SUSTAINABLE PRODUCTION OF HYDROGEN AND CHEMICAL COMMODITIES FROM BIODIESEL WASTE CRUDE GLYCEROL AND CELLULOSE BY BIOLOGICAL AND CATALYTIC PROCESSES

Biniam Taddele Maru

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Biniam Taddele Maru

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Thesis

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Supervised by

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To my Father Girazmach Taddele Maru, grandparents Fitewerrari Bitsue WeldeGewergis and Weizero Bafena Atsibeha.

SUMMARY

Energy is a critical input to the functioning of today's society and economy. Our society highly demands sufficient and uninterrupted supply of energy. This high demand is impossible to satisfy through traditional fossil energy sources. Currently, traditional fossil energy sources such as oil are expensive and at the same time scarce to meet the growing demand. Accordingly, the gap between supply and growing demand for energy needs an alternative renewable energy sources.
Renewable energy sources should incorporate the traditional energy sources to be more sustainable. Thus, we must employ new efforts to make the alternative sources to avoid the increasing risk of supply disruptions, price volatility, air pollution, climate change, and global impact. One product that can fulfill this requirement is hydrogen. Hydrogen is a clean energy carrier, sustainable and can be produced from any primary energy source that holds great promise for a secure supply of energy and that will reduce the effects of climate change.

Hydrogen has a high-energy yield (122 kJ/g), which is about 2.75 times greater than that of hydrocarbon fuels. Untimely energy production from sustainable hydrogen sources has the advantage of closing a cycle in which carbon dioxide is not released while obtaining net energy flows. This fact is the significant contribution of current fossil fuel based energy sources to anthropogenic climate change. Furthermore, H_2 has many applications mostly in industrial sectors such as chemical plants and food production. There is also high demand for its use in fertilizers. However, these days, 96% of the total hydrogen production is mainly based on the fossil fuels, releasing carbon dioxide and consequently is not sustainable. However, the biological H_2 production from bacteria satisfies these requirements. Biological H_2 production delivers clean H_2 with an elegant and simple technology and is more suited for the conversion of waste organic matters in small-scale applications as compared to the other thermo chemical processes. In addition, biohydrogen production has advantages mainly due to simpler technology operated at ambient temperature and atmospheric pressure, higher evolution rate of H_2 and a wide spectrum of substrate utilization. The major deterrent of this biohydrogen production process stems from its lower achievable yields. This yield appears too low to be economically viable as an alternative to the existing chemical or electrochemical processes of H_2 generation.

Thus, this problem needs to be addressed in optimizing the microbiological and catalytic processes to meet higher yields. This makes it competent with specific needs for fuel flexibility.
Besides, finding new substrates and new biological activities is decisive to make more economically feasible resources for hydrogen production. In response to the challenges of finding renewable and economically feasible sources and processes of hydrogen production, this thesis investigates microbial hydrogen production by anaerobic mesophilic and thermophilic microorganisms via dark fermentation from biodiesel waste crude glycerol (vastly abundant, cheap and renewable inevitable byproduct of biodiesel manufacturing process) and cellulose (abundant and cheap

renewable resources from agricultural and wood industries).

Therefore, the main aim of this thesis is to contribute new understanding on the production of H_2 for energy from glycerol and cellulose sources using highly productive microorganism for optimal H_2 production and amendment to dark fermentation for energy and environmentally benign H_2 production processes.

*This thesis has eight chapters. A general introduction covering the different topics of the thesis is presented in **Chapter 1**. **Chapter 2** presents a review of hydrogen production potential of crude glycerol. **Chapters 3, 4** & **5** are related the investigation and selection of high H_2 yielding microorganisms from glycerol. **Chapter 5**, deals with improvement on dark fermentation from glycerol using different carrier assisted materials, while, chapter 6 and 7 comprise the studies carried out on applying biodiesel waste (crude glycerol) and WSFs from catalytic degrading of a cellulose use for useable product and H_2 production.*

*Specifically outlining the works achieved in this thesis**: chapter 2** assesses the current relationship between the market, availability and production of both biodiesel and glycerol. Besides, it also discusses the influence of the growing biodiesel production on the commercial prices of glycerol. The characteristics and production of crude glycerol generated from biodiesel manufacturing processes in general and in particular, what is used in this thesis as case study is discussed briefly. Additionally, it presents the potential of crude glycerol as a carbon source for biohydrogen. Previous studies on biohydrogen production and biochemical from pure glycerol and crude glycerol as substrate is also discussed. Accordingly, these studies are used to compare with the work done in this thesis using different mesophilic and thermophilic strains. An overview on the possible metabolic pathways and routes of glycerol biochemical transformation is provided in this thesis. The chapter also briefly discusses the shortcomings of crude glycerol bioconversion to hydrogen production and suggests its improvement methods. Finally, it lays out potential future research areas that need further investigation.*

***Chapter 3** explains the hydrogen production from glycerol by dark fermentation using three strains of bacteria: namely, Enterobacter spH1, Enterobacter spH2, and Citrobacter freundii H3 and a mixture thereof (1:1:1). The study findings show that when an initial concentration of 20 g/L of glycerol was used, all three strains and their mixture produced substantial amounts of hydrogen ranging from 2400 to 3500 mL/L, being highest for C. freundii H3 (3547 mL/L) and Enterobacter spH1 (3506 mL/L). The main nongaseous fermentation products were ethanol and acetate, albeit in different ratios. For Enterobacter spH1, Enterobacter spH2, C. freundii H3, and the mixture (1:1:1), the ethanol yields (in mol EtOH / mol glycerol consumed) were 0.96, 0.67, 0.31, and 0.66, respectively. Compared to the individual strains, the mixture (1:1:1) did not show a significantly higher hydrogen level. This indicates that the absence of synergistic effect. Enterobacter spH1 was selected for further investigation because of its higher yield of hydrogen and ethanol.*

***In chapter 4**, the production of biohydrogen from glycerol, by the hyperthermophilic bacterium Thermotoga maritima DSM 3109, was investigated in batch and chemostat systems. T. maritima converted glycerol to mainly acetate, CO_2 and H_2. Maximal hydrogen yields of 2.84 and 2.41 H_2 per glycerol were observed for batch and chemostat cultivations, respectively. For batch cultivations: i) hydrogen production rates decreased with increasing initial glycerol concentration, ii) growth and hydrogen production was optimal in the pH range of 7-7.5, and iii) a yeast extract concentration of 2 g/L led to optimal hydrogen production. Stable growth could be maintained in a chemostat, however, when dilution rates exceeded 0.025 h^{-1} glycerol conversion was incomplete. A detailed overview of the catabolic pathway involved in glycerol fermentation to hydrogen is presented for T. maritima. Based on comparative genomics the ability to grow on glycerol can be considered as a general trait of Thermotoga species.*

***Chapter 5** provides an improvement of the dark fermentation of glycerol by surface immobilization of co-culture (Enterobacter spH1 and Citrobacter freundii H3) on assisted carriers. Four different carriers were employed and such as maghemite (Fe_2O_3), activated carbon (AC), silica gel (SiO_2) and alumina (γ-Al_2O_3). The effect of the presence of iron was studied by its impregnation over AC and SiO_2. The glycerol conversion and the maximum H_2 production ($P_{max,H2}$), H_2 production rate ($R_{max,H2}$) , H_2 yield (Y_{H2}) were dependent on the specific surface area (S_{BET}) of the support and the presence iron species. The order of the maximum H_2 production was: Fe/AC (S_{BET}= 736 m^2/g)> AC (S_{BET}= 1195 m^2/g)> Fe/SiO_2 (S_{BET}= 440 m^2/g)>SiO_2 (S_{BET}= 685 m^2/g)>Fe_2O_3 (S_{BET}= 205 m^2/g)> γ-Al_2O_3 (S_{BET}= 253 m^2/g)> Free Culture (FC). The glycerol conversion in all cases was higher than that obtained from FC. The metabolites were mainly composed of 1,3-propanediol, ethanol, lactate, H_2 and CO_2. A progressive enhancement in the H_2 production was clearly visible comparing the Fe_2O_3, Al_2O_3, SiO_2 and AC supports. The H_2 production on iron impregnated AC and SiO_2 supports was enhanced comparing with the production achieved with the correspondent bare supports. These results indicate that support enhance the productivity of H_2. This may be due to specific surface area attachment, biofilm formation of the microorganism and hydrogenase enzyme activation by iron species.*

***Chapter 6** shows, how an application of waste glycerol from biodiesel (crude glycerol) as a substrate for H_2 production using a mixed culture of Enterobacter spH1 and Escherichia coli CECT432. Enterobacter spH1 was selected as the best hydrogen and ethanol producer in an earlier comparative study (chapter 3). The same procedure as in chapter 3 was followed for making a selection between the strains of E. coli CECT432, E. coli CECT434 and Enterobacter cloacae MCM2/1. E. coli CECT432 was selected due to its higher productivity of H_2 (1307 mL/L). The co-culture of Enterobacter spH1 and E. coli CECT432 was expected to have a higher productivity of H_2: i) similarity of fermentation end product formation such as ethanol and especially small amount of 1,2-propanediol, ii) co-culture of these strains may simultaneously metabolize the impurities present in crude glycerol. Indeed a microbial co-culture (1:1) of Enterobacter spH1 and E.coli*

CECT432 showed a higher H_2 productivity (4767 mL/L) from pure glycerol (220.1 mM). This synergistic effect of the co-culture was also tested for H_2 production using waste glycerol from biodiesel. The composition of the crude glycerol was investigated and found to consist of (w/v): glycerol 47.5%, water 40.5%, ash content 4.8% and Material Organic Non-Glycerol (MONG) 7.2%. The amount of total soluble organic carbon (TOC) in the crude glycerol was 316.6 g/L.

A maximum H_2 yield and ethanol yield of 1.21 and 1.53 mol/mol glycerol was obtained on the waste glycerol, respectively. These yields are the highest reported to date using mesophilic strains. This indicates that the co-culture has a strong synergistic effect.

*The use of crude glycerol was also tested for T. maritima. It showed growth. The yield observed was 3.21 mol H_2 /mol glycerol and the rate of H_2 was 2.38 mmol / L*h .These yield and rate were higher than the for pure glycerol.*

The ability to produce H_2 production without prior purification of the waste glycerol is attractive because it avoids extra costs.

*In **chapter 7** A two-step integrated system consisting of heterogeneous catalysis followed by dark fermentation was investigated for the production of biohydrogen. Hydrolysis of cellulose in the aqueous phase was carried out in an autoclave reactor with ZrO_2 catalysts modulated by three different promoters: sulfate, fluoride, and phosphate. The resultant water-soluble fractions (WSFs) derived from the catalytic cellulose hydrolysis were then submitted to dark fermentation without any additional treatment. The dark fermentation step tested three different microorganisms, Enterobacter spH1, Citrobacter freundii H3 and Ruminococcus albus DMS 20455, for their ability to produce H_2 from cellulose and glucose and the liquid product derived from cellulose hydrolysis. The two enteric bacteria (Citrobacter freundii H3 and Enterobacter spH1) effectively fermented the WSFs, producing H_2 and other organic compounds as metabolites. For the WSFs derived from cellulose hydrolysis with ZrO_2-P and ZrO_2-S catalysts, Enterobacter spH1 exhibited values of 1.40 and 1.09 mol H_2/mol hexose, respectively.*

The research that underpins this thesis, provides new insights on: 1) the fermentative behavior of anaerobic mesophilic and thermophilic hydrogen producing organisms from glycerol and cellulose, 2) how to integrate the dark fermentative system with catalytic degrading of a cellulose and use biodiesel waste (crude glycerol) for useable product and H_2 production, and 3) the advantage of using a solid support carriers to increase surface immobilization and ultimately increasing hydrogen production. These insights will contribute to the general understanding of microbial hydrogen production, application of waste management disposal, and hopefully it will lead to sustainable hydrogen production from biodiesel wastes crude glycerol in the future

RESUMEN

La obtención de nuevas Fuentes de energía es un punto crítico para el funcionamiento de la sociedad y la economía actual. El avance de nuestra sociedad depende de una suficiente e ininterrumpido suministro de energía. Las fuentes de energía fósiles tradicionales como el petróleo son limitadas, los precios muy elevados y la brecha creciente entre el aumento de la demanda y menor oferta, en un futuro no muy lejano, tendrá que ser suplida, cada vez más, por las fuentes de energía alternativas y renovables. Debemos esforzarnos por hacernos más sostenibles para gestionar eficazmente el creciente riesgo de interrupciones en el suministro y la volatilidad de los precios, así como reducir sustancialmente la contribución, de los sistemas energéticos actuales, reduciendo la contaminación atmosférica, el cambio climático y los impactos que están asociados.

En el frente de la tecnología, el hidrógeno, un portador de energía limpio y sostenible que se puede producir a partir de cualquier fuente de energía primaria, es una opción atractiva. El hidrógeno es por consiguiente una futura promesa para aliviar de una manera muy singular nuestras preocupaciones sobre la seguridad del abastecimiento y del cambio climático. El hidrógeno se cita a menudo como combustible limpio, "verde" del futuro. Este tiene un alto rendimiento de energía (122 kJ/g), que es aproximadamente 2,75 veces mayor que el de los combustibles de hidrocarburos. La producción de energía a partir de fuentes sostenibles de hidrógeno tiene la ventaja de cerrar un ciclo en el que no se libera dióxido de carbono, mientras se obtienen flujos netos de energía; un hecho importante acerca de la contribución significativa de las actuales fuentes de energía basadas en combustibles fósiles al cambio climático antropogénico. Además, el hidrógeno tiene muchas aplicaciones, en su mayoría en los sectores industriales, tales como en la fabricación de productos químicos y la producción de alimentos y tiene una gran demanda para su uso en fertilizantes.

Para que el hidrógeno sea una fuente de energía ambientalmente limpia y sostenible, que pueda ser alternativa a los combustibles fósiles, tanto en su origen como en producción, tiene que ser producido a partir de recursos renovables. Sin embargo, el 96% de la producción total de hidrógeno se hace a partir principalmente de combustibles fósiles liberando dióxido de carbono y por tanto, no puede ser sostenible. Al contrario de éstas, la producción biológica de hidrógeno a partir de bacterias satisface estos requisitos. La producción biológica de hidrógeno da lugar a hidrógeno limpio con una tecnología elegante y simple. Este proceso es más adecuado para la conversión de la materia orgánica de desecho en aplicaciones a pequeña escala en comparación con otros procesos termoquímicos. Además, la producción de biohidrógeno tiene ventajas, debido, principalmente, a la tecnología más simple, que opera a temperatura ambiente y presión atmosférica, una mayor tasa de evolución de hidrógeno y un amplio espectro de utilización de sustrato. El principal elemento de disuasión de este proceso de producción de biohidrógeno deriva de sus inferiores rendimientos alcanzables. Este rendimiento parece demasiado bajo para ser económicamente viable como una alternativa a los procesos químicos o electroquímicos existentes de generación de hidrógeno. Por lo tanto este problema debe ser abordado en la optimización de los procesos microbiológicos y mediante catálisis para cumplir con mayores rendimientos que sean competentes con las necesidades específicas de flexibilidad de combustible.

Para que este proceso sea más económicamente viable, se deben encontrar nuevos sustratos y nuevas actividades biológicas de mejora. En respuesta a los retos de la búsqueda de fuentes y procesos de producción de hidrógeno renovable y económicamente posible, en esta tesis se ha investigado la producción de hidrógeno microbiano por microorganismos mesófilos y termófilos anaerobios mediante la fermentación oscura del glicerol bruto contenido en residuos de biodiesel (muy abundante, barato, renovable e inevitable subproducto del proceso de fabricación del biodiesel) y celulosa (abundante y barata para ser utilizada como recurso renovable de la agricultura y las industrias de la madera).

El objetivo principal de esta tesis es contribuir a una nueva comprensión de la producción de hidrógeno para producir energía a partir de fuentes de glicerol y celulosa, utilizando microorganismos altamente productivos.

Esta tesis se divide en ocho capítulos. Una introducción general sobre los diferentes temas de la misma se presenta en el capítulo 1. El ***capítulo 2*** *presenta una revisión del potencial de producción de hidrógeno a partir de glicerol en bruto. Los capítulos* ***3, 4 y 5*** *están relacionados con la investigación y selección de microorganismos de alto rendimiento para la producción de* H_2 *a partir de glicerol. El* ***capítulo 5****, se refiere a la mejora de la fermentación oscura a partir de glicerol utilizando diferentes materiales como soportes, mientras que, los* ***capítulos 6 y 7*** *comprenden los estudios llevados a cabo en la aplicación de los residuos de biodiesel (glicerol en bruto) y la fracción líquida de la degradación catalítica de celulosa para la producción de hidrógeno y otros productos.*

Delineando específicamente las tareas logradas en esta tesis: el ***capítulo 2*** *analiza la relación existente entre el mercado, la disponibilidad y la producción de biodiesel y glicerol. También se discute la influencia de la creciente producción de biodiesel en los precios comerciales de glicerol. Las características y la producción de glicerol bruto generados en los procesos de fabricación de biodiesel, en general, y en particular lo que se utiliza en esta tesis como estudio de caso se discute brevemente. Además, se presenta el potencial del glicerol en bruto como fuente de carbono para biohidrógeno. Se compilan estudios previos realizados en la producción de biohidrógeno a partir de glicerol puro y glicerol crudo como sustrato y se intenta comparar estos estudios con el trabajo realizado en esta tesis con diferentes cepas mesófilas y termófílas. Se presenta también en esta tesis, una visión general de las posibilidades de las vías de transformación bioquímica del glicerol. Se discuten las deficiencias de la bioconversión del glicerol bruto para la producción de hidrógeno y se sugieren métodos de mejora. Finalmente se decribe decriben nuevas líneas de investigación futura que necesitan ser llevadas a cabo para una mejora global del proceso.*

En el ***capítulo 3****, se estudia la producción de hidrógeno a partir de glicerol mediante la fermentación oscura con tres cepas de bacterias: a saber, Enterobacter SPH1, Enterobacter spH2 y Citrobacter freundii H3 y una mezcla de los mismos (01:01:01). Se encontró que, cuando se utilizó una concentración inicial de 20 g / L de glicerol, las tres cepas y su mezcla produjeron cantidades sustanciales de hidrógeno que van 2400 a 3500 mL / L, siendo la más alta de C. freundii H3 (3,547 mL / L) y Enterobacter SpH1 (3506 mL / L). Los principales productos de fermentación no gaseosos fueron etanol y acetato, aunque en diferentes proporciones. Para Enterobacter spH1, Enterobacter spH2, C. freundii H3, y la mezcla (01:01:01), los rendimientos de etanol (EtOH en moles / mol de glicerol consumido) fueron 0,96, 0,67, 0,31, y 0,66, respectivamente. En comparación con las cepas individuales, la mezcla (1: 1: 1) no mostró un nivel significativamente más alto de producción de hidrógeno, lo que*

indica que no hubo efecto sinérgico. Enterobacter spH1 fue seleccionada para una mayor investigación debido a su mayor producción de hidrógeno y etanol.

