (reaction 3) and butyrate (reaction 14). Although it has been reported that ATP can be synthesized through propionate production, the amount of ATP per mole of propionate is inconsistent and often negligible (7); thus, reaction 5 does not show ATP formation in Figure (2.6) **(Lee et al., 2008).**

The reactions that produce lactate, propionate, acetaldehyde, ethanol, and butyrate (reactions 4, 5, 8, 9, and 11-14, respectively) consume $NADH_2$ and release NAD+. The$NADH_2$ needed for these reactions must come from reaction 1 or from conversion of pyruvate to acetyl-CoA (reaction 2A). The production and consumption of $NADH_2$ among these reactions must be balanced **(Seeliger et al., 2002).**

H_2 production can occur in two catabolic steps. One is the decarboxylation of pyruvate into acetyl-CoA (reaction 2), which generates reduced ferredoxin (Fdred), a direct e donor for proton reduction to H_2 gas; for example, *Clostridium sp.* utilize this pathway for producing H_2. Since reactions 2A, B compete for Fdred, generation of H_2 eliminates the generation of $NADH_2$, or vice versa. The other is formate cleavage (reaction7), which is the dominant mechanism for H_2 generation in facultative anaerobes, such as *Enterobacter* and *Klebsiella* **(Nakashimada et al., 2002).**

Fermenting bacteria produce different distributions of reduced products in response to environmental conditions, of which pH is significant. Fdred generated in reaction 2 can lead to $NADH_2$ or H_2 . The competition for Fdred between NAD+ and H+ must be a primary control over H_2 yields. It seems likely that low pH stimulates the coupling reaction of Fdred and H+ to form H_2. On the other hand,

Coupling oxidation of Fdred to NAD+ generates$NADH_2$, which is essential for biomass synthesis, as well as for driving the many reactions in Figure (2.6) that consume $NADH_2$. Consumption of $NADH_2$ for these other functions may pull electron equivalents away from reaction 2B. Lactate and propionate often are dominant products at conditions close to neutral pH, which can be important at acidic pH, depending on substrate types or inocula, and they come directly from reduction of pyruvate (reactions 4 and 5); propionate can be produced from another pathway (i.e., the methylmalonyl-CoA pathway), but we do not show it in Figure (2.6)since we focus on final e- sinks and thermodynamics. Competing reactions from the pyruvate node produce acetyl-CoA and Fdred (reaction 2) or formate (reaction 6) **(Thauer et al., 1977).**

Since production of lactate and propionate prevents formation of Fdred and formate, both of which lead to H_2 generation (reactions 2B and 7), and also consume $NADH_2$, their formation (at neutral pH) ought to lower H_2 generation. Ethanol and butyrate become significant at acidic pH; normally, ethanol is abundant at around pH 4–4.5, and butyrate is predominant at slightly higher acidic pH than ethanol . Ethanol and butyrate are produced through reduction of acetyl-CoA (beginning with reactions 8 and 10, respectively), which consumes $NADH_2$ and may lower H_2 generation via reaction 2A. Ethanol production does not involve ATP synthesis, while ATP is generated in butyrate

production. Because acetate is generated by hydrolysis of CoA from acetyl-CoA (reaction 3), acetate production does not involve $NADH_2$ or H_2 formation. Acetate is common for a wide range of pH presumably because the bacteria conserve chemical energy as ATP **(VanGinkel et al., 2001).**

In principle, glucose could be fermented into 12 mol H_2 and 6 mol CO_2, but acetate cannot be fermented in dark fermentation. Converting acetate into H_2 requires exogenous energy, such as light (photofermentation) or electrical energy (microbial electrolytic cells). Thus, the commonly accepted maximum bioH_2 yield is 4 mol of H_2/(mol of glucose), when acetate is the only organic fermentation product (without considering biomass growth). However, most bioH_2 research has shown actual H_2 yields close to or lower than 2 mol of H_2/(mol of glucose) in mesophilic conditions **(Li &Fang, 2007) .**

Regulation, kinetics, bacterial community structure, thermodynamics, or a combination causes the bacteria to invest electron equivalents into products other than H_2 and acetate. Among them, perhaps the most fundamental is thermodynamics. In a general sense, generation of H_2 can be thermodynamically unfavorable, since ($\Delta G°$) (pH7) for H+ reduction to H_2 is +79.4 kJ/(mol of H_2) (+39.9 kJ/(e- equiv)). Thus, electron flow to protons could be an energetic drain. However, the thermodynamics can be made favorable when the H_2 concentration is low enough **(VanGinkel & Logan, 2005a, b).**

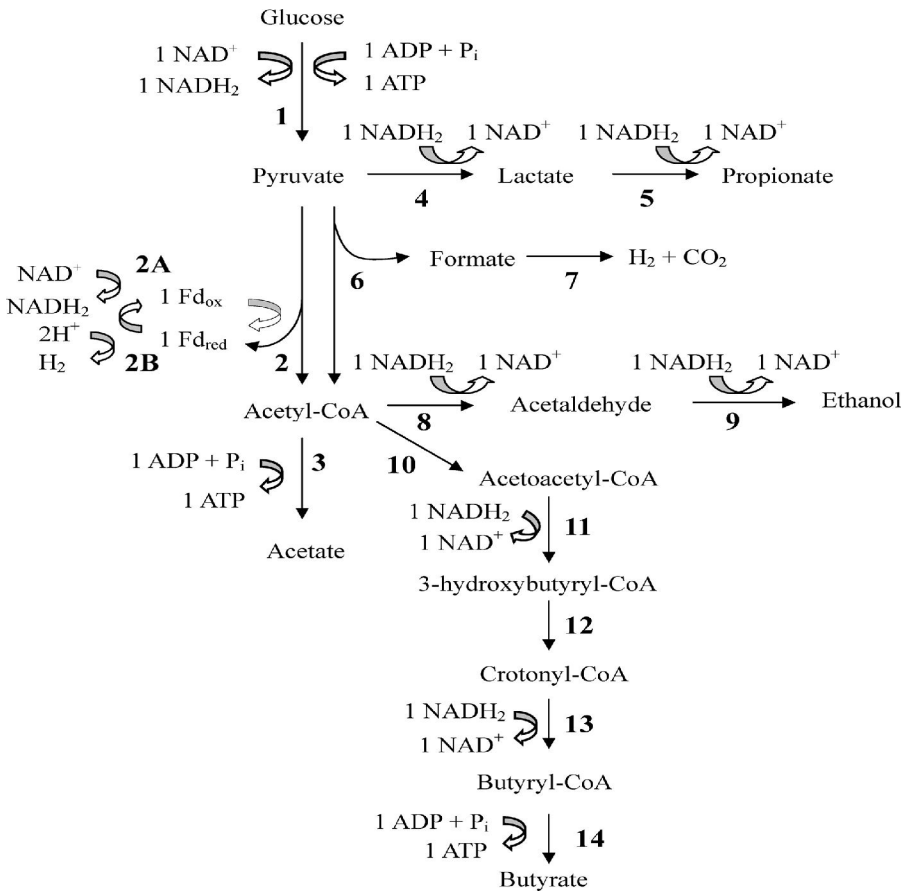


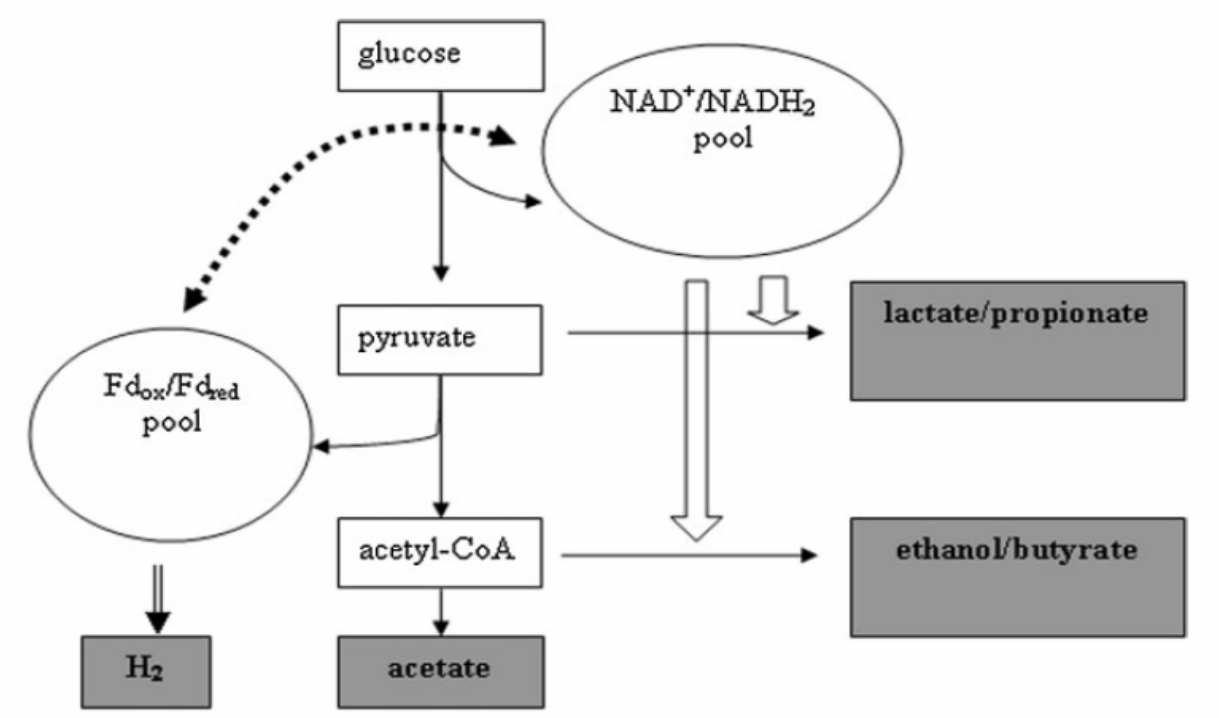
Figure 2.6: Catabolic pathways of mixed-acid fermentation from glucose. The pathways illustrate the electron flows, key fermentation products, and formations of NADH2, NAD+, ADP، and ATP: Fdox, oxidized form of ferredoxin; Fdred, reduced form of ferredoxin; 2A, Fdred/Fdox oxidation coupled to NAD+/NADH2 reduction; 2B, Fdred/Fdox oxidation coupled to H+/H_2 reduction. NADH2 is NADH + H+. NADH2 (or NAD+) and ATP yields are based on 1 mol of the reaction product **(Lee et al., 2008).**

2.7- Eectron flow model:

A complementary way for understanding interaction in complex mixed-culture systems is to track electron flow. An electron-flow study was performed in a pure-culture fermentation using electron equivalence (e- equiv) balances and known pathways**.**

Electron-flow model is based on two central principles. The first principle is that all e- equivalence removed from substrate (e.g. glucose) must be accounted for in the fermentation products, such as H_2, acetate, butyrate, and ethanol. The second central principle is that the bacteria must balance $NADH_2$ production with $NADH_2$ consumption. $NADH_2$ is mainly produced during glycolysis in glucose fermentation. $NADH_2$ is consumed by the production of ethanol, butyrate, lactate, and propionate. Likewise, the electron carrier Fdred must have equal production and consumption. Figure (2.7) is a

schematic diagram of the electron-flow model from glucose. The electron equivalence generated by glycolysis and pyruvate decarboxylation accumulates in the NAD+/NADH2 and Fdox/Fdred pools, respectively. The reduced Fd is then oxidized by Fd-dependent hydrogenase which transfers the electrons to protons resulting the formation of H_2. $NADH_2$ generated from glycolysis can be oxidized by $NADH_2$-Fd reductase in order to generate constant reducing equivalents for the catabolic process. Reducing equivalents can also be generated when $NADH_2$ is oxidized in the ethanol pathway and by lactate dehydrogenase. When electrons of $NADH_2$ or Fdred remain, these electrons can move between the NAD+/$NADH_2$ and Fdox/Fdred pools (dotted-line arrow, as shown in figure 2.6). The direction of this intra-electron flow depends on e- equiv and H_2 relative to e-equiv of Fdred **(Lee et al., 2009).**



2.7 - Schematic diagram of the electron-flow model. Electron equivalence are generated at glycolysis and pyruvate decarboxylation and accumulates as NADH2 and Fdred, respectively, in each electron carrier pool. Gray boxes are end products. The dotted arrow indicates electron flow between NAD+/$NADH_2$ and Fdox/Fdred pools. The dotted arrow indicates electron flow from Fdred to proton, releasing H_2. Block arrows indicate $NADH_2$ utilized for producing reduced liquid end products (lactate, propionate, ethanol and butyrate) **(Lee et al., 2009) .**

2.8- Hydrogenases:

Hydrogen metabolism in microorganisms is carried out by metalloenzymes, namely nitrogenases and hydrogenases. Nitrogenases release H_2 as a byproduct during nitrogen fixation. Hydrogenases catalyze the simplest chemical reaction: $2H^+ + 2e^- \leftrightarrow H_2$. The reaction is reversible, and its direction depends on the redox potential of the components able to interact with the enzyme. In the presence of H_2 and an electron acceptor, it will act as a H_2 uptake enzyme; in the presence of an electron donor of low potential, it may use the protons from water as electron acceptors and release H_2. The production of H_2 is one of the specific mechanisms to dispose excess electrons through the activity of hydrogenases present in H_2 producing microorganisms. Hydrogenase activity can be measured in vitro, using artificial or natural electron carriers. The in vivo function of the hydrogenases depends on the current redox status of the cell **(Ivanova, 2008).**

Hydrogenases have various physiological roles. They have a different localization as well as a different subunit composition in the cell. The first classification of these enzymes was based on the identity of specific electron donors and acceptors, namely, NAD. Until 2004, hydrogenases were classified according to the metals at their active sites. Three main classes were recognized: iron-only ((FeFe) hydrogenases), nickel-iron ((NiFe) hydrogenases), and "metal-free" hydrogenases. In 2004, Lyon et al. showed that the metal-free hydrogenases in fact contain iron. Thus, those enzymes previously called "metal-free" are now named "ironsulfur- cluster-free" hydrogenases, since they contain no inorganic sulfide in contrast to the Fe-only enzymes. In some (NiFe) hydrogenases, one of the Ni-bound cysteine residues is replaced by selenocysteine. On the basis of sequence similarity, however, the (NiFe) and (NiFeSe) hydrogenases belong to the same superfamily. The (NiFe) hydrogenases are heterodimeric proteins consisting of small (about 30 kDa) and large (about 60 kDa) subunits. The small subunit contains three iron-sulfur clusters while the large subunit contains a nickel-iron centre. Periplasmic, cytoplasmic, and cytoplasmic membrane-bound hydrogenases have been found. The (NiFe) hydrogenases, when isolated, are found to catalyse both H_2 evolution and uptake, with low-potential multihaem cytochromes such as cytochrome C3 acting as either electron donors or acceptors, depending on their oxidation state **(George et al., 1989).**

The hydrogenases containing Fe-S clusters and no other metal than iron are called Fehydrogenases. Three families of Fe-hydrogenases are recognized:

I. Cytoplasmic, soluble, monomeric Fe-hydrogenases, found in strict anaerobes such as Clostridium pasteurianum and Megasphaera elsdenii. They are extremely sensitive to inactivation by dioxygen (O_2) and catalyse both H_2 evolution and uptake;

II. Periplasmic, heterodimeric Fe-hydrogenases from Desulfovibrio spp., which can be purified aerobically and catalyse mainly H_2 oxidation;

III. Soluble, monomeric Fe-hydrogenases, found in chloroplasts of green alga Scenedesmus obliquus, which catalyse H_2 evolution. The (Fe_2S_2) ferredoxin functions as natural electron donor linking the enzyme to the photosynthetic electron transport chain. Ni-Fe and Fe-only hydrogenases have some common features in their structures: each enzyme has an active site and a few Fe-S clusters. The active site is also a metallocluster, and each metal is coordinated by carbon monoxide (CO) and cyanide (CN^-) ligands **(Adams, 1990).**

2.9-Thermodynamics of hydrogen formation:

Thermodynamics plays an important role in chemistry, chemical engineering and in chemical process development. The use of thermodynamic methods for the predictions of the true yield and stoichiometry of bacterial reactions has been widely applied in biotechnology. However, these findings are sometimes very far from experimental results where many complicating factors include experimental errors, maintenance energy estimates, and simplifying assumptions, are present. Although as much as 12 mol H_2 can theoretically be derived from glucose, there is no known natural metabolic pathway that could provide this yield, due to the presence of other products. Assuming that glucose is the substrate and acetic acid is the main product, the theoretical ratios of H_2 yield to substrate in a typical dark fermentation process may reach up to 4 moles of H_2 per mole of glucose utilized , if the main aqueous product is butyrate only 2 moles of H_2 are produced **(Thauer et al., 1977).**

Acetic acid: $C_6H_{12}O_6 + 2\,H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4\,H_2$

Butyric acid: $C_6H_{12}O_6 + 2\,H_2O \rightarrow CH_3\,(CH_2)_2\,COOH + 2CO_2 + 2\,H_2$

However, in a bacterial consortium there will be different microbial fermentation pathways, resulting in a mixture of products and the amount of H_2 generated will be determined by the acetate/butyrate ratio. In addition, the high partial pressure of hydrogen may result in metabolic shift towards the production of more reduced products (e.g. alcohols, lactate, butyrate, propionate etc) which will affect the final gas yield obtained **(Bartacek et al., 2007; Hyung et al., 2008).**

It is clear that the H_2 production in fermentation associated with low H_2 yield is the result of large quantities of by-products formed. For optimal hydrogen yields formation of products like ethanol, lactate, propionate and others that consume hydrogen during their production must be avoided. The following reactions represents some of the metabolic reactions that bypass the major H_2 - producing reactions in carbohydrate fermentation, and some of these reaction uses H_2 to form more reduced fermentation by-products **(Masilela, 2011).**

Propionic acid production with hydrogen

$$C_6H_{12}O_6 + 2\,H_2 \rightarrow 2CH_3CH_2COOH + 2\,H_2O$$

Ethanol production with hydrogen

$$CH_3COOH + H_2 \rightarrow CH_3CH_2OH + H_2O$$

Fermentation to ethanol

$$C_6H_{12}O_6 \rightarrow 2\,CH_3CH_2OH + 2CO_2$$

The H_2 yields and production rates of thermophilic bacteria, growing at temperatures above 60 ºC, often show higher values as compared to those of mesophilic bacteria. At elevated temperatures H_2 formation is thermodynamically more feasible and can produce up to 83-100 % of the theoretical maximum H_2 yield. This is due to the fact that an increase in temperature would enhance H_2 productivity and thermodynamic conditions which results in less undesired side products formation. These conditions, allows the bacteria to degrade acids to form H_2 and CO_2. Thermodynamically, acetate can be only oxidized to CO_2 at a very low hydrogen partial pressure, at elevated temperatures, provided methanogens are inhibited and when the hydrogen partial pressure is kept low **(Hussy et al., 2003).**

Syntrophic propionic acid oxidation:

$$CH_3CH_2COOH + 2H_2O \rightarrow CH_3COOH + 3H_2 + CO_2$$

Syntrophic butyric acid oxidation:

$$CH_3CH_2CH_2COOH + 2H_2O \rightarrow 2CH_3COOH + 2H_2$$

Syntrophic acetic oxidation

$$CH_3COOH + 2H_2O \rightarrow 4H_2 + 2CO_2 \quad .$$

The chemical reactions that expected to take place in dark fermentation process and their Gibbs free energy can be summarized as follows:

Table 2.9 - Reaction stoichiometries of dark fermentation of glucose.

Reaction	**Stoichiometry**	**$G^{0,}$ (kJ reaction^{-}**	**References**
Complete oxidation of glucose	$C_6H_{12}O_6+12H_2 \rightarrow 12H_2+6HCO_3^-+6H^+$	+3.2	(Thauer et al., 1977)
Acetate production	$C_6H_{12}O_6+4H_2O \rightarrow 2CH_3COO^-+4H_2+2HCO_3^-+4H^+$	-206.3	(Thauer et al., 1977)
Butyrate production	$C_6H_{12}O_6+2H_2O \rightarrow CH_3CH_2CH_2COO^-+2H_2+2HCO_3^-+3H^+$	-254.8	(Thauer et al., 1977)
Ethanol production	$C_6H_{12}O_6+2H_2O \rightarrow 2CH_3CH_2OH_2+2HCO_3^-+2H^+$	-235.0	(Ren & Gong, 2006)
Acetate and ethanol production	$C_6H_{12}O_6+3H_2O \rightarrow CH_3CH_2OH++CH_3COO^-+2H_2+2HCO_3^-+3H^+$	-215.716	(Hwang et al., 2004 ;Ren & Gong, 2006)
Lactate production	$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOO^-+2H^+$	-198.1	(Kim et al., 2006)
Butanol production	$C_6H_{12}O_6+2H_2O \rightarrow 2CH_3CH_2OH+2HCO_3^-+2H^+$	-280.5	(Chin et al., 2003)
Propionate production	$C_6H_{12}O_6+2H_2 \rightarrow 2CH_3CH_2CH_2COO-+2H_2O+2H^+$	-359.0	(Hussy et al., 2003)
Valerate production	$C_6H_{12}O_6+H_2 \rightarrow CH_3CH_2CH_2COO^-+HCO_3^-+H_2O+2H^+$	-330.9*	(Ren & Gong, 2006)
Acetogenesis	$4H_2+2HCO_3^-+H^+ \rightarrow CH_3COO-+4H_2O$	-104.6	(Thauer et al., 1977)
Acetogenesis	$C_6H_{12}O_6 \rightarrow 3CH_3COO^-+3H^+$	-310.6	(Kim et al., 2006).
Acetate fermentation to H_2	$CH_3COO^-+4H_2O \rightarrow 4H_2+2HC_3^-+H^+$	+104.6	(Thauer et al., 1977; Stams, 1994)
Butyrate fermentation to H_2	$CH_3CH_2CH_2COO^- +10H_2O \rightarrow 10H_2+4HCO_3^-+3H^+$	+257.3	(Thauer et al., 1977 ; Stams, 1994)

2.10- Microorganisms used in Hydrogen dark fermentation studies and their hydrogen yields:

A variety of microorganisms, including bacteria, archaea and yeast, in a wide temperature range, are capable of H_2 production by dark fermentation. The organisms used in H_2 dark fermentation studies include obligate anaerobes, facultative anaerobes and aerobes (in anaerobic conditions) **(Nandi & Sengupta, 1998).** Clostridia and enteric bacteria are the most studied bacterial genera in dark fermentative H_2 production. *Clostridia* are obligate anaerobic, Gram-positive, rod-shaped and spore-forming bacteria **(Chen et al., 2002).** Enteric bacteria are facultatively anaerobic, oxygen tolerant, Gram-negative and non-sporulating rods **(Madigan et al., 2000).**

The use of facultative anaerobes together with obligate anaerobes in hydrogen fermentation process is beneficial since facultative anaerobes reduce the oxygen to water and create an anaerobic environment for the O_2-sensitive obligate anaerobes, and thus avoid the addition of reducing agents in the growth medium **(Yokoi et al., 1998).**

Hydrogen dark fermenting microorganisms can be easily enriched from various natural and engineered environments. Different waste treatment processes have been the most used sources of microorganisms for dark fermentative H_2 production system. Heat or extreme pH can be used to enrich spore-forming H_2 –fermenters and to inactivate H_2-consuming methanogens **(koshenin, 2008).**

2.11- Factors affecting dark fermentative hydrogen production:

Hydrogen fermentation has been extensively studied because it has the potential for providing sustainable and renewable energy for the future. It has been reported that the temperature, pH, HRT, hydrogen/carbon dioxide partial pressure, volatile fatty acids and inorganic content are the main parameters that affect the anaerobic hydrogen fermentation process **(Liu, 2006).**

2.11.1: Temperature.