*En el **capítulo 4**, se investigó la producción de biohidrógeno a partir de glicerol, por la bacteria hipertermófila Thermotoga maritima DSM3109, en los sistemas por lotes y chemosta. La T. maritima convirtió glicerol principalmente en acetato, CO_2 y H_2. Se observaron rendimientos de hidrógeno máximos de 2.84 y 2.41 moles H_2 por moles glicerol de los lotes y cultivos chemostat, respectivamente. Para cultivos por lotes: i) la tasa de producción de hidrógeno disminuyó al aumentar la concentración de glicerol inicial, ii) el crecimiento y la producción de hidrógeno fue óptima en el intervalo de pH de 7-7,5, y iii) una concentración de extracto de levadura de 2 g/L dio lugar a la producción de hidrógeno óptima. El crecimiento estable podría mantenerse en un chemosta, sin embargo, cuando las tasas de dilución superaron 0,025 h^{-1}, la conversión de glicerol fue incompleta. También se da una descripción detallada de la ruta catabólica utilizada en la fermentación de glicerol a hidrógeno por parte de T. maritima. Basándose en la genómica comparativa, la capacidad de crecer en glicerol puede ser considerada como un rasgo general de las especies Thermotoga.*

*En el **capítulo 5**, una mejora de la fermentación oscura de glicerol fue propuesta mediante inmovilización del co-cultivo (Enterobacter spH1 y C. freundii H3) en diferentes materiales inorgánicos que hace de soportes de dichos microorganismos. Para ello, se emplearon cuatro soportes diferentes: maghemite (Fe_2O_3), carbón activado (AC), gel de sílice (SiO_2) y alúmina (γ-Al_2O_3). El efecto de la presencia de hierro se estudió mediante su impregnación sobre el AC y SiO_2. La conversión de glicerol y la máxima producción de H2 (Pmax, H2), la tasa de producción de H_2 (Rmax, H_2) y el rendimiento de H_2 (YH_2) fueron dependientes de la superficie específica del soporte y de la presencia de especies de hierro. El orden de la máxima producción de H_2 ha sido: Fe / CA (SBET = 736 m^2 / g)> CA (SBET = 1.195 m^2 / g)> Fe/SiO2 (SBET = 440 m^2 / g)> SiO_2 (SBET = 685 m^2 / g)> Fe_2O_3 (SBET = 205 m^2 / g)> γ-Al2O3 (SBET = 253 m^2 / g)> Cultivo Libre (CL). La conversión de glicerol en todos los casos fue superior a la obtenida a partir de CL. El metabolismo se compone principalmente de 1,3-propanodiol, etanol, lactato, H_2 y CO_2. Un aumento progresivo de la producción de H2 se observa claramente comparando el Fe_2O_3, Al_2O_3, SiO_2 y soportes AC. La producción de H_2 en los soportes de AC y SiO_2 impregnados con hierro se ha mejorado en comparación con la producción obtenida con los soportes originales correspondientes. Estos resultados indican que la presencia de hierro en los soportes mejora la productividad de H_2, lo cual puede ser debido a un efecto sineégico entre la fijación específica en la superficie del soporte, facilitando la formación de la biopelículas del microorganismo y/o la activación de la enzima hidrogenasa por dichas especies de hierro.*

***Capítulo 6**. Una aplicación de glicerol en residuos de biodiesel (glicerol bruto) como sustrato se utilizó para la producción de H_2 usando un cultivo mixto de E. coli CECT432 y Enterobacter spH1. El Enterobacter spH1 fue seleccionado como el mejor productor de hidrógeno y etanol a partir de nuestro estudio comparativo anterior (capítulo 2). El mismo procedimiento que en el capítulo 2 fue seguido para la selección entre las cepas de E. coli CECT432, E. coli CECT434 y E. cloacae MCM2/1. La E. coli CECT432 se seleccionó debido a su mayor productividad de H_2 (1306,6 mL/ L). El co-cultivo de Enterobacter spH1 y E. coli CECT432 se propuso para una mayor productividad de hidrógeno debido a la similitud de la formación de productos finales de la fermentación, tales como el etanol y especialmente una pequeña cantidad de 1,2-propanodiol y a que el co-cultivo de estas cepas puede metabolizar simultáneamente*

las impurezas presentes en el glicerol en bruto. Por lo tanto, se diseñó un co-cultivo microbiano (01:01) de E. coli CECT432 y Enterobacter spH1 para tener mayor producción de H_2 (4767.12mL / L) a partir de glicerol puro (220,1 mM). Este efecto sinérgico de co-cultivo se probó para la producción de H_2 con glicerol en residuos de biodiesel, facilitado por una empresa de biodiesel en Barcelona, España. La caracterización del glicerol crudo se investigó a fondo para ver el efecto de la fermentación oscura. Este se compone principalmente de (w / v): glicerol 47,5%, agua 40,5%, contenido de ceniza 4,8% y el material orgánico no-glicerol (MONG) 7,2%. La cantidad de carbono orgánico soluble total (TOC) en el residuo de glicerol fue 316.6 g / L. Se obtuvo un rendimiento más alto de H_2 (Y_{H2}) y de etanol (Y_{EtOH}) de 1,21 y 1,53 moles / mol glicerol, del glicerol en bruto, respectivamente. Los rendimientos obtenidos son de los más altos alcanzados usando cepas mesófilas que se haya informado hasta la fecha. Esto muestra que el co-cultivo tiene un fuerte efecto sinérgico para la producción de H_2. La producción de H_2 lograda sin purificación de los residuos de biodiesel que contiene glicerol es atractiva debido a que se evitan costes adicionales.

*En el **capítulo 7** se investigó un sistema integrado de dos etapas que consiste en la catálisis heterogénea seguido por fermentación oscuro para la producción de biohidrógeno . La hidrólisis de la celulosa en la fase acuosa se llevó a cabo en un reactor autoclave con catalizadores de ZrO_2 modulados por tres promotores diferentes : sulfato , fluoruro , y fosfato. Las fracciones hidrosolubles resultantes derivadas de la hidrólisis de la celulosa catalítica se sometieron a continuación a la fermentación oscura sin ningún tratamiento adicional . La etapa de fermentación oscura estudio la capacidad de producir hidrogeno a partir de celulosa, glucosa y el producto líquido derivado fe la hidrólisis de la celulosa por parte de tres microorganismos diferentes , Enterobacter SPH1 , Citrobacter freundii H3 y Ruminococcus albus DMS 20455. Las dos bacterias entéricas (Citrobacter freundii H3 y Enterobacter SPH1) fermentan de manera efectiva las fases hidrosolubles, produciendo H2 y otros compuestos orgánicos como metabolitos. Para las fases hidrosolubles derivadas de la hidrólisis de celulosa con catalizadores de ZrO_2 -P y ZrO_2 -S, Enterobacter SPH1 exhibió valores de 1,40 y 1,09 mol H_2/mol hexosa , respectivamente.*

La investigación que sustenta esta tesis, proporciona nuevos conocimientos sobre: 1) el comportamiento fermentativo de microorganismos anaerobios mesófilos y termófilos productores de hidrógeno a partir de glicerol y celulosa 2) la forma de integrar el sistema de fermentación oscura con la degradación catalítica de la celulosa y el uso de residuos de biodiesel (crudo glicerol) para la producción de H_2 y otros productos de valor añadido y 3) la ventaja de utilizar soportes portadores sólidos para aumentar la superficie de inmovilización y, finalmente, aumentar la producción de hidrógeno. Estas ideas contribuirán a nuestra comprensión general de la producción microbiana de hidrógeno, su aplicación en la eliminación de residuos, y con suerte, dará lugar a la producción de hidrógeno sostenible, en el futuro, a partir de residuos de biodiesel y celulosa.

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LIST OF ABBREVIATIONS

$R_{max,H2}$	Maximum rate of hydrogen production, mmol /L *h
$R_{max,i}$	Maximum rate of product I formation, mmol /l*h
1.3-PDO	1,3-propanediol
3HPA	3- hydroxypropionaldehyde
AC	Activated carbon
AC-SO3H	Sulfonated Activated-Carbon Catalyst
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
B	Volatile suspended solids, g VSS/l
BDDT	Brunauer, Deming, Deming and Teller
BDP	biodiesel peninsular
BHP	Biological hydrogen production
CDW	Cell dry weight
CG	Crude glycerol
CHP	Combined heat and gas system
DAD	Diode array
DHA	Dihydroxyacetone
DHAK	Dihydroxyacetone kinase
ED	Entner Doudoroff
EM	Embden Meyerhof
FAD	Flavin adenine dinucleotide
FAMEs	Fatty acid ethyl esters ()
Fd	Ferredoxin
Fe/AC	Iron over Activated carbon
Fe/SiO2	Iron over Silica gel
Fe_2O_3	Maghemite
FTIR	Fourier Transformation Infra-red
G	Glycerol concentration, mol
G_0	Initial glycerol concentration, mol
GC	Gas chromatograph
GC-MS	Gas chromatograph mass spectrophotometer
GHG	Greenhouse gas
GldA	Glycerol dehydrogenase
H_2	Cumulative hydrogen, mmol / L_{medium}
HER	Hydrogen evolution rate
H_{max}	maximum cumulative hydrogen, mmol / L_{medium}
HMF	Hydroxymethylfurfural
HPLC	High performance liquid chromatograph
ICDD	International center for Diffraction date

MCF	Microbial fuel cell
MONG	Material organic non glycerol
MYG	Malt yeast extract glycerol /Glucose medium
NADH	Nicotinamide adenine dinucleotide
NB	Nutrient broth medium
OD	Optical density
PEP	Phosphoenolpyruvate
PG	Pure glycerol
P_i	Concentration of product formation i, mmol / L
PPDK	Phosphate dikinase
PPO	Pure plant oil
Q	Adsorption capacity
R^2	Coefficient of determination
RE	Renewable energy
RID	Refractive index
$R_{max,\,S}$	maximum rate of substrate consumption, mmol / L*h
RT	Room temperature
S	Substrate concentration, mmol / L
S_0	Initial substrate concentration, mmol / L
S_{BET}	Specific surface area
SEM	Scanning electron microscope
SiO_2	Silica gel
S_{max}	Maximum concentration of substrate consumption, mmol / L
SR	Steam reforming
T	Fermentation time, h
TCD	Thermal conductivity detector
TDP	Temperature dependent programming
VFAs	Volatile fatty acids
WAF	Waste animal fats
WEO	World energy outlook
WVO	Waste vegetable oil
X	Cumulative hydrogen, mol
XRD	X-ray diffraction
Y	Substrate yield of metabolite (mol metabolite/mol glycerol)
Y_M	Specific substrate yield
Zr_2O_3-***PS***	Zirconium oxide phosphate
Zr_2O_3-***S***	Zirconium oxide sulfate
γ-Al_2O_3	Alumina
$\Delta P_{max,i}$	Maximum concentration of product i formation, mmol / L
λ	Lag time, h
WSFs	Water-soluble fractions

THESIS AIM AND SCOPE

The use of glycerol as a carbon source for H_2 production using different organisms has yet to be thoroughly examined via dark fermentation. Therefore, significant task remains in identifying and isolating more H_2 -producing strains and forming co-cultures for given media and different fermentation conditions, which could achieve the maximum hydrogen production (3 mol H_2 /mol glycerol).

The overall aim of this thesis was to contribute to new understanding on the production of H_2 for energy from crude glycerol (vastly abundant, cheap and renewable inevitable byproduct of biodiesel manufacturing process) and catalytically degraded cellulose (abundant and cheap renewable resources from agricultural and wood industries) using highly productive microorganism for optimal H_2 production and amendment to dark fermentation for environmentally benign H_2 production processes. To achieve this aim the specific tasks and objectives undertaken for this thesis were:

1. Investigate and select high H_2 yielding microorganisms. **(Chapter 3)**

2. Assess the potential of glycerol for producing H_2 under anaerobic conditions (i.e., dark fermentation) using the newly isolated strains *Enterobacter* spH1, *Enterobacter* spH2, *Citrobacter freundii* H3, and their co-culture (1:1:1). **(Chapter 3)**

3. Investigate in detail, biohydrogen production from glycerol by *T. maritima* including the optimum growth parameters and cultivation conditions for *T. maritima as* well as a putative glycerol catabolic pathway leading to hydrogen is presented, and the unusual thermodynamics and biochemistry of high yield H_2 formation from glycerol are discussed. **(Chapter 4)**

4. Develop and assess the improvement on dark fermentation using different support matrices to increase surface immobilization of microorganisms. **(Chapter 5)**

5. Evaluate H_2 production and other valued products from pure glycerol (PG) as a raw material using different strains like *Escherichia coli* CECT432, *Escherichia coli* CECT434 and *Enterobacter cloacae* MCM2/1, in dark fermentation. Compare H_2 and valued by products from pure glycerol (PG) from crude glycerol (CG) using the strain *E. coli* CECT432, and mixture of higher H_2 producer of the three strains *E.coli* CECT432, *E.coli* CECT434 and *Enterobacter* spH1 mixed in a ratio of 1:1. Determine the kinetics of the H_2 production, end metabolites and carbon balance using the crude glycerol and pure glycerol for the co-culture. As well as, characterize of the CG was thoroughly investigated to see the

effect of the impurities on the biohydrogen production and usable value added products. **(Chapter 6)**

6. Investigate the activation effect of zirconium oxide with different acid promoting the catalyst behavior, acidity, and conversion capacity and on the selectivity versus glucose. Then finally study the growing and fermenting capacity as well as hydrogen production and by-product formation of *Enterobacter* spH1 and *Citrobacter freundii* H3 in the products of the reactions using the of the different activated catalysts. **(Chapter 7)**

THESIS OUTLINE

Chapter 1 provides general introductory background of the energy problems related to demand and supply of fossil fuels and their negative environmental impacts. It discusses on addressing this problem by looking for an alternative renewable energy sources. On the frontier fermentative biohydrogen production from renewable primary sources is outlined, as a sustainable method

Chapter 2 presents systematic and comparative review of available reports on bio-hydrogen production from pure glycerol and crude glycerol as a substrate. Hydrogen production potential of crude glycerol, factors affecting the productivity (pH, temperature, pressure and concentration), various pretreatment methods, bioreactor systems used for microbial hydrogen production as well as the glycerol bioconversion potential of different microorganisms was described. Short comings of crude glycerol bioconversion, limitation of studies are discussed in detail and various strategies for improved hydrogen production have been suggested. In addition this chapter presents, the characteristics of crude glycerol generated from BDP biodiesel manufacturing company which we used in this study was reviewed as a case study of glycerol resource.

In **chapter 3** we investigate the potential for H_2 production from pure glycerol using mesophilic microorganisms. This chapter also presents results of a comparative analysis of biohydrogen production and other byproducts of glycerol using *Enterobacter* spH1*, Enterobacter* spH2*, Citrobacter freundii* H3, and mixture thereof (1:1:1) to observe if there is any synergetic increase in H_2 productivity.

In **chapter 4** we investigate the Extreme and hyperextreme microorganisms for their H_2 production potential from biodiesel and pure glycerol. This investigation is based on previous studies that reported *Thermotoga maritima* contains coding sequences for a complete pathway for the uptake and conversion of glycerol, and a positive signal indicating oxidation of glycerol by *T. neapolitana* was found in a microplate assay. However, until now, there has been no research on H_2 production from glycerol by *T. maritima.* Henceforth our study was of the first to examine *T. maritima* for H_2 production from glycerol and discusses the unusual thermodynamics and biochemistry of high yield H_2 formation.

Chapters 5 examine on how different supported materials improved production of biohydrogen and usable chemical products using *mixed cultures of Citrobacter freundii* H3 *and Enterobacter* spH1.

In **chapter 6** we explore the potential of crude glycerol (biodiesel waste containing crude glycerol derived from waste vegetable oil (WVO) and waste animal fat (WAF)) for H_2 production under anaerobic conditions i.e. dark fermentation. Other mesophilic type was used for the production of

H_2 from crude glycerol. This type consists of *E.coli* CECT432, *E.coli* CECT434 and E. *cloacae* MCM2/1. A co-culture of *E. coli* CECT432 and *Enterobacter* spH1 is used also to see if the H_2 production can be increased.

Chapter 7 deals with how to integrate systems of the catalytic process of degrading the cellulose to smaller compounds such as glucose, HMF, cellobiose and ethanol and use this in the system of the dark fermentation for biohydrogen production

Chapter 8 provides general concluding remarks and highlights future research and development works.

CHAPTER 1

1. GENERAL INTRODUCTION

1. General Introduction

1.1 Energy demand, supply and fossil fuels

Energy plays vital role in global economic growth. All our work, leisure, and our economic, social and physical welfare depend on the sufficient, uninterrupted supply of energy. The growths of global population, economic expansion and increased energy-based standards of living [1] have drawn to higher energy demand. In 2008, the total global energy consumption was in the range of 515-530 EJ (1 Exajoules= 1018J) [2,3]. The total world energy requirement is increasing due to population growth, which is estimated to reach to 8.5 billion by 2035 [2]. Consequently, the total world energy consumption is still expected to increase in absolute terms to 700-810 EJ by 2035 [2, 4] which will be a double of 1990's consumption.

Fossil fuels such as oil, coal and natural gas remain the principal sources of energy worldwide and are responsible for up to 85% of world's energy (figure 1.1 [5]). However, many fossil fuels reserves are at their peak of extraction and their production is rigorously controlled by a small cartel of very powerful nations who decide on pricing schedules [6]. Although estimation of the depletion of the fossil fuel sources is difficult, it has been estimated that it will be depleted by the year 2100, which makes the need for alternative fuels solutions [7]. Crude oil production will approach a theoretical depletion near 2060-2070, and the theoretical depletion for natural gas is close for crude oil [8,9]. From these supply and demand observations, our dependence on fossil based energy production is unsustainable [10, 11]. Furthermore, fossil fuels are recognized as nonrenewable sources of energy.

1.2 Environmental effects

The combustion of fossil fuels for energy production, electricity generation, transportation, or other industrial processes releases carbon dioxide (CO_2) and other greenhouse gases into the atmosphere, thereby impacting negatively on the environment [12]. In recent years, global warming and associated climate change have been found to be mainly due to the increase of CO_2 concentration into the atmosphere [13]. For instance, over the last three decades, GHG emissions have increased by an average of 1.6% per year with carbon dioxide (CO_2) emissions from the use of fossil fuels growing at a rate of 1.9% per year [13]. This has become a matter of growing concern all over the World. There has been a significant international effort to support long lasting solutions to reduce anthropogenic greenhouse gases emissions. The Kyoto Protocol adopted in Kyoto, Japan, in 1997 has been seen as an important first step towards a truly global emission reduction regime that will stabilize GHG emissions [12]. In 2010, worldwide GHG increased by 31% against the 1990 levels. However, the signatories of the first commitment period between 2008 to 2012 (37 industrialized countries) have collectively reduced by 22% of the 1990 base levels [14]. Despite all efforts and concerns, the problems with these GHG are far from solved. The

combination of the situation presented above i.e. the pending global energy crisis and environmental impacts from GHG emissions are stimulating a rapid growth in search for alternative energy sources to complement or possibly to substitute the conventional fossil fuels.

1.3 Renewable energy sources

Renewable energy (RE) refers to resources that are replenished in a relatively short period of time. Renewable energy sources include hydropower, wood biomass (used to generate heat and electricity), alternative biomass fuels (such as ethanol and biodiesel), organic wastes (biomass, industrial), geothermal, wind, and solar [2]. These RE sources have great potential to meet energy needs of the future. The use of renewable energy was 1684 million tonnes of oil equivalent (Mtoe) in 2010, accounting for 13% of global primary energy demand (Figure 1) [2,15]).

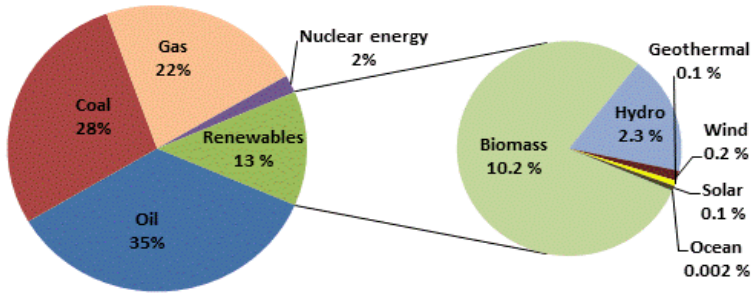

Figure 1.1. The distribution of energy sources and the shares of renewable energy (adapted from [5]).

A lot more is needed to replace fossil fuels, especially when it is compared with the total increasing energy consumption. To facilitate this replacement, an estimate of $6.4 trillion investment in RE production is required over the period from 2012 to-2035 [15]. Furthermore, integration of less mature technologies, including biofuels produced through new processes (also called advanced biofuels or next-generation biofuels), fuels generated from solar energy, solar cooling, ocean energy technologies, fuel cells and electric vehicles, will require continued investments in research, development and demonstration (RD&D), capacity building and other supporting measures [15].

Nuclear power can be considered as an alternative to fossil fuel based energy production particularly because of its high energy output. However, nuclear power generation has significant safety risks both on its operation and disposal of harmful radioactive waste. Accidents that occurred in recent years such as in Fukushima Daiichi, Japan are reminders of the risks associated

with the use of nuclear power plants and a possible deterrent for its expansion at least in the near future [15].

Different countries in the world are supporting a move to RE production. In 2009, the European Union released the Renewable Energy Directive, which set legally binding targets for the share of renewable energy (covering electricity, heat and biofuels) in gross final energy consumption of each member state by 2020, equating to 20% in total [15].
RE sources have a large potential to displace emissions of GHG from the combustion of fossil fuels and, thereby, to mitigate climate change. If implemented properly, renewable energy sources can contribute to improved energy access and diversity, a secure and sustainable energy supply, and a reduction of negative impacts of energy provision on the environment and human health as well as to broad social and economic development.

1.4 Renewable sources: opportunities and challenges for energy production

1.4.1 Direct solar energy

Solar power is a very favorable alternative energy to fossil fuels. It is obtained from the sun and is the most abundant and cleanest renewable source available [16, 17]. With a small portion of the total radiation (3,850,000 exoJoule per year) [18] of the sun that could be captured, it would be enough to fulfill the current energy demand (474 exojoule per year in 2008) [2].
There are several technologies used to harness energy from the sun irradiance to produce electricity, to produce thermal energy, to meet direct lighting needs and, potentially, to produce fuels that might be used for transport and other purposes [15]. Although solar energy has great potential, by now it is not enough to substitute fossil fuels due to its high initial investment costs and the large areas needed for its application [19].

1.4.2 Geothermal energy

Geothermal energy is the energy achieved from the accessible thermal energy of the Earth's interior. In geothermal areas water sinks below the earth surface and warms up. The water is used either as hot water or as steam to drive turbines that produce electricity [20]. Hydrothermal power plants and thermal applications of geothermal energy are mature technologies. When used to generate electricity, geothermal power plants typically offer constant output however this output will be not fulfill the needed consumption [5]. Geothermal heat is considered to be clean and renewable energy although there are some arguments about this issue; some scientists claim that geothermal energy is not completely renewable [21].

1.4.3 Hydropower

Hydropower is harnessing the energy of water moving from higher to lower elevations, using power engines primarily to generate electricity. Hydropower projects encompass dam projects with reservoirs, run-of-river and in-stream projects and cover a continuum in project scale. It has many benefits; high availability, without CO_2 emissions, and because of the simple technologies, it has a long history of usage [22, 23]

Currently, this is the second largest contributor of renewable energy next to biomass and it represented 2.3% (Figure 1) [5]. Hydropower technologies are mature. The operation of hydropower reservoirs often reflects their multiple uses, for example, drinking water, irrigation, flood and drought control, and navigation, as well as energy supply [5].
There are still some obstacles producing hydropower, e.g. high capital cost regarding buildings and the water lagoons require huge space and may have negative impact on the environment. But when installed, it has relatively low operational cost and is a very clean energy source [23].

1.4.4 Wind energy

Wind as an energy source has been used for many years. Initially, it was used only to propel boats but since 1880´s wind power has been used to make electricity [24]. Wind energy is another type of solar power, since wind is created when the sun shines and heats up the atmosphere creating a temperature gradient. Wind is also caused by the rotation of the earth and its irregular surface [24]. The production of electricity from wind is almost fully developed and competitive with other renewable energy sources. Wind is a completely pollution free technology and is used in many places around the world. Energy from wind is converted to electricity or mechanical energy by wind turbines which create power by driving a generator [16]. There are few flaws concerning the use of wind energy, the cost of building and installing the turbines is still higher than for generators used for fossil fuels as well as the instability in energy source. In addition, it has been criticized because of the environmental disruption of wildlife, especially bird, and also because of noise and visual effects [24].

1.4.5 Ocean energy

Ocean energy derives from the potential, kinetic, thermal and chemical energy of seawater, which can be transformed to provide electricity and thermal energy. A wide range of technologies are possible, such as barrages for tidal range, submarine turbines for tidal and ocean currents, heat exchangers for ocean thermal energy conversion, and a variety of devices to harness the energy of waves and salinity gradients. Ocean technologies, with the exception of tidal barrages, are at the demonstration and pilot project phases and many require additional R&D. Some of the technologies have variable energy output profiles with differing levels of predictability (e.g., wave,

tidal range and current), while others may be capable of near-constant or even controllable operation (e.g., ocean thermal and salinity gradient) [5].

1.4.6 Bioenergy (biofuel) from biomass

Biomass is organic matter which is produced by plants, animals and microorganisms [25]. Energy from the sun is converted to organic matter e.g. carbohydrates of short carbon cycle such as sugars, starch and cellulose by green plants, algae and photosynthetic bacteria [26, 27]. During photosynthesis, the carbohydrates in the biomass respond to oxygen and form carbon dioxide and water. When it burns completely, the same amount of carbon dioxide is formed since it is fixed during its growth [27]. The main difference with fossil fuels is the short carbon cycle. This is because the carbon in this fuel is extracted from carbon cycle million years back.

Bioenergy (biofuel) from biomass can be produced from a variety of biomass feedstocks, including forest, agricultural and livestock residues; short-rotation forest plantations; energy crops; the organic component of municipal solid waste; and other organic waste streams. Through a variety of processes, these feedstocks can be directly used to produce electricity or heat, or can be converted in to fuels in the form of liquids, gasses or solids. [22].

The variety of bioenergy technologies is broad and the technical maturity varies substantially. Some examples of commercially available technologies include small- and large-scale boilers, domestic pellet-based heating systems, and ethanol production from sugar and starch.

Advanced biomass integrated gasification combined-cycle power plants and lignocellulose-based transport fuels are examples of technologies that are at a pre-commercial stage, while liquid biofuel production from algae and some other biological conversion approaches are at the research and development (R&D) phase. Bioenergy technologies have applications in centralized and decentralized settings, with the traditional use of biomass in developing countries being the most widespread current application. Bioenergy typically offers constant or controllable output. Bioenergy projects usually depend on local and regional fuel supply availability, but recent developments show that solid biomass and liquid biofuels are increasingly traded internationally [5].

Direct combustion has been done for centuries but it is not the most efficient method of biomass utilization because of energy loss due to incomplete combustion, low efficiency and pollution. Therefore, it would be more feasible to convert the biomass to other fuel forms such as gaseous or liquid which are better to handle and pollute less when used. Examples of such fuels are hydrogen, methane, methanol, butanol and ethanol. Biomass fuels are still considerably more expensive than fossil fuels but emerging technologies will decrease this cost in coming years [28].

The methods of converting biomass to other energy carriers can be divided into thermal and biochemical conversions. During thermal conversion, heat is the main mechanism to convert the biomass into other biofuels, and commonly used methods are combustion (heat/electricity), pyrolysis or liquefaction (bio-oils) and gasification (syngas).

Biochemical conversions are all the processes in which the enzymes of micro-organisms or the organisms itself, are used to convert the biomass into other forms of biofuel like biogas, bioethanol, biodiesel or biohydrogen. This includes anaerobic digestion (CH_4) or fermentation to ethanol, butanol or hydrogen [29]. Figure 1.2 presents more details on biohydrogen and more discussion on biomass as a source for biohydrogen is presented in section 1.4.6.4.

Types of biofuel from biomass. Biofuel biomass is a substantial renewable source, which can be used as a fuel for producing electricity or converted in other forms of energy such as biofuels. As is the case for fossil fuels, different types of fuels are needed. There are options needed for replacing liquid car fuels (e.g. biodiesels, bioalcohols) as well as for instance gaseous fuels to replace natural gas. The most common types of biofuels will be shortly described below.

1.4.6.1 Bioethanol

Bioethanol is the most commonly produced biofuel worldwide. It is also commonly biofuel as a potential resource of renewable energy. (Bio)Ethanol is a colorless, flammable and volatile liquid which boils at 78.4°C and freezes at -114.1°C [30]. Bioethanol, together with propanol and butanol are called the bioalcohols. Although bioethanol is the most common biofuel, especially because of high production in Brazil, biobutanol is claimed to be the best replacement for gasoline, as it can be easily used by normal gasoline engines present in most cars and will produce more energy when combusted than bioethanol. Bioethanol has higher octane number [31], burns faster and has higher evaporation temperature than gasoline. These factors results in a higher compression ratio and shorter burn which leads to better energy efficiency compared to gasoline [32]. Use of bioethanol as an additive in gasoline is steadily increasing with a common mixture at 10% ethanol and 90% gasoline (E10) [33]. Higher concentrations of ethanol e.g. 85% (E85), requires special engines and hybrid cars [33]. Additionally, the proportion of O_2 in ethanol is higher as compared to gasoline and the blended fuel burns better and smaller amount of carbon monoxide is formed, which is formed mainly by incomplete combustion [30, 34]. There are several disadvantages to use bioethanol as a fuel. The energy released by burning ethanol is only 65-69% of the energy released by burning the same amount of gasoline. Also, ethanol has low flame luminosity and low vapor pressure which results in engine ignition difficulties in cold weather. Despite these disadvantages, bioethanol is considered an attractive biofuel that is renewable and reduces greenhouse gas emissions (NO_x, SO_x, CO and CO_2) [32].

In most cases bioalcohol is produced through fermentation of mainly wheat, corn and sugar cane

by microorganisms or by enzymes derived from them. Bioethanol derived from sugar and starch based biomass is called first generation ethanol. This opens the discussion again about the food for fuel competition, and more and more research is done on the use of biomass from waste streams and from non-competitive biomass sources for fuel such as lignocellulosic biomass [35]. Ethanol from cellulosic biomass is called second generation ethanol as it is made from sugars derived from cellulose and hemicellulose, which are the main building blocks in complex biomass. Cellulosic bioethanol production has recently emerged but its production is much less as compared to first generation ethanol. However, it is considered to be more sustainable fuel than corn and sugar based ethanol in the near future [36]. In line with this, in recent years, increasing attention has been directed to bioethanol production by microorganism using different carbon sources of biomass.

1.4.6.2 Biodiesel

It is produced by enzymatic or chemical transesterification of vegetable oils or animal fats [37]. Production of biodiesel is mainly from oil rich plants such as rape oil and soybeans [38], but also from algae which is a potential viable option. Algae species can range from small single-celled organisms (microalgae) to multi-cell organisms with complex structures. The ratio of lipid/oil by weight of algae varies widely (from 2 to 70%) but it is among the highest ratio found in living organisms [39].
Biodiesel is quite similar in composition as fossil diesel and consists mainly out of fatty acid ethyl esters (FAMEs). Biodiesel has theoretically 5-8% less energy compared to conventional diesel. However, because of better lubrication properties the actual energy difference is only 2% lower, or about 35 MJ $L^{-}1$ [38]. More detail about biodiesel production process in relation with glycerol is presented in chapter 2 under the review of glycerol for biohydrogen production and other biochemical.

1.4.6.3 Biogas

Biogas is produced by anaerobic digestion of biomass, and mainly consists of methane and CO_2. This gas can be used by a combined heat and gas system (CHP) to produce heat and electricity, or can be used directly as car fuel, or for cooking and heating. For this latter application the biogas can be mixed into the natural gas network, though the biogas needs to be purified first. An interesting possibility is to mix animal manure and crop residues to produce biogas on farms.

Methane is an odorless gas composed of one carbon and four hydrogen atoms (CH_4). It occurs naturally as a part of the natural gas coming up from the ground: it is produced microbiologically by methanogens in anaerobic environments like swamps, in garbage dumps and in the digestive systems of many animals. It is lighter than air, highly flammable and non-toxic unless presented in large amounts in confined spaces where it may cause suffocation [40]

Methane is considered to be a greenhouse gas: it has 21 times more greenhouse effect then carbon dioxide (CO_2). Biogas (CH_4 and CO_2) produced in landfills has been collected for many years and used either directly as an energy source (burning) or the methane is separated from CO_2 (and other gases) and used as vehicle fuel. More commonly, methane is produced by anaerobic digestion from wastewater and agricultural residues, and has been broadly applied both in pilot and large scale facilities, mainly in Denmark and Germany [41].

1.4.6.4 Biohydrogen

The use of renewable biomass as a major feedstock for hydrogen production has received considerable attention in recent years. Two types of biomass feedstock are available to be converted into hydrogen [42]: (i) dedicated bioenergy crops, and (ii) less expensive residues, such as organic waste from regular agricultural farming and wood processing (biomass residues). In particular, as resumed in Figure 2, the production process that is available using biomass can be summarized. The methods available for the hydrogen production from biomass can be divided into two main categories: thermo chemical and biological routes. This process can involve different routes based on the biomass resource to biohydrogen production for instance via reforming reactions (autothermal reforming, steam reforming, partial oxidative steam reforming). The different H_2 production system from biomass and other sources will be discussed later

1.4.7 Challenges of Renewable Energies from biomass

The other issues about RE especially the bioenergy resources are the land-use competing with food production. Recently, important discussion on, the food versus fuel debate, indicates the use of biomass for energy also has its drawbacks. The assessment by WEO, 2012 [15] indicates that global bioenergy resources are more than sufficient to meet projected demand without competing with food production, although the land use implications will have to be managed in a sustainable manner. Diverting farmland or crops for biofuel production should not harm the food supply, especially in developing countries. Another point of attention is that the total CO_2 reduction of the overall process should be calculated. This is the reason why, nowadays, biofuels are divided into three different groups, first, second and third generation biofuels. Although multiple definitions are used for these groups, in general biofuel generated from crops which are only grown for fuel purposes are called first generation [44]. Some examples of these first generation crops are different grain species, corn and sugar cane. The use of these resources will affect the food supply mainly due to limitation of arable land and is an important factor in this food for fuel debate. For instance, in US alone, more than 80% of the arable land available would only fulfill the need for 50% of the vehicle fleet [45, 46].

Therefore, increased interest is now on the use of lignocellulosic biomass for the production of second generation biofuels. The second generation biofuels are made from biomass which is non-edible, lignocellulosic biomass like trees, stems, leaves and husks [44]. This is non-food materials

from biomass and waste and is expected to be less harmful on land usage, cost and CO_2 emission reduction [47].

The use of fast growing crops which need less water and nutrients can increase the biomass yield per hectare of land and reduces the production costs. Technological breakthroughs are still needed to make these second generation biofuels cheaper than fossil fuels, but this is only a matter of time [44].

All biofuels coming from algae are called third generation biofuels. This field gained a lot of attention in the recent years. The advantage of algae is that they grow relatively fast, but as direct sunlight is needed, sufficient mixing is needed which can be difficult and expensive. Although the results for algae to produce biofuel are promising, more research is needed to improve this technology.

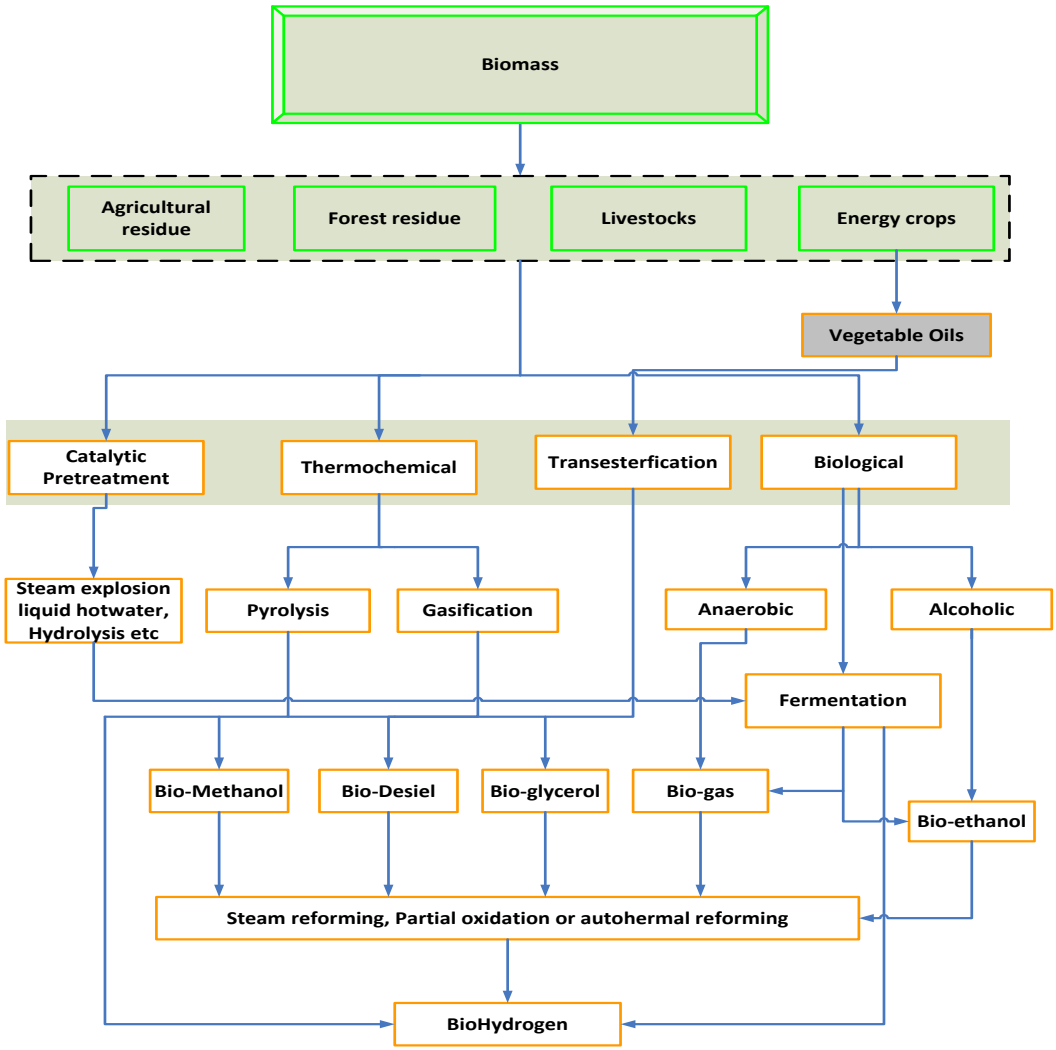

Figure 1.2. Selected hydrogen production technologies from various biomass (adapted from [43].

1.5 Hydrogen production processes

One of the advantages of H_2 as energy carrier is that all primary resources such as fossil fuels, renewable energy sources (solar, wind, hydro, geothermic, biomass) and nuclear power could be used for its production [48]. In particular, H_2 can be extracted from any substance containing hydrogen atoms, such as hydrocarbons, water and even some organic matter. Thus, the different technologies utilize mainly these compounds as starting materials for the final H_2 molecule formation. In addition, it can be readily produced from synthesized hydrogen carriers such as methanol, ammonia and synthetic fuels. Of these renewable sources the bioH_2 production from biomass will be discussed later.