The temperature affects the hydrogen producing bacteria activities and hydrogen production rate. Dark hydrogen fermentation reactions can be operated at different temperatures: mesophilic (25-40°C), thermophilic (40-65°C), extreme thermophilic (65-80°C) or hyperthermophilic (>80°C) **(Hussy et al., 2003).**

Up to now, most of dark fermentation experiments are conducted at 35-55° C. The extreme thermophilic process provides a number of advantages compared with the mesophilic and thermophilic. Firstly, the hydrogen production is much higher at extreme-thermophilic conditions than at mesophilic and thermophilic conditions. It has been reported that extreme-thermophilic anaerobic hydrogen fermentation can achieve more hydrogen production and higher hydrogen production rates than mesophilic hydrogen fermentation. It has been reported that at exteme-thermophilic condition (70°C), hydrogen yield reached the theoretical maximum of 4 moles hydrogen per mole glucose, where the ones at mesophilic and thermophilic conditions were normally less than 2 mole hydrogen per mole glucose. Secondly, it has much better pathogenic destruction for digested residues performed at high temperatures. Thirdly, it minimizes the contamination by hydrogen consumers such as methanogens, solventogens **(Chin et al., 2003).**

Hallenbeck, (2005) reported that at high fermentation temperature it was thermodynamically favorable for a hydrogen-producing reaction as the high temperature resulted in the increase in the entropy term, and made dark hydrogen fermentation more energetic while the hydrogen utilization processes were negatively affected with temperature increase **(Hallenbeck, 2005)**. Extreme thermophilic bacteria show a better tolerance to high hydrogen partial pressures which will cause a metabolic shift to non-hydrogen producing pathways, such as solvent production **(Liu, 2006).**

2.11.2- pH.

pH level has an effect on enzyme activity in microorganisms, since each enzyme is active only in a specific pH range and has maximum activity at its optimal pH . It has been accepted in hydrogen research that pH is one of the key factors affecting the hydrogen production. Hydrogen fermentation pathways are sensitive to pH and are subject to end-products. Many studies have been conducted to produce hydrogen from solid wastes. Results indicated that the control of pH was crucial to hydrogen production. It has been reported that under unoptimal pH, the hydrogen fermentation process shifted to solvent production, or prolonged the lag phase. The lactate production was always observed together with sudden change of environment parameters, such as pH, HRT, and temperature, which indicated the culture, was not adapted to the new environment conditions **(Ren & Gong, 2006).**

2.11.3-Hydrogen partial pressure.

The hydrogen concentration in the liquid phase, related to hydrogen partial pressure, is one of the key factors affecting the hydrogen production. The partial pressure of H_2 (p H_2) is an extremely important factor especially for continuous H_2 synthesis. Hydrogen synthesis pathways are sensitive to H_2 concentrations and are subject to end-product inhibition. As H_2 concentrations increase, H_2 synthesis decreases and metabolic pathways shift to production of more reduced substrates such as lactate, ethanol, acetone, butanol, or alanine. As the temperature increases, however, conditions that favor hydrogen formation reactions are less affected by H_2 concentration . Continuous H_2 synthesis requires p H_2 of 50 kPa at 60° C, 20 kPa at70°C, and 2 kPa at 98°C under standard conditions **(Hussy et al., 2003; Kim et al., 2006; Kraemer & Bagley, 2006).**

2.11.4- Carbon dioxide partial pressure.

In case of carbon dioxide, high CO_2 concentration can favor the production of fumarate or succinate, which contributes to consume electrons, and therefore decrease hydrogen production. It has been reported that the removal of CO_2 can improve the hydrogen production in dark fermentation. After CO_2 was removed, the hydrogen production was doubled. Furthermore, when removing the CO_2 from the liquid with sparging of argon gas and hydrogen gas, they also found, compared to hydrogen partial pressure, the CO_2 partial pressure had higher inhibition effect to the dark fermentation process (**Kim et al., 2006; Hawkes et al., 2002; Tanisho et al., 1998)**

2.11.5- Organic acid concentration.

It has been reported that high concentration of the organic acids result in a collapse of the pH gradient across the membrane and cause the total inhibition of all metabolic functions in the cell. It has been claimed that both the total acetate or butyrate acid concentration and the undissociated form of these acids can inhibit the dark hydrogen fermentation process **(Chin et al., 2003; VanNiel et al., 2003).**

A near-complete H_2 production inhibition was observed by VanGinkel Logan (2005b) with added acetic acid to give undissociated acid concentrations in the reactor of 63 mM, which occured at pH 5.5 and 165 mM acetate addition They reported that the fermentation pathway changing from organic acid and hydrogen to solvent was not

detected. It also has been reported that the total acetate concentration is a strong inhibitor to hydrogen fermentation process **(VanGinkel & Logan 2005b; Liu, 2006).**

2.11.6-Inorganic elements:

Recent research indicates elements such as iron and nitrogen, and compounds such as carbonate and phosphate can affect the hydrogen production in dark fermentation process as well **(Kim et al., 2006; Hawkes et al., 2002; Li &Fang, 2007)**

2.11.7- Iron concentration.

Hydrogenases are important enzymes as they directly involved in the hydrogen production during hydrogen fermentation process. It has been reported that by increasing iron concentration, the hydrogen production increases significantly In the process of fermentative hydrogen production, Fd, an iron–sulfur protein, functions primarily as an electron carrier and is involved in pyruvate oxidation to acetyl-CoA and CO_2 and in proton reduction to molecular H_2 **(Kim et al., 2006) .**

Vanacova et al. (2001) demonstrated that iron could induce metabolic change and be involved in the expression of both Fe–S and non-Fe–S proteins operating in hydrogenase. Therefore, the authors presumed that the addition of iron had some effects on the growth of fermentative organisms and the rate of hydrogen production **(Vanacova et al., 2001).**

2.11.8-C/N ratio.

The carbon/nitrogen (C/N) ratio is also important for dark fermentation process stability. It has been reported that proper C/N ratio can increase the hydrogen production in mesophilic hydrogen fermentation from sewage sludge. They found at the C/N ration of 47, the hydrogen production was 5 times higher than the one at C/N ratio 40 **(Lin &Lay, 2004).**

Table 2.11- Main Factors Affecting Bioydrogen Production.

Factor	Effect(s)	References
Temperature	- affects fermentation metabolism, activity and microbial composition.	(Hussy et al., 2003; Chin et al., 2003)
pH	- affects fermentation metabolism, activity and microbial composition. - low pH decrease H_2 and increase solvent(e.g. ethanol) production(inhibition of hydrogenase activity). - Extreme pH(low or high) can be used to select spore-forming organisms. -Affects cell membrane charge and the transport of compounds through the membrane. - Affects enzyme activity. - Affects toxicity of harmful substances.	(Ren & Gong, 2006; hwang et al., 2004)
Substrate concentration and loading rate(Food to microorganism (F/M)ratio)	- Affects fermentation metabolism, activity and microbial composition - High substrate loading may decrease H_2 production and increase solvent(.e.g. ethanol) production. (substrate inhibition, improper F/M-ratio)	(Ren & Gong, 2006; Hussy et al., 2003; Chen et al., 2006)
Hydraulic retention time(dilution rate)	- Affect the fermentation mechanism, activity and microbial composition - Generate "hydraulic selective pressure", which effect the microbial composition, and granulation - Low HRT favors H_2 production - Too low HRT may result in washout of H_2(or ethanol)producers - Too low HRT decrease H_2production due to substrate inhibition and improper F/M ratio - Low HRT can be used to wash out methanogens or homoacetogens	(Hussy et al., 2003; Hawkes et al., 2002; Li &Fang, 2007; Zhang et al., 2006)
Hydrostatic pressure (effecting partial pressures of gases).	-Affects fermentation metabolism.	(Bothun et al., 2004)
Partial pressure of hydrogen (ρH_2).	- Affects fermentation metabolism, activity and microbial composition. - Increased pH_2 decreases H_2 production. - Increase in pH_2 decreases the regeneration of NADH leading to the formation of reduced products.	(Hussy et al., 2003; Kim et al., 2006; Kraemer&Bagely, 2006)
Partial pressure of CO_2	-May affects fermentation metabolism and activity -May affect the activity of acetogens and methanogens.	(Kim et al., 2006; Hawkes et al., 2002)
Fermentation products (organic acids).	- High concentrations of undissociated acids may decrease H_2 production and increase solvent production. - High acid concentration may lead to cell lysis -Sensitivity depends on organism and on acids, longer acids generally more toxic. - May affect the fermentation metabolism, activity	(Chin et al. 2003; VanNiel et al., 2003)

	and microbial composition. - The passage of undissociated acids through cell membrane followed by their dissociation uncouples proton motive force. - Sensitivity to organic acids depends on pH(pH affects the dissociation of acids) - Bacteria generally more vulnerable to self-produced acids than externally added.	
Inhibitory compounds in complex substrates or waste streams.	- Sensitivity to inhibitory compounds is strain-dependent. - Compounds (oragnic acids, alcohols, alhedydes etc.) released in the hydrolysis of lignocellulosic substrates may be toxic to microorganism. Toxicity increase with increasing hydrophobicity of compounds - Na- ion. Inhibitory at high concentrations. - Heavy metals, e.g., Cu, Ni, Zn, Cr, Cd, Pb. Micrountrients, but inhibitory at high concentrations.	(Li &Fang, 2007)
Composition of growth media -Buffers - Nutrients - Growth factors	- Buffers, phosphate and carbonate. Needed to resist pH change. Carbonate buffers release CO_2 - Macronutrients(e.g., N, P , S, Mg ,Ca, Na.). Essential for cell growth. Needed in synthesis of macro and micromolecules in cells.	(Kim et al., 2006; Hawkes et al., 2002; Li &Fang, 2007)

2.12- Operation strategies:

2.12.1- Batch and Semicontinuous Processes.

Under laboratory experiments conducted in batch mode to examine characteristics of H_2 producing bacteria and to optimize culture- operating conditions, microbial cultures were found to perform inefficiently, leading to lower H_2 production rates (0.06- 0.66 1/1.h). Enhancing H_2 production efficiency, stability and sustainability is thus a major challenge to batch hydrogen systems **(Han& Shin, 2004; Zhang et al., 2005).**

Han&Shin (2004) developed a semi continuous mode for anaerobic H_2 production from food waste. Pretreated seed sludge and food waste were loaded into an anaerobic leaching bed reactor, and dilution water was continuously fed to reactor by a peristaltic pump at different dilution rates. Microbial reaction was considered accomplished as biogas production ceases, which generally took around 7 days. Appropriate control of dilution rate could enhance H_2 fermentation efficiency by improving degradation of not readily degradable matters. Also, dilution rate might delay shift of predominant metabolic flow from H_2 and acid forming pathway to solvent- forming pathway **(Han &Shin, 2004).**

Valdez- Vazquez et al. (2005) examined H_2 production from municipal solid wastes in another semi continuous pattern. Reactors which were fed with substrate twice a week in a draw and fill mode in an anaerobic chamber and operated continuously at 35°C and 55°C for 40 days demonstrated that H_2 was produced steadily **(Valdez- Vazquez et al., 2005).**

A high rate anaerobic sequencing batch reactor (ASBR) has been used to evaluate H_2 Productivity of and acid enriched sewage sludge microflora at 35°C .A 4 h cycle, including feed reaction, settle, and decant steps, was operated on 5 L ASBR. Sucrose substrate concentration was kept at 20g COD/L, and HRT was maintained initially at 12-120h and thereafter at 4-12h. Reaction/settle period ratio was maintained at 1.7. Hydrogenic activity of sludge microflora was found HRT dependent, and that proper pH control was necessary for a stable operation of bioreactor. Peak hydrogenic activity was noted at an HRT of 8h and an organic loading rate (OLR) of 80 kg COD/m^3. day. Each mole of sucrose in reactor produced 2.8 mole of H_2 and each gram of biomass produced 39 mmol of H_2 per day. Very low HRT might deteriorate H_2 productivity. Concentration ratios of butyric acid to acetic acid, as well as VFA and soluble microbial products to alkalinity can be used as monitoring indicators for hydrogenic bioreactor (**Lin & Chou, 2004)**.

2.12.2- Continuous suspended sludge process.

2.13.2.1- Continuous stirred tank reactor.

In CSTR, which is frequently used for continuous H_2 production, H_2 producing bacteria are well suspended in mixed liquor and less suffered from mass transfer resistance. Because of its intrinsic structure and operating pattern, a CSTR is unable to maintain high levels of biomass inventory **(Chen&Lin, 2003; Horiuchi et al., 2005).**

Depending on operating HRTs, biomass measured in terms of volatile suspended solids (1-4 g- VSS/L) is commonly reported. Washout of biomass may occur at short HRTs, and thus H_2 production rates are considerably restricted. Highest H_2 production rate of CSTR culture fermenting sucrose with a mixed H_2 Producing culture was reported as 1.12 L/L.h **(Chen &Lin, 2003).**

Vanderhaegen et al. (1992) found that granular sludge disappeared within three weeks when CSTRs were incubated statically instead of being shaken **(Vanderhaegen et al., 1992).**

Spontaneous granulation of H_2 producing bacteria can occur with reduced HRT in CSTR **(Yu &Mu, 2006; Yu et al., 2003).**

In such a conventional system, H_2 producing bacteria are well suspended in mixed liquor and less suffered from mass transfer resistance, but washout of biomass may occur at shorter HRTs. H_2 production rates are thus restricted considerably by a low CSTR biomass retention and low hydraulic loading **(Lay et al., 1999; Yu &Mu, 2006).**

Show et al.(2007) and Zhang et al.(2007a) found that formation of granular sludge significantly increased overall reactor biomass to as much as 16.0g- VSS/L, which enabled CSTR to operate at an OLR of up to 20 g- glucose/L.h and hence enhanced performance in H_2 production **(Show et al., 2007; Zhang et al., 2007a).**

2.12.3- Membrane Bioreactor.

One method for increasing reactor biomass concentration is the use of a membrane in a chemostat to control biomass concentration. At a HRT of 3.3h, Oh et al. (2004a) demonstrated that biomass concentration increased from 2.2 g/L in a control reactor (no membrane chemostat) to 5.8 g/L in an anaerobic membrane bioreactor (MBR). This was achieved by controlling sludge retention time (SRT) at 12 h, corresponding to a slight increase in H_2 production rate from 0.50 to 0.64 L/L.h. Increasing SRT can further enhance biomass retention, which favors substrate utilization, but may result in a decrease in H_2 production rate **(Oh et al., 2004a).**

By summarizing several studies of H_2 production by MBR, Li &Fang, (2007) found that H_2 production rates were achieved between 0.25 – 0.69 L/L.h in MBR system. This process has not shown any advantage compared to other high efficiency H_2 production systems. In addition, membrane fouling and high operating cost would limit the use of MBR process in anaerobic H_2 fermentation **(Li &Fang, 2007).**

2.12.4- Immobilized Cell Processes and Methods.

Immobilized – Cell systems, in comparison to suspended cell systems in continuous operations, are more capable of maintaining higher biomass concentration and can be operated at high dilution rates without biomass washout **(Show et al., 2007; Zhang et al., 2008b).**

Biomass immobilization can be achieved through forming granules, biofilm or gel-entrapped bioparticles. Many researchers immobilized pure or mixed cultures of H_2 producing bacteria by gel entrapment in a form of biogels such as *Clostridum butyricum* strain IFO 13949 in agar gel **(Yokoi et al., 1997a),** *Enterobacter aerogenes* strain HO-39 in K- carrageenan, calcium alginate or agar gel**,** sewage sludge in calcium alginate beads, or alginate bead with adding activated carbon powder, polyurethane and acrylic latex/ silicone **(Wu et al., 2002)**, and sewage sludge and activated carbon powder fixed by ethylene vinyl acetate copolymer **(Wu et al., 2005).**

Peak H_2 production rates obtained by continuous gel- immobilized sludge ranged from 0.090 L/L.h in a chemostat with stirring **(Yokoi et al., 1997b)** to 0.93 L/L.h in a fluidized bed reactor **(Wu et al., 2005).**

Biofilm attachment on solid and porous support carriers seems to be superior to gel entrapped bioparticles in continuous H_2 production. In continuous cultures without any pH control, H_2 production and glucose consumption rates with *C. butyricum* immobilized on porous glass beads were higher than corresponding values with cells immobilized in agar gel at HRTs of 3h and 5h **(Yokoi et al., 1997a).** In another study with *E. aerogenes* strain HO-39 culture, H_2 production rate for agar gel and porous glass beads increased with decreasing HRT, this did not occur in free cells. H_2 Production rate of glass beads was superior to that of agar gels at HRT < 3h, and was maximum (0.85 L/L.h) at HRT of 1h. Biofilm attached on solid or porous supports had an advantage in improving H_2 Production rate compared to that of gel entrapped bioparticles, where in inferior performance is attributed to low mass transfer efficiency, stability and durability of bioparticles. Hence, this may not be technology of choice for fermentative H_2 production **(Yokoi et al., 1997a).**

Granular sludge has some advantages over biofilm sludge in continuous dark H_2 fermentation. Firstly, fast growing characteristics of H_2 producing cultures might cause system upset of fixed bed biofilm processes. Maximum specific growth rate (0.17-0.5 h) **(Horiuchi et al., 2002; Chen et al., 2001; VanGinkel & Logan, 2005a)** and biomass specific growth yield (0.08- 0.33 g-V SS/g- COD) **(Kim et al., 2006; Chen et al., 2001; Yu &Mu, 2006)** of H_2 producing bacteria indicated that H_2 Producing bacteria would increase rapidly if a higher OLR was employed.

OLR for immobilized sludge H_2 production was reported as high as 80 g glucose/L.h **(Wu et al., 2006).**

Nicolella (2002) mentioned that biofilm reactors are not particularly useful when dealing with fast growing organisms with maximum specific growth rate faster than 0.1h .

Rapid buildup of H_2 Producing biofilms could result in system upset due to mass transfer limitation **(Nicolella et al., 2000).**

Oh et al. (2004b) reported microbial growth of H_2 producing bacteria too excessive under mesophilic condition, causing system upset just after one week of operation. On the other hand, a packed bed reactor using cylindrical activated carbon as support matrix exhibited steady and efficient H_2 production **(Oh et al., 2004b).**

Fed with synthetic sucrose at 20g COD/L system was operated at 0.5-5 h HRT and 35° C for 15 days. Reduction of bed porosity from 90% to 70% would result in a decrease in H_2 production performance, and pressure drop was higher when bed porosity was lower. System stability of such a biofilm based process may be challenged by long term operation. System upset might occur once interstitial void spaces in pack bed reactor are clogged with biomass **(Lee et al., 2003).**

Washout of support carriers might be an intrinsic drawback of biofilm processes. Zhang et al. (2008c) investigated H_2 production by granular sludge and biofilm sludge growing on granular activated carbon in two fluidized bed reactors at a pH of 5.5 and on OLR of 40 g- glucose/ L.h. A similar performance in H_2 production was observed with two immobilized cultures, both were tested at different HRTs (0.125- 3h) and influent substrate concentrations (5-120g/L). Biofilm sludge was washed out substantially and

reactor biomass was replaced by granular sludge after 50 days of operation. But H_2 Production was not affected during transition **(Zhang et al. 2008c).**

Severe washout of support carriers is presumably attributed to fast-growing characteristics of H_2 -producing bacteria, wherein maximum specific growth rate and cell yield coefficient were determined to be 0.5/ h and 0.12 g- VSS/ g-glucose, respectively. A large amount of support carriers is normally required to support microorganism growth in biofilm processes. Carriers occupy a considerable space in reactor and reduce effective volume for biomass- substrate interactions, resulting in lower reactor performance and efficiency. Supporting carriers need to be replaced periodically due to wear and tear. Cost of material replacement could be a major economic consideration in maintenance **(Zhang et al., 2008b).**

Granular sludge processes generally exhibit long startup. A complete development of H_2- producing granules may take several months**.** During startup of an UASB H_2-producing reactor, Mu&Yu (2006) found that small granules (dian 400-500 1/4m) were formed at reactor bottom after 140 days of operation. Granules developed further to sizes larger than 2.0 mm upon 200 days. Although, reactor reached steady- state H_2 production and substrate degradation after 5 months of startup operation, development and accumulation of mature and stable granular sludge were only completed beyond 8 month of operation **(Mu &Yu, 2006).**

Chang&Lin (2004) noted that a UASB reactor took 39 days to achieve constant gas production at a HRT of 24 h and granules become visible after 120 days of operation. A longer period (180 days), however, was required for further development of granules **(Chang&Lin, 2004).**

Granulation of H_2-producing cultures can be markedly accelerated. Packing of a small quantity of carrier matrices significantly accomplished sludge granular sludge bed (CIGSB) bioreactor. Column reactors were initially packed with cylindrical activated carbon, spherical activated carbon, sand or filter sponge at a bed height of 4-8 cm and with bed porosities of 90-99%. Granulation of seed sludge could take place in all carrier-packed reactors as HRTs were shortened to 4-8 h, dependent on carrier type **(Lee et al., 2006).**

By adding cationic polymer (cationic polyacrylamide) and anionic polymer (silica sol), rapid granulation of H_2- producing culture could be accomplished within 5 min. As sludge has a negative charge of -26 mV, high molecular weight cationic polymer (MW, 15,000,000) with 0.7% (w/w) of dry sewage digester sludge was added and stirred at 200 rpm for 2 min to neutralize sludge. Since residual cations may cause detrimental effect on microorganisms, anionic silica sol of 0.7% (w/w) of dry sewage digester sludge were added and stirred at 200 rpm for 2 min. Total time required for granulation was about 5 min. When granular sludge was operated in a stirring reactor, granular shape was maintained stably, its size ranged from 1.0 to 3.0 mm and maximum concentration granular sludge was found to be approx. 7 g/L**(Kim et al., 2005).**

Zhang et al. (2008c) developed an approach of acid incubation to initiating formation of H_2- producing granules rapidly in a CSTR. H_2- producing granules were formed rapidly within 114 h as seed microbial culture was subjected to a 24 h period of acid incubation at a pH of 2.0. Changing culture pH would result in improvement in surface physicochemical properties of culture favoring microbial granulation **(Zhang et al., 2008c)**.

Table 2.12: Main fermentation processes used in dark hydrogen fermentations and some of their benefits and draw backs

Reactor Type	Benefits (+) and drawbacks (-)	References
Continuously Stirred tank reactor (CSTR).	+ simple process, easy to operate and control.	(Kim et al., 2006; Li &Fang, 2007).
	- low biomass retention.	
Up flow anaerobic sludge blanket reactor (UASB).	+ Good retention of biomass.	(Yu&Mu, 2006).
	- Slow development of granules (long start- up period).	
Fluidized-bed reactor (FBR).	+ Good retention of biomass.	(Zhang et al., 2007b; Masilela, 2011).
	+ Good mass transfer due to efficient mixding.	
	+ No clogging.	
	- Instability of H_2 production.	
	- Volume occupied by carrier(less volume available for biomass).	