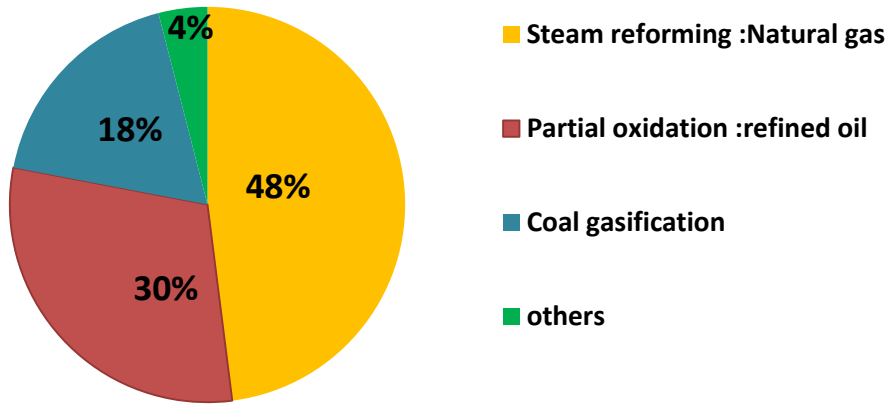

Figure 1.3. World hydrogen production process adapted from Corbo et al 2011[49]

H_2 can be produced by a number of physico-chemical processes, among them chemical and thermochemical processes are used at industrial and commercial scales. Almost 96% of the total production of H_2 is covered by this [49]. Almost half of the hydrogen used worldwide comes from steam reforming (SR) of natural gas (48%). The other contributions to H_2 production are based mainly on partial oxidation of refinery oil (about 30%) and coal gasification (18%) and the rest 4 % H_2 derives by water electrolysis (Figure 1.3) [49]. However, the main H_2 production process (96 %) is recognized as non-environmentally friendly and non-sustainable due to CO_2 emission, expensive or energy intensive.

Biological production of H_2 is seen to be a potential and more attractive way especially if organic wastes and biomass could be used as raw material [50].Generation of H_2 from biological materials, especially lignocellulosic materials, has become the focus of current research. This represents a potential route towards the development of sustainable energy production processes [51, 52] Description about the biological H_2 production process will be described more in detail later.

1.5.1 Chemical and thermochemical H_2 production

Thermal chemical processes require the use of thermal energy to favor the advance of chemical reactions, providing hydrogen as direct product. Thermal chemical approaches involve, as reactants, various resources which contain hydrogen atoms as part of their molecular structure, such as hydrocarbons or water. The conversion advance aimed at directly obtaining high hydrogen yield, can be further improved by catalyst addition (hydrocarbon reforming) or should require chemical compound usage (water splitting by thermochemical cycles). Steam reforming of natural gas or hydrocarbon, partial oxidation of hydrocarbon, coal gasification and electrolysis of H_2O are processes used for H_2 production. A short description of these processes is presented below.

1.5.2 Steam reforming

Currently, steam reforming (SR) process is the major industrial process for the manufacture of H_2 [53]. It involves the conversion of natural gas (CH_4) or hydrocarbon into H_2 and CO_2 in the presence of H_2O vapor. This reaction is carried out in two steps. The first step is a catalytic conversion of hydrocarbon into syngas which is a mixture of carbon monoxide (CO) and H_2. It's an endothermic reaction and heat is often supplied from combustion of some of the hydrocarbon in the feed (Reaction 1.1).

$$CH_4 + H_2O \rightarrow CO + 3\ H_2 \qquad \textbf{Reaction (1.1)}$$

The second step consists on a reaction called a "water gas shift" which simultaneously converts CO produced in the first step into CO_2 and H_2 by reaction with H_2O according to the exothermic equation at 130°C (Reaction 1.2) [54,55].

$$CO + H_2O \rightarrow CO_2 + H_2 \qquad \textbf{Reaction (1.2)}$$

The process temperature and pressure vary respectively between 800–900°C and about 0.1–0.3 MPa [48]. This process has been developed at large scale and used for many years despite of being so energy intensive. The efficiency of SR process is in the range of 65-75%. The only disadvantage is CO_2 emission into the atmosphere [56].

1.5.3 Hydrocarbon Partial Oxidation

In this process, H_2 is produced through a catalytic partial combustion of hydrocarbon with pure O_2 gas. Carbon monoxide and H_2 are produced and then CO is further converted to CO_2 and H_2 by the "water gas shift" reaction as in steam reforming (Reaction 1.2). The theoretical H_2 to CO ratio results lower than that of SR (about 2/3), as the main oxidant is O_2 instead of H_2O. As it is an

exothermic reaction, there is no need for an external reactor heating system [54]. The process efficiency is around 50%. It is also a process that releases CO_2 into the atmosphere [56].

1.5.4 Coal and Biomass gasification

Gasification process is converting a solid fuel (coal) into a gaseous CO/H_2-based synthetic gas (syngas), which can be subsequently treated to produce a clean fuel suitable for combined cycles, H_2. Gasification process is carried out in chemical reactors (gasifiers), where the following main reactions occur:

$$C(s) + ½O_2 \rightarrow CO \qquad \textbf{Reaction (1.3)}$$

$$C(s) + O_2 \rightarrow CO_2 \qquad \textbf{Reaction (1.4)}$$

$$C(s) + H_2O \rightarrow CO + H_2 \qquad \textbf{Reaction (1.5)}$$

$$CO + H_2O \rightarrow H_2 + CO_2 \qquad \textbf{Reaction (1.6)}$$

This process is comparable to the partial oxidation of hydrocarbon. H_2 is then produced by "water gas shift" reaction [56]. In this process, the carbonaceous particles (coal) are heated and volatilized at temperatures ranging from 1000 to 1500°C producing simultaneously CO_2 and H_2 gaseous mixtures and char (pyrolysis) [48]. In addition, biomass-derived materials could be converted in gasifiers by applying heat under pressure in the presence of steam and a controlled amount of oxygen, very similar to coal gasification process. This could permit the problem of carbon dioxide emissions to be solved. Current research on biomass gasification focuses on reducing the amount of CO_2 released by the process.

1.5.5 Electrolysis of water

Electrolysis is an electro-chemical method to obtain hydrogen using electricity by splitting of water molecule into H_2 and O_2 according to reaction 1.7. This reaction is carried out by electron displacement between electrodes immersed in cells containing an electrolyte (H_2O mixed with some salt in order to enhance its conductivity) [57]. This process is useful when highly pure H_2 is required by end users. The only problem is the availability of electricity which makes the H_2 produced expensive [11, 58] hence cannot compete with H2 produced from fossil fuels. In the future this can change, especially if the electricity used can originate from biofuels.

$$2H_2O \rightarrow O_2 + 2H_2 \qquad \textbf{Reaction (1.7)}$$

1.5.6 Biological H_2 production

The development of renewable H_2 production technologies has all their specific advantages and disadvantages in terms of potential, efficiency, scale and foreseen production cost. Most technologies for the production of renewable H_2 are still in the R&D stage and world-wide subject of increased research efforts.

Several microbial driven biochemical reactions, mainly in anaerobic fermentation processes produce H_2 from organic material such as biomass and waste materials like crude glycerol. In addition, certain microorganisms can synthesis enzymes that can produce H_2 from water if an outside energy source, like sunlight, is provided to them. Such production is called biohydrogen production [59].

Biological H_2 production delivers clean H_2 with an elegant and simple technology, more suited for the conversion of a wide spectrum of substrate utilization and is more sustainable method. Specific ways in which microorganisms can produce biohydrogen are described below [60].

i. Photofermentation and Biophotolysis of water using green algae and blue-green algae (cyanobacteria)
ii. Dark fermentation
iii. Hybrid systems, using dark fermentative and photofermentative

1.5.6.1 Biohydrogen production using two-stage fermentation or Hybrid fermentation (dark fermentative and photofermentative)

Hybrid fermentation technology might be one of the promising routes for the enhancement of H_2 production yields. The synergy of the process lies in the maximum conversion of the substrate which otherwise fails to achieve a complete conversion due to thermodynamic limitations [61].
Thus, in this system the light independent bacteria and photosynthetic bacteria provide an integrated system for maximizing the H_2 yield [62]. In such a system, the anaerobic fermentation of carbohydrates (or organic wastes or industrial waste like crude glycerol) produces intermediates, such as low molecular weight organic acids, which are then converted into H_2 by the photosynthetic bacteria in the second step in a photo-bioreactor. The overall reactions of the process for glycerol substrate can be represented as in the following equations and Figure 1.4.

I. Stage I. Dark fermentation (facultative or strict anaerobe bacteria)

$$C_3H_8O_3 + H_2O \rightarrow CH_3COOH + CO_2 + 3H_2 \qquad \textbf{Reaction (1.8)}$$

II. Stage II. Photo-fermentation (photosynthetic bacteria):

$$CH_3COOH + 2H_2O \rightarrow 4H_2 + 2CO_2 \qquad \textbf{Reaction (1.9)}$$

So, theoretically it is evident that using glycerol as the sole substrate in the dark anaerobic fermentation, where acetic acid is the predominant metabolite, a total of 7 mol of H_2 could be expected in a combined process from one mol of glycerol. For the maximum 3 moles H_2 production using the dark fermentation system it is more explored in chapter 2 and 3.

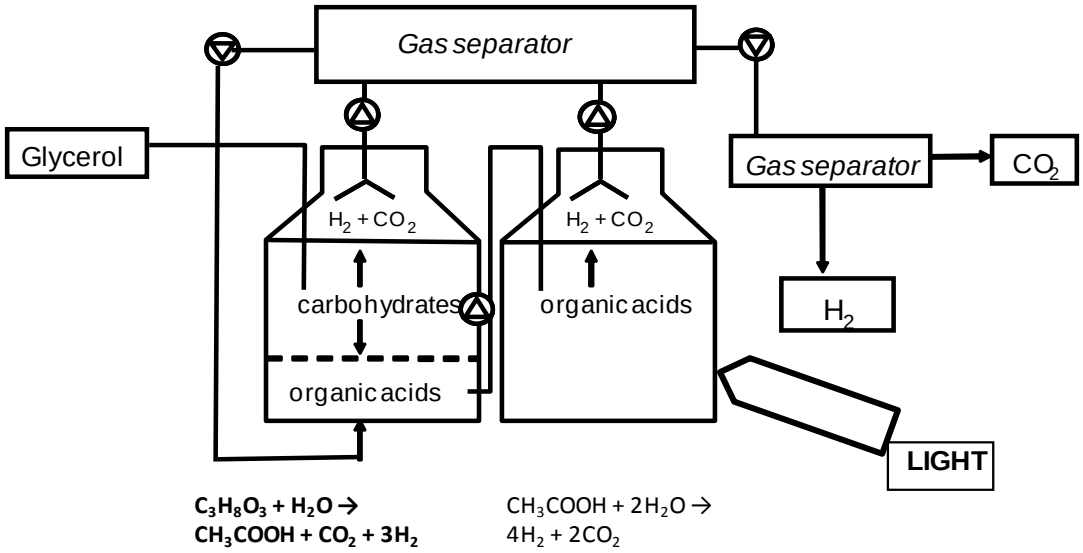

Figure 1.4. Biohydrogen production using two-stage fermentation using glycerol (adapted from Reith et al, 2003 [62]).

1.5.6.2 Photobiological H_2 production

By performing a dark fermentation a maximum of 3 moles of hydrogen can be produced from one mole of glycerol reaction 8. The rest of the potentially available energy will stay trapped in the organic acids as it is thermodynamically not possible to oxidize these anaerobically without input of extra energy (Reaction 1.9) [63]. However this can be further processed by photofermentation. This conversion is performed by photosynthetic bacteria such as genus *Rhodobacter,* which obtain energy from light to combat the thermodynamic barrier of anaerobic organic acids (acetic, lactic, and butyric) oxidation [11, 41, and 64].

The other way of producing H_2 is using Microalgae and cyanobacteria (photoautrophic microorganisms), which use radiation from light to split H_2O molecules into H_2 and O_2 by photosynthesis. This can be termed as biophotolysis [64]. Photobiological H_2 production may be considered the most economic process utilizing simply H_2O, but it can only be operated during daytime. Also, production of O_2 from the process may decrease the H_2 efficiency by inhibiting the H_2O splitting reaction [10, 11, 65-67]. To perform optimally, the design of the photofermenter is very important because it requires a large surface area to collect light energy and proper mixing inside the fermentor. So far, the production rates in photofermenters are much lower than in dark fermentation fermentors. It had been reported by Das, 2008 [68] that 3-10 % photochemical efficiencies had observed using this process. A possible alternative to this might be the utilization of solar collectors [10].

1.5.6.3 Dark Fermentation for H_2 production

This process utilizes obligate and facultative anaerobic microorganisms to convert organic materials such as biomass, organic wastes, industrial wastes etc. into H_2 from general anaerobic metabolism. The anaerobic production of H_2 involves the partial oxidation (in acidogenesis phase) of organic materials as an example, Reaction 1.8. The anaerobic biohydrogen production process is not only stable, but also more rapid and it can be carried out in the absence of light compared to the photofermentation process [10, 69]. More emphasis has been placed on the dark fermentative production of H_2 because it is renewable, environmentally friendly and less energy intensive. Other advantages lie in the utilization of waste materials. This process can couple H_2 production from various substrates in industrial and/or agricultural wastes to other forms of energy such as butanol and ethanol. Other end products of the process could also include high valued fine chemicals (biochemical) [11, 70-74]. The main advantage of dark fermentative biological hydrogen production (BHP) is that the hydrogen evolution rate (HER) (mmol/ L *h) is higher in contrast to other BHP [59, 75].

Major known drawbacks of dark fermentative BHP are the low yield of H_2 per substrate consumed (Y (H_2/S))[mol/mol], which is due to metabolic fundamentals [76]. Moreover, concomitant production of carbon rich metabolites (i.e. organic acids, alcohols) and CO_2 is produced [77] and must be individually evaluated for each strain. CO_2 can be removed or separated from H2, sequentially stored in biomass [78] or converted to other substances, such as CH_4 [79,80]. Basic microbiological investigations and bioprocess engineering research was performed to increase the overall strain performance of BHP during fermentation of pure microorganisms [81-83].

This fermentation can be performed by different groups of organisms at different temperatures. Mesophilic fermentation takes place between 15 and 40 °C, while thermophilic hydrogen production takes place at temperatures between 45 and 80 °C. Over the use of these two different groups of microorganism more is described in chapter 2 and 3.

1.5.6.3.1 Raw materials for H_2 dark fermentation production

Renewable mass is the most versatile non-petroleum based resource for H_2 production. It is basically vegetable raw materials but also can be generated from various industries as waste material. The different kind of potential vegetable raw biomass for the hydrogen production can be categorized as lignocellulosic biomass (i.e. grass, wood, straw), starchy biomass (i.e. potato, cereals, food, starch-based wastewater) and sucrose containing biomass (i.e. sugar beet, sugar cane, sweet sorghum). From the waste materials also bio-diesel industry waste can be added to the raw material list as well.

Earlier in section 1.4.6.6 it was outlined that biomass can be used in different production processes for H_2 production (Figure 1.3). Here more details on the biomass type are discussed. As it was discussed before, H_2 production ranges from domestic organic waste to more defined agro-industrial residues and finally to well-defined product from energy crops such as corn and sugarcane. The later ones are easily degradable biomass upon hydrolysis usually yield glucose and sucrose. Lignocellulosic biomass has a more complex structure and, thus, requires additional pre-treatment in the form of heat, strong acids or bases, or enzymes such as cellulases and hemicellulases [84].

Sugars biomass

The most used sugars for biofuel production are glucose (hexose) and sucrose (a disaccharide). Most microorganisms ferment sugars easily via the Embden-Meyerhof pathway. Examples of biomass that are rich in sugars are corn, sugar-cane, sugar beet, sweet sorghum and many fruits [26]. Using sucrose containing biomass (sugar beet, sugar cane, sweet sorghum, pressed beet pulp) H_2 can be produced, more or less, same amounts than starchy biomass (potato). Sucrose containing biomass still has the drawback that the usable crop needs to be farmed, and this makes it competitor with the food farming. It is neater not profitable to use crops for the H_2 production when there is possible feed stocks available from the waste.

Starchy biomass

Starch-based biomass covers from the vegetable raw materials for example potato and cereal and from food wastes of the industry and household. These substrates contain high levels of carbohydrate and protein. Several species of starch rich plants are suitable for biofuel production, e.g. corn starch and sweet potatoes [84].

Lignocellulosic biomass

Lignocellulose is composed of cellulose; hemicellulose and lignin, which form the structural component of plant cell wall (Figure 1.5). Lignocellulose is available in bulk as lignocellulosic wastes of agricultural and wood industries, and also in the raw biomass like grass, wood and straw. It is available in immense amount and is present in all plants [85]. The production of lignocellulose on earth is about 2 to 5 $\times 10^{12}$ tons every year [26, 86]. Lignocellulolytic materials are the largest renewable sources of hexose and pentose sugars with potential use for industrial fermentation especially for ethanol production and biohydrogen [26, 86]. However, the limitation is the pre-treatment process involved to degrade cellulose to simple sugars. In addition, the removal of the lignin has also to be done before fermentation. Lignocellulosic biomass is an example of bioenergy source avoids the complications related with the biomass for biofuel versus food.

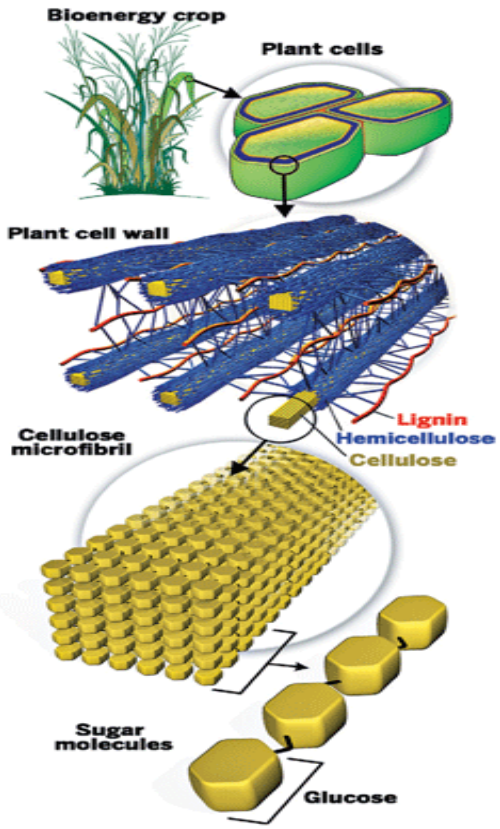

Figure 1.5. Structure of lignocellulose [87].

Pretreatment of lignocellulosic biomass The goal of biomass pretreatment is to break down the basic units of the lignocellulose into monosugars, to separate the components of lignocellulose and improve the accessibility and susceptibility of the cellulose and hemicellulose. This is done by reducing biomass particles size and change the biomass structure. Nowadays there are different types of pretreatment in use. Some of these are steam explosion, liquid hot water, acid, alkaline and biological. Studies have shown that pretreatment of lignocellulose is the major factor determining the recoveries of sugars from the hydrolysis of cellulose and hemicellulose [32].

For pretreatment of lignocellulosic material to be economical feasibility the following four factors should be considered [32]:

1. Good monosaccharide yields
2. Minimum loss and degradation of carbohydrates
3. Minimum formation of substances that may have inhibitory effects on the hydrolysis and fermentation process
4. Lower operational costs.

Formation of inhibitory compounds by pretreatment. Depending on the pretreatment used, a portion of the sugars and other organic compounds present can be converted to other substances, such as furfural, hydroxymethylfurfural (HMF), acetic acid, syringic acid, p-hydrobezen acid, and vanilline. These compounds have and inhibitory effects on growth and metabolism of microorganisms [88]. Therefore, efforts are usually made to minimize the formation of these chemicals or to remove them from the hydrolysates before fermentation [89]. Furfural and HMF have similar activity; they retard the fermentation of hemicellulose hydrolysates by yeast or other biocatalysts and must be removed or mitigated. Furfural is considered more toxic [90]. Relatively low concentration of these substances has inhibitory effects on microbes [91].

Removing inhibitory compounds from hydrolysates (water soluble fraction (WSF)). For higher yields of fermentation of hydrolysates needs detoxifying if inhibitors like furfural and HMF are present. The inhibitors act as strong barriers for microbial metabolism. Consequently, it is important to remove or neutralize these compounds from the hydrolysates before fermentation [88]. However, this process incurs cost. Taking into account the chemical composition of hydrolysate, several detoxification methods such as biological, physical and chemical have been used to convert inhibition compounds into inert material or reduce their concentration [92]. On the other hand, a study done by Gerhard et al, 1983 [93] for sulphate reducer *Desulfovibrio sp*. strain F-1 showed that furfural was used as sole source of carbon and energy. Boopathy et. 1993 [94] showed that furfural and also most likely HMF are reduced by enteric bacteria.

1.5.6.3.2 Dark fermentation from Glucose

Organisms degrade organic compounds to gain both energy and carbon. This can be processed to gain energy either through respiration, photosynthesis or fermentation. Respiratory organisms use oxygen as the final electron acceptor but fermentation occurs under anaerobic conditions and is more common in prokaryotes than eukaryotes [95]. Rearrangement (oxidation and reduction reactions) of the organic compounds used as carbon source leads to release of energy from high energy compounds and ATP is formed by substrate level phosphorylation from ADP and inorganic phosphate. The amount of energy produced under anaerobic conditions is much less as compared to respiration, e.g. fermentation of glucose to ethanol and lactate only leads to production of 2 ATP as compared to maximum of 38 ATP´s from glucose oxidation in respiration [96]. The main reason is the excretion of these compounds out of the cells instead of a complete oxidation to CO_2 as in respiration. Most anaerobic bacteria use glycolysis (Embden-Meyerhof pathway) to break down glucose into two units of pyruvate in a series of ten enzymatic reactions. Glycolysis is also the first part of the degradation process of glucose by aerobic bacteria [96, 97].

Thus, glucose in dark fermentation is degraded to two moles of pyruvate, glycolytic (Embden-Meyerhof) pathway, which is further converted to various end products, H_2 being one of them. Pyruvate is further oxidized to acetyl-CoA, which can be converted to acetyl phosphate and results

in the generation of ATP and the excretion of acetate (Figure 1.6). This oxidation to acetyl-CoA requires a ferredoxin (Fd) reduction. Reduced Fd is oxidized by hydrogenase which regenerates Fd(ox) and releases electrons as molecular H_2 [98,99]. The principal H_2 pathway is through pyruvate ferredoxin oxidoreductase (PFOR) [100]. The overall reaction of the processes can be described as follows:

$$\text{Pyruvate} + \text{CoA} + 2\text{Fd(ox)} \rightarrow \text{Acetyl-CoA}^{+} + 2\text{Fd(red)} + \text{CO}_2 \qquad \textbf{Reaction (1.10)}$$

$$2\text{H}^{+} + \text{Fd(red)} \rightarrow \text{H}_2 + \text{Fd(ox)} \qquad \textbf{Reaction (1.11)}$$

Despite having higher evolution rate, the yield of H_2 from the fermentation process is lower than that of other chemical/electrochemical processes. Theoretically, H_2 yield is 4 mol of H_2/mol of glucose when the end product is acetic acid, while 2 mol of H_2/mol of glucose will be obtained if the metabolic end product is butyric acid. In practice, the yields are low since the end products contain both acetate and butyrate [98]. Besides, as yields increase the reaction becomes thermodynamically unstable. Another constraint of the process is the low conversion efficiencies of the substrate used.

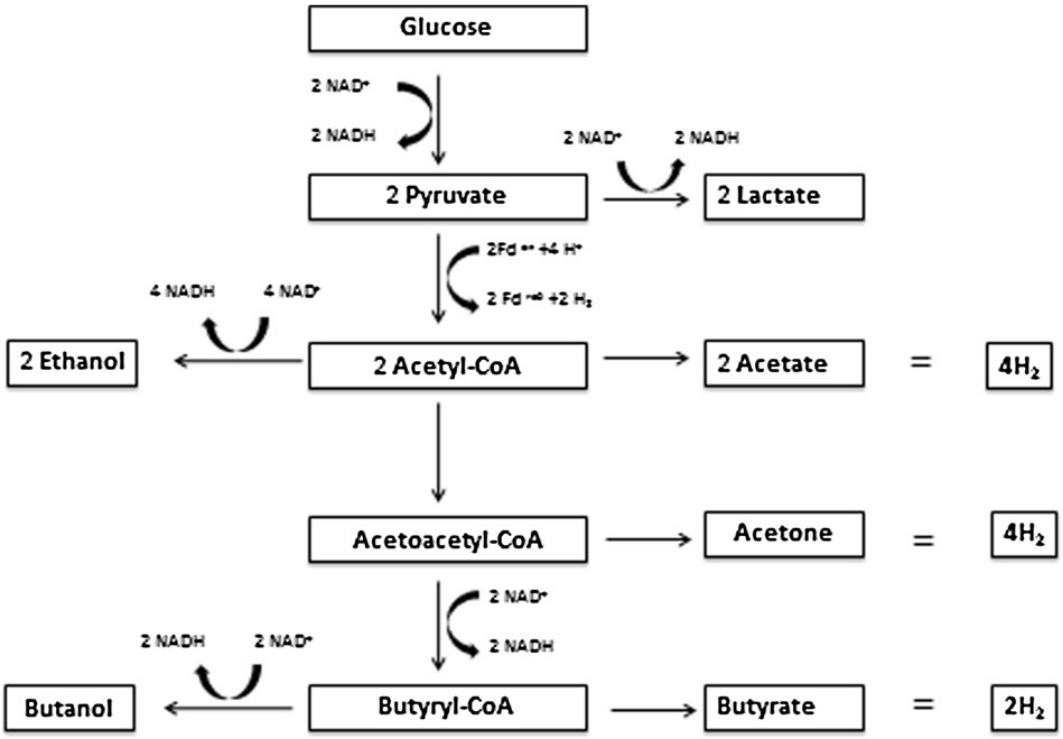

Figure 1.6. Anaerobic metabolism of glucose and pyruvate and H_2 production (adapted from [101]

1.5.6.3.3 Dark fermentation from glycerol

To enhance the economy of the dark fermentation it is important to explore potential substrates which can be utilized by broad range of H_2 producing bacteria. Several substrates, mainly carbohydrate sources such as glucose [102,103], xylose [104,105], sucrose [106] and starch [107] have been tested in an attempt to maximize biohydrogen through dark fermentation. However, these carbon sources are very costly. Recently the feasibility of using organic wastes or waste waters [108-111], lignocellulosic agricultural residues, starch-based materials and tofu-processing [11,103,112] has been widely studied for biohydrogen production.

In recent times glycerol waste from the biodiesel industry has emerged as a promising substrate for bioconversions [113]. The world biodiesel production is increasing; in 2010 the total annual production capacity in the US and EU was 6.9 million tonnes and it was expected to be doubled in 2012 [114]. This rapid increase in biodiesel production will result in a considerable surplus of glycerol waste, because about 1 kg is generated for every 10 kg of biodiesel produced. In this regards, it is an attractive carbon source for dark fermentation. Although it has been known for decades that H_2 can be generated through glycerol fermentation, dark fermentative H_2 production from biodiesel-glycerol waste has been studied and practiced very little. Some studies have shown that glycerol can be used as an alternative for H_2 gas production by anaerobic fermentation [113,115-117]. Early studies [115,118] focused on hydrogen and ethanol production using *Enterobacter aerogenes* HU-101 and glycerol-containing wastes discharged from biodiesel manufacturing. Besides, pure cultures, various mixed micro-flora and co-cultures have also been examined for their ability to produce H_2 from carbohydrates [10, 66, 119]. In this regard, glycerol waste is considered to be a major carbon source for biohydrogen production via anaerobic fermentation.

1.5.7 Microorganisms involved in biological hydrogen production

Many microorganisms have been identified for anaerobic fermentation. H_2 gas is synthesized by a large group of microorganisms that include both obligate and facultative anaerobic bacteria. Hydrogen-producing microbes have been found in environments with a wide range of temperature, including mesophiles (25-40 °C) [120,121], thermophiles (40-65 °C), extreme thermophiles (65-80 °C), or hyperthermophiles (>80 °C) [106]. Cultures are selected either as single or multiple strains, especially for their adaptation to a substrate or raw material. Some experimental results supporting the hypothesis of co-culturing have given a higher yield of H_2.
Species from *Thermotoga* (obligate) and *Enterobacteriaceae* (facultative) families have been widely used in biohydrogen production. There are numerous types of microorganisms that are found to produce hydrogen during anaerobic condition. Strictly anaerobic bacteria are the most common class of bacteria that produced hydrogen. They have relatively high hydrogen production yield. However, a few facultative bacteria have been identified as hydrogen producers when the

hydrogenase enzyme was found in these bacteria. However, cultivation of strict anaerobic bacteria was rather difficult as trace amounts of oxygen inhibited their growth. The anaerobic, facultative anaerobic, thermophilic and co- and mixed-culture bacteria are discussed further below.

1.5.7.1 Thermotoga species

The genus of the *Thermotoga* was first described in 1986 when uniquely thermophilic bacteria were isolated from the geothermal heated sea floors in Italy and the Azores [122]. Today nine different species have been identified; *T.elfii, T. hyphogea, T. lettinhae, T. maritima, T. neapthophila, T. neapolitiana, T. petrophilia, T. subterranean* and *T. thermaram* [123].

These rod shaped bacteria are anaerobic, extermophilic (65-80 °C), that are chatertaerized by an outer sheetlike structure called toga [122]. Members of the thermotoga feremt various sugars, maily to acetate , CO_2 and H_2. H_2 production has been extesively studied for *T. elif , T.maritima* and *T. neapolitana* [124,125]. They have been identified as a potential process that favorable to reaction kinetics, avoiding contamination by H_2 consuming bacteria [126].

In several hyperthermophilic bacteria belonging to the genus *Thermotoga*, yields of hydrogen on glucose is higher and may approach the theoretical maximum yield of 4 mol H_2 mol $^{-1}$ glucose. Schroder et al. [127] reported that *Thermotoga maritima* converted 1 mol glucose into 2 mol acetic acid and 4 mol of H_2. Takahata et al. [128] found yields of 3.7 and 4 mol H_2 mol $^{-1}$ glucose in *T. petrophila* and *T. naphtophila*, respectively, while van Niel et al. [129] found a yield of 3.8 mol H_2 mol $^{-1}$ glucose in *T. elfii*. In *T. neapolitana*, which has been extensively characterised with respect to hydrogen production, van Ooteghem et al. [102] reported H_2 yields at or even above 4 mol H_2 mol $^{-1}$ glucose. Also in other hyperthermophilic bacteria, hydrogen yields on glucose may approach the theoretical maximum [126]. Most studies on hydrogen production in *Thermotoga* have used glucose as carbon source although hydrogen productions at a large scale will have to be based on cheaper substrates, such as plant biomass or waste streams like for example mash from the fermentation industry or biodiesel crude glycerol. A single report also describes hydrogen production in *T. neapolitana* on waste glycerol [130]. Yet, to date, there is no study on the conversion of glycerol to H2 using a *T. maritima*.
Henceforth the ability of this organism especially the *T. maritima* is dissicussed in more detail in chapter 4.

1.5.7.2 Enterobacter species

Microorganisms of the *Enterobacteriaceae* family are facultative anaerobes, gram- negative, rod shaped and recognized as glucose fermenters. They utilise a wide range of carbon sources. They have been used in many studies using glucose as carbon source and found butanediol

fermentative pathway with mixed acid products [131]. *Enterobacter cloacae*, *Enterobacter aerogenes*, *Citrobacter freundii*, *and Citrobacter intermedius* have been used in some experiments and they conducted to a high yield of H_2. Concomitantly with H_2 the metabolites such as acetate, ethanol, 2,3-butanediol, acetone, ethanol and CO_2 are mostly produced [132-135]. Facultative anaerobe produces ATP by aerobic respiration if oxygen is present and is capable of switching to anaerobic fermentation. Therefore, it has an advantage compared to anaerobic bacteria, which is sensitive to the presence of oxygen. Facultative bacteria can consume oxygen by aerobic respiration, leaving anaerobic condition that favors to hydrogen production. *Enterobacter* sp. is the most common gram negative and facultative anaerobe with the ability to produce hydrogen. For the newly isolated strains of *Enterobacter*, *Citrobacter* and commercially *E. coli* 432 will be discussed more for glycerol fermentation in more details in chapter 2 in the review and in chapter 3.

1.5.8 Parameters and factors influencing fermentative biological hydrogen production

Environmental factors such as temperature, pH, H_2 partial pressure, feedstock (carbohydrates or carbon source), substrate concentration, inoculum, nutrients and other soluble metabolites formed can influence biological hydrogen production process. The main factors that affects of this process are discussed below.

1.5.8.1 Temperature

Microorganisms are capable to produce hydrogen in a large range of temperature 15–85^{0}C. Usually for H_2 production mesophilic and thermophilic bacteria are used. The efficiency of H_2 production is temperature-dependent due to the strong reliance of chemical (biochemical) reactions such as enzymatic activity and cellular maintenance upon temperature [136]. Temperature is one of the most important factors affecting other parameters such as pH, oxido-reduction potential, electron transfer, the rate of microbial growth, and consequently the rate of metabolites formation and biogas production in an anaerobic digestion process [137]. The effect of different temperatures on growth rate could be predicted in terms of the activation energy required for growth as in enzyme-catalyzed chemical reactions [138]. An optimum temperature exists at which each micro-organism can survive and grow depending on the microbes. Studies using mesophilic cultures indicated that, although H_2-producing bacteria are able to perform at ambient temperature conditions. Hence, increasing temperature in the mesophilic regime always improves the H_2 production, while further increasing culture temperature beyond mesophilic range may cause a decrease in H_2 production.

Above the optimum temperature for the specific group, cell degradation can become probably dominant over growth processes. With temperature below the optimum, cell growth can proceed

slowly or not at all because the cell membrane is not fluid to be penetrated by nutrients needed for growth [139].

Most of biohydrogen productions by anaerobic processes operate at ambient temperature (30-40°C) with the advantage of being efficient and less energy intensive [137]. However, thermophilic processes for biological H_2 production have been successful especially when contaminants had to be removed from the liquid organic materials and in the case of wastewaters containing high strength organic matter [140]. High temperatures are known thermodynamically to encourage increasing biochemical reactions [126]. The temperature at which the reaction takes place affects the thermodynamics, according to $\Delta G^0 = \Delta H - T\Delta S^0$ [141]. At higher temperatures the Gibbs free energy change for the overall reaction from glucose to acetate (Reaction 7) becomes more favourable. Consequently, operations are performed at high nutrient loading rates which lead to high products formation and better process efficiency [142,143]. However, the energy required to maintain high temperature is the only economic problem [140]

1.5.8.2 pH

The pH has a significant impact on the performance of anaerobic processes. It determines the degradation pathway of organic matter and has an effect on microbial activities as in biochemical operations [131,144]. Value of pH of the environment of the process may affect the hydrogenase activity as well as the metabolism pathway. Increasing pH could increase the ability of hydrogen-producing bacteria to produce hydrogen during fermentative process, but pH at much higher levels could decrease it.

Microorganisms have an optimum pH value from which any deviation can cause change in their behavior. pH can be maintained at its optimal range by addition of sufficient buffers like bicarbonates [144, 145].

A pH between 6.0 and 7.4 has been found as acceptable for the activity of the hydrolytic microorganisms [144,145]. The optimum pH range to achieve the maximum H_2 yield or specific H_2 production rate was found between 5.0 and 6.0 in most studies using acid-producing pure or mixed cultures of bacteria in continuous process [140,144-149]. Concomitantly, an increase on the production of VFAs, particularly acetic acid, butyric acid and propionic acid has been observed [140,149]. These soluble metabolites determine the pathway which enhances the H_2 production [148].

An increase above this range to pH 8.0 tends to favor the growth of methanogens which inhibit the growth of acidogenic bacteria, lowering the H_2 production [144,150-152]. A lower pH to 4.5 shifts the VFAs-producing pathway to an alcohol-producing pathway which lowers the H_2 yield [66,137].

1.5.8.3 H_2 partial pressure and soluble metabolites

H_2 partial pressure is another factor which has an influence on biohydrogen production process. When the amount of H_2 rises, its partial pressure rises also. This situation causes the decrease of H_2 production. It has also a direct effect on the proportion of the various intermediate products of the anaerobic reactions [152-154].

As presented above, fermentative H_2 production by anaerobic process is a partial oxidation of organic materials. During the anaerobic fermentation the hydrogenase reaction, involving enzyme-catalysed transfer of electrons from an intracellular electron carrier molecule to protons, is thermodynamically unfavourable and depends on the range of H_2 partial pressure [82, 126, 155, 156]. The transfer of electrons from the electron donating carbon skeletons to inorganic electron acceptors such as protons, in the liquid phase, is facilitated by the electron carriers such as nicotinamide adenine dinucleotide (NADH, E NADH 'o = -320 mV) and ferredoxin (Fd, EFd'o = -400 mV). With the redox potential of the proton/dihydrogen couple EH_2= -414 mV, H_2 partial pressures have to be lower than 40 Pa (0.3 atm) or 60 Pa (6x10-4) to allow electrons to be released as molecular H_2 from NADH or ferredoxin. Consequently, a low H_2 partial pressure promotes H_2 generation with production of acetate and CO_2 as co-products rather than ethanol or butyrate [126,144, 154]. In contrast, high H_2 partial pressures stimulate the accumulation of propionate, reduced fatty acid compounds and alcohols in the liquid phase with decrease in the H_2 production rate and H_2 yield [136,155]. Therefore, the H_2 partial pressure has to be maintained at a low level to allow H_2 synthesis during a continuous fermentation process. It means that in order to maintain H_2 production higher it is necessary to remove excess of H_2 from the system.

Many strategies of removal or separating excess H_2 gas have been developed to avoid the negative effect of the H_2 accumulation in the gas phase and in order to increase H_2 production rate and H_2 yield such as sparging the reactor with nitrogen (15x higher hydrogen production rate) or argon or CO_2 into the head space of the bioreactor, and addition of KOH in the liquid phase [82,126,156-158]. More on improvement of H_2 production using argon purging for lowering H_2 partial pressure will be discussed in **chapter 3**.

Soluble metabolites. Biological H_2 production is usually accompanied by soluble metabolites production (VFAs and solvent). The production of these intermediate products reflects changes in the metabolic pathway of the microorganisms involved. A better knowledge of such changes could improve the understanding of conditions favourable for H_2 production [66, 137, 155].

The major VFAs detected are acetate, butyrate, propionate, succinate, lactate and formate [159].The first three VFAs are the most commonly found in biological H_2 production and used to assess the process performance [11,126, 150]. Theoretically 4 moles and 2 moles of H_2 gas can be generated from a mole of hexose when acetic and butyric acids are end-products respectively.

Thus high H_2 yields are associated with a mixture of acetate and butyrate fermentation products [66]. Propionate production is a H_2 dependent pathway (it consumes H_2 when present into the reactor) [140,157]. Preventing the commencement of this pathway will help to increase the H_2 production rate [66,110, 157,].

Mostly ethanol, butanol, butanediol, acetone accompany VFAs formation during anaerobic H_2 production. It is known that the accumulation of alcohol into the bioreactor decreases the H_2 production rate and H_2 yield [157,160]. This is due to the fact that reduced fermentation end-products containing H_2 which has not been liberated as H_2 gas and also electron donors produced during fermentation processes (important for hydrogenase enzymes), are mostly consumed by these products [66, 161]. Therefore, to maximize H_2 yield, bacterial metabolism during fermentation process must be directed away from alcohols and reduced acids formation towards VFAs [66, 131, 157].

1.5.9 Bioreactors used in H_2 production

The choice of the bioreactors depends on the type of substrate such as organic waste materials, crude glycerol, strength of wastewater and they are mostly used in streams containing soluble organic wastes which could be converted by microorganisms in organic acids, alcohols and biogas [139,144]. According to the type of process which is used during hydrogen production, different kind of reactors is designed. The following are the most commonly used reactors for H_2 production. Such as 1) photo-bioreactors, 2) dark fermentation bioreactors: a) Continuous stirred tank reactor b) Anaerobic sequencing batch reactor c) Membrane bioreactor d) Fixed-bed bioreactor e) Upflow anaerobic sludge blanket bioreactor): 3. Microbial electrolysis cells, 4. Hybrid bioreactors, 5. Multi-stage bioreactors.