	- strong shear forces can detach biomass.	
	- Energy needed for biomass fluidization.	
Packed- bed reactor (PBR).	+ No need for mechanical mixing.	(Chang et al., 2002; Lee et al., 2003).
	+ Good retention of biomass.	
	- Clogging.	
	- Lower mass transfer than in FBR.	
	- Gas hold- up.	
	- Volume occupied by carrier (less volume available for biomass)	
Tricking biofilter reactor (TBR).	+ Good biomass retention.	(Cohen, 2001).
	- High mass transfer between liquid and gas phase (reduced gas hold-up).	
	- Clogging.	
	- Long start up period.	
Granular bioreactors, e.g. carrier induced granular sludge bed bioreactor (CIGSB) and immobilized – cell-seeded anaerobic bioreactor (ICSAB).	+ Excellent biomass retention (allows very high loading and short HRT).	(Wu et al., 2006).
	+ Rapid sludge granulation (short start- up time).	
	+ Maximized space available for biomass (no or low amount of carrier).	
	- Mass transfer can be poor.	
	- Channeling of flow and formation of gas pockets (if no mixing).	
Membrane bioreactor (MBR).	+ Efficient retention of biomass.	(Cohen, 2001; Li &Fang, 2007).
	+ Disinfection and high quality of the treated water (no bacteria).	
	+ low sludge volume.	
	- Fouling and clogging of membranes.	
	- High capital costs of the membrane.	
	- High energy requirements to push liquid through membranes.	

2.13-Reactor type:

2.13.1- Fixed- bed Reactor.

Fixed – or packed- bed reactor is operated under lesser extent of hydraulic turbulence, thus its microbial cultures usually encounter mass transfer resistance resulting in lowered rates of substrate conversion and H_2 production.

Kumar& Das (2001) investigated H_2 production by *Enteroboacter cloacae* attaching on coir in packed-bed reactors at a HRT of 1.08 h, and found that rhomboid bioreactor with convergent –divergent configuration gave maximum H_2 production (1.60 1/1.h) as compared with tapered reactor (1.46 L/L.h) and tubular created by reactor geometry favoring mass transfer and reduced gas hold-up **(Kumar &Das, 2001).**

Rachman et al. (1998) found that high H_2 molar yield could not be maintained consistently in a packed-bed reactor, although pH in effluent was controlled at > 6.0. This is because pH gradient distribution along reactor column resulted in a heterogeneous distribution of microbial activity. In order to overcome mass transfer resistance and pH heterogeneous distribution, fluidized-bed or expended-bed reactor system with recirculation flow was recommended to be more appropriate in further enhancing H_2 production rate and yield. Increasing slurry recycle ratio can alleviate mass transfer resistance in a packed- bed reactor **(Rachman et al., 1998).**

Kumar & Das (2001) observed that both H_2 production and substrate conversion rates of a packed-bed reactor increased with recycling ratio. Maximum H_2 production rate (1.69 L/L.h) was noted at a recirculation ratio of 6.4 **(Kumar& Das, 2001).**

Support materials have important effects on biomass retention and consequently H_2 production in fixed- bed reactors. Chang et al. (2002) immobilized acclimated sewage sludge on surfaces of porous supports using loofah sponge, expanded clay, and activated carbon for continuous H_2 fermentation in fixed bed reactor. Besides loofah sponge, other carriers exhibited better biomass yields. By comparing two support carriers favoring biomass yield, activated carbon was found a better choice of support carriers used in H_2-producing fixed- bed reactors, with which maximum H_2 production rate (1.32 L/L.h) was reached at a HRT of 1 h and a sucrose concentration of 20 g/L **(Chang et al., 2002).**

Kumar & Das (2001) assessed effect of support materials on immobilization of *Enterobacter cloacae IIT-BT 08* in packed- bed reactors and found that coir, with bigger

surface area due to its fibrous and corrugated properties, is best carrier compared to rice straw and bagasse in terms of cell retention (0.44 g dry carrier), packing density (100 g/L reactor volume), cell loading (44 g dry cell/L reactor volume) and H_2 production rate (62 m mol/L. h). Therefore, packing materials of higher surface area are preferred in packed- bed reactors for H_2 production **(Kumar& Das, 2001).**

2.13.2- Fluidized Bed Reactor (FBR).

H_2 efficiently in a three- phase FBR operated at a HRT between 1-6 h with a maximal steady-state rate (0.93 L/L.h) and an optimal yield of H_2 (2.67 mol/mol sucrose), which was highest value reported in gel- immobilized culture systems **(Wu et al.,2003).**

Zhang et al.(2007b) obtained higher H_2 production by biofilm culture (pH 4.0) growing on granular activated carbon in an anaerobic FBR at HRTs of 0.5-4 h and influent glucose concentrations of 10-30 g/l. at operating pH, biofilm sludge concentration was retained up to 21.5 g- VSS/l. H_2 might be produced efficiently in an anaerobic FBR as H_2 production rate reached a maximum rate of 2.36 L/L.h **(Zhang et al., 2007b).**

2.13.3- Upflow anaerobic sludge blanket Reactor.

UASB reactor system has been applied in H_2 production due to its potential of high biomass concentration and treatment efficiency. Chang &Lin (2004) found that H_2 yield stabilized at 1.5 mol H_2/ mol sucrose at HRT of 8-20 h in a UASB granular reactor. The yield drastically decreased at a HRT of 4 or 24h. H_2 production rate (0.25 L/L.h) and specific H_2 production rate (53.5 mmol H_2/g-VSS.day) peaked at a HRT of 8 h **(Chang &Lin, 2004).**

Biomass concentration reached maximum value of 7.2 g-VSS/l at a HRT of 24 h, but decreased to 5.0 g/l at optimum HRT of 8 h. Yu &Mu (2006) studied H_2 production (yield 0.49 -1.44 mol-H_2/mol- glucose) from synthetic sucrose wastewater in UASB reactor with granular sludge operated at 38°C and a pH of 4.4 $\pm$0.1 for over 3 years. H_2 production rate increased with increasing substrate concentration form 5.33 to 28.07 g-COD/l, but decreased with increasing HRT from 3 to 30 h. However optimum operating conditions only gave rise to a low H_2 production rate (0.2 L/L.h) **(Yu &Mu, 2006).**

Yu et al. (2002) investigated H_2 production from rice winery wastewater in an up flow anaerobic reactor inoculated with mixed anaerobic cultures at various HRTs (2-24h),

substrate concentrations (14-36 g-COD/L) and temperatures (20-55◦C). H_2 yield (1.37-2.14 mol –H_2/ hexose) attained optimum H_2 production rate (0.16l/L.h) and specific H_2 production rate (8.02 L H_2/g VSS d) under following testing conditions: biomass concentration, 2.50 g-VSS/L; HRT, 2h; COD, 34g/L; and temp., 55°C. Due to a low level of biomass retention, UASB granular system with or without granular sludge did not show advantages in H_2 production rate of specific H_2 production rate compared over other systems such as fixed- bed reactor of FBR**(Yu et al., 2002).**

2.13.4 -CSTR Granular Sludge Reactor.

Fang et al. (2002) demonstrated that H_2- producing acidogenic sludge could agglutinate into granules in a well- mixed CSTR reactor treating a synthetic sucrose-containing wastewater at 26°C, pH 5.5 and HRT of 6h. Formation of granular sludge enhanced biomass growth up to 20 g/L and consequently H_2 production rate up to 0.54 L/L.h with 97% sucrose being degraded **(Fang et al., 2002).**

In a similar CSTR system with granular sludge fermenting glucose wastewater (10 g/l) at a pH of 5.5 and 37°C, a maximum H_2 yield (1.81 mol-H_2/mol-glucose) and a maximum H_2 production rate (3.20 L/L.h) were obtained at a HRT of 0.5 h **(Zhang et al., 2007a).**

Wu et al. (2006) further developed such a granular-sludge based CSTR system. CSTR system was initially seeded with silicone-immobilized sludge at 40°C and pH 6.6 ± 0.2, and reactor performance was examined at a HRT of 0.5-6h and an influent sucrose concentration of 10-40 g- COD/l. Self-flocculated granular sludge occurred at a HRT of 0.5 h, reached a concentration of up to 35.4 g-VSS/L, and resulted in a significant increase in H_2 production rate (15 L/L.h.) A two-fold increase in specific H_2 production rate was found after formation of self- flocculated granular sludge due to transition in bacterial community structure **(Wu et al., 2006).**

Several other high- rate H_2-producing systems based on granular sludge techniques have been developed. Packing of a small quantity of carrier matrices at the bottom of upflow reactor significantly stimulated sludge granulation that can be accomplished within 100 h in a novel carrier-induced granular sludge bed (CIGSB) bioreactor. CIGSB bioreactor, started up with a low HRT of 4-8 h (corresponding to an OLR of 2.5-5 g COD/l.h), enabled stable operation at an extremely low HRTs (0.5 h) without experiencing

biomass washout. Granular sludge was rapidly formed in CIGSIB supported with activated carbon, reaching a maximum concentration of 26/g/L at a HRT of 0.5 h. Ability to maintain high biomass concentration at low HRTs corresponding to high OLRs highlights remarkable H_2 production rate at 7.3 L/L.h (7.15 mol/L.d) and a maximum H_2 content and substrate conversion exceeded to 40 and 90%, respectively. H_2 production rate of CIGSB system further improved (9.3L/L.h) by optimizing reactor column height and diameter at a ratio of 12 and with agitation. After altering physical configuration of CIGSB bioreactor, concentration of granular sludge increased to 40 g-VSS/L **(Lee et al., 2006).**

2.14- Optimization of H_2 production by bioprocess engineering.

2.14.1- Mass transfer.

In H_2 production bioreactors, efficient mass transfer is especially important to enable good contact between microorganisms, substrates and nutrients, and to enable efficient separation of gases from the system **(Kraemer & Bagely, 2006).**

Packed (carrier material) or granular bioreactors are prone to suffer from gag hold-up**,** and from formation of gas pockets which result in decreased H_2 production. Mass transfer can be increased by mixing and by proper bioreactor design. Efficient mixing can be achieved by mechanical stirring, recycling of gases or liquids, or by gas purging depending on the reactor type and configuration. Mass transfer can be further enhanced by applying proper bioreactor shapes**,** and by optimizing bioreactor dimensions such as the height-to diameter ratio **(Kumar& Das, 2001).**

2.14.2- Biomass retention.

High biomass concentration, enabling the use of high organic loading, is a prerequisite for high-rate H_2 production. However, the quality of bacteria is even more important. Different retainment strategies affect both the quality and quantity of biomass **(Wu et al., 2005).** The cell retainment strategies applied for dark fermentative H_2 production are listed in following table:

Table 2.14.2- Cell retainment strategies applied for dark fermentative H_2 production (koshinen, 2008).

Strategy	Bioreactor	Comments	VSS (gl^{-1})
Biofilms on carrier material			
Porous glass beads.	PBR	Higher H_2 production obtained than by agar entrapment.	N.A.
Loofah sponge, expanded clay, activated carbon.	PBR	Activated carbon resulted in the highest H_2 production rates.	15
Lignocellulosic materials; rice straw, bagasse and coir.	FBR	The best cell retention and H_2 production rate obtained with coir.	N.A.
Urethane foam.	PBR		N.A.
Synthetic commercial sponge.	PBR		N.A.
Brick dust.	Batch	Immobilization increased H_2 production Higher H_2 production obtained with brick dust than with calcium- alginate entrapment.	N.A.
Activated carbon pellets.	FBR	High biomass content in attach-growth phase (21.5 $gVSSL^{-1}$), no granulation observed.	21.5
Polyvinyl alcohol.	CSTR	Low H_2 production and process stability, likely due to mass transfer limitations with biofilm and carrier material.	N.A.
Cell entrapment wihtin matrix			
calcium alginate.	Batch		N.A.
Agar matrix.	PBR		N.A.
Ethylene-vinyl acetate copolymer+ AC powder.	Batch	H_2 production stable in repeated batch assays.	N.A.
Sodium alginate + AC powder + polyurethane or acrylic latex/silicone	Batch	Cell entrapment increased H_2 production, crylic latex/silicone entrapment provided the best mechanical strength and durability in repeated batch assays.	N.A.
Sodium alginate + AC powder + acrylic latex/silicone.	FBR		N.A.
Granulation, induction method or self granulation			
Acid-treatment (pH 2,24h)	CSTR	Granules formed at HRT 2h within 5d after acid treatment. No granules formed without acid treatment.	32.2
Addition or cationic polyacrylamide and anionic silica sol.	CSTR	Granulation occurred within5 min. Higher H_2 production and better stability achieved than with biofilm reactor.	N.A.
Cylindrical AC pellets.	FBR	Efficient cell granulation achieved, best H_2 production with lowest (70%) bed porosity (more space for granules). Granulation occurred at GRT_s2to 4h.	N.A.

Spherical or cylindrical AC, sand or filter.	CIGSB	Spherical AC most effective inducer, granulation occurred within 100h at 2h HRT. Carrier type affected the time and HRT required for granulation.	26.1
Cylindrical AC.	CIGSB	Addition of $CaCl_2$ improved the mechanical strength. Of granules, liquid and gas refluxing increased H_2 production	~ 15
Cylindrical AC.	CIGSB	Applying agitation and optimization of reactor H/D-ratio increased biomass retainment and H_2production.	~ 40
Silicone + AC powder-immobilized sludge.	CSTR	Efficient granulation(35.4gvss l^{-1}) and H2 production achieved(15.09 L h^{-1} L^{-1})	34.5
Cylindrical AC or silicone+ AC powder- immobilized sludge.	CSTR	Granulation affected H_2 production through quantity and quality of microorganism. Granular sludge reactors had higher biomass, less diverse community structure compared to a suspended-cell reactor.	10.3
Self-granulation.	CMCR	Self-granulation was achieved at 10 h HRT. Organism *E. aerogenes.*	N.A.
Self-granulation.	UASB	Self-granulation was achieved at 12 h HRT, formation of granules took 120 days.	3.1
Self-granulation.	UASB	Addition of calcium increased the size of granules, total biomass and H_2 production.	~9
Self-granulation.	CSTR	Granules formed within 15 d at HRT 6h.	20
Self-granulation.	CSTR	Granules formed within 15 d at HRT of 2.2h.	N.A.

2.14.3- Granulation.

Granulation has been the most effective means of biomass retention in hydrogen dark fermentation bioprocesses. Further, granulation can improve H_2 production by altering the microbial community structure **.**In H_2 fermentation bioreactors; granulation has been obtained through self- flocculation or through induction by the addition of entrapped cells or inert carriers**.** The microbial granulation process is not fully understood and several granulation theories exist. Granulation is a complicated process involving physicochemical, biological, and hydrodynamic factors **(Zhang et al., 2008a).**

Granulation is a process whereby suspended bacterial consortia agglutinates either to themselves, or to suitable carrier particle or growth nuclei to form discrete well defined granules or biofilm. Anaerobic granules are characterized by their dense and strong microbial structure, regular, smooth round shape, ability to endure high flow rates and high organic loading rates .Granulation has been considered as the most effective means of ensuring biomass retention in hydrogen dark fermentation processes with biomass concentration of up to79 gVSS/l reported in mesophilic systems. Efficient cell retention enables high organic loading rates, and therefore, high H_2 production rates have been achieved with granular cell based reactors using mesophilic microorganisms. Hydrogen productivities up to 15.1L/h/L for sucrose 7.5 L/h/L for glucose have been obtained. Formation of bacterial granules in these reactors is a complex process, involving different trophic bacterial groups, and their physico-chemical and microbial structural interaction **(Masilela, 2011).**

Table 2.14.3- Factors affecting granulation (koshinen, 2008).

Physicochemical
Gravitation force.
Electrostatic forces(opposite change attraction).
Hydrodynamic shear forces.
Surface tension.
Diffusion.
Van der Waals forces.
Thermodynamic forces (Brownian motion).
Biological
Microbial community composition.
Microbial morphology, physiology and genetic competence.
Quality and quantity of extracellular polymeric substances excreted.
Microbial signaling (quorum sensing).
Cell charges of microorganisms (cell hydrophobicity).
Cell mobility.
Process conditions
Characteristics of waste water (substrates, nutrients, inhibitors).
Chemicals supplied (e.g. positively charged ions Al^{3+}, Ca^{2+} Fe^{3+} Mg^{2+})
Bioprocess design(reactor type, configuration and dimensions).
Hydrodynamic conditions and organic load.
Temperature, pH, redox-state.
Mixing.

2.14.4- Biofilms.

In general, lower H_2 production rates have been obtained with biofilm reactors compared to granular reactors. In the high- rate H_2- producing FBRs, bacterial granulation have been observed, and the majority of biomass retained in granules compared to biofilms. This phenomenon led to the development of carrier- induced sludge bed reactors (CISBR) with high H_2 production rates obtained. Biofilm reactors may have lower stability in H_2 production than granular reactors. Kim et al. (2005) compared the performance of biofilm and granular bioreactors, and reported that biofilm reactor lower stability and performance of transfer within the biofilm reactor due to the production of propionate. They suggested that the poor mass transfer within the biofilm and carrier material created optimal environment for propionate producers (high pH and suitable pH). The change in propionate production was irreversible. Optimization of carrier material is crucible in biofilm reactors. **(Kim et al., 2005).**

2.14.5- Gas separation.

The effects of H_2 on the metabolism and the fermentative pattern of anaerobic bacteria have been demonstrated. A H_2 -producing mixed culture produces more H_2 when it is removed by nitrogen gas. A H_2 yield of 0.85 mol/mol consumed glucose was obtained after 5 HRT with the gas produced being 53.4% H_2. With nitrogen sparging at a flow rate approximately 15 times the H_2 production rate, the H_2 yield was 1.43 mol/ mol consumed glucose. However, this method has a disadvantage in that a recirculation gas implies strong dilution with an excess amount of stripping gas to a low mole fraction. Thus its application at an industrial scale is not economically feasible **(Hussy et al., 2003; Mizuon et al., 2000).**

Logan et al. (2002) examined the biological production of H_2 with two techniques: an intermittent pressure release method (Owen method) and a continuous gas release method using a bubble measurement device (respirometric method). Under otherwise identical conditions, the respirometric method resulted in the production of 43% more H_2 gas from glucose than the Owen method. In the respirometric method, total pressure in the headspace never exceeded ambient pressure and H_2 typically composed as much as 62% of the headspace gas. This procedure only seemed to be adequate when from initial stages of the fermentation, the H_2 concentration was elevated. In opposition to this, the H_2 concentration increased with time in most of the fermentations. This is the reason why it is

recommendable to concentrate the biogas until suitable levels to recover it later. Otherwise, a biogas can be obtained with a very variable H_2 composition. Methods of reducing hydrogen partial pressure were studied before. The high concentration of hydrogen within the bioreactor and dissolved liquid can results to metabolic shift to more fermentation end products **(logan et al., 2002).**

Therefore, the influence of hydrogen partial pressure within the anaerobic H_2 production process is inevitable and is considered as an important approach towards improvement of hydrogen productivity.

In Masilela (2011) study, rapid removal of H_2 produced within the bioreactor bed and the gas-desengager was promoted by gas stripping. Efficient removal of H_2 from the bioreactor was achieved by means of recycling of degassed effluent at a high flow rate through the bioreactor bed. For hydrogen yield (HY) above the theoretical threshold of 4.0 mol H_2 / mol glucose would require the anaerobic oxidation of acetate, butyrate and propionate in the absence of H_2 consuming bacteria. Under suitable thermodynamic conditions characterized by thermophilic temperatures > 50 C and H_2 partial pressures < 20 Pa, the syntrophic anaerobic oxidation of acetate, propionate and butyrate is facilitated. The reduction of hydrogen partial pressure was achieved by hydrogen mass transfer from liquid to gas phase which was facilitated by combination of high effluent recycling rate and well design bioreactor gas-disengager **(Masilela, 2011).**

2.15- Hybrid processes.

The effluent of H_2 dark fermentation process contains still plenty of chemical oxygen demand (COD) (organic acids and alcohols) and needs, in both environmental and economical point-of view, to be further treated **(Logan, 2004).**

In hybrid processes, organic acids and alcohols produced by dark fermenters are converted to H_2 by photofermenters or microbial fuel cells, or alternatively to CH_4 by methanogens. The substrate range for photofermentors is wide meaning that low H_2 production efficiency in dark fermentation is compensated by a higher H_2 production in the consecutive photofermentation **(koshinen, 2008; Kraemer & Bagley, 2005).**

The effluent from anaerobic digestion has high N and P content and could be used as a fertilizer, e.g., for energy crop production **(Hawkes et al.2002).**

There are several examples of combining dark fermentation with photofermentation or methanogenesis, but only a few reports, so far, on combining dark fermentation with microbial fuel cell (EAMC)**.** Gassanova et al. (2006) propose a three-stage process of using cyanobacteria in the 1st stage for production of biomass and consumption of CO_2 followed by 2nd stage methanogenesis and 3rd stage photofermentation for the production of fuel gases (CH_4 and H_2) from the cyanobacterial biomass **(Gassanova et al., 2006; Kyazze et al., 2007)**

Photo and dark fermentations can be combined within one reactor vessel (photo- and dark fermenters mixed), or in a tow-stage process (dark fermentation in 1st stage, photofermentation in 2nd). Combination of photo and dark fermentations increase significantly the H_2 yields compared to dark fermentation only, with HYs of up to 7.2 mol-H_2mol-hexose^{-1} obtained in 2-stage dark and photo fermentation system **(Fang et al., 2004)**.

Hawkes et al. **(2007)** reviewed the publications on two-stage hydrogen-methane process and found most of them reported a higher total efficiency on waste treatment and energy recovery than the traditional one stage process **(Hawkes et al., 2007)**.

In a two-stage hydrogen methane fermentation process with household solid waste as substrate at mesophilic temperature in **koshinen** study (2008), HRT was controlled at 2 and 15 days in hydrogen stage and methane stage, respectively. It was found that the hydrogen production was 43 ml H_2 /gVSS added, and the methane production was 500 mL CH_4 /g VSS added. The methane production was 21% higher than the one in one-stage process Photo and dark fermentations can be combined within one reactor vessel (photo- and dark fermenters mixed), or in a tow-stage process (dark fermentation in 1st stage, photofermentation in 2nd). Combination of photo and dark fermentations increase significantly the H_2 yields compared to dark fermentation only, with HYs of up to 7.2 mol-H_2mol-hexose^{-1} obtained in 2-stage dark and photo fermentation system **(koshinen, 2008)**.