The range of types of reactors that had been used it starts from batch reactor 10 ml working volume [162], a 2.5-l fermentor, to continuous stirred tank reactor, upflow anaerobic sludge blanket and the anaerobic Fluidized bed bioreactor have been used in the anaerobic treatment of wastewater for H_2 production.

1.5.10 Kinetic models for biological hydrogen production

Models used for prediction and elucidate, kinetic constants, analysis, design and operation of the production process. Kinetics models can be used for batch fermentative H_2 production to see effects of substrate and inhibitor concentration, T, pH etc. on the process. Common models used for biohydrogen production are the following: Gompertz model describes the progress of a batch fermentative hydrogen production process, growth of hydrogen-producing bacteria, substrate degradation; Monod Model analysis the effects of substrate concentration (without inhibitor effects) on the rates of substrate degradation, H_2 producing bacteria growth, H_2 production;

Arrhenius model describes the effects of temperature on fermentative H_2 production; Han–Levenspiel model describes the effects of inhibitor concentration (salt or hydrogen on the intercellular pH) on fermentative hydrogen production, mostly batch, Andrew model analysis effects of H^+ concentration on the specific hydrogen production rate; Luedeking–Piret Model shows relationship between the hydrogen-producing bacteria growth rate and the product formation rate.

1.5.1 Modified Gompertz Model

$$\boldsymbol{H_2\,(t) = H_{max,H_2} \times exp\left\{-exp\left[\frac{R_{max,H_2} \times e}{R_{max,H_2}}\left(\lambda_{H_2} - t\right) + 1\right]\right\}} \qquad \textbf{Eq. (1.1)}$$

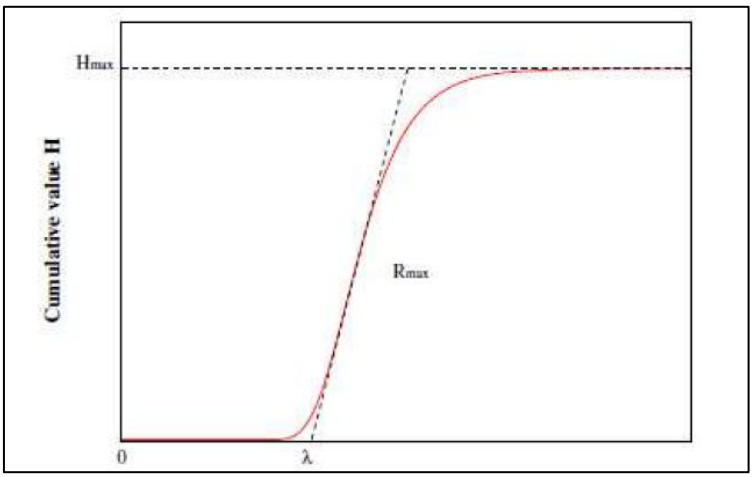

Figure 1.7. Fitting using modifies Gompertz model

Description:

Equation 1.1 and Figure 1.7 describes the cumulative value (H) over a certain time (degradation or growth). λ is called lag time and gives information about the actual start of the process. When λ is reached the rate (R) increases rapidly until finally reaching the maximal cumulative value (H_{max}).
Of these Gompertz model chosen in this thesis because it gives the following advantages. Easy (not complicated), omnipotent (progress, growth, production of hydrogen and some soluble metabolites), obtains constants that have biological meaning better understanding of a process, widely used (several studies about different processes and substrates already), correlation coefficient near 1 (0.95-1.0) and perfect model to describe batch fermentation processes [57,110].

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CHAPTER 2

2. GLYCEROL: AN ATTRACTIVE AND VERSATILE, CARBON AND ENERGY SOURCE FOR BIOHYDROGEN AND BIOCOMMODITIES : A REVIEW

Manuscript in preparation:

2.1 Abstract

This chapter reviews the use of glycerol, an inevitable by-product of biodiesel manufacturing process, to produce hydrogen and other biochemical products through dark fermentation. It assesses the current relationship between the market, availability, and production of both biodiesel and glycerol, as well as the influence of the growing biodiesel production on the commercial prices of glycerol is discussed. Besides, a brief discussion of the characteristics and production of crude glycerol generated from biodiesel manufacturing processes in general and a case study used in this thesis is included in this chapter. Additionally, it presents the potential of crude glycerol as a carbon source for biohydrogen and main feedstock for other applications, pretreatment methods, and reaction condition used as well as the glycerol bioconversion potential of different microorganisms.

Furthermore, it incorporates previous studies on biohydrogen and biochemical production from pure glycerol and crude glycerol as substrate. This thesis uses the previous studies to compare it with the works conducted in this thesis using different *mesophilic* and *thermophilic* strains. An overview on the possible metabolic pathways and routes of glycerol biochemical transformation is also provided. It also incorporates a brief discussion of the short comings of crude glycerol bioconversion to hydrogen production and possible improvement mechanisms. Finally, it lays out possible research areas that need further exploration.

2.2 Introduction

Glycerol (1,2,3-propanetriol also glycerin) (figure 2.1) is a highly reactive tri-alcohol molecule, which has two primary and a secondary hydroxyl groups. Physically it is soluble, colorless, odorless, viscous, and hygroscopic; with a specific gravity of 1.261 g mL^{-1}, melting temperature of 18.2 °C, and a boiling temperature of 290 °C and has high energy density [1, 2]. Chemically, it is able to react with a stable alcohol under most operational conditions; it is non-toxic, non-volatile, and non-flammable [3, 4]. The unique combination of physiochemical properties, its compatibility with other substances, and easy handling offers glycerol to have more than 1500 end-users or large volume applications. Of these, it is usually used for personal care, food production and in a multitude of products. Additionally, it is often used as: humectant, plasticizer, emollient, thickener, solvent, dispersing medium, lubricant, sweetener, and antifreeze [3,4]. Furthermore, important commodities and high-added value products of industrial interest, such as organic acids, bioplastics, polyunsaturated fatty acids, carotenoids etc. can be produced from raw glycerol.

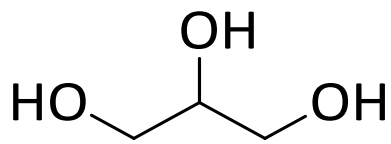


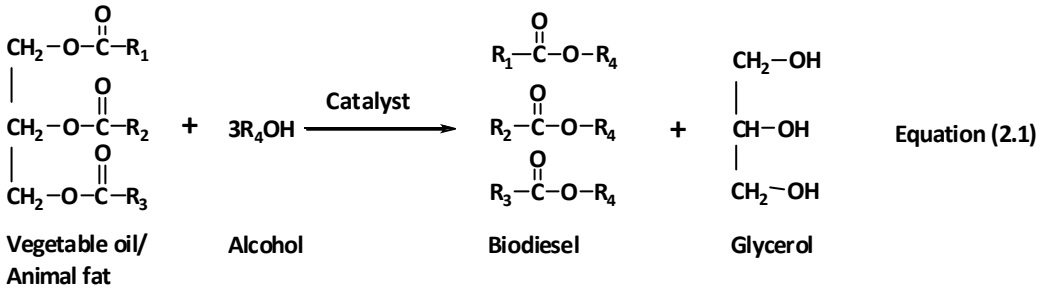
Figure 2.1. Molecular structure of glycerol

Glycerol is a chemical commodity obtained mainly as by-product in the oleochemical and biodiesel industry. Naturally combined with triglycerides in all animal fats and vegetable oils, it represents about 10% of these materials. It is derived from fats and oils during the production of fatty acids and soap production, or by the transesterification process with alcohols for biodiesel synthesis. Although glycerol can also be produced synthetically through petrochemical processes from epichlorohydrin and using propylene as raw material, such processes are no longer conducted at the industrial level [5, 6].

In general, commercially glycerol can be categorized mainly in three basic groups: i) Crude glycerol ranging from 50-90 wt % (high water content and presence of MONG (Matter Organic No glycerol). It is brown and mainly used as energy intake for cattle. ii) industrial glycerol, content of 90-95%, (low water content and the presence of MONG) is gray and it is used in all types of intermediate industrial chemical processes and iii) refined glycerol (USP or FFC / PhEur), content above 98%, is colorless, transparent and minimum presence of MONG and water. It is used in the cosmetics, personal care, and pharmaceutical industries.

Of the three, bio-glycerol (crude glycerol) is the principal by-product obtained during transesterification of vegetable oils and animal fats from biodiesel industry [7, 8, 9, 10, 11]

(Equation 2.1) [12]. This Equation 2.1 shows the transesterification of large branched triglyceride molecule to biodiesel and glycerol. In theory, from this process, three moles of bio-glycerol can be produced accompanied by one mole of biodiesel. Currently, the biodiesel production is expanding rapidly worldwide. As a result, the market is being flooded with excess crude glycerol during the transesterification process of the oil with methanol or ethanol. However, the crude glycerol is not pure and it is not cost-effective to purify this waste stream for use in the food, pharmaceutical, or cosmetic industries. Accordingly, there are various alternative methods developed for utilizing this crude glycerol. If this waste stream can be utilized economically, for instance for producing H_2 for fuel cells in large scale; the biodiesel production process will become more profitable and more prevalent.



Equation (2.1)

Biotechnology can provide a broad range of methods for the valorization of glycerol. This is due to the glycerol's nature of being a simple carbon source that can easily assimilated by numerous eukaryotic and prokaryotic microorganisms. This compound, however, had been neglected as substrate for microbial fermentations for many years mainly due to its high cost. Nevertheless, recent developments in the fuel market, which led to the production of biodiesel derived from vegetable oil in large scale, reversed this situation. Thus, the utilization of glycerol as a sole carbon and energy source for microorganisms attracted attention to the potential use in bioconversion of abundant glycerol produced from biodiesel [7, 9, 13].

This chapter presents a review of a systematic and comparative study of currently available reports on bio-hydrogen and concomitantly biochemical production from crude glycerol as a substrate. Mostly, the characteristic of crude glycerol generation from BDP biodiesel manufacturing company, which we use in this study, is reviewed as a case study of glycerol resource. The most important issues related to the glycerol industry are elucidated. H_2 production potential of crude glycerol by biotechnological production processes has been rectified. Various pretreatment methods, reaction conditions by microbial for H_2 production as well as the glycerol bioconversion potential of different microorganisms is described. Shortcomings of crude glycerol bioconversion, limitation of study and various strategies for improved H_2 production is also explored in detail.

2.2.1 Biodiesel production (Biodiesel Industry)

Biodiesel, defined as a clean burning fuel used for diesel engines, is manufactured from renewable sources (vegetable oils, animal fats, or used cooking oils) and short chain alcohols (methanol, ethanol, or butanol), via transesterification process. This process is uses methanol, ethanol or butanol to produce a methyl, ethyl or butyl esters fatty acids, respectively. Vegetable oil usually contains up to 14 different kinds of fatty acids [14]. In this process, glycerol is an important byproduct. Biodiesel is considered as a renewable fuel source. Currently most of the biodiesel is produced using methanol, which is petrochemically obtained. This dependence on methanol could be considered as non-renewable basis. Accordingly, different efforts to produce biodiesel from ethanol are carried out to generate a renewable process [15, 16, 17].
The use of biodiesel has many advantages. In a global energy and environmental context, it decreases the reliance on petroleum fuel imports, promote rural development, and reduce greenhouse gas emissions.

2.1.1 World biodiesel and crude glycerol production

The EU is the world's largest biodiesel producer, where this is also the most important biofuel representing about 70 %, on basis of volume of the total biofuels market in the transport sector. The second producer is the U.S. [18]. Currently the world's capacity for biodiesel production is increasing dramatically (Figure 2.2). The expected product of biodiesel, last year, was around 20.751 million tonnes. In turn, the expected production of glycerol, in total, was 2.751 million tonnes of glycerol (Figure 2.2) because for each of 10 tons of biodiesel produced there is always a side product of 1 tons of glycerol [19].

As shown in Figure 2.2 there is a steady growth in the biodiesel production each year. It is also expected that the biodiesel market will grow from $8.6 billion in 2009 to $12.6 billion in 2014 [24]. According to ''Global Biodiesel Market'' (2009-2014), by 2014, the total global biodiesel market is expected to be worth of US$12.6 billion [24]. In 2010 the US market for biodiesel is expected to reach 6453 million litres [25]. Hence, in the near future the global crude glycerol will increase rapidly.

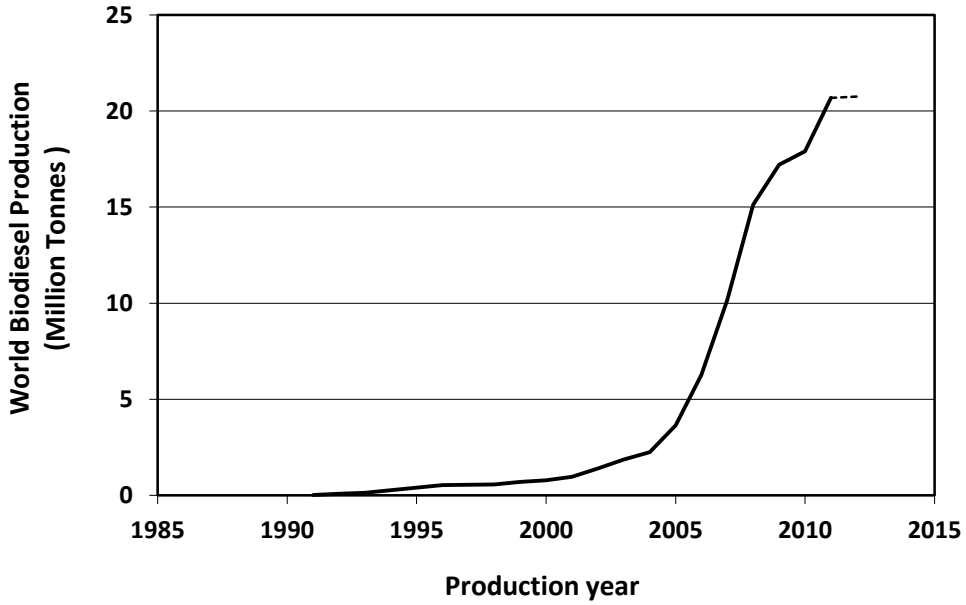


Figure 2.2. World Biodiesel Production, 1991-2012 *Note: 2012 was a projection, representation in tonnes is done by taking the density of biodiesel 0.88kg/L adapted from [20-23].

2.2.2 Renewable feed stock for biodiesel production

The cost of biodiesel production process is highly dependent upon the feedstocks price. Through the transesterification process biodiesel can be produced using a variety of feedstocks divided into: Pure plant oil (PPO), waste vegetable oil (WVO), waste animal fat (WAF) and algae oil. Recycled WVO and WAF are also gaining more attention since they are advantageous in two ways. These advantages are that they are cheap and using them eliminates the need for troublesome waste disposal. Therefore, the biodiesel production cost makes it more competitive with the fossil diesel [26].

The waste product of cooking vegetable oil exists in mass worldwide. The US only produces around 9 million tons of WVO per year [27] and countries **in** the EU produce approximately 0.7-1.0 million tons/yr. [27]. China, on the other hand, is generating more than 4.5 million tons of WVO annually. Roughly half of this could be collected through the establishment of an integrated collection and recycling system [28]. Apart from the feedstock (fat or oil), several chemicals are used to produce biodiesel for the esterification and transesterification process such as alcohol (methanol or ethanol), catalyst (KOH or NaOH) and neutralizer (HCl or H_2SO_4 or H_3PO_4) [29]**.**

2.2.3 Glycerol a byproduct of biodiesel production

Transesterification process can be carried out in two ways, chemically or biocatalytically catalyzed. Chemical catalysis has other two alternatives, alkali and acid catalysis. Industrial biodiesel production (chemical transesterification), conventionally, triglycerides, such as vegetable oils and animal fats are mixed with methanol in a reactor. Sodium hydroxide or potassium hydroxide is added as catalyst and the mixture is agitated and heated to the boiling temperature of methanol [30-32]. Fig. 2.3 represents a schematic overview of transesterification and biodiesel production process for the BPD plant. (For more information on the process flow and the plant production system it can be referred in the Index case study).

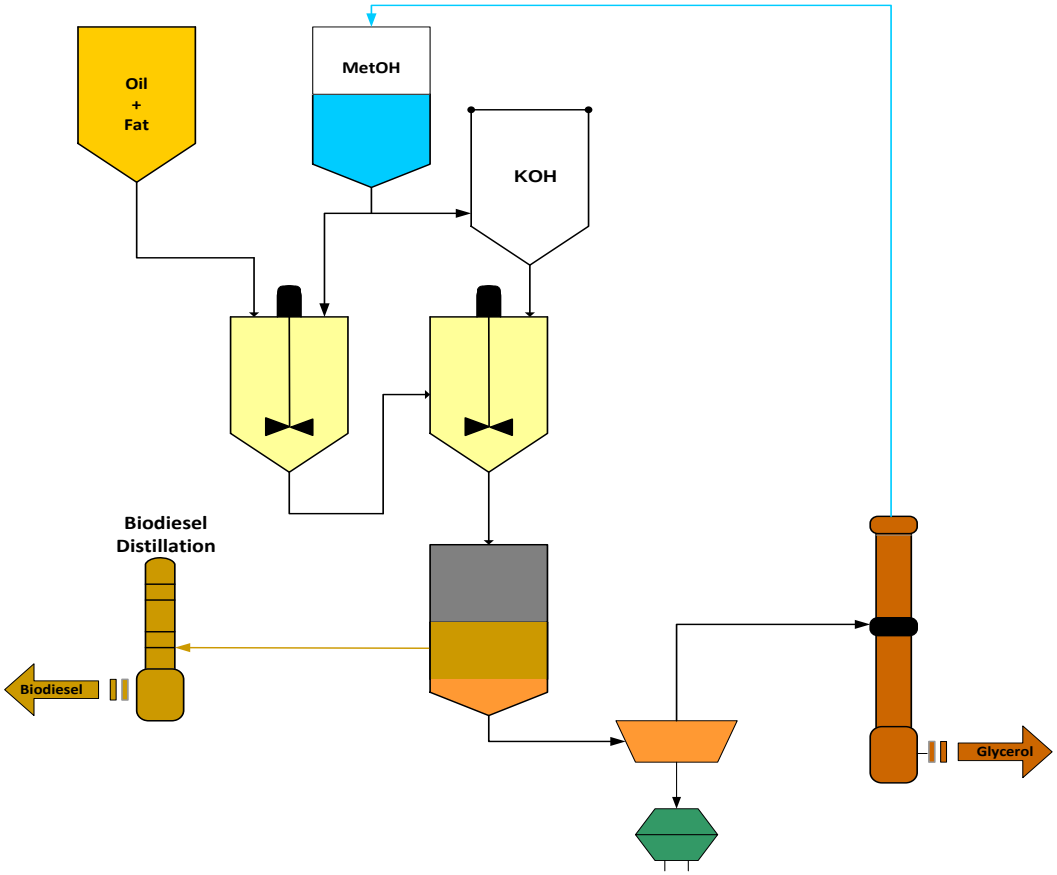


Figure 2.3. Production process in stocks del vallles (with the permission of the company)

After transesterification, the resulting two phase of methyl ester/glycerin have to be separated and further processed. The upper layer is methyl ester of fatty acids which is biodiesel and the lower layer, is crude glycerol. Excess alcohol can be recovered from the transesterified mixture or from each phase after separation. The separated glycerol contains residual alcohol, traces of catalyst and water, insolubles, unreacted solid substances present in the raw materials and some

esters. Generally, WVO and WAF will contain different amounts of proteins, ketones and aldehydes, sulphur compounds etc. which end up in the glycerol phase.

Glycerol, depending on the production plant size and the economy, can be sold as crude or refined onsite. The glycerol produced in the transesterification is of crude grade and thus, it has low value. There are different approaches to its utilization. Small producers usually limit the glycerol treatment to dehydration and either sell it to the refiners or burn it onsite for steam production. On the other hand, a refined glycerol can constitute an important economic variable for the production plant. Therefore, most big production plants refine glycerol, at least to a technical grade [33].

The crude glycerol phase generally contains almost 75% glycerol [30]. However, glycerol content in biodiesel manufacturing waste may vary for different manufacturing plants. (More detail is presented in the section of the crude glycerol composition).

Over all, the types of glycerol produced currently differ significantly in the content of water, fatty acid residues, esters, and other organic wastes. These differences are more likely due to the use of diverse feedstocks for biodiesel production. Although, most of the first use oils lead to not big differences in the glycerol layer, a completely different behavior was observed for the glycerol obtained from WVO represented by low concentration of glycerol and methanol with a high content of fats.

2.2.4 Glycerol market, production and its oversupply problem

The availability of crude glycerol has almost double since 2003, due to the increased in production of biodiesel. However, its demand has remained almost unchanged [34]. Annually nearly 160000 tons of glycerol is used for technical applications and it is expected to grow at a rate of 2.8% every year [34, 35].

Thus, this combined effect of supply excess and limited demand of raw glycerol led to low sale prices.

Although pure glycerol is an important feedstock in many industrial sectors, large-scale producers must refine raw glycerol. In order to remove impurities such as fatty acids, alcohol and catalyst, it needs to use a separation processes (filtration, chemical additions, and fractional vacuum distillation). Generally these processes are expensive and economically unfeasible for small and medium scale plants [36].

Since 2006, the glycerol oversupply forced biodiesel producers to set sales prices of 2 cents per pound or even lower for the raw product. On mid-2007, however, the price reached between 6

and 10 cents per pound [17]. On the other hand, depending on the quality and purity of the glycerol, its price showed similar trend, which was as low as 20-30 cents per pound [17,35]. As a consequence, the raw glycerol market will remain weak while a large amount of this raw component is available. Therefore glycerol is nowadays a key problem in biodiesel production. Its low sale price could convert this by-product in a residue which, then the biodiesel producers must be should find alternative uses to avoid the continue falling on the glycerol price.

2.2.4.1 Prices of crude glycerol

As biodiesel production skyrockets, the market is being flooded with crude glycerol. In US crude glycerol prices have dropped from 25 cents/lb in 2004 to 2.5-5 cents/lb in 2006 [37,38] because the current demand for glycerol is not large enough. This shows that new uses for this byproduct are clearly needed.

Until very recently, purified glycerol was considered as a high-value chemical with prices as high as $2/kg. Because 1 kg of glycerol is produced for every 10 kg of biodiesel produced. As a result, this has created a glut in the glycerol market causing sharp decrease in the price of glycerol which is now estimated to be around $0.1/kg. In addition, biodiesel production units are facing increasing production cost due to the fact that glycerol, a major income generator, has become a waste stream. This is due to the fact that the glycerol produced by a biodiesel production facility contains significant amounts of salts, heavy metals, and water. Besides, the cost of purification is way expensive than the current price of glycerol [39].

Once considered a desirable co-product that could contribute to the economic viability of biodiesel production, many now regard crude glycerol as a ‘waste stream’ with a disposal cost associated to it. For example, an analysis of the feedstock and processing costs in the production of biodiesel from soybean oil yields a gross processing margin about $0.079 per gallon of biodiesel (including a glycerol credit of $0.021, but excluding any interest expense, tax credits or fixed costs) [40]. Clearly, the development of processes to convert crude glycerol into higher value products is both an urgent need and a ‘target of opportunity’ for the development of biorefineries. Such technologies could be readily integrated into existing biodiesel facilities, thus, establishing true biorefineries and revolutionizing the biodiesel industry by improving its economics. Moreover, waste streams containing high levels of glycerol are generated in almost every industry that uses animal fats or vegetable oils as starting feed stocks (please refer Table 6.1 in chapter 6). For example, the oleochemical industry generates waste streams containing 55–90% glycerol [41]. Such glycerol surplus will not only result in a further reduction in prices, but the disposal of these streams will become a major issue [5].

2.2.5 Crude glycerol composition

The crude glycerol produced during the biodiesel production process is impure. The impurities include methanol and soaps. Biodiesel producers use excess methanol to drive the chemical transesterification and do not consume in the reaction the entire methanol. Therefore, it is present in the glycerol layer. Also, free fatty acids present in the initial feedstock can react with the base to form soaps that are soluble in the glycerol layer. In addition to methanol and soaps, crude glycerol also contains a variety of elements such as calcium, magnesium, phosphorous, or sulphur [42]. It has been reported that glycerol makes up anywhere from 65% to 85% (w/w) of the crude glycerol streams [43, 44]. In a research conducted by Selembo et al. 2009 [45] they found the presence of 69.5% (w/v) glycerol in the waste generated by a biodiesel manufacturing plant. Similarly, Ito et al. [17] have reported the presence of 41% (w/v) glycerol in biodiesel waste collected from biodiesel manufacturing factory, Hiroshima prefecture, Japan. The crude glycerol from BPD biodiesel production plant technical contains about 47.5 % (w/v). Full analysis is presented in chapter 6. This is used as a case study.

The remaining weight in the crude glycerol streams is mainly methanol and soaps [42]. The wide range of the purity values can be attributed to different glycerol purification methods used by the biodiesel producers and the different feedstocks used in biodiesel production. For example, [42] have characterized the glycerol produced from various biodiesel feedstocks. The findings show that the crude glycerol from any feedstock is generally between 60 and 70 % (wt) glycerol. Mustard seed feedstocks had a lower level (62%) of glycerol, while soy oil feedstock had 67.8 % glycerol and waste vegetable had the highest level (76.6 %) of glycerol. Thompson and He (2006) [42] also investigated the elemental composition of crude glycerol. The elements present in the glycerol produced from most feedstocks (such as mustard seeds, canola, soybean, and waste vegetable oil) were similar. Calcium was in the range of 10-20 ppm, magnesium was 3-7 ppm, phosphorous was 10-60 ppm, and sulfur was 14-21 ppm. It should be noted that when crambe, an oilseed crop, was used as feedstock the crude glycerol contained the same elements but with vastly different concentrations. Schröder and Südekum (1999) [46] have also reported the elemental composition of crude glycerol from rapeseed oil feedstock. Phosphorous was found to be between 1.05 % and 2.36 % (w/w) of the crude glycerol. Potassium was between 2.20 % and 2.33%, while sodium was between 0.09% and 0.11%. Besides, the content of cadmium, mercury, and arsenic were all below detectable limits.

The University of Guelph Laboratory Services, Soil and Nutrient Laboratory (Guelph, ON) [47] also studied the characterization of crude glycerol shows a compositional analysis in $\mu g \cdot g^{-1}$ calcium, 4.3; magnesium, 1.3; phosphorus 7.8; potassium, 28,000; sodium 230; sulfur, 1,400; nitrogen 190; and $g \cdot L^{-1}$ glycerol, 280; methanol, 260; free fatty acids, 297.

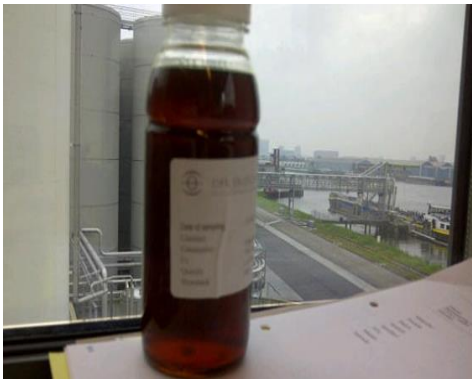


Figure 2.5. Biodiesel waste (Crude glycerol)

2.2.6 Application of crude glycerol

Glycerol has many industrial applications. Figure 2.6 shows the applications of glycerol in different sectors such as: pharmaceutical (18%), personal care (toothpaste and cosmetics 16%), polyether/polyols manufacture (14%), food (11%), triacetin (10%), alkyd (8%), snuff (6%), detergents (2%), cellophane (2%), and explosives (2%). The remaining share (11%) is used in the manufacture of lacquers, varnishes, inks, adhesives, plastic synthetics, regenerated cellulose, and other industrial uses [48].

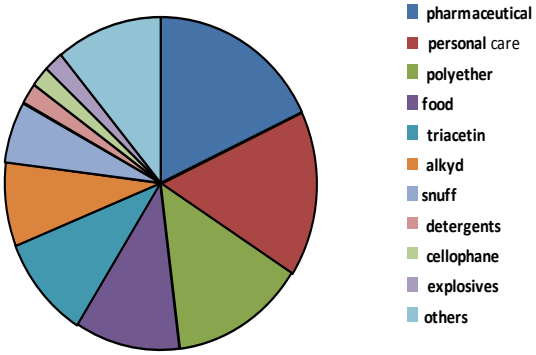


Figure 2.6. Application of glycerol

However, as mentioned earlier, purification of crude glycerol is costly [49] and hence their application as seen in figure 2.6 is not economically significant. For economic reasons crude glycerol can be utilized through a variety of methods such as combustion [37], composting, or anaerobic digestion [50]. Crude glycerol has also different uses as a feed additive for various animals such as pigs [51], broiler chickens [52], and laying hens [53]. Studies indicate that the metabolizable to digestible energy ratio of glycerol is similar to that of corn or soybean oil when fed to pigs [51]. Birds fed 2.5 % to 5% glycerol-diets had higher breast yield than the control group [52]. Crude glycerol has also been used to feed dairy cows in order to prevent ketosis, but the

result was not positive [54]. Alternatively, it can be used as a substrate for bioconversion or raw material for thermochemical to valuable products.

For biological conversions of crude glycerol, the glycerol serves as a feedstock in various fermentation processes. Different researchers have investigated bioconversion of crude glycerol to numbers of valuable products. For example, [10] Barbirato et al. (1998) reporte bioconversion of crude glycerol to 1,3-propanediol. Ying et al. [55] have also reported bioconversion of crude glycerol (85% w/v), collected after lipase-catalysed transesterification of soybean oil. Lee et al. [56] have used glycerol in the fermentation by *Anaerobiospirillum succiniciproducens* for the production of succinic acid. *E. coli* ferments glycerol leading to the production of a mixture of ethanol, succinate, acetate, lactate, and hydrogen [57]. Glycerol can also be converted to citric acid by the yeast *Yarrowia lipolytica*. It has been reported that this organism produces the same amount of citric acid when grown on glucose or on raw glycerol [58]. Rymowicz et al. [59] found that acetate mutant strains of *Y. lipolytica* can produce high levels of citric acid while producing very little isocitrate. Furthermore, it has been shown that *Clostridium butyricum* can utilize biodiesel-derived glycerol to produce 1,3-propanediol (an important chemical building block with many industrial uses) in both batch and continuous cultures. During the fermentation process, the organism also produces byproducts of acetic and butyric acid [60].

It has been also reported that glycerol can be thermochemically converted into propylene glycol [61,62], acetol [63], or a variety of other products [37]. Cortright et al. [64] have developed an aqueous phase reforming process that transforms glycerol into H_2. Virent Energy Systems is currently trying to commercialize this technology and claim that sodium hydroxide, methanol, and high pH levels within crude glycerol help the process [65].

The above discussion clearly shows how crude glycerol was successfully used for different bioconversion processes. However, there is a need to find out another economically attractive and environmentally sound bioconversion technology for crude glycerol. Production of hydrogen using bioconversion of crude glycerol may be a suitable option because, bio-hydrogen has high energy content and it is a pollution free source of energy. This gives it the potential to be an alternative to increasingly depleting fossil fuels.

2.2.7 Glycerol for biological hydrogen production

Carbohydrates, mainly glucose, are the preferred carbon sources for dark fermentation. However, due the cost of using glucose several substrates are in an attempt to look for other cheap carbon sources.

On this frontier glycerol is the best candidate. Its wide availability, cheap, more reduced nature, the rapid increase in biodiesel production which will result in a considerable surplus of glycerol in the near future and all other advantages described above, allows to use glycerol as a sole carbon and energy source. In some industrial fermentation processes, this may substitute traditional

carbohydrates such as sucrose, glucose and starch, [7,8,13]. Therefore, using this surplus of biodiesel waste to generate biofuels such as H_2 or bioethanol provides numerous benefits for the world. Glycerol can be utilized by many microbes for their growth. Energy content of pure glycerol is 19.0 MJ/kg. However, it is 25.30 MJ/kg for crude glycerol which may be due to presence of methanol and traces of biodiesel [66].

Glycerol is not only cheap and abundant but also its greater degree of reduction than sugars offers the opportunity to obtain reduced chemicals such as succinate, ethanol, xylitol, propionate, hydrogen, etc. at higher yields than those obtained using sugars [57]. For example, conversion of glycerol into the glycolytic intermediates phosphoenolpyruvate (PEP) or pyruvate generates twice the amount of reducing equivalents produced by the metabolism of glucose or xylose (Figure 2.7). Fermentative metabolism would then enable higher yield of fuels and reduced chemicals from glycerol compared with those obtained from common sugars such as glucose or xylose. The advantages of glycerol are evident when the synthesis of a reduced compound, such as succinic acid, is considered. Although production from glycerol can be achieved through a redox-balanced pathway, the use of glucose or xylose results in a shortage of reducing equivalents that clearly limits succinic acid yield (Figure 2.7).

Such high energy content of crude glycerol indicates its high potential to be an effective substrate for hydrogen production. Additionally, unlike most cellulosic waste materials it does not require additional pretreatment to make it available for the hydrogen-producing microorganisms. Moreover, substrates such as whey and molasses have high demand due to their wide range of application in industrial fermentations. Further, substrates such as food waste generally contain solid materials of different origin and need proper grinding and mixing before subjecting it to fermentation. Hence, for large scale hydrogen production, crude glycerol seems to be the ideal substrate without having the aforementioned constraints.

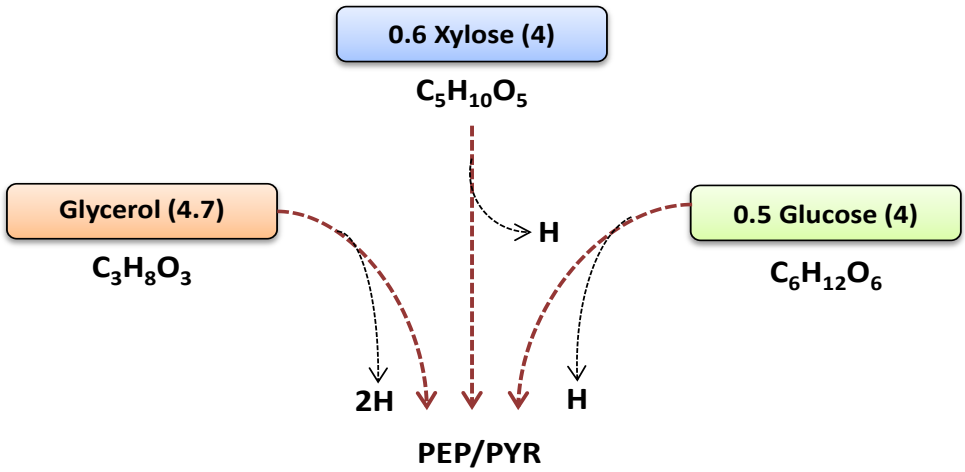


Figure 2.7. Generation of reducing equivalents during the conversion of glucose, xylose and glycerol into the glycolytic intermediates phosphoenolpyruvate (PEP) or pyruvate (PYR) in bacteria. The degree of reduction per carbon, ε, is indicated in parenthesis (adapted from [67]).

Different researchers have investigated biohydrogen and biochemical productions converted from pure and crude glycerol to H_2. We have summarized in Table 2.1 these investigations. To our best knowledge none of them have tried strains of *Citrobacter freundii* H*3* species and the *T. maritima* for H_2 production using glycerol. The use of these strains for H_2 production is studied in this thesis for the first time in chapter 3 and 4.. More details on the study done of *Citrobacter freundii* H3*, T. maritima, Enterobacter* spH1 and *E.coli* 432 are presented in other chapters of this thesis.

Ito et al [17] studied H_2 production from pure glycerol using different concentration (1.7–25g/L) using Table 2.1. Using *E. aerogenes* HU-101 as culture, they have reported a maximum H_2 production rate of 80 mmol / L*h in continuous, packed-bed reactor of 60 ml at dilution rate of 0.1 h^{-1} using 10 g/L pure glycerol in the complex medium (5.0 g/l yeast extract, 5.0 g/l tryptone). They have also reported a higher yield of 1.12 mol H_2/ mol glycerol using low amount of pure glycerol concentration 1.7 g/L in batch cultures. In addition they [17] also reported the biochemical end metabolites such as ethanol, acetate, 1,3 propanediol and formate. Wu et al. [68] also have studied the potential of glycerol as a substrate in continuous system using a lower concentration of glycerol (1,6 mmol/L) for H_2 production by *Klebsiella* sp. HE1. In their report they indicated that 0.3 mol H_2 /mol glycerol with 0.42 mol ethanol /mol glycerol and 0.3 mol 1,3-propanediol/mol glycerol. Similarly, Gonzalez-Pajuelo et al [69] study shows a conversion of higher pure glycerol concentration of 58 g/L in batch and continuous system by *Clostridium butyricum* achieved a yield of 0.6 mol 1,3 propanediol / mol glycerol and very small yield of 0.02 mol H_2 /mol glycerol was observed. The ability of glycerol conversion for *E. coli* was also tested at 10 g/L in batch system by Murarka et al.[67] and they have found a yield of 0.94 mol H_2/mol glycerol, 0,923 mol ethanol/mol glycerol and 0,012 mol acetate /mol glycerol. HU et al. [70] also showed capacity of an evolved *E.coli* HW2 bioconversion of pure glycerol to H_2 and ethanol. Summarily, Kivisto¨ et al. [71] have demonstrated H_2 production by *Halanaerobium saccharolyticum* utilizing pure glycerol as a substrate. In another study by Escapa et al. [72] shows H_2 production from glycerol using a microbial fuel cell (MFC). In addition, Seifert et al. [73] have evaluated pure glycerol as a substrate for H_2 production in a 60 ml glass reactor with working capacity of 30 ml in batch system and using anaerobic digested sludge as an inoculum. In this case, a maximum 0.41 mol H_2 per mol glycerol was obtained for a medium containing 10 g l^{-1} glycerol. *Selenomonas acidaminovorans* DSM 6589, *Clostridium butyricum* LMG 1212 t2, *Anaerosinus glycinii* DSM 5192, *Anaerovibrio lipolytica* L 1641, *Anaerovibrio lipolytica* lL 1741 are some of the strains which are reported to produce H_2 from pure glycerol. However, it was difficult to quantify the H_2 production ([74] referred here in).

We have also demonstrated that using the potential of pure glycerol for the different mesophilic strains of *Enterobacter, Citrobacter, E. coli* and thermophilic *T. maritima* for biohydrogen and biochemical products. *T. maritima* has shown highest yield of almost the theoretical maximum possibly produced with a value of 2.86 mol H_2 / mol glycerol.

However, pure glycerol is expensive and its use as a substrate for commercial production of H_2 will not be economically efficient. An alternative to this problem is crude glycerol. As described above, crude glycerol is a waste by-product of biodiesel and is produced in excess amount due to the high demand of biodiesel and it needs proper treatment prior to its disposal [75, 76]. Meanwhile, it is a good carbon source at the same time it has the potential to support microbial growth as sole carbon source.