Similarly, a two-stage hydrogen-methane process developed by Sapporo Breweries Ltd. Together with Shimadzu Corp. and Hiroshima University successfully produced H_2

and CH_4 from bread waste, and achieved 10% more methane production compared to traditional one-stage process**.** The Energy Technology Research Institute of the National Institute of Advanced Industrial Science and Technology in Japan operated a semi-pilot scale two-sage hydrogen-methane plant using kitchen waste, paper waste and food waste. When an overall HRT was reduced from 25 to 15 days, the decomposition of organic wastes was increased from 60–65% to 80% and energy recovery increased from 40–46% to 55% in comparison to traditional one-stage methane fermentation**.** These proved the two-stage process could achieve not only hydrogen production but also higher methane production by enhancing the hydrolysis in the hydrogen stage **(Liu, 2006 Cooney; et al., 2007).**

Table 2.15a: Hydrogen production with processes combining dark and photofermentation (koshinen, 2008).

Reactor Type	Microorganism	Conditions			Substrate (concentration (g/L)	H2 production	
		T (^{0}C)	PH	HRT (h)		HY (mol H_2 mol-Substrate^{-1})	HPR (mmol h^{-1} L^{-1})
ASBR	*Clostridium butyricum+ Rhodobacter sp.*	30	6.5	N.A.	Starch (5)	6.6	N.A.
Batch (two-stage)	*Clostridium butyricum (I), Rhodobacter sp. (II)*	30	N.A.	N.A.	Starch (5)	3.7	N.A.
ASBR (two stage)	*Clostridium butyricum+ Enterobacter aerogenes (I), Rhodobacter sp. (phase II).*	37 (I) 35(II)	5.25(I); 7.5(II)	24(I) 120 (II)	Sweet potato residue	7.0	N.A.
ASBR(two-stage)	*Clostridium butyricum+ Enterobacter aerogenes (I), Rhodobacter sp (II).*	37 (I) 35(II)	5.25(I); 7.5(II)	24(I) 120 (II)	Sweet potato residue	7.2	N.A.
Batch	*Loctobacillus delbrueckii+ Rhodobacter sphaeroides*	30	N.A.	-	Glucose	7.1	N.A.
CSTR (two-stage)	Mixed communities		55(I)	6 (I)	Glucose and Sucrose	3.8	N.A.
Batch	*Clostridium butyricum + Rhodobacter sp*	N.A.	8	-	Glucose	7.0	N.A.

Batch (two stage)	*Community (I) + Rhodobacter sphaeroides (II)*	38(I) 30(II)	N.A.	-	Sucrose (18)	6.63	N.A.
Batch (two stage)	*Community (I) + Rhodobacter sphaeroides (II)*	35(I)	6.8 (I) 6.7 (II)	-	Olive waste water	Mill 29L_{H2} L^{-1}_{waste}	0.33
Batch	*Cleeulomonas sp+ Rhodopseudomonas capsulate (mutant)*	33	N.A.	-	Cellulose (5)	6.2	N.A.
Batch	*Vibrio fluvialis + Rhodobium marinum*	30	N.A.	N.A.	Starch (4.05)	~2.1	N.A.

Table 2.15 b: Performance of mixed- culture processes combining hydrogen dark fermentation and methanogenesis (koshinen, 2008).

Reactor Type	**Conditions**			**Substrate (concentration (g/L)**	**H_2 and CH_4 Production**		**VSS (g L^{-1})**
	T (^{0}C)	**PH**	**HRT (h)**		**Maximum yield**	**PR (mmol h^{-1} L^{-1})**	
CSTR (two phase)	55 (I, II)	N.A.	29 (I) 576(II)	Olive pulp	1.6 $mmol^{H2}$ grs^{-1} 19 mmol CH_4 grs^{-1}	0.58 (H_2) 0.28 (CH_4)	N.A.
ASBR	37 (I;II)	5.0- 55 (I)	48 (I) 360 (II)	Houshold solid waste	1.8 $mmol_{H2}$/ gvs^{-1} 20.8 mmol $CH_{4\ gvs\text{-}1}^{-1}$	2.8 (H_2) 4.3 (CH_4)	N.A.
Leachin bed reactors (I); UASB (II)	37 (I;II)	N.A.	144 (I) 14.4(II)	Food waste	12.9 mmol $H_{2/gvs}^{-1}$ 8.8 mmol $CH_{4\ gvs}^{-1}$	6.3 (H_2) 3.0 (CH_4)	N.A.
CSTR (I) PBR (II)	55(I) 60(II)	5.9(I) ~8 (II)	28.8 (I) 148.8(II)	Food+ paper waste	2.4 (6.6 L_{H2} G_{cod}^{-1}) (I)	9.3 (H_2) 10.6 (CH_4)	N.A.
CSTR (I) TRF (II)	35 (I.II)	5.2-5.3 (I)	12 (I) 48 (II)	Sucrose (20)	1.47 (0.721 H_2 g_{COD}^{-1}) 0.294 L $_{CH4}$ g_{COD})	13.3 (H_2) 4.4 (CH_4)	3.0 (I)
CSTR (I) CSTR (II)	35 (I,II)	5.5 (I) 7.5 (II)	9.6 (I) 72.7(II)	Glucose (60)	0.34 (H_2) 0.02 (CH_4)	3.5 (H_2) 0.21 (CH_4)	N.A.
CSTR (I) UASB (II)	35 (I) 28 (II)	5.5 (I) 6.9–7.2 II)	10 (I) 64 (II)	Glucose (15)	0.115 $gH_{2COD}g$ feed $_{COD}^{-1}$	11.3 (H_2)[a] 3.2 (CH_4)[a]	0.8 (I) 0.9 (II)

Chapter III

Material and methods

3.1- Materials

3.1.1-Bioreactor nutrient medium formulation:

A modified Endo medium formulation **(Endo et al., 1982)** was used as the bioreactor influent medium in this study. The modification involved the reduction in the concentration of sodium bicarbonate from 6.72 g l^{-1} to 3. 36 g l^{-1}.

The inorganic minerals of the medium consisted (g l^{-1}): NH_4HCO_3 (3.490); $MnSO_4$ (0.015); $CaCl_2$ (0.2); K_2HPO_4 (0.699); $NaHCO_3$ (3.36); $MgCl_2 6H_2O$(0.015); $FeSO_4$ $7H_2O$(0.0225); $CuSO_4$ $5H_2O$ (0.005); and$CoCl_2$ H_2O (1.24). The medium was supplemented with 17.8 g sucrose l^{-1} (equivalent to 20 g COD l^{-1}) in dissteled water.

3.1.2-- Inoculum collection.

An anaerobic thermophilic bacterial consortium was isolated from a mixture of sewage sludge and fresh wet goat manure. Sewage sludge was collected from an anaerobic sludge digester at Gaza municipal wastewater treatment works. Fresh goat dung was collected from grass-fed cattle at El Mughraqa (Gaza Strip).

3.2- methods.

3.2.1- Inoculum preparation.

Collected dung and sewage sludge samples (50% w/w)were mixed in a 500 ml Schott bottles. The inoculum mixture was pre-conditioned by acid and heat-shock treatment to enrich or select for anaerobic thermophilic hydrogen producing bacteria. Acid treatment involved lowering the pH of the inoculum mixture to 2 with 1 M Hcl and incubating at pH 2.0 for 24 h at room temperature to inhibit the activity of themethanogens. Following the acid treatment, the pH of inoculum mixture was adjusted to7.0 by mixing 50% v/v with Endo medium before heating at 90 °C in a water bath for 2 hours to remove non-sporulating hydrogen-consuming microorganisms, such as methanogenic microorganisms.

After the acid and heat treatments 250 ml samples of inoculum mixture was inoculated into 1 L Schott bottles containing 250 ml Endo medium and incubated at 65 °C

in a shaking incubator set at rpm 86. Cultures were maintained by subculturing into fresh Endo medium every 2 to 3 days **(Masilela, 2011).**

3.2.2- Bioreactor Design and Set-up:

Basically the bioreactor consists of a number of components:

An influent and recycled effluent inlet manifold or diffuser, tubular bioreactor compartment (B) that housed the bacterial granular bed and a tubular gas disengager (G) into which the hydrogen saturated effluent is discharged. The dimensions of B, and G (diameter and length) is given in as follows:

Bioreactor dimensions (B): Radius: 4 cm, Length: 20 cm. Volume: 1L

Gas disengager dimensions (G): Radius: 3 cm. Length: 54.5 cm.Volume: 1.54 L.

Pipe volume: 0.76 L.

Total volume: 3.3L.

Stainless steel hollow tube will be used for the construction of B, and G.

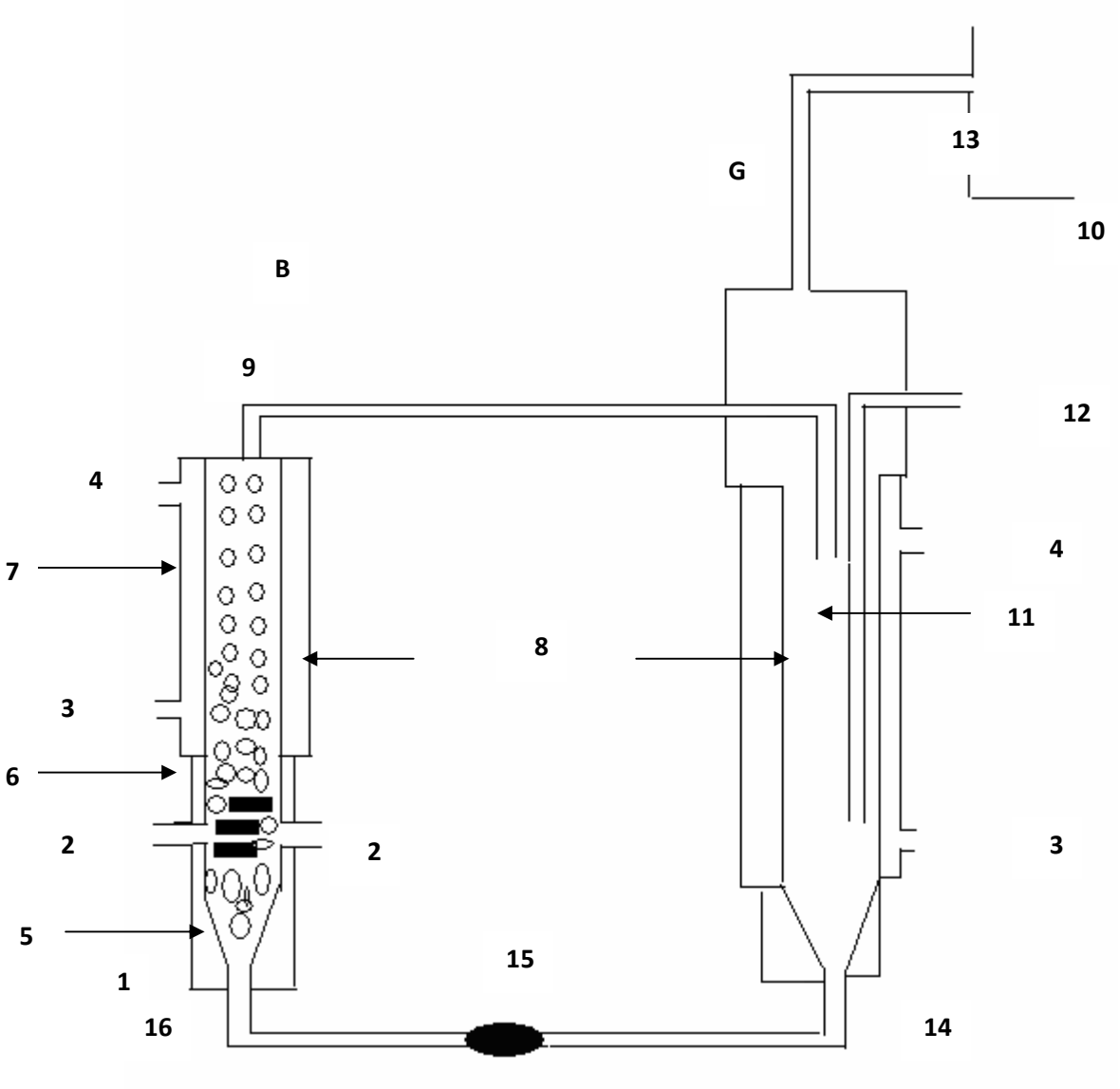


Figure 3.2.2- Modified AFGB system. Diagram labels: 1 - inlet manifold or diffuser; 2 - influent inlets; 3 - water jacket inlet for heat exchanger; 4 - water jacket outlet for heat exchanger; 5 -bed of glass beads in effluent/influent diffusion and for bubble generation through cavitation; 6 - activated carbon for inducing granulation; 7 - fluidized bacterial granular bed (B); 8 - water jacket for heat exchanger; 9- effluent connecting pipe to gas disengager (P); 10- gas collector; 11- effluent gas disengager tube (G); 12 - effluent outlet overflow pipe; 13 - gas outflow pipe;14- effluent recycle outlet pipe (P); 15 - effluent recycle pump; and 16 -effluent recycle inlet (P). Total AFGB volume: V= B+G+P.

The effluent gas disengager (H: 54.5cm and ID:6.0 cm) has two effluent outlets one at the bottom that is connected to a variable peristaltic pump (0.4 kW , WG 600S +YZ 35 peristaltic pump from Xi an and Heb Biotechnology Co., Ltd. China) which is used to recycle degassed effluent into the bioreactor via the diffuser. The second effluent outlet drains the excess effluent overflow from the gas disengager. The liquid-gas separator or effluent gas disengager has a working volume of 1.54 L. At the base of the bioreactor (B)

the stainlesssteel cylinder is connected to a conical shaped diffuser (ID: 8 cm and H: 15 cm) made from stainless steel which functioned as the primary inlet for the effluent recycle stream. A stainless steel sieve (32 mesh) is fixed over the inlet of the diffuser. Above the stainless steel sieve the conical diffuser is filled with a 10 cm layer of 5 mm glass beads. Positioned at the upper end of the diffuser will be 4 inlet ports (ID 5 mm) with each inlet arranged at 90° with respect to the two other inlets on each side. Nutrient medium (influent stream) is supplied directly into the upper glass bead layer via the 4 inlet ports. Bioreactor and gas disengager temperatures will be maintained at the various operational temperatures (65 C°) by circulating heated water from a heated water bath through the bioreactor and gas disengager water jackets. A variable peristaltic pump (BT/F 100F +YZ15 peristaltic pump from Xi an and Heb Biotechnology Co., Ltd. China), with a power less than 40W will be used to pump the Endo nutrient into the bioreactor.

3.2.3- Operation Strategy:

On top of the glass bead, a 10 cm bed of cylindrical activated charcoal (CAC) particles (diameter = 2.5 mm and length = 5.0 mm) was used to facilitate the induction of bacterial granulation in the bioreactor .Prior to its use, the CAC was first washed with distilled water to remove all the suspended fine particles and then sterilised by autoclaving for 20 minutes. Sufficient CAC was added to the bioreactor to give a settled bed height of 10 cm. Endo medium 2x (0.75 L) and treated seed inoculum (0.25 L) were added to the bioreactor system. Following inoculation, the bioreactor was operated on a batch effluent-recycle mode for 48 h at 65 °C to acclimatize the bacteria and allow for their attachment to the CAC. After this acclimatization period, the bioreactor operation was switched to continuous effluent recycle mode with an initial HRT of10 h. The HRT was then gradually decreased over 2 day intervals by increasing the nutrient medium supply rate. With further decreases in the HRT below 4h, the biofilm growth increased and bacterial granules began to form and accumulate at the surface of the expanded CAC bed. Once granule formation had been initiated, further reductions in the HRT to between 2 and 1 h resulted in the rapid growth and expansion of the granular bed. Granule induction, growth and development were carried out at 65 °C. The HRT was then gradually decreased over 2 day intervals by increasing the nutrient medium supply to a final rate of 3.3L/h.

The gas disengager has three outlets: one at the end wich is connected to the pump and used for effluent recycle the other is on the top and connected to 12L calibrated

cylinder filled with water and used to measure the total gas produced by water displacement method . The third inlet is used to collect the excess over flow.

As reported in a previous study the effluent gas disengager is used to reduce the concentration of H_2 trapped in the effluent to its thermodynamic equilibrium concentration. This was accomplished by facilitating the maximum transfer or release of H_2 from the liquid phase within the gas disengager to the vapour phase, which in turn was being continuously exhausted from the gas disengager.

High rates of effluent recycling between the bioreactor and the gas disengager generated a high degree of fluid turbulence and cavitations within the gas disengage tube. This process facilitated the release of super saturated dissolved H_2 through bubble production. Efficient removal of H_2 trapped in the effluent phase by the gas disengagement would increase the efficiency of the bioreactor**.**

3.3- Analytical Techniques:

3.3.1- Gas analysis:

Gas analysis was performed by volumetric method. Twenty ml of the collected gas was taken by a syringe from the top of the gas collecting cylinder that has rubber cover and the gas is injected through a series of scotch bottels tightly closed:

The first contains 10% NaOH to precipitate CO_2 gas and the other contains 5% lead acetate to precipate H_2S. The third bottle contains H_2O which is collected in measuring cylinder upon displacement and accounted for H_2 gas. A standard with pure hydrogen gas was made to account for errors.

The following formula was used for converting total bioreactor gas flux (L/h) to mmol:

$$\frac{\Delta H_2}{\Delta t} = \frac{p_a \left[\%H_2{}^{vm} G_T \right]}{RT_a}$$

where, ($\frac{\Delta H_2}{\Delta t}$) mmol H_2 /h; (p_a) atmospheric pressure (101.3 kPa "Gaza area"); ($\%H_2{}^{vm}$) the percentage hydrogen content from volumetric measurements (vm); (G_T) (L/h)

represents the total gas production rate from the gas meter measurements; R is the gas constant (8.314 J/K/mol); (T_a) 298.15 K (the temperature at which the gas flow from the gas meter was monitored) **(Obazu et al., 2012; Masilela ., 2011; Milne. et al., 2002).**

3.3.2- Volatile fatty acids analysis (VFAs):

Detection of VFAs (acetate, propionate, and butyrate) produced during fermentation in the bioreactor was performed by HPLC chromatography(Chrom Tech) using the AQUASIL C18, 5 µm, 250 x 4.6 mm .Eluent: 1% ACN / 99% 0.05M KH_2PO_4, pH 2.8 .Flow: 1.25 ml/min. Detection: UV @ 210 nm. Before performing any liquid measurements, samples were subjected to filtration using a 0.22 µm membrane filters **(Exceptional Chromatography of Polar Compounds. Thermo Scientific AQUASIL C18 HPLC Columns. Technical Guide Version 2).**

3.3.3- Determination of sucrose concentration.

The concentration of sucrose in the reactor effluent and feed was measured colorimetrically using the sucrose-resorcinol method. A solution of resorcinol reagent is to be prepared by dissolving 0.1 g resorcinol in 100 ml of 95% ethanol and 30% HCl is also made. A sucrose stock solution was prepared by dissolving 17 g of commercial sucrose into 1l d H_2O. Thereafter, sucrose standard curves were then generated by mixing a known dilution of this standard (sucrose standard solution) with d H_2O to a total volume of 1 ml in 10 ml test tubes. For the sucrose colorimetric assay, each sucrose standard curve dilution (1 ml) is mixed with 0.75 ml of 30% HCl and 0.75 ml of the resorcinol reagent and then incubated in at 80°C for 8 min, after which 2 ml d H_2O was added to the sample. A spectrophotometer set at 520 nm was used for sucrose measurement against blank made with d H_2O water as reference. Before performing colorimetric sucrose test, bioreactor effluent samples was subjected to a filtration using 0.22 µm membrane filters , then 1 ml of sample is used for sucrose determination according to above described method **(Obazu et al., 2012; Masilela, 2011; Milne et al., 2002).**

3.3.4-Total bioreactor bacterial biomass determination:

The total biomass concentration in the reactor was determined gravimetrically. Up on opening the bioreactor at the end of experiment, the bioreactor content suspended with bacterial cell was removed from the bioreactor and passed through 0.22 µm membrane

filters. The residue collected on the filter is dried in an oven at 65 °C set at rpm 86 for 48 hours. Thereafter the filter is weighed after dried to determine the total mass of the biomass within the bioreactor **(Obazu et al., 2012; Masilela, 2011; Milne et al., 2002).**

Chapter IV

Results

4.1-Bioreactor set up and design.

In this study, it is necessary to implement a number of **modifications** in the bioreactor design and operation strategy, from old anaerobic fluidized bed bioreactor (AFBR) prototype (Wits bioreactor prototype) in order to improve both the Hydrogen productivities (HPs) and hydrogen yields (HYs). **Firstly,** the total volume of the original bioreactor system had to be reduced substantially to 3.3l. **Secondly,** following the reduction in the volume of the bioreactor system; the HPs and HYs was improved by bioreactor operational strategy. This was done by operating the bioreactor at increasing effluent recycle rate from 0.4L/min to 3.6L / min, the bioreactor for most of the duration of the experiment the effluent recycle rate was maintained at 3.6L /min with simultaneous increases in the influent dilution rate (or reduction in HRT). **Thirdly,** the gas disengager was designed to assist in the reduction of the hydrogen concentration trapped in the liquid bulk phase. This is accomplished by facilitating stripping of the H_2 from the liquid phase within the gas disengager to the vapour phase, which is being continuously exhausted from the gas disengager. The above mentioned modifications aim to improve the ability of the reactor to reduce the hydrogen partial pressure within the reactor.

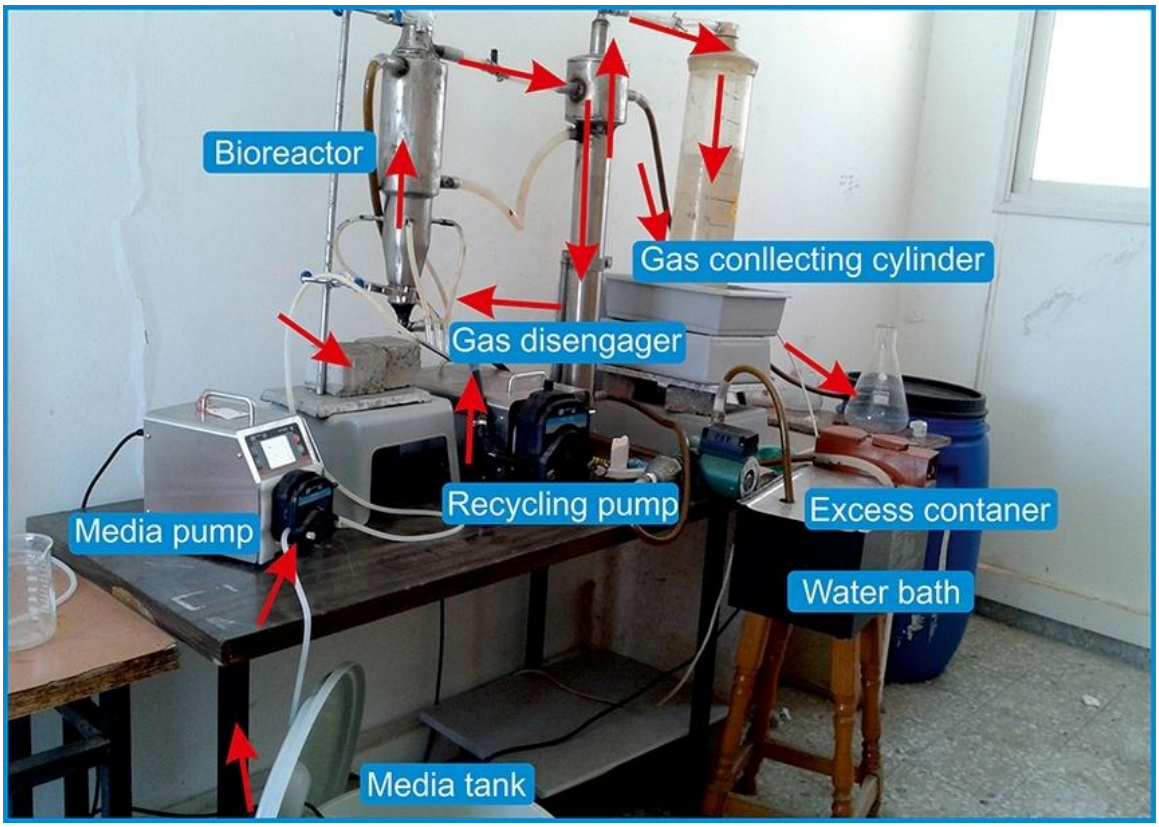


Figure 4.1 - Modified AFGB system installed.

4.2-Granule growth:

Following the inoculation of the bioreactors, granule formation is expected to take place within 3 days after the Endo medium supply rate or influent rate had reached 0.33L/h (HRT of 10 h) when hydrogen gas can be detected. To grow the granular bed, the influent rate was then increased every two days. By the end of the run (24 days), the settled bed had grown to occupy the full of the bioreactor and that was accompanied by increasing in hydrogen production and yield. This corresponded to a total granule dry mass of 7.41 g/L.

Upon opening the bioreactor, the granules were almost filling the bioreactor. It was white in color indicating the absence of hydrogen sulfide and it had different shapes : spherical, epileptical, and hard mature granules.

The bacteria on the granules showed predominant rod shaped bacteria. It also cultured under aerobic and anaerobic conditions. The spore of *clostridium* species after 7 days culture under anaerobic conditions was shown.

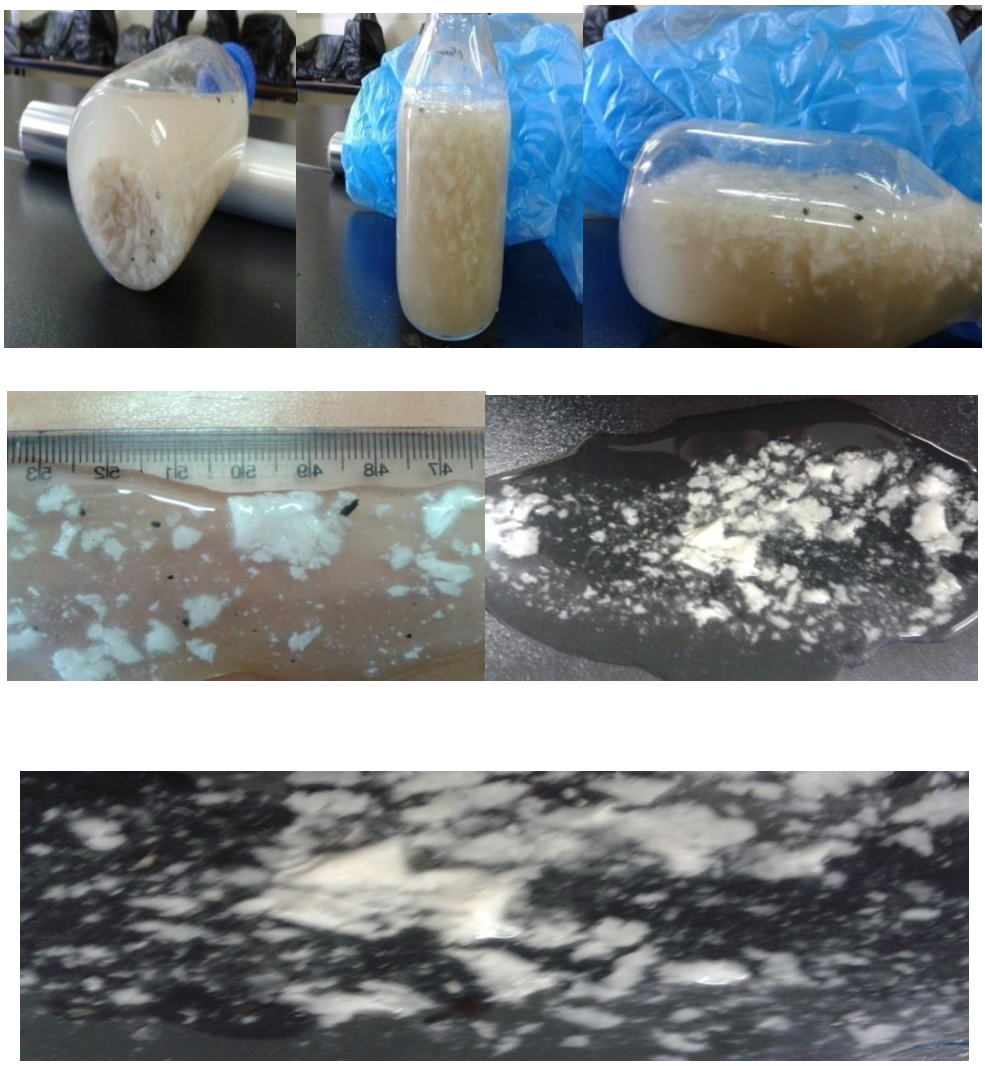


Figure4.2- Bacterial granules in the bioreactor.

4.3-Thermophilic bioreactor performance.

Hydrogen production was detected starting from day 3 (at HRT 10 h), as the HRT was gradually decreased from 8 h to 4.h the hydrogen production rate(HPR) and hydrogen yield increased from 0.85 L H_2/h and 3.1 mol H_2 /mol glucose to 1.75L H_2/h and 3.9 mol H_2/mol glucose, respectively. The effect of effluent recycle rate on both HPR and hydrogen content (%) is shown in tables (4.3.1,2,3,4), as the effluent recycle rate was increased from 0.4 L/min to 3.6 L/min and a decrease in the HRT from 10 h to 1h resulted in an HPR of 7.57 L H_2/h, while hydrogen content of 69% was found at 1 h HRT.

Table 4.3.1- Thermophilic bioreactor performance with respect to hydrogen production rate (HPR), hydrogen Productivity (HP)and hydrogen yield (HY), during 24 days of operation. (The experiment was condacted on summer June , Ougust 2013)

day	Actions taken	HRT(h) feeding rate (L/h)	Recycle rate (L/min)	Total gas produced (L)	pH	% Hydrogen	hydrogen production rate (L/h)	HP (mol//h)	HY mol H /mol glucose
1	Sewage and dung were brought, mixed in scotch bottle and the ph was reduced to 2 using 1 M HCL, and left for 24h.	-							
2	The ph of the Inoculum was raised to 7.2 using Endo Media half by half (volume). the Inoculum is heat shocked at 90 c for 2 h and left over night.								
3	The system was operated in a batch manner. The column was fed with the Inoculum and		0.4 L/min	1L/h		No hydrogen was detected			

	3x endo media .The recycle was kept at .o4 l/h. This was operated for 3 days.								
4-6	**The system was shift to continuous process.**	**10 h 0.33 L/h**	**0.4 L/min**	**1.7L/h**	**3.3**	**35%**	**0.595 L/h**	**0.0244**	**2.904**
7-9	**HRT decreased , recycle rate increased.**	**8 h 0.4 L/h**	**1.5 L/min**	**2.3 L/h**	**6**	**37%**	**0.851 L/h**	**0.0348**	**3.8443**
10-12	**HRT decreased , recycle rate increased**	**6h 0.55 L/h**	**2 Lmin**	**2.9 L/h**	**5.7**	**42 %**	**1.218 L/h**	**0.0450**	**3.98**
13-15	**HRT decreased , recycle rate increased**	**4h 0.825 L/h**	**2.5L /min**	**3.9 L/h**	**4.9**	**45 %**	**1.755 L/h**	**0.0720**	**4.79**

16-18	**HRT decreased , recycle rate increased**	**2h 1.1 L/h**	**3L/min**	**5.1 L/h**	**6.2**	**50 %**	**2.55 L/h**	**0.105**	**5.398**
19-21	**HRT decreased , recycle rate increased**	**1.5 h 2.2 L/h**	**3.4L/min**	**8.1L/h**	**7.3**	**63 %**	**5.122 L/h**	**0.2100**	**5.648**
22-24	**HRT decreased , recycle rate increased**	**1h 3.3 L/h**	**3.6 L/min**	**10.98L/h**	**7.8**	**69%**	**7.57L/h**	**0.310**	**5.827**

Table 4.3.2- Thermophilic bioreactor performance with respect to: Sucrose conversion rate, distribution of soluble metabolites, during 24 days of operation.

HRT/feeding rate	Effluent recycler rate	Concentration of sucrose in the feed g/Lh (calculated)	Concentration of sucrose in the effluent g/L	%of conversion	Concentration of sucrose in the effluent Mm	Concentration of acetate in the effluent mM	Concentration of butyrate in the effluent mM	Concentration of propionate in the effluent mM	HP mol/L/h	HY mol H /mol glucose
10 h 0.33 L/h	**0.4 L/min`**	**5.88**	**1.23**	**98%**	**3.6**	**4.39**	**4.85**	**8.34**	**0.0244**	**2.904**
8 h 0.41L/h	**0.4 L/min**	**7.30**	**3.01**	**85%**	**8.79**	**3.406**	**1.96**	**8.14**	**0.0348**	**3.8443**
6h 0.55 L/h	**1.5 L/min**	**9.79**	**3.4**	**79%**	**9.96**	**3.3**	**2.12**	**5.36**	**0.0450**	**3.98**
4h 0.825 L/h	**2 L/min**	**14.69**	**4.12**	**70%**	**12.036**	**-**	**-**	**-**	**0.0720**	**4.79**

2h 1.1 L/h	**2.5 l/min**	**19.58**	**4.2**	**68%**	**12.27**	**2.8**	**2.13**	**3.65**	**0. 105**	**5.398**
1.5 h 2.2 L/h	**3 L/min**	**39.16**	**4.75**	**65%**	**13.88**	**2.49**	**1.70**	**2.48**	**0.2100**	**5.648**
1h 3.3 L/h	**3.6 L/min**	**58.74**	**5.01**	**62%**	**14.6**	**2.29**	**0.48**	**1.75**	**0.310**	**5.827**

Table 4.3.3-Thermophilic bioreactor performance with respect to sucrose conversion.

Sucrose concentration in the feed (mM)	Sucrose concentration in the effluent (mM)	Sucrose utilized (mM)	% converted to H2	% Conversion rate
51.65	3.6	48.05	35	98
57.76	8.79	49.00	37	85
61.03	9.96	51.34	42	79
78.73	12.00	66.73	45	70
89.99	12.2	77.79	50	68
131.9	13.88	118.02	63	65
168.79	14.6	154.37	69	62

Table 4.3.4-Thermophilic bioreactor performance with respect to total bioreactor volume and recycle rate.

Bioreactor volume (B) L	Total volume (V) L	Effluent recycle rate L/min	Total volume : effluent recycle rate ratio	H_2 yield mol/mol	comment
5	12.6	3.5	3.61	1.84	(Obazu et al., 2012).
3.27	10.5	3.5	3	2.84	(Obazu et al., 2012).
5.072	7.5	3.5	2.14	3.905	(Masilela, 2011).
3.27	5.74	3.2	1.79	3.55	(Obazu et al., 2012).
1	3.3	3.6	0.91	5.8	This study

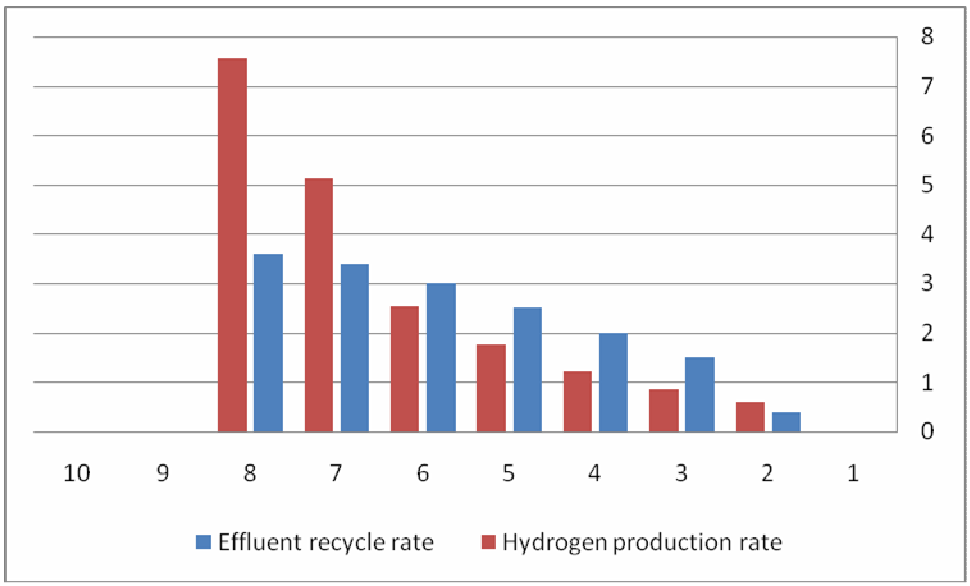


Figure 4.3.1: Effect of effluent recycle rate on hydrogen production rate. The effluent recycle rate increased every third day during the 24 days run. As the rate increases from 0.4L/min, to 3.6L/min, the hydrogen production rate increases from 0.595L/h to 7.57L/h.

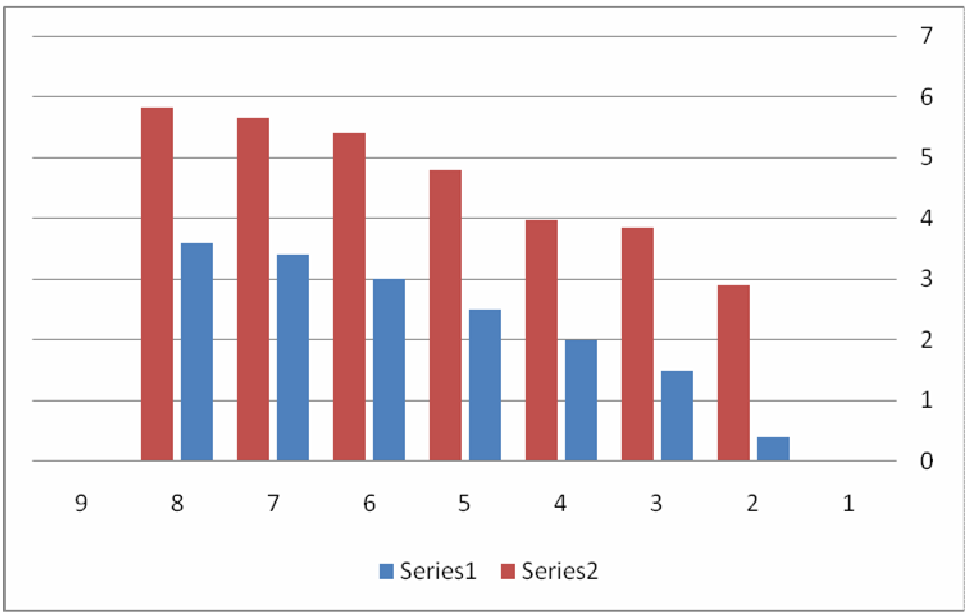


Figure 4. 3.2: Effect of effluent recycle rate on hydrogen yield. The effluent recycle rate increased every third day during the 24 days run. As the rate increases from 0.4L/min, to 3.6L/min, the hydrogen yield increases from 2.904 to 5.827 mol hydrogen/mol glucose.

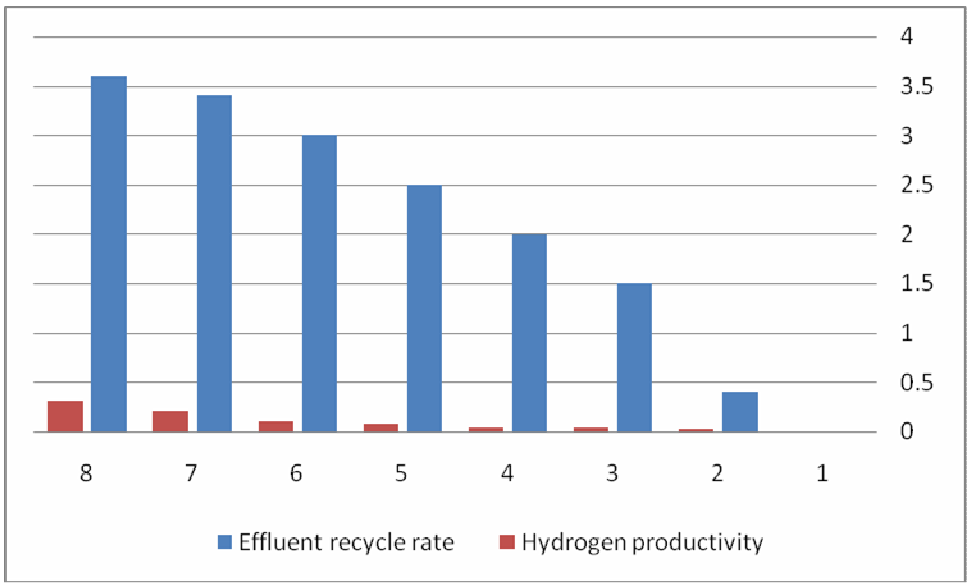


Figure 4. 3.3: Effect of effluent recycle rate on hydrogen productivity. The effluent recycle rate increased every third day during the 24 days run. As the rate increases from 0.4L/min, to 3.6L/min, the hydrogen productivity increases from 0.0244 to 0.310 mol hydrogen /h.

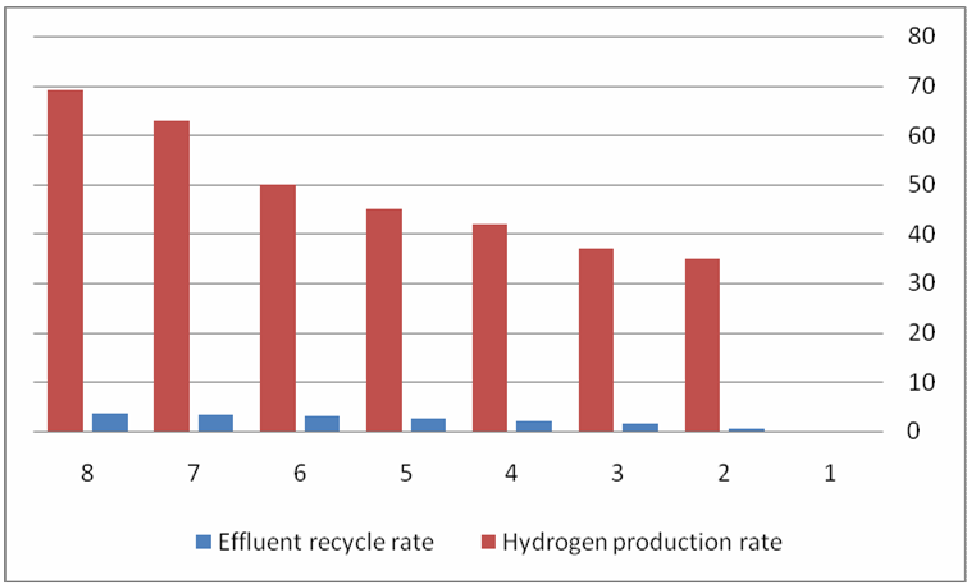


Figure 4.3.4: Effect of effluent recycle rate on hydrogen content. The effluent recycle rate increased every third day during the 24 days run. As the rate increases from 0.4L /min, to 3.6L/min, the hydrogen content increases from 35% to 69% hydrogen in the total gas.

Hydrogen (H_2) and carbon dioxide (CO_2) were produced as gaseous products, and no methane (CH_4) was expected during the course of the experiments, because the acid and heat pretreatment methods were effective at inhibiting the activity of methanogenic bacteria in the anaerobic sludge. Moreover, the growth rates of methanogens and homoacetogenes are generally lower than those of H_2 producers (**chen et al., 2002)**.

Methanogenes and homoacetogenes can, therefore, be washed out by using short HRTs. During the bioreactor operation, heat shocks (temperature 70^{0}C used to reselect spore forming bacteria**(chang et al., 2002).**

In this study a maximum hydrogen productivity of 3100 mmol H_2 /h was achieved. The experimental results showed that both HPR and hydrogen yield increased significantly with the shortened HRT, giving the maximum at the shortest HRT of 1h of 7.57 l H_2 / h and 5.8 mol H_2 / mol glucose , respectively (both plots are shown in figures 4.3.5&4.3.6).

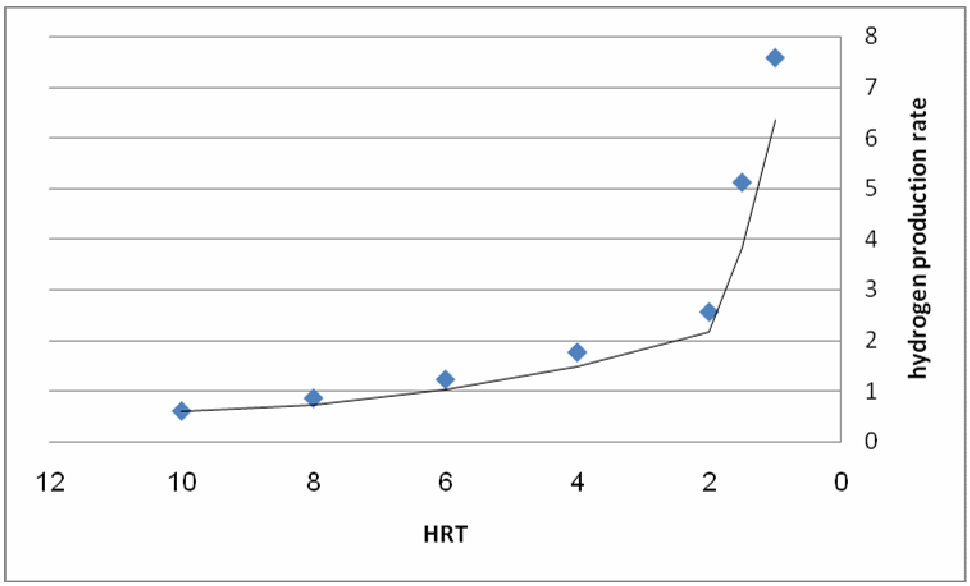


Figure 4. 3.5: Effect of HRT on hydrogen production rate. HRT decreased every third day during the 24 days run. As the HRT decreases from 10 h to 1h, the hydrogen production rate increases from 0.595L/h to 7.57L/h.

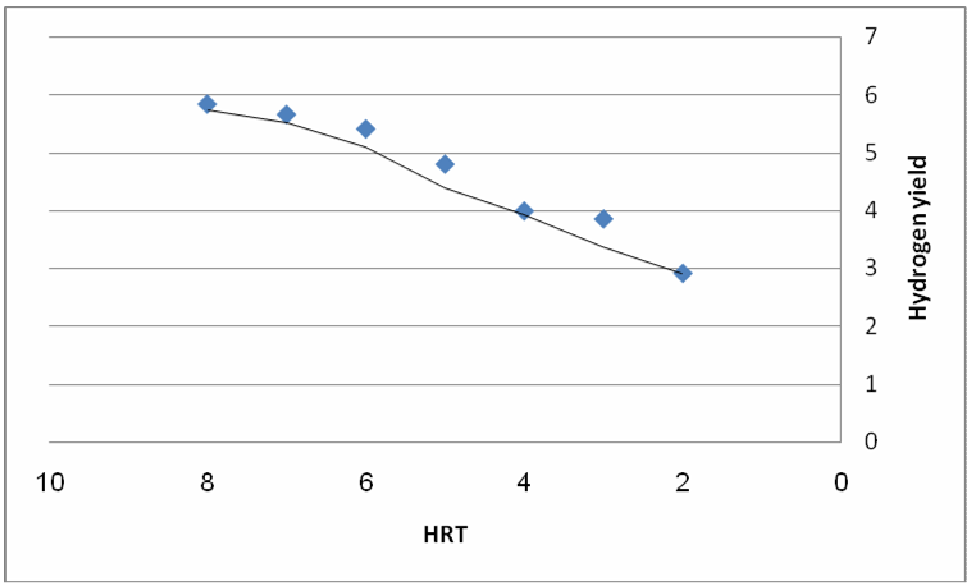


Figure 4. 3.6: Effect of HRT on hydrogen yield. HRT decreased every third day during the 24 days run. As the HRT decreases from 10h to 1h, the hydrogen yield increases from 2.904 to 5.827 mol hydrogen/mol glucose.

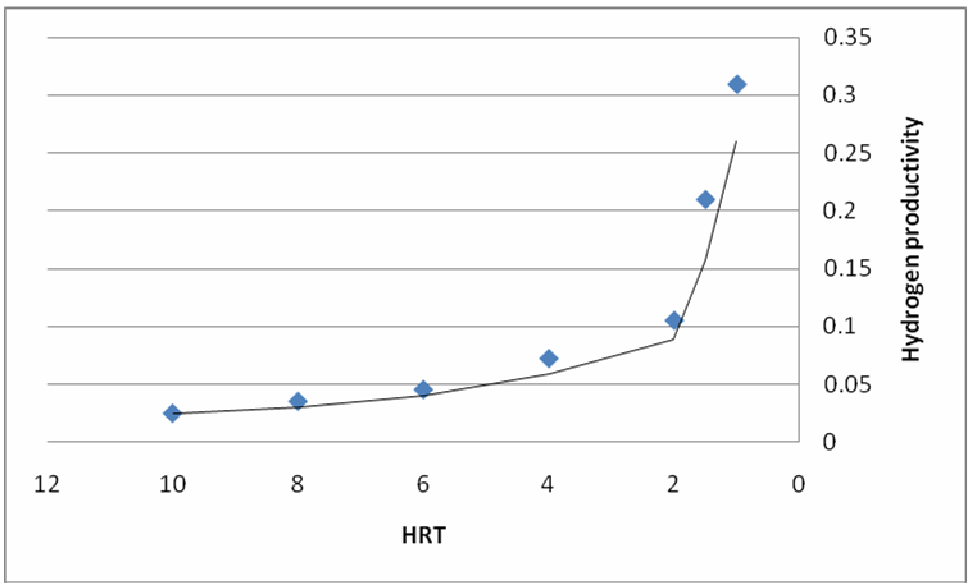


Figure 4. 3.7: Effect of HRT on hydrogen productivity. HRT decreased every third day during the 24 days run. As the HRT decreases from 10h to 1h, the hydrogen productivity increases from 0.0244 to 0.310 mol hydrogen /h.

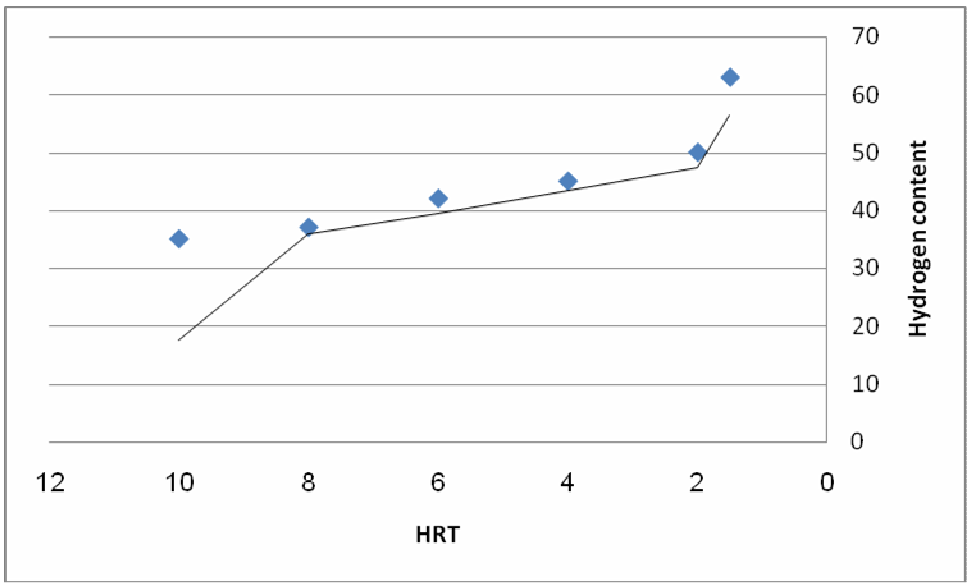


Figure 4. 3.8: Effect of HRT on hydrogen content. HRT decreased every third day during the 24 days run. As the HRT decreases from 10h to 1h, the hydrogen content increases from 35% to 69% hydrogen in the total gas.

However, the sucrose conversion rate decreased apparently, from 98 % at 10h to 62% at 1 h HRT . Regardless of decrease in sucrose conversion efficiency the production of hydrogen increased and also hydrogen composition increased with the reduction in HRTs.

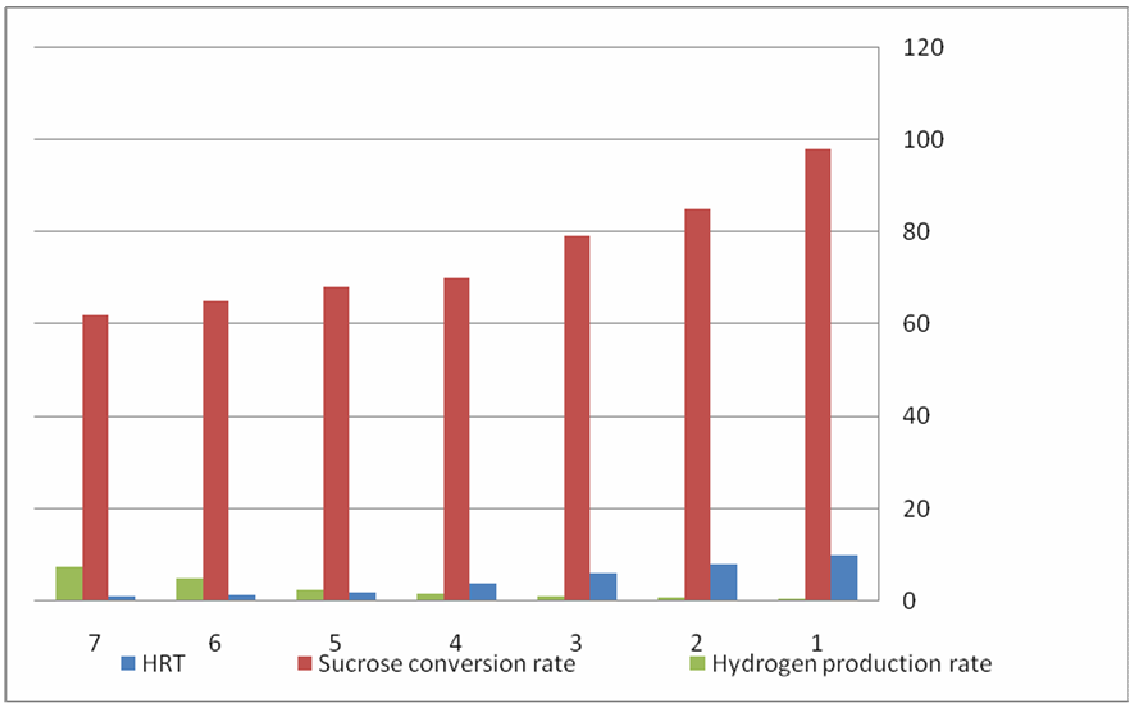


Figure 4. 3.9: Effect of HRT on hydrogen production rate and substrate conversion. HRT decreased every third day during the 24 days run. As the HRT decreases from 10h to 1h, sucrose conversion rate calculated as

the % of the utilized substrate converted to hydrogen, decreases from 98% at 10h HRT 62% at 1h HRT. The hydrogen production rate increases from 0.595L/h to 7.57L/h.

The changes of the hydrogen production at different pH values from 4 to 7.4 are shown in table (4.2.3a). During the course of the experiments the hydrogen production increased with increasing pH, with the maximum hydrogen production rate of 7.57 L H_2 /h attained at pH 7.8.

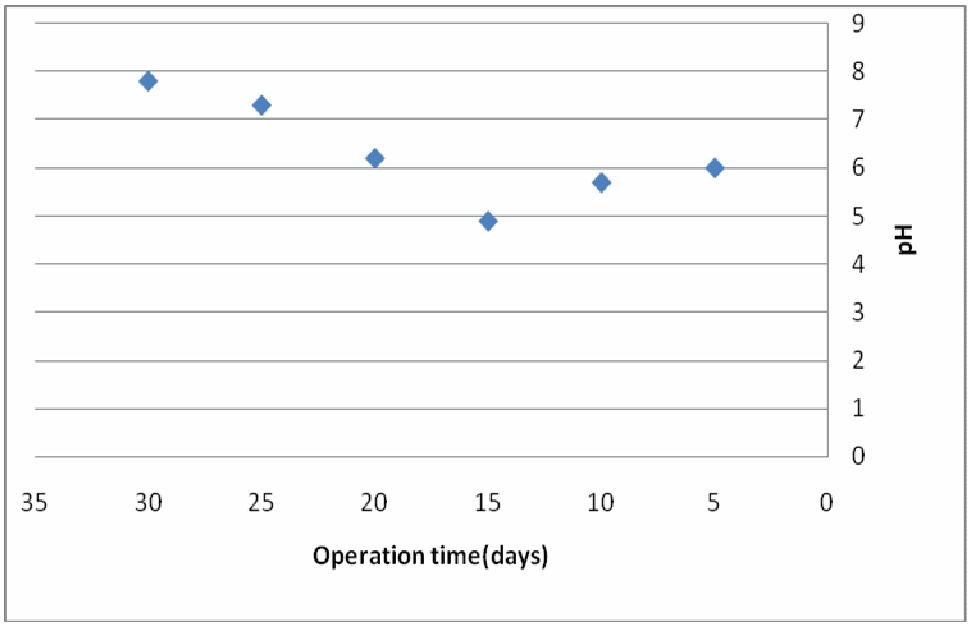


Figure 4.3.10: Time course profile of the pH in AFBR during 24 days. The stability of the AFBR during 24 days run. The temperature was kept at 65 ^{0}C , the pH of the effluent was measured and it appears that the pH increases with increasing the effluent recycle rate and HRT from 3.3 to 7.8 and that was accompanied with increasing in the performance of the system regarding the hydrogen productivity , yield, and production rate.

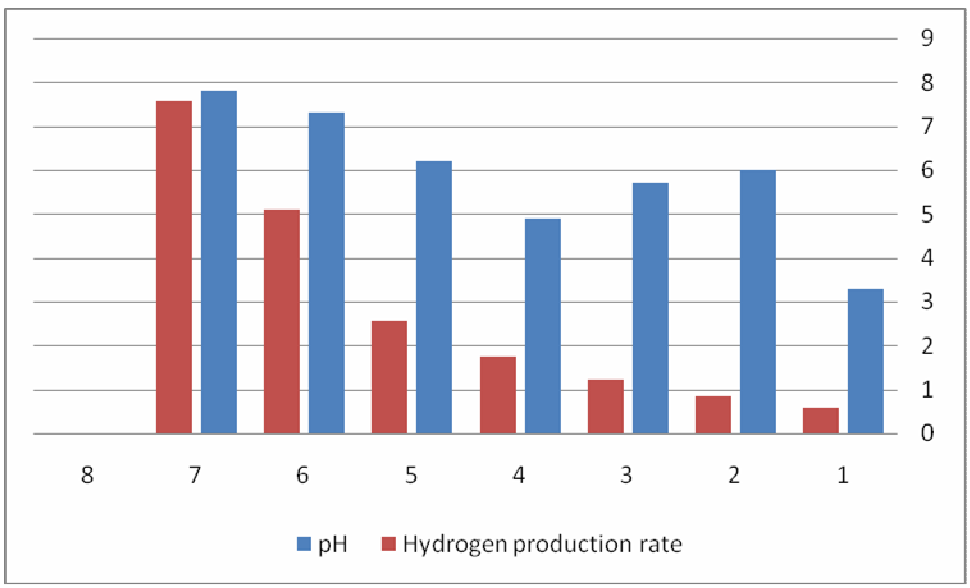


Figure 4. 3.11: The relation ship between pH and hydrogen production (L/h) during 24 days operational course. The pH increases with increasing the effluent recycle rate and HRT from 3.3 to 7.8 and that was accompanied with increasing in the performance of the system regarding the hydrogen production rate.

We suspect that the sharp increase in the effluent pH was as a result of low concentration of the acetate, propionate and butyrate in the bioreactor effluent. The effluent was mainly composed of acetate, propionate and butyrate; the results showed that the concentration of acetate, propionate, and butyrate declined from 4.39 mM, 8.34 mM and 4.85 mM to 2.299 mM, 1.75 mM and 0.84 mM, respectively (table 4.2.3 b).

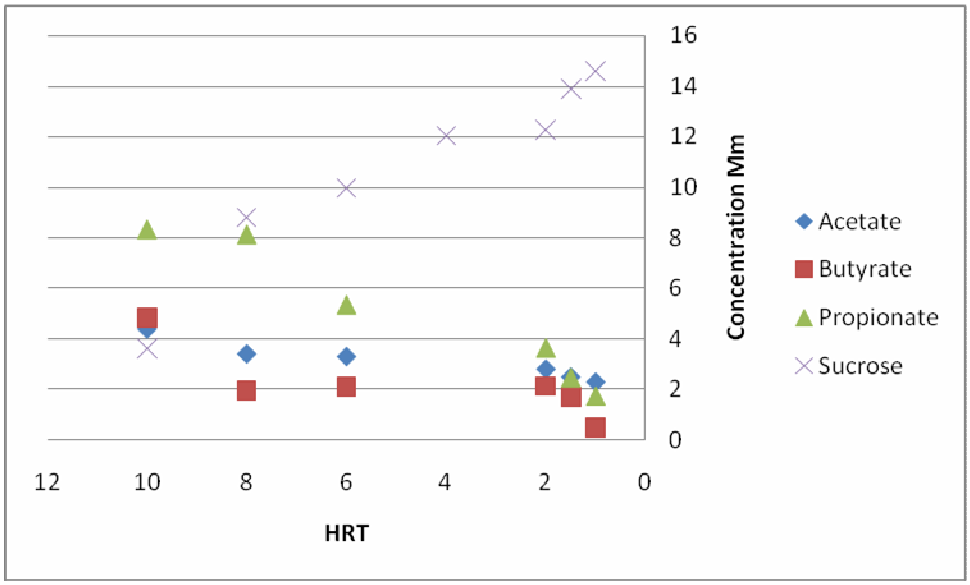


Figure 4.3.12: The distribution of soluble metabolite in the AFBR with respect to HRT. The concentration of acetate, putyrate, propionate decreases and the concentration of sucrose increases with the decrease in its conversion rate, indicating that there may be change in the metabolic bath ways of the consortium in the direction of utilizing volatile fatty acids for hydrogen production.

The substantial decrease in the bioreactor working volume to degassed effluent recycle ratio (V/F_{er}) leads to increase in HY. V/F_{er} of different bioreactors volumes from previous studies compared with this study was shown in table (4.3.13)

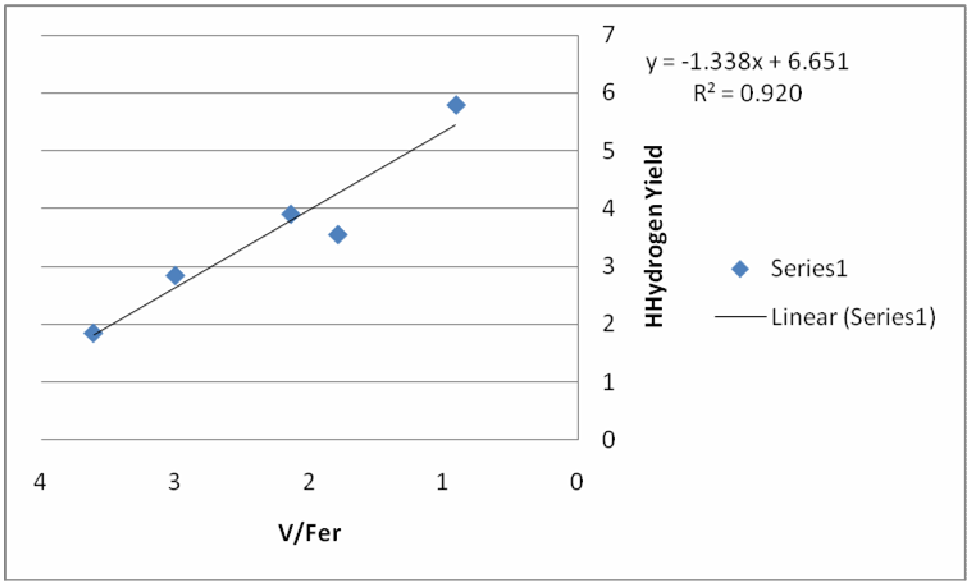


Figure 4.3.13: The relationship between V/F_{er} and hydrogen yield. As the ratio between total bioreactor volume (V) and the effluent recycle rate (F_{er} **)** decreases, hydrogen yield increases.

Chapter V
Discussion

5.1-Bioreactor Design and Strategy:

The realization of hydrogen as a clean fuel for the future has triggered research around the world to look for novel methods for its production from renewable resources. In the past two decades, many studies investigating the efficiency of dark anaerobic biohyhdrogen production have been conducted under mesophilic temperatures. In spite of large number of studies conducted so far, H_2 yield have been quite low and stagnant.

This study claims that , certain modifications in the anaerobic fluidized bed bioreactor (AFBR), and operation strategy in dark fermentative biohydrogen production using mixed bacterial cultures in a thermophilic (65°C) anaerobic fluidized bed bioreactor (AFBR), will facilitates maximum hydrogen production and productivity .

To date the thermodynamic or substrate conversion efficiency of dark anaerobic biohydrogen production for all anaerobic fluidized granular bed bioreactors (AFGBs) has not exceeded 3.55-3.905 mol H_2/mol glucose **(Obazu et al. 2012; Masilela, 2011; Kraemer & Bagley, 2007).** The reason for this is that the experimental conditions under which high hydrogen productivities (HPs) have been achieved do not favor the simultaneous achievement of hydrogen yields (HYs) greater than 2 mol H_2/mol glucose.

Achievement of HYs greater than 2.0 mol H_2/mol glucose in bioreactor experiments have been dependent on the following operation conditions: monocultures, thermophilic temperatures, low substrate loading rates, low dilution rates, gas stripping by sparging with N_2, maintenance of low partial pressures (<100 Pa) and low bacterial biomass densities. Bioreactor experiments that achieved high HPs have depended on the following operational conditions: undefined multispecies bacterial consortia, high substrate loading rates, high partial pressures, high dilution rates, and high bacterial biomass densities **(De Vrije et al., 2007).**

All the necessary and sufficient conditions that are most likely to facilitate the simultaneous achievement of high HYs and high HPs can be explicitly stated as follows:
1. High microbial volumetric biomass density. This would require bacterial granulation.

2. High rates of microbial biomass retention within the bioreactor. This would require bacterial granules with good settling properties.
3. Low hydraulic retention times or high dilution rates.
4. High organic substrate loading rates.
5. Maintenance of the lowest possible partial H_2 pressure within the fluidized bacterial granular bed.
6. Efficient effluent gas-disengagement.
7. High rates of de-gassed effluent recycling though the fluidized granular bed.
8. Maintenance of thermophilic temperatures within the bioreactor increase HY.
9. Minimisation of dead volume within the bioreactor system.
Conditions 1 to 9 were the premises of the working hypothesis for this investigation undertaken in this study.

5.2- Effect of thermophilic tempreture, HRT, and effluent recycle rate on hydrogen yield and productivity.

Recently, thermophilic fermentations are gaining increasing attention around the world, due to high hydrogen yields associated with them **(Hallenbeck, 2005).**

Using (extreme) thermophiles, 1 mol of glucose can be converted to 4 mol of H_2 and 2 mol of acetic acid as the main product **(Zeidan & van Niel, 2010),** which is considered as the maximum theoretical yield achievable.

Thermophilic hydrogen fermentation has major advantages: Higher product yields, sanitation and therefore elimination of pathogenes and avoidance of hydrogen consuming organisms like methanogenes.

In this study, the application of influent rate of 3.3 L/h (HRT 1h) and effluent recycle rate of 3.6 L/min resulted to the attainment of maximum hydrogen production rate and Hydrogen yield of 7.57 L H_2 /h and 5.8 mol H_2 / mol glucose, respectively.

The reported hydrogen yield value in this study is very high and more than theoretical yield, this yield was achieved because of the better thermodynamic conditions in our thermophic bioreactor (such as high substrate loading rates, low hydrogen partial pressure and high bacterial biomass densities). Comparable, similar findings were reported by Zeidan& van Niel (2010) who reported that in a thermophilic fermentation with

Caldicellulosirupor owensensis an HY of 4.0 mol H_2 / mol glucose has been achieved **(Zeidan & van Niel, 2010).** Table 5.2 shows a comparison of hydrogen production rates and yields achieved in this study and those reported in literature.

The plug flow nature of AFBRs aids the even distribution of biofilm in the reactor. The recycle and plug flow of AFBRs provides an advantage in the treatment of inhibitory wastes where the high flow rates and even distribution across the bed ensure the biomass is exposed evenly and for a short duration while the recycling mode ensures the breakdown of compounds. Other benefits of recycling include partial neutralization of pH of the incoming influent, a reduction of the alkalinity (buffering carbonate / CO_3^{-2} ions) required, reduction of the effects of incoming influent shock loads and compensation for the fluctuations in the influent flow rate **(Thompson, 2005).**

Table 5.2: Summary of Bioreactor operation and performance data for Different high performance AFGB Systems.

Bioreactor system	Substrate	Concentration (g/l)	T (^{0}C)	HT (h)	PH	% H_2	HP (mmol H_2/Lh)	HY (mol H_2/mol glucose)	SHP (mmol H_2/g/h)	Bioreactor Volume (L)	Bacterial granule biomass density (g/L)	References
BF AFBR	Glucose	10	37	0.25	5.5		310.7	1.71	8.96	0.6[a] 1.4[b]	61-65[c] 34-37[d]	(Zhang et al, 2008a,c)
GS ARBR	Glucose	10	37	0.25	5.5		269.8	1.66	8.77	0.6[a] 1.4[b]	61-65 34-37[d]	(Zhang et al, 2008a,c)
UASB	Sucrose	20	60	0.75	5.0	42	152.5	1.3	9.53	0.22	16	(O-Thong et al., 2008)
CIGSB SAC	Sucrose	17.8	35	0.5	6.7	38.1	299.6	1.52	11.45	1.0	26.1	(Nogma et al, 2011)
CIGSB CAC	Sucrose	17.8	35	0.4	6.7	35.6	288.6	1.19	11.57	1.0	26.1	(Lee el al, 2004)
CIGSB	Sucrose	17.8	30	0.5	6.7	34.9	160.6	1.19	5.44	0.88	30-40	(lee et al , 2005)
CIGSB	Sucrose	17.8	35	0.5	6.7	40.5	280.8	1.56	7.73	0.88	30-40	(lee et al , 2005)
CIGSB	Sucrose	17.8	40	0.5	6.7	40.1	313.1	1.58	9.12	0.88	30-40	(lee et al , 2005)
CIGSB	Sucrose	17.8	45	0.5	6.7	32.9	215.8	1.33	7.11	0.88	30-40	(lee et al ,

												2005)
CIGSB	Sucrose	17.8	35	0.5	6.7	40.5	280.8	1.56	7.64			(Lee et al, 2006)
CIGSB[e]	Sucrose	17.8	35	0.5	6.7	41.7	380.6	1.96	9.50		40	(Lee et al, 2006)
AFGB HER	Sucrose	17.8	45	0.37	5.4	45	298.7 (at 85 Kpa)	1.24	20.07	5.0[b] 10.5	19.5	(Nogma et al, 2011)
AFGB HER	Sucrose	17.8	70	0.37	5.5	67	507.5 (at 85 Kpa)	2.2	29.59	5.0[b] 10.5[f]	22.7	(Nogma et al, 2011)

AFBR: anaerobic fluidized bed reactor, AFBR GAC: AFBR with bacteria biofilm attached to granulated activated carbon' AFGB HER: anaerobic fluidized granular bed reactor with high rate effluent recycling AFBR: biofilm anaerobic fluidized bed reactor, CIGSB CAC: carrier induced granular sludge bed with cylindrical activated carbon; CIGSB SAC: with spherical activated carbon; AFBR granular sludge anaerobic fluidized bed; and UASB: upflow anaerobic sludge bed.

a Bioreactor working volume corresponding to bed biofilm or granular height.

b Bioreactor working volume

c Biomass in corresponding to bed height.

d Biomass in total bioreactor working volume.

e CIGSB with bed agitation.

f Total bioreactor system volume.

The results of this study indicate that high hydrogen production rate and yield can be simultaneously achieved in the anaerobic fluidized bed bioreactor. Let's look at the below reaction:

$$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 2HCO_3^- + 4H^+ + 4H_2$$

$$\Delta Go = -206.1 \text{ kJ/mol}$$

Given the strongly negative ΔGo for the above reaction, it seems possible that of the electron equivalents (e- eq) of glucose, 8 e- eq should end up in H_2 with the remaining 16 e- going to acetate. Because of internal bioreactor thermodynamic constrains, dark fermentation hydrogen yields are usually below 4 mol H_2 /mol glucose **(Rittmann, 2008).** Theoretically acetate could be further oxidized under anaerobic conditions to yield 4 H_2 and $2CO_2$ in the absence of methanogens if the partial pressure is reduced.

It appears that for the anaerobic oxidation of glucose to hydrogen and acetate (reaction above) the decrease in the ΔGo from -206.1 kJ/mol at 25 ^{0}**C** to -223.7 kJ/mol at 60^0**C** was insufficient for overcoming the thermodynamic barrier necessary to achieve HY of 3.0 mol H_2 / mol glucose in a high rate thermophilic granular bed bioreactor **(O-Thong et al., 2008)** with an HP of 152 mmol H_2 /(L.h).

The above ΔGo at 60^0**C** was based on a calculated enthalpy of 61.6 kJ/mol for the overall reaction and on estimated entropy of 513.5J/ (mol.K) for H+ ion production under cellular ionic and pH conditions, the latter value is consistent with this reaction:

$$CH_3COOH + 2H_2O \rightarrow 4H_2 + CO_2$$

being an entropic driven process with (ΔG0 = +104.6 kJ mol-1).

Whether or not a practical viable anaerobic single or multi-stage bioprocess could be engineered, possibly with the application of external work in the form or another, that would remove the potential energy barriers preventing the complete oxidation of glucose to 12 H_2, remain an interesting, but controversial consideration **(Hallenbeck, 2009; Hallenbeck&Ghosh, 2009).**

In this study, we succeeded to overcome the thermodynamic constraints preventing the simultaneous attainment of both high HPs and high HYs by combination of external

parameters such as thermophilic temperature, low HRTs and high recycles of de-gassed effluent.

5.3- microbial growth and induction of granulation.

FBRs retain microbial growth on granular support media ((activated carbon granules (GAC)) which in turn is kept in suspension by drag forces exerted by up flowing influent**.** Influent is pumped though a bed of inert support media at sufficient velocities or flow rates to induce suspension, termed fluidization**.** Once the bed is fluidized, each particle provides a large surface area for microbiological biofilm formation. It is this factor that allows AFBRs to have such a large treatment capacity **(Thompson, 2005; DeAmorim et al., 2009).**

Granulation is an efficient means of bacterial biomass retention in dark fermentation bioreactors, and thus enables high organic loading and H_2 productions **(Wu et al., 2006; Zhang et al., 2007a).**

In this study, carrier induced thermophilic bacterial granulation has proven to be helpful in enhancing H_2 yield and providing stability to the process. The granules were suspected to be formed within a period of 5 to 9 days, and during days up to 24, in response to an influent rate of 3.3 L/h and effluent recycle rates of 3.6 L/min the fluidized granule bed occupied the full bioreactor working volume of 1 L, giving a fluidized bacterial dry mass density of 7.41 g /L.

Volumetric hydrogen productivity is directly proportional to the bacterial biomass density. Recent advances in the capacity to initiate the induction, growth and development of anaerobic bacterial granules has made it possible to achieve the bacterial dry mass necessary for the achievement of HPs greater that 120 mol H_2/(L.h). The granules had good settling ability and density which facilitated the bioreactor to be operated at a very low HRT of 1h with minimum bacterial biomass washout in the AFBR. This was evidence that induction of microbial granulation by short HRT is considered to be related to the hydrodynamic (and organic loading) selective pressures **(Lee et al., 2006).**

Because of high microbial cell retention in the reactor, it was evidence that formation of granules resulted to improved hydrogen production efficiencies. Without the granules possessing the tensile strength sufficient to withstand the corrosive action resulting from the exposure to the high forces, generated by the combine effect of high

influent rates and the high degassed effluent recycle rates, it will not be possible to operate a thermophilic fluidized bed bacterial granular bed bioreactors under these operational conditions. Clearly, granulation played an important role in maintaining the stability of the bioreactor, such as enhancing biomass retention and generation of micro-environment that favors interspecies syntrophic interaction among bacteria involved in metabolism **(Masilela, 2011)**.

5.4- Assessment of gas disengagement.

Another important advantage of this bioreactor is the improving of H_2 liquid-to-gas mass transfer of hydrogen. The mass transfer of highly soluble gases is not limited in the usual conditions occurring in anaerobic fermentors (low-intensity mixing). Conversely, the limitation is important for poorly soluble gases, such as hydrogen. Hydrogen could be over concentrated to as much as 80 times the value at thermodynamic equilibrium. Such over concentrations bring into question the biological interpretations that have been deduced solely from gaseous measurements **(Thompson, 2005).**

The low solubility and low mass transfer coefficients of gases like hydrogen can delay the attainment of thermodynamic equilibrium between the different phases of the AFGB system. Gaseous fluxes from the AFGB system involves hydrogen mass transfer in different states of motion **(Nogma et al, 2011).**

In the AFGB system we have the co-existence of a quasi-static solid phase, a mobile bulk fluid or liquid phase and a mobile gaseous phase. The quasistatic solid phase consists of the fluidized bacterial granular bed which functions as the generating biocatalyst. The mobile liquid phase consists of the nutrient influent and recycled degassed effluent. The mobile gaseous phase consists of gas bubbles generated though the process of cavitation or bubble nucleation within the bioreactor. The latter two mobile phases also shift the anaerobic oxidation processes away from the thermodynamic equilibrium state by removing hydrogen from the quasi-static solid phase or from the biocatalyst surface. In a majority of the AFGB systems the bacterial granules consists of a multispecies microbial consortium. See figure 5.4 **(Obazu et al., 2012).**

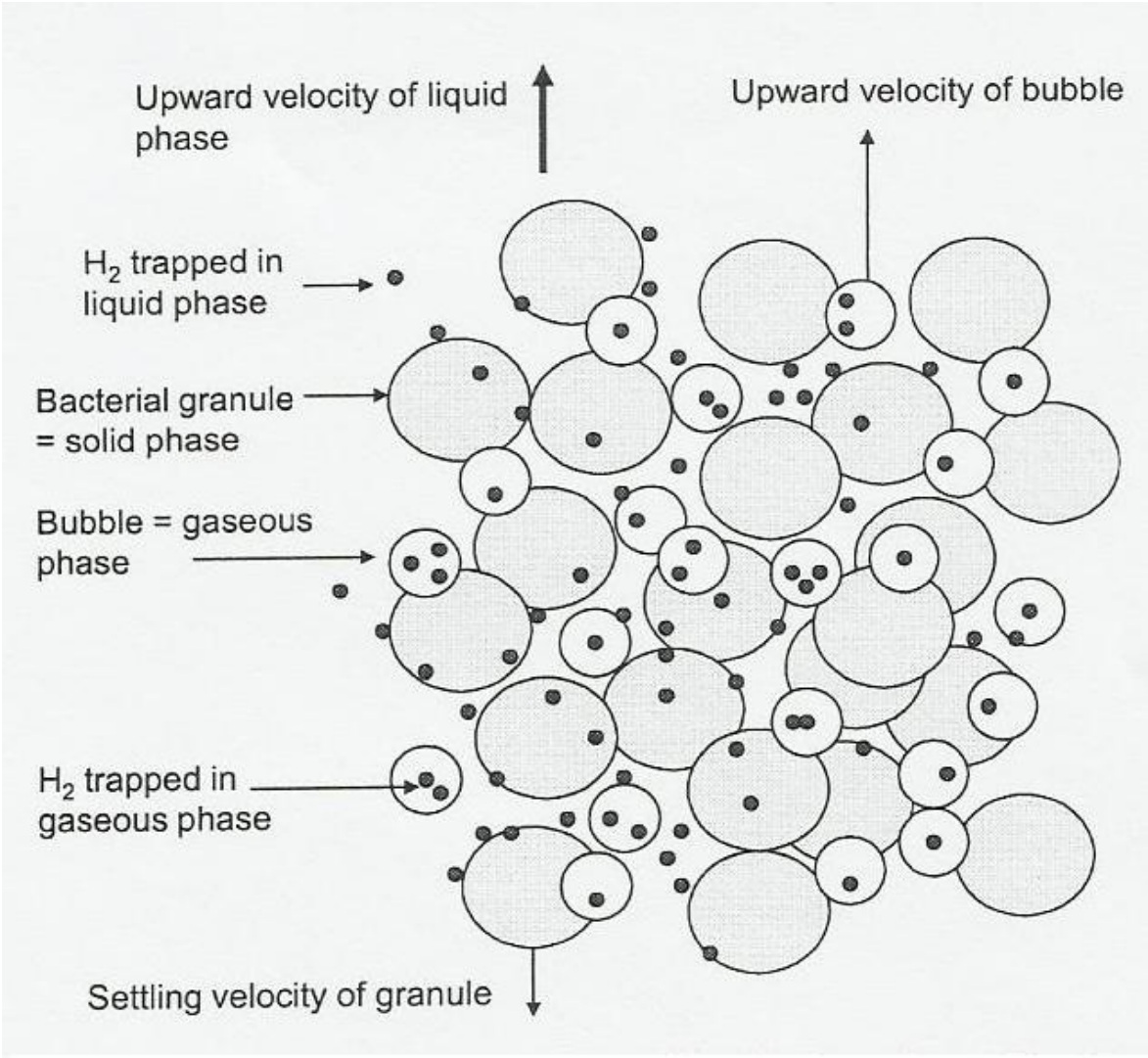


Figure 5.4: The partitioning of non-dissolved and soluble H_2 between the three different phases in the AFGB system: a quasi-static solid phase comprised of a fluidized bed of bacterial granule particles, a mobile bulk fluid or liquid phase and a mobile gaseous phase. The mobile liquid phase consists of the two fluid fluxes, the nutrient influent flux and the recycled degassed effluent flux. The mobile gaseous phase consists of gas bubbles filled with water vapor, H_2 and CO_2 **(Obazu et al., 2012).**

In this study, it was proposed that high rates of effluent recycling achieved two essential process goals which were necessary for increasing hydrogen yield:

The first process goal was the rapid physical removal of H_2 trapped within fluid phase surrounding the bioreactor fluidized granular bed. The second process goal was the efficient effluent gas disengagement brought about by discharging the effluent at a high flow velocity from the bioreactor into the gas disengager tube. For effluent gas disengagement to be efficient, it would have been necessary for the process to remove most of the supersaturated concentration of dissolved hydrogen from the effluent before it

was recycled back into the bioreactor. The H_2 content of the effluent recycled back into the bioreactor would correspond to the thermodynamic equilibrium dissolved hydrogen concentration, which would have been impossible to remove completely from the effluent stream in the gas disengager. So it was assumed that the effluent recycled back into the bioreactor would contain the thermodynamic equilibrium concentration of dissolved hydrogen. In about one minute, the entire fluid volume supporting the fluidized granular bed was displaced from the bioreactor with fresh degassed effluent mixed with influent nutrients, while the granular bed remained behind in the bioreactor.

Recycling of degassed effluent at high velocity though the fluidized granules brings about the continuous and rapid displacement of H_2 containing bubbles and dissolved H_2 from the bioreactor bed. Thus the physical rate of the removal of H_2 trapped in the liquid phase was considerably greater than the rate at which H_2 was being generated by the granules embedded in the liquid phase. Also an increase in temperature inhibits the H_2 consuming hydrogenases, thereby increasing the net flux of H_2 from the granules into the mobile fluid phase **(Ngoma et al., 2011).**

In addition, according to Le Chatelier's principle, under these operating conditions, the high rates of H_2 removal by the mobile fluid phase would make the ΔG^0 more negative not only for the anaerobic oxidation of glucose, but also for the anaerobic oxidation of acetate, propionate and butyrate, in the absence of H_2 consuming bacteria. This would also add to the net flux of H_2 into the mobile liquid phase from the quasi-static fluidized granular bed. Thus the combination of high temperatures and a low V/ (F_{er}) quotient appears to be the necessary bioreactor operation conditions for the simultaneous achievement of high HPs and HYs.

5.5- Effect of total bioreactor volume on biohydrogen yield and production.

Moreover, Space Time Yield (STYs) which represents the mass of a product P formed per volume of the reactor and time is low for biohydrogen production and this discouraged the production of hydrogen at commercial level. To overcome this constrain, it is essential to increase both HP and HY simultaneously.

The STYs is given by this equation:

STY = mass of product P in kg/ volume of the reactor in $m^3 \times$ time in S.

From the equation it is supposed that by decreasing the total of liquid volume of the AFBG system (V) relative to the degassed effluent recycle rate a simultaneous increase in both HP and HY can be achieved **(IUPAC Recommendations, 1993).**

The total volume (V) of an AFGB system consists of the sum of the working bioreactor volume (B), the volume of the gas disengager (G) and finally the volume of the piping (P).

This hypothesis would predict that for some critical value X, where $X = V/(F_{er})$,HP will be some factor greater than120mmole hydrogen/L/h , and HY will be equal to or greater than 3.0 mol H_2 /mol glucose. A commercially viable STY per unit volume should aim at achieving the HPs greater than 120 mmol H_2 /L/h and HYs greater than 3 mol H_2 /mol glucose. Simultaneous achievement of high volumetric productivities (HP = 231.3 mmol H_2 /L/h) and high hydrogen yields (HY = 3.55 mol /mol glucose) was obtained by increasing the temperature to 70° C and by reducing the total bioreactor system volume (V) to 5.74 L and increasing the degassed effluent recycle rate ($(F_{er})$to 3.2 L/min, giving a $V/(F_{er})$ value of 1.8 min in a study by Obazu et al.(2012) and they claimed that the rate of physical removal of trapped gas in the bulk liquid phase surrounding the fluidized granules reduced the thermodynamic constraints preventing the simultaneous achievement of high HPs and high HYs in the anaerobic fluidized granular bed bioreactor **(Obazu et al., 2012).**

In this study the further reduction in the $X = V/(F_{er})$ to 3.3/ 3.6 leads to increase in both hydrogen production and yield to (HP = 0.310 mol H_2 /L/h) and high hydrogen yields (HY = 5.8 mol /mol glucose).

5.6- A Relationship between hydrogen and soluble metabolites.

With increasing temperature, concentrations of the VFA (acetate, butyrate and propionate) decreased. The rise in the HY and pH was consistent with decline in the concentration of the VFAs. Low HYs and high H_2 partial pressures are associated with the accumulation of the VFAs (acetate, propionate and butyrate). Under high H_2 partial pressures the aggregated reaction for the anaerobic oxidation of glucose by a multispecies bacterial consortium can be expressed as follows:

$C_6H_{12}O_6 + tH_2O \rightarrow uCH_3COO^- + vCH_3CH_2COO^- + wCH_3CH_2CH_2COO^- + xH^+ + yHCO_3^- + zH_2$.

With the different VFAs being produced in proportions corresponding to u:v:w, where u, v, and w are never zero. For the anaerobic oxidation of glucose, a decline in the values of u, v, and w for VFAs is consistent with a rise in the value of z for H_2 .

In this study , at low HRT (between 1.5 and 1 h) and with substrate concentrations equal to or greater than 10 g/L, the average concentration of acetate, propionate and butyrate were, 2.299 mM, 1.75 mM and 0.84 mM, respectively.

High butyrate and propionate concentrations were generally associated with low HYs. Increasing the HY above the theoretical theshold of 4.0 mol H_2 / mol glucose would require the anaerobic oxidation of acetate, butyrate and propionate in the absence of H_2 consuming bacteria. The anaerobic oxidation of the anions of alkanoic acids such as propionate and butyrate to acetate and H_2 becomes endergonic when the partial pressures of H_2 exceed 4 Pa or the dissolved H_2 concentration exceeds 0.024 lM **(Lee & Zinder, 1988).**

With a reduction in the H_2 partial pressure below 4 Pa, the oxidation of alkanoic acids and acetate becomes exergonic in the absence of H_2 consuming bacteria **(Adams et al., 2006),** and HYs should then exceed the theoretical limit of 4.0 mol H_2 /mol glucose.

A reduction in the H_2 partial pressures within the bioreactor to the levels necessary for achieving HYs equal to or greater than 4.0 mol H_2 /mol glucose represents a major challenge in the design and operation of AFGBs.

5.7- A relationship between pH and soluble metabolites.

Another important factor in metabolic shifting is pH, the monitoring and control of the pH in a H_2 producing reactor is important not only for the control of metabolic pathways **(Lay, 2002)**, but also because pH serves as an inhibition mechanism for methanogens (**Masilela, 2011**).

The choice of pH is important not only for the optimal production of H_2, but also for the production of volatile fatty acids (VFA) and control of bacterial biomass growth. The accumulation of VFAs causes rapid drop in pH which is unfavourable to H_2 production. Moreover, some VFA can be toxic or inhibitory to the H_2- producing microbial population (**Zheng& Yu, 2005**).

As discussed by VanGinkel& Logan (2005b), butyric acid could be more toxic than acetic acid in H_2 fermentation process, although there is no agreed theshold value for shifting from acidogenesis to solventogenesis **(VanGinkel& Logan 2005b).**

The optimum pH reported for solventogenesis is around 4.5 while for acidogenesis, it is 5.5 or higher **(Ferchichi et al., 2005).**

In this study, hydrogen production rate and yield increased with simultaneous increase in pH from 4.5 to 7.8, and after day 3, these observations were consistent with the decrease in all values of the concentration of VFAs.

These results suggest that a change in pH value leads to the change in fatty acids concentration or composition thus driving more NADH for the formation of hydrogen.

Importantly, a change of pH in fermentation system causes the shift of bacterial metabolites, and the carbon flux at high pH value has more trends to production of more acetate and eventually, results increased hydrogen production **(Tang et al., 2008).**

5.8-Syntrophic microcolony model and VFAs:

According to syntrophic microcolony model, a close synergistic relationship among different microbial groups is essential for efficient breakdown of the complex organic compounds. In fact, syntrophic microcolonies provide kinetics and thermodynamic requirements for intermediate transference and therefore efficient conversion **(Schink & Thauer, 1988).**

Synergistic requirements would drive bacteria to form granules, in which different species function in a synergistic way and can easily survive. This model in H_2 production process is associated with improved or more efficient substrate utilization.

In addition the presence of individual species may provide with crucial metabolic functional characteristics. These microorganisms can be easily obtained in natural environment where they co-exist, for example in sewage sludge.

In this study claims that sucrose substrate is converted by hydrogen producing microorganisms within the granule boundary layer and organic acids (acetate) are produced in the process, the presence of acetogens within the granule boundary layer could facilate oxidation of acetate to H_2 under favorable bioreactor conditions (e.g very low hydrogen partial pressure). VFAs which are produced are transported by random diffusion in all directions and thus penetrate the acetogens cluster within the granule, and thus converted to hydrogen since methanogens were successfully inhibited.

The produced H_2 is stripped away from the granule by high de-gassed effluent recycle rate from the bulk liquid. Indeed, in this study the effect of increased effluent recycle rate, thermophilic temperature and increased bacterial biomass resulted to thermodynamically favorable conditions within the reactor that facilitated high hydrogen production rate and yield.

Chapter VI

Conclusion and Further suggestions

6.1-Conclusion.

This study achieved simultaneously increase HY and HP when the bioreactor system and operational conditions had been modified in two significant ways:

Firstly, the total volume of the bioreactor system relative the effluent volume recycles flux was reduced substantially.

Secondly, following the reduction in the volume of the bioreactor system, the effluent recycle rate for a bioreactor with a working volume of 3.3 L was maintained at 3.6 L / min and the dilution rate was increased. All these modifications resulted in an increase in the HY (5.8 mol H_2 /mol glucose) confirming that high HYs and HPs could be simultaneously achieved. In addition, when the temperature was high at high influent flow rates and at high effluent recycles rates the following results where obtained:

(1) High HYs and HPs were simultaneously achieved.

(2) Volatile fatty acids (VFAs) such as acetate, butyrate and propionate decreased, indicating that at temperatures greater than 55^0C under these bioreactor operation conditions the oxidation of acetate, butyrate and propionate became thermodynamically favourable.

Carbon balance analysis in terms of sucrose concentration in influent and effluent streams confirmed that VFAs oxidation was taking place at temperatures greater than 55^0C. Under these operational conditions VFAs were being oxidized to hydrogen. This result is consistent with further experimental results that gave HYs greater than 4 mol H_2 / mol glucose.

6.2- Further suggestions.

With the almost complete oxidation of acetate, butyrate and propionate, this bioreactor operational system brings about the almost complete conversion of hexose substrate into bacterial biomass, H_2 and CO_2.

Sucrose conversion efficiency will be increased if a system consisting series of connected small boioreactors would be designed. The effluent overflow of the first bioreactor is passed though from the first to the next in the series. Each bioreactor in the

series will be connected to gas disengager with the ratio of degassed effluent recycling rates to bioreactor volume not being less than 1.0. This will result in the complete conversion of hexoses to into bacterial biomass, H_2 and CO_2. The effluent output of last bioreactor will contain no VFAs, only bacterial biomass.

The bacterial biomass in the effluent of the final bioreactor can be used as the feed stock for the production of methane in an upflow anaerobic sludge bed bioreactor. This operational system allows for high rate conversion of hexose into H_2 CH_4 and CO_2.

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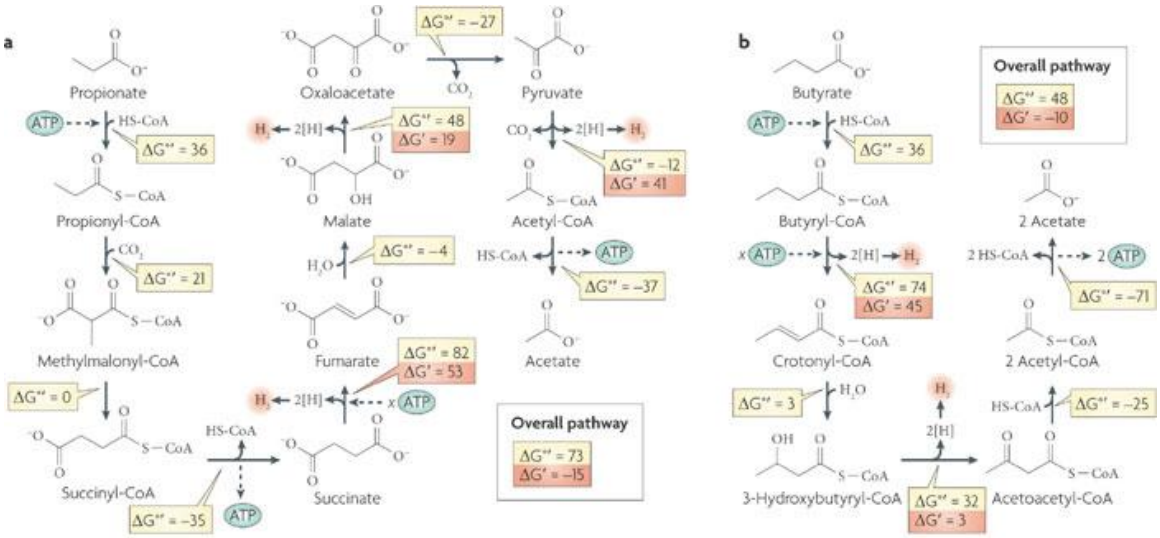
Appendices:

Appendix1- Oxidation of volatile fatty acids metabolic pathways



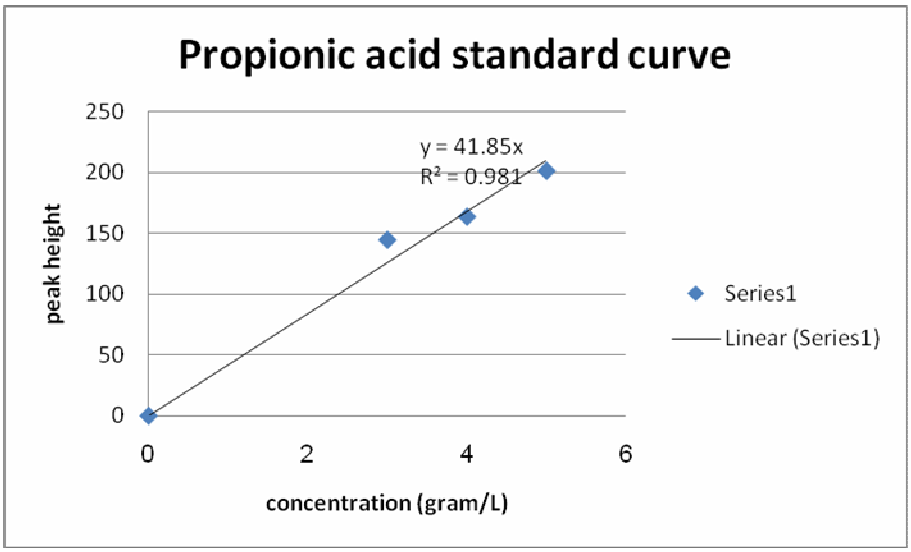
Appendix 2- Composition of endomedia:

A modified Endo medium formulation (Endo et al., 1982) used in this study with some modifications.

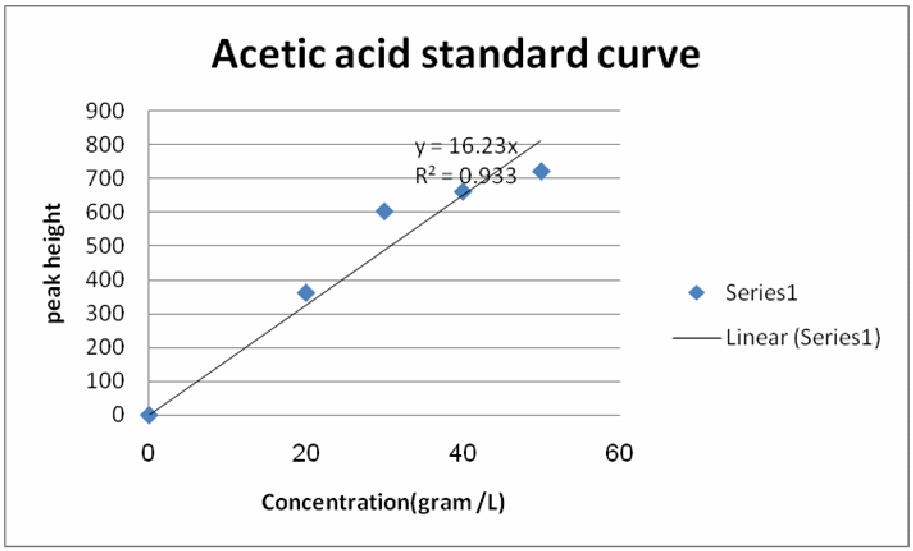
Chemical	Components g/L
Sucrose	17.63
NaHCO3	3. 36
NH4HCO3	3.490
MnSO4	0.015
CaCl2	0.2
K2HPO4	0.699
NaHCO3	3.36
MgCl2 6H2O	0.015
FeSO4 7H2O	0.0225
CuSO4 5H2O	0.005
CoCl2 H2O	1.24 x 10-4

Appendix 3- Standard curves:

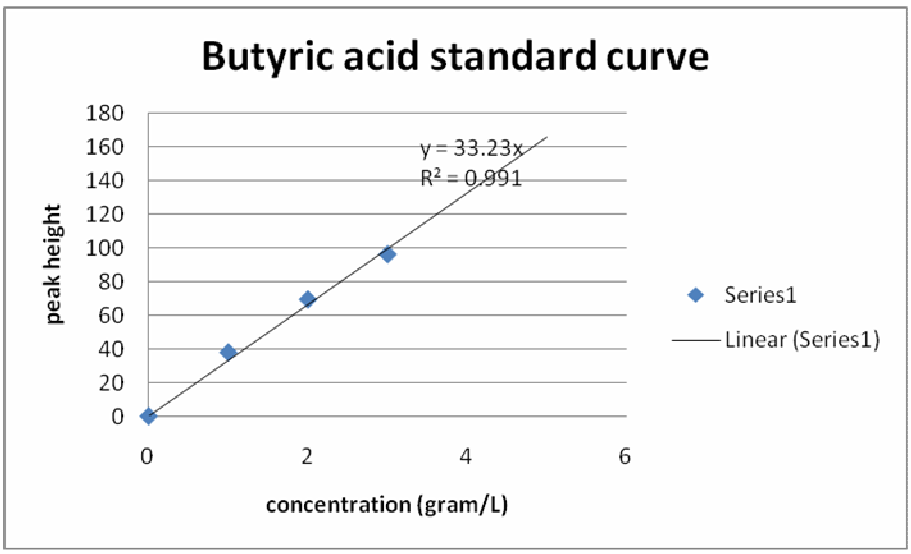
3.1- Propionic acid standard curve.



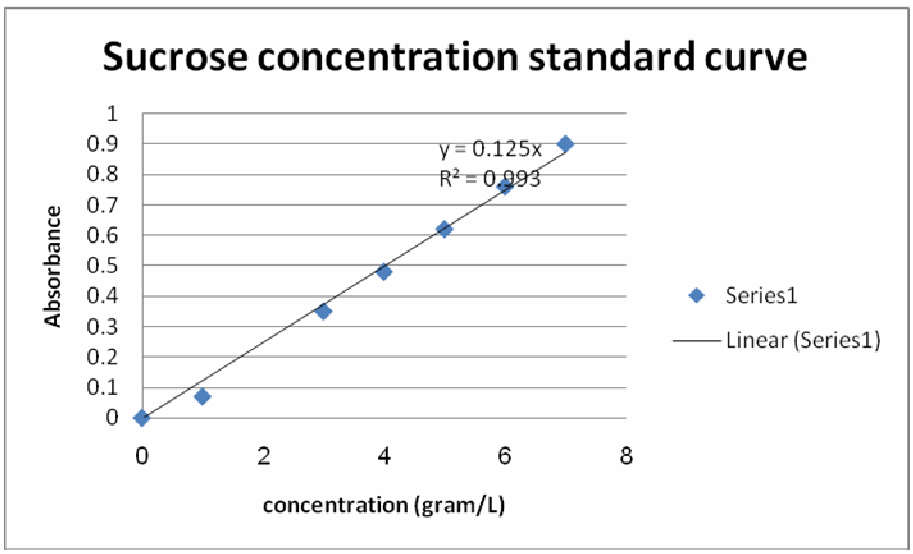
3.2- Acetic acid standard curve.



3.3- Butyric acid standard curve.



3.4- Sucrose acid standard curve.



Appendix 4- Oxidation of volatile fatty acids and hydrogen partial pressure

Appendix 4.1: The effect of H_2 partial pressure and temperature on ΔG for anaerobic acetate oxidation

Hydrogen Partial Pressure (Pa)

T	ΔG	0.1	1	5	10	20	30	60
25	104.76	-37.21	-14.38	1.58	8.45	15.33	19.35	26.22
30	102.49	-41.86	-18.64	-2.42	4.57	11.56	17.08	24.90
35	100.13	-46.60	-23.00	-6.51	0.59	7.70	11.85	18.95
40	97.77	-51.34	-27.00	-10.60	-3.38	3.83	8.06	15.28
45	95.41	-56.08	-31.72	-14.69	-7.36	-0.03	4.26	11.60
50	93.05	-60.83	-36.08	-18.79	-11.34	-3.89	0.47	7.92
55	90.68	-65.57	-40.44	-22.88	-15.31	-7.75	-3.32	4.24
60	88.32	-70.31	-44.80	-26.97	-19.29	-11.61	-7.12	0.56
65	85.96	-75.05	-49.16	-31.06	-23.27	-15.47	-10.91	-3.12
70	83.60	-79.80	-53.52	-35.15	-27.24	-19.33	-14.71	-6.80

Gibbs Free Energy (KJ/mol)

Acetate= 50 mM

HCO_3 = 84 mM

H^+ = pH 7.0

Minimum quantum of Gibbs free energy for ATP production "-20 KJ/mol

Bioreactor operational parameters for thermodynamic feasibility for dark anaerobic acetate oxidation

Appendix 4.2: The effect of H_2 partial pressure and temperature on ΔG for anaerobic acetate oxidation

Hydrogen Partial Pressure (Pa)

T	ΔG	0.1	1	5	10	20	30	60
26	76.30	-38.94	-21.81	-9.85	-4.69	0.46	3.48	8.63
30	74.39	-42.78	-25.37	-13.20	-7.96	-2.72	1.57	5.59
35	72.44	-46.66	-28.97	-16.60	-11.27	-5.94	-2.83	2.60
40	70.48	-50.55	-32.57	-20.00	-14.58	-9.17	-6.00	-0.59
45	68.53	-64.44	-36.17	-23.40	-17.90	-12.40	-9.18	-3.68
50	66.67	-58.33	-39.77	-26.80	-21.21	-15.62	-12.35	-6.77
55	64.62	-62.21	-43.37	-30.20	-24.52	-18.85	-16.53	-9.86
60	62.66	-65.10	-46.97	-33.59	-27.84	-22.08	-18.71	-12.95
65	60.71	-69.99	-50.57	-36.99	-31.15	-25.30	-21.83	-16.04
70	58.75	-73.88	-54.17	-40.39	-34.46	-28.53	-25.06	-19.13

Gibbs Free Energy (KJ/mol)

Propionate = 50 mM

Acetate = 4 mM

HCO_3 = 84 mM

H^+ = pH 7.0

Minimum quantum of Gibbs free energy for ATP production "-20 KJ/mol

Bioreactor operational parameters for thermodynamic feasibility for dark anaerobic acetate oxidation

Appendix 4.3: The effect of H_2 partial pressure and temperature on ΔG for anaerobic butyrate oxidation

Hydrogen Partial Pressure (Pa)

T	ΔG	0.1	1	5	10	20	30	60
25	48.24	-42.60	-31.18	-23.20	-19.76	-16.33	-14.32	-10.88
30	44.93	-47.43	-35.82	-27.71	-24.22	-20.72	-17.63	-10.88
35	41.62	-52.26	-40.47	-32.22	-28.67	-25.12	-23.04	-19.49
40	38.31	-57.10	-45.11	-36.73	-33.12	-29.51	-27.40	-23.79
45	35.00	-61.93	-49.75	-41.24	-37.57	-33.90	-31.76	-28.09
50	31.68	-66.77	-54.39	-45.75	-42.02	-38.30	-36.12	32.39
55	28.37	-71.60	-59.04	-50.56	-45.47	-42.59	-40.48	-36.70
60	25.06	-76.44	-53.68	-64.76	-50.93	-47.09	-44.84	-41.00
65	21.75	-81.27	-68.32	-59.27	-55.38	-51.48	-49.20	-45.30
70	18.44	-86.10	-72.97	-63.78	-59.83	-55.87	-53.66	-49.60

Gibbs Free Energy (KJ/mol)

Butyrate = 50 mM

Acetate = 4 mM

HCO_3 = 84 mM

H^+ = pH 7.0

Minimum quantum of Gibbs free energy for ATP production

"-20 KJ/mol

Bioreactor operational parameters for thermodynamic feasibility for dark anaerobic acetate oxidation

Appendix 6: Chromatoghraphy curves:

WorkDAD---REPORT

General Inforamtion

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Experiment Condition

Material:	Hyper 0DS2 C18	Flowrate:	1.0 ml/min
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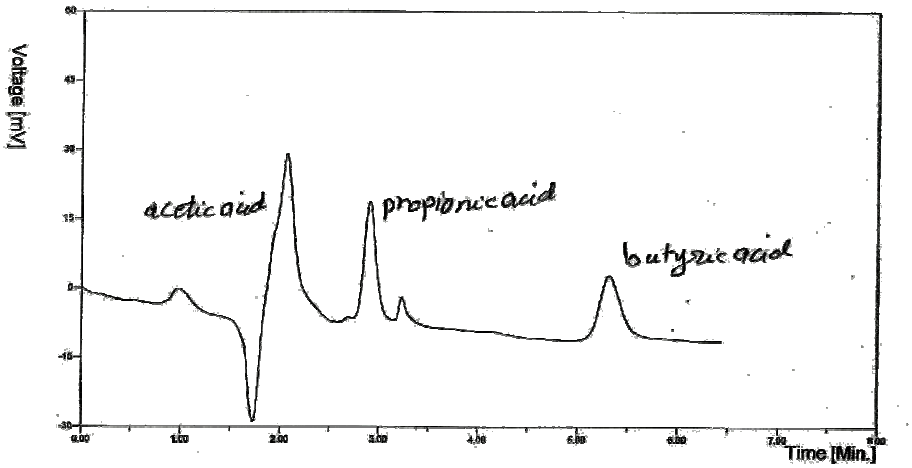
Length:	250 mm	Detector:	DAD
Diameter:	4.6 mm	Volume:	10 ul



WorkDAD---REPORT

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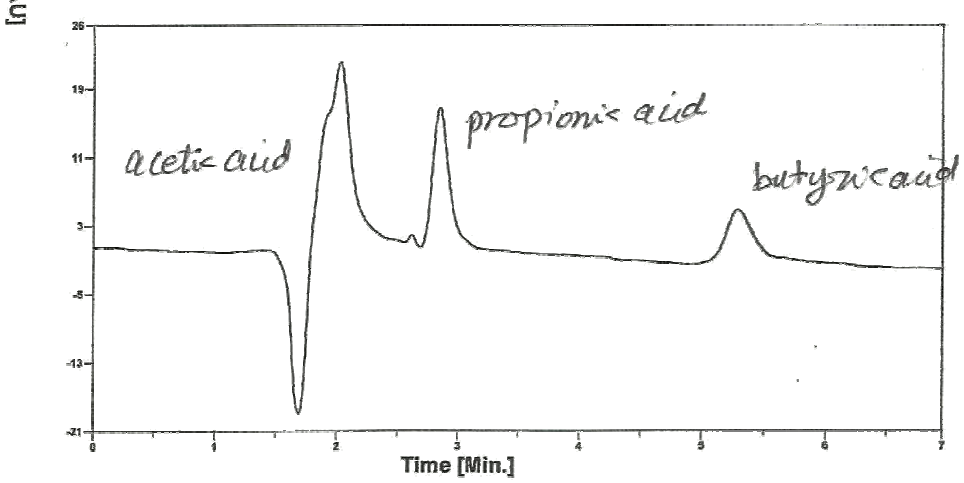
Operator:Tom

Material:Hyper 0DS2 C18	Flowrate:1.0 ml/min
Mobil Phase:MeOH	Pressure:10.0 MPa
Column Length:250 mm	Detector:DAD
Column Diameter:4.6 mm	Sample Volume:10 ul



Calculations

Substrate conversion rate:

Substrate utilized= Sucrose con. In the feed as measured by analysis- sucrose con. In the effluent as measured by analysis.

But because not all that quantity is converted into hydrogen, this con. is multiplied by the % of hydrogen in the total gas as measured by volumetric analysis.

The quantity of substrate converted into hydrogen from this calculation is divided by the con. Of sucrose in feed calculated according to the rate of feeding /h and this would be the substrate conversion rate.

Hydrogen productivity (mole/h):

From this equation:

$$\frac{\Delta H_2}{\Delta t} = \frac{p_a \left[\%H_2{}^{vm} G_T \right]}{RT_a}$$

Hydrogen yield:

Then hydrogen productivity value is divided by sucrose calculated from the feed multiply by the conversion rate and then divided by 2 because each mole sucrose equal to 2 moles glucose and that would be the yield (mole hydrogen/mole glucose).

Bioreactor elements and dimensions:

	Radius(cm)	Height(cm)	Volume(L)
Conical diffuser	4	15	0.75L
Bioreactor(B)	4	20	1L
Disengage(G)	3	54	1.54L
Pipes(P)	1.25	155	0.76L

Total volume= B+G+P=3.3L

Appendix 7: Glossary of terms.

Anaerobic digestion: Decomposition of biological wastes by micro-organisms, usually under wet conditions, in the absence of air (oxygen), to produce biogas.

Biofuel: Fuel produce directly or indirectly from biomass. The term biofuel applies to any solid, liquid, or gaseous fuel produced organic (once living) matter. The word biofuel covers a wide range of products, some of which are commercially available today, and some of which are still in the research and development phase.

Biogas: A combustible gas derived from decomposing biological waste under anaerobic condition. Biogas normally consists of 50-60% methane, 25-50% carbon dioxide, and other possible elements such as nitrogen, hydrogen or oxygen.

Biomass: Organic matter available on a renewable basis. Biomass includes forest and mill residues, agricultural crops and wastes, wood and wood wastes, animal wastes, livestock operation residues, aquatic plants, fast-growing trees and plants, and municipal and industrial wastes.

Bioreactor: A bioreactor is a vessel in which a biochemical process occurs. This usually involves organisms or biochemically active substances derived from such organisms.

Charcoal: Solid residue derived from carbonization distillation, pyrolysis, and torrefaction of fuelwood.

Combustion: The transformation of biomass fuel into heat, chemicals, and gases through chemical combination of hydrogen and carbon in the fuel with oxygen.

Digester: An airtight vessel or enclosure in which bacteria decompose biomass in wet Condition to produce biogas.

Fuel cell: A fuel cell is a device that converts the chemical energy from a fuel into electricity through a chemical reaction with oxygen or another oxidizing agent. Hydrogen is the most common fuel, but hydrocarbons such as natural gas and alcohols like methanol are sometimes used. Fuel cells are different from batteries in that they require a constant source of fuel and oxygen/air to sustain the chemical reaction; however, fuel cells can produce electricity continually for as long as these inputs are supplied.

Effluent: Effluent liquid or gas discharge from a process or chemical reactor, usually containing residues from that process.

Energy crops: Crops grown specifically for their fuel value. These include food crops such as corn and sugar-cane, and non-food crops such as poplar trees and switchgrass.

Feedstock: A feedstock is any biomass resource destined for conversion to energy or biofuel. For example, corn is a feedstock for ethanol production, soybeans oil may be feedstock for biodiesel and cellulosic biomass has the potential to be a significant feedstock source to bioethanol.

Fermentation: Conversion of carbon containing compounds by microorganisms for production of fuels and chemicals such as alcohols, acids or energy-rich gases. It is a biochemical reaction that breaks down complex organic molecules (such as carbohydrates) into simpler materials (such as ethanol, carbon dioxide, and water). Bacteria or yeast can ferment sugars to bioethanol.

Fossil fuel: solid, liquid, or gaseous fuels formed in the ground after millions of years by chemical and physical changes in plant and animal residues under high temperature and pressure. Oil, natural gas, and coal are fossil fuels.

Greenhouse effect: The effect of certain gases in the Earth□s atmosphere in trapping heat from the sun.

Hydrocarbons: Any chemical compound containing hydrogen, oxygen, and carbon Hydrogen: Simple molecule conceivable, with a molecular formula of H_2. Gaseous fuel that can be produced from fossil fuels, biomass and electricity.
Methane: Methane is a combustible chemical compound with the molecular formula CH_4. It is the principal component of natural gas.

Organic matter: Matter that comes from once living-living organism.

Particulate: A small, discrete mass of solid or liquid matter that remains individually dispersed in gas or liquid emissions. Particulate take the form of aerosol, dust, fume, mist, smoke, or spray. Each of these forms has different properties.

Sludge: Sludge is formed in the reaction basin during biological waste water treatment process and separated by sedimentation. Sludges can be converted into biogas via anaerobic digestion.