Therefore, it can be used as an alternative feedstock for biological H_2 production. Various investigators have studied the H_2 production potential of crude glycerol and reported very high hydrogen yield. For instance, Ngo et al. [77] have investigated H_2 production by *Thermotoga neapolitana* DSM 4359 using crude glycerol as a substrate. We have also studied in serum bottle 240 mL for the first time the potential of using crude glycerol from waste animal fat and waste vegetable oil by *T. maritima* with the highest yield of 2.86 mol H_2/mol glycerol with acetate as higher end product (Maru et al 2013a)[78]. Therefore, a two-stage fermentation of crude glycerol is proposed where dark fermentation will be followed by photo fermentation using suitable photosynthetic organism since the higher acetate can be used as a carbon source. Accordingly, Guillaume and Patrick [79] have reported a maximum yield of 6 mol of H_2 per mole of glycerol consumed using photo fermentation of pure and crude glycerol by *Rhodopseudomonas palustris*. This amount is 88.8% of the theoretical maximum, 7 mol H_2 production per mole glycerol [79]. This indicates that, intermediate products such as acetic acid, ethanol and butyric acid were further metabolized to H_2, which is otherwise accumulated during dark fermentation. In another study by Hsien-Long et al [80], they have also pointed out that intermediate products, such as organic acid can be further metabolized to CO_2 and H_2 by photosynthetic organisms.

Researchers have investigated the bioconversion of glycerol using monoculture or mixed microbial consortia. As it is shown in Table 2.1, *E. aerogenes* is the most studied organism for hydrogen production by crude glycerol. Mixed microbial culture from environmental sources is the other mostly used inoculums for glycerol bioconversion. However, some constraints of process stability do exist and might be considered especially when industrial wastewaters with continuous composition variation are used. Sarma et al 2012[84] however, argue that the use of co-culture of two suitable strains for bioconversion of crude glycerol to H_2 is not been explored completely. They have also suggested that use of co-culture for glycerol bioconversion to H_2 may play a significant role in improving hydrogen yield. Similarly, some species of *Klebsiella*, *Escherichia*, *and Enterobacter* are known to have soap degradation potential [85]. Therefore, a co-culture of hydrogen producing bacteria and methanol or soap degrading bacteria may be helpful to improve bioconversion of crude glycerol to H_2. However, soap or methanol degradation efficiency of proposed co-culture should be verified in presence of crude glycerol as alternative substrate, before using it for glycerol bioconversion. Accordingly, we have demonstrated the use of selected co-culture such as *Enterobacter* spH1 with *E.coli* have a higher H_2 (1.52 mol H_2 /mol glycerol

consumed) and ethanol (1.21 mol ethanol/glycerol consumed) production for crude glycerol. Sakai and Yagishita [81] have used *E. aerogenes* NBRC 12010 to produce H_2 from crude glycerol by using bio-electrochemical cells. Bruna et al. [82] also indicated production of H_2 by anaerobic sludge using crude glycerol as a substrate

At high temperature, H_2 production is more exergonic, extreme- and hyper-thermophiles show resistance to high hydrogen partial pressures [83] which otherwise would cause a metabolic shift to production of more reduced products. One advantage of fermentation at extreme temperatures is that the process is less sensitive to contaminations. On the other hand, it is complicated to achieve a positive economical relation between the energy used to heat and maintain the reactor at high temperatures and the H_2 production. Moreover, extreme thermophilic anaerobic bacteria usually grow low densities resulting in low production rates. Statistically based evidence shows that thermophilic strains comprise high substrate conversion efficiency, but mesophilic strains achieve high volumetric productivity. Moreover, microbes of *Thermotoga* have to be preferred when aiming to achieve high substrate conversion efficiency in comparison to the families *Clostridiaceae* and *Enterobacteriaceae* [74].

From the above discussion and based on Table 2.1, it is clear that bioconversion of crude glycerol for H_2 production is gaining wide attention. This is because it is a cheap and having higher reduced nature of carbonaceous materials as a substrate for H_2 production and good hydrogen yield achieved by thermophilic microorganisms such as *T. maritima* and *T. neapolitana* through the dark fermentation.

Table 2.1- Yields of end products from glycerol by different microorganisms in batch dark fermentation system.

Organism	Substrate	Substrate concentration (g/L)	Mode of operation	pH/temperature	H_2		Other end products							Ref
					Yield (mol-H2 mol-1 glycerol)	Rate or specific H_2 production	Ethanol	Lactate	Acetate	1,3-propanediol	Formate	Butyrate	CO_2	
*Enterobacteraerogenes*HU-101		1.7	batch	6.8 /37°C	1.12		0.96	ND	0.2	0.2	0.14			
		3.3	batch	6.8/37°C	0.9		0.83	0.05	0.1	0.22	0.2			
		10	batch	6.8/37°C	0.71		0.67	0.11	0,09	0.12	0.19			
		25	batch	6.8/37°C	0.71		0.56	0.17	0,06	0.17	ND			
	Biodiesel waste		continuous	pH nc/37°C		63 mmol-H_2 l^{-1} h^{-1}	0.85							
		5	batch	6.8 /37°C	1.05		1	0.06	0.07	0.06	0.1			
		10	batch	6.8/37°C	0.89		0.86	0.14	0.09	0.16	0.12			
		25	batch	6.8/37°C	0.82		0.8	0,12	0.02	0.14	0.01			
	Pure glycerol		continuous			80 mmol-H_2 l^{-1} h^{-1}								[17]
Enterobacter aerogenes ATCC 13048	Crude Glycerol	10	batch	6.22/37°C		0.608L								
		20	batch	6.22/37°C		0.625L								
	Pure glycerol	10	batch	6.22/37°C		0.71L							345.0 cm^3CO2 dm^{-3} medium	[86]
Enterobacter aerogenes	Pure glycerol	10	batch	37°C	0.62		0.64	0.18	0.02	NR	NR			[87]
Escherichia coli	Pure glycerol	10		37°C	0.94		0.92		0.01	0				[67]
Clostridium acetobutylicum	Pure glycerol	87%(w/v)	Fed-batch Continuous	6.5/35°C	0					0.64				[87]
Colstridium butyricum	Pure glycerol	87%(w/v)	Fed-batch Continuous	6.5/35°C	0					0.69				[88]

Continued **Table 2,1**

Organism	Substrate	Substrate concentration (g/L)	Mode of operation	pH/temperature	H_2		Other end products							Ref
					Yield (mol-H2 mol-1 glycerol)	Rate or specific H_2 production	Ethanol	Lactate	Acetate	1,3-propanediol	Formate	Butyrate	CO_2	
Clostridium butyricum	Pure glycerol 87%	30.2g/l							0.024	0.6				
		58g/l	continuous	6.5/35°C					0.066	0.62				
		39.6g/l	batch	35°C	NR		NR.	0.026	0.072	0.58	NR			
	Crude Glycerol 92%	35g/l							0.031	0.57				
		60.6g/l	continuous	6.5/35°C					0.061	0.6				
		44.1g/l	batch	35°				0.023	0.073	0.51				
	Crude Glycerol 65%	30.8g/l							0.031	0.62				
		62.1g/l	continuous	6.5/35°C					0.059	0.61				
		33.5g/l	batch	35°	NR		NR	0.011	0.087	0.56	NR			[69]
Klebsiella pneumonia	Pure glycerol	1.6mmol/L	continuous	6.55	0.61		0.3		0.17	0.42				[89]
Mixed (wastewater)	Pure glycerol	4g/L	chemostat	8	0.05		0,67		0.04	0.14	0,75			[90]
Mixed (wheat soil)	Pure glycerol	3g/L	batch	6.2/30°C	0.28			<1mM		0.69	<1mM			
	Crude Glycerol	3g/L (70% Gly)	batch	6.2/30°C	0.31			<1mM		0.59	<1mM		39% CO_2 in gas phase	[75]
Enterobacter aerogenes	Pure glycerol	9.9g/L	batch	6,0/30°C	0.69		0.84			NR				[81]
Klebsiella sp HE1	Pure glycerol	50g/L	batch	6/35°C	0.345		0.42			0.3				[68]
Escherichia coli HW2	Pure glycerol	10g/L	batch	37°		0 68 +/- 0 16 mmol/L/h	1,48mmol/L/h							[70]
Mixed microflora	Pure glycerol	10g/L	batch	6/37°C	0.41		NR	NR	NR	NR	NR			[73]

Continued **Table 2,1**

Organism	Substrate	Substrate concentration (g/L)	Mode of operation	pH/temperature	H_2		Other end products							Ref
					Yield (mol-H2 mol-1 glycerol)	Rate or specific H_2 production	Ethanol	Lactate	Acetate	1,3-propanediol	Formate	Butyrate	CO_2	
Enterobacter aerogenes	Pure glycerol	2%(v/v)	batch	37°	0.89		0.001	NR	NR	NR	NR			[91]
Thermotoga neapolitana DSM 4359	Crude Glycerol	5g/L	batch	6.8-7.5/75°	2.73 ± 0.14			0.014	0.71					[77]
Halanaerobium saccharolyticum DSM 6643	Pure Glycerol	2.5g/L	batch	7.4/37°C	0.6 ± 0.02		NR		0.14	0,22		NR	0.58 ± 0.03	
Halanaerobium saccharolyticum DSM 6643(subspecies	Pure Glycerol	2.5g/L	batch	7/37°C	1.6± 0.28				0.29	NR			1.11 ± 0.21	[71]
Heat-treated anaerobic sludge	Pure glycerol		continuous	7/25°C		0.6 l-H_2 l^{-1} d^{-1}								[72]
	Crude glycerol		batch	7/30°C		0.41 ± 0.1 m^3-H_2 m^{-3} d^{-1}								
Domestic wastewater	Pure glycerol		batch		3.9									[45]
	Pure glycerol		batch	d/30°C	6		d							
Rhodopseudomonas palustris	Crude glycerol		batch	d/30°C	4									[79]
Mixed micro-flora obtained from fixed-bed anaerobic reactors	Crude glycerol		batch	5.5/25.0 ± 0.50°C		200 ml-H_2 g^{-1} COD	d							[82]
Enterobacter aerogenes NBRC 12010	Crude glycerol		batch	6/30°C	0.77								0.80 mol-CO2 mol^{-1} glycerol	[81]
Mixed micro-flora of organic waste or soil	Pure glycerol		batch	6.5/35°C		11.5-38.1 ml-H_2 g^{-1} COD	1,3-propanediol							[92]
Klebsiella sp. HE1	Pure glycerol		batch	6/35°C	0.345									[68]

Continued **Table 2,1**

Organism	Substrate	Substrate concentration (g/L)	Mode of operation	pH/temperature	H_2		Other end products							Ref
					Yield (mol-H2 mol-1 glycerol)	Rate or specific H_2 production	Ethanol	Lactate	Acetate	1,3-propanediol	Formate	Butyrate	CO_2	
Anaerobic digested sludge	Pure glycerol		batch	6/37°C	0.41								0.784 ± 0.063 l-CO_2 l^{-1} media	[73]
Bacillus coagulans IIT-BT S1	Pure glycerol		batch	6.5/37°C	2.13									[93]
Enterobacter aerogenes ATCC 15038 glycerol wastes (108mM), 1 mM thionine	Biodiesel waste		batch	6.5/37°C	0.63-0.77									[81
Escherichia coli ATCC 700926	Pure glycerol		batch	NR	0.05-0.19									[94]
Escherichia coli MG1655	Pure glycerol		batch	6.3/37°C	0.935									[67]
Clostridium pasteurianum LMG 8285	Pure glycerol		chemostat	6.8/37°C	0.87									[95]
Caloramator viterbensis DSM 13723	Pure glycerol		batch	6/60°C	0.401									[96]
Clostridium butyricum DSM 5431	Pure glycerol		chemostat	7/33°C		36.5mmol g^{-1} h^{-1}								[97]
Escherichia coli BL21(DE3) pFEGA	Pure glycerol		batch	30°C		0.026 mmol L^{-1} h^{-1}								
Escherichia coli BL21(DE3) pFEGApISC	Pure glycerol		batch	30°C		0.018mmol L^{-1} h^{-1}								
Escherichia coli BL21(DE3) ΔIscRpFEGA	Pure glycerol		batch	30°C		0.076mmol L-1 h-1								[98]
Escherichia coli BW25113 Δ*frdC*	Pure glycerol		batch	6.3/37°C		0.07mmol L-1 h-1								[70]

Continued **Table 2,1**

Organism	Substrate	Substrate concentration (g/L)	Mode of operation	pH/temperature	H_2		Other end products							Ref
					Yield (mol-H2 mol-1 glycerol)	Rate or specific H_2 production	Ethanol	Lactate	Acetate	1,3-propanediol	Formate	Butyrate	CO_2	
Escherichia coli HW1	Pure glycerol		batch	6.3/37°C		0.04 mmol $L^{-1} h^{-1}$								
Escherichia coli HW2	Pure glycerol		batch	6.3/37°C		0.92mmol $L^{-1} h^{-1}$								
Klebsiella pneumoniae DSM 2026	Pure glycerol		chemostat	7/37°C		15 mmol $g^{-1} h^{-1}$								[99]

2.2.7.1 Microbial metabolism of glycerol

Glycerol, as carbon and energy source has been used for production of H_2 and different biochemical products. Glycerol metabolisms are a dismutation process occurring through coupled oxidative and reductive pathways [100]. Both oxidative and reductive of glycerol are known for different species [101,102]. As shown in Figures 2.8 and 2.9 in the oxidative pathway, glycerol is first converted to dihydroxyacetone with the formation of $NADH_2$ [103]. This intermediate is then phosphorylated by the glycolytic enzyme dihydroxyacetone kinase. Finally, the phosphorylated product is metabolized through glycolysis to pyruvate which then may be oxidized to different end-products [101]. Ethanol, butanol, 2,3-butanediol, acetate, butyrate and lactate are some of the possible metabolites of the oxidative metabolism of glycerol (Figure 2.8 and 2.9) [15,101,104-106]. The glycerol bioconversion pathway to H_2 is based on a simple redox reaction: $2H+ + 2e- \leftrightarrow H2$ [107]. Enzymes that emanate from hydrogen producing organisms catalyze this reaction. Three of the main such enzymes are nitrogenases, [NiFe]-hydrogenases, and [FeFe]-hydrogenases [107,108]. As it is shown in Figure 2.8, the oxidative metabolism of glycerol, first pyruvate is produced and then converted to different metabolites and H_2 via different pathways. Pyruvate is broken down to acetyl-CoA via reduction of a ferredoxin (Fd) catalyzed by pyruvate ferredoxin oxidoreductase. Reduced ferredoxin (Fd) is then oxidized by a hydrogenase that reproduces oxidized Fd and hydrogen gas [107,109].

In the reducing pathway, glycerol is finally converted to 1,3-PDO via production of the intermediate product 3-hydroxypropionaldehyde. Conversion of glycerol to 3-hydroxypropionaldehyde is catalyzed by B_{12}-dependent glycerol dehydratase and related diol dehydratases, which is then reduced to 1,3-PDO by 1,3-propanediol dehydrogenase [101,110,111]. For species unable to synthesize 1,3-PDO, such as *E. coli*, the reductive pathway takes place through a respiratory pathway that requires an external electron acceptor. Alternatively, Gonzalez et al. [112] and Ko et al [109] reported that 1,2-PDO can be synthesized from the glycolytic intermediate dihydroxyacetone-phosphate (DHAP) in *E. coli*.

$$C_3H_8O_3 + H_2O \rightarrow CH_3COOH(\text{Acetic acid}) + CO_2 + 3H_2$$
$$C_3H_8O_3 \rightarrow C_2H_5OH(\text{Ethanol}) + CO_2 + H_2$$
$$2C_3H_8O_3 \rightarrow C_4H_8O_2(\text{Butyric acid}) + 2CO_2 + 4H_2$$
$$2C_3H_8O_3 \rightarrow C_4H_{10}O(\text{Butanol}) + 2CO_2 + H_2O + 2H_2$$

Equation (2.2)[84]

Equation (2.2) Stoichiometric equations showing hydrogen yield during glycerol bioconversion. From Equation (2.2), a theoretical maximum of 3 mol H_2 can be produced per mole of glycerol when acetate is the fermentation end product. However, only 2 or 1 mol H_2 per mol glycerol can be generated during butyrate and ethanol production respectively. For reduced end-products such as diols and lactic acid H_2 generation can be even lower [107, 113].

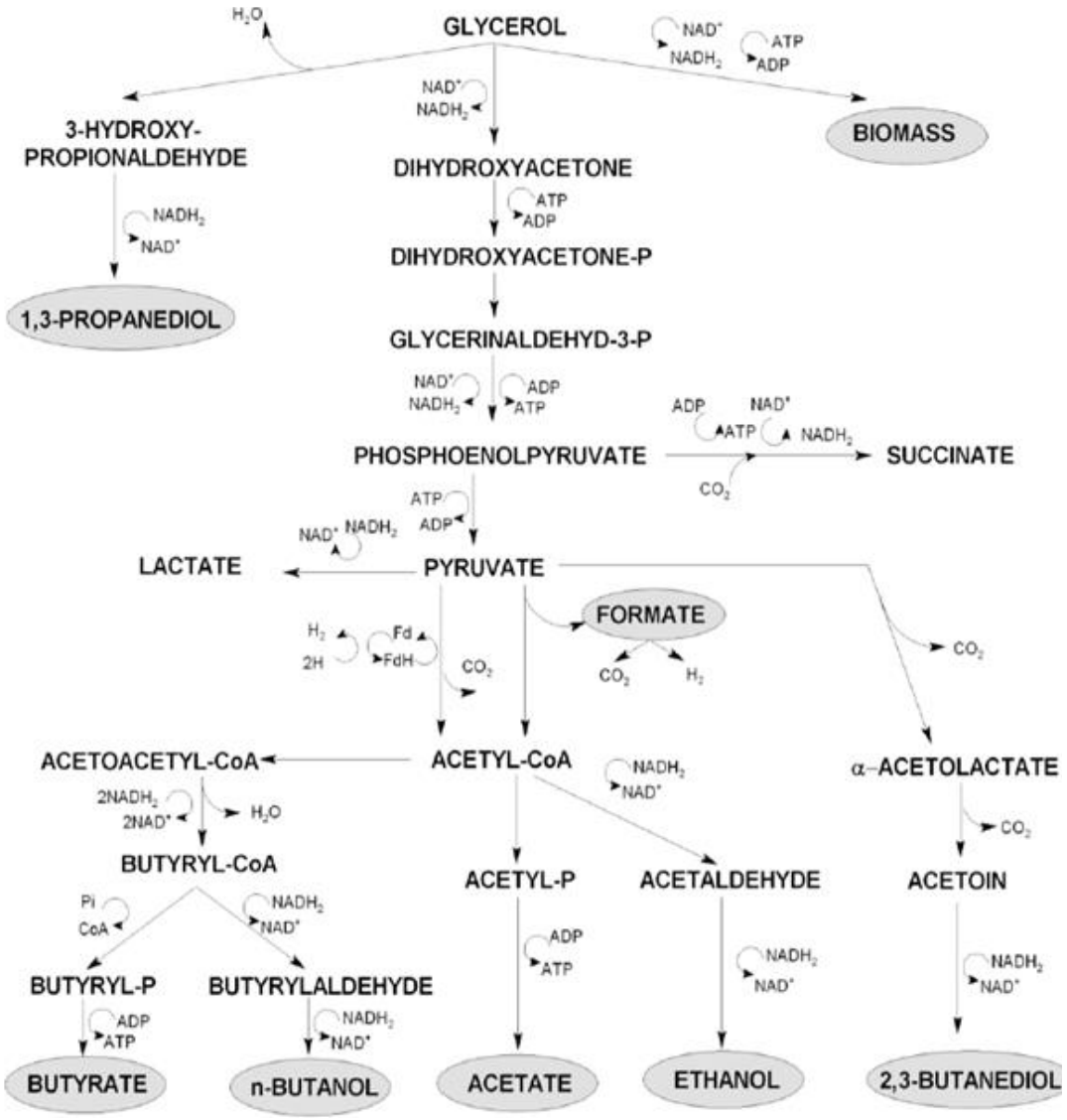


Figure 2.8. Biochemical pathways of glycerol fermentation of representative microorganism (from [89,100].

2.2.7.2 Enzymes and genes involved in metabolic path way for glycerol uptake by bacteria

A number of microorganisms can grow anaerobically on glycerol as the sole carbon and energy source. *Klebsiella* spp., *Citrobacter* spp., *Clostridium* spp., and *Enterobacte*r spp. metabolize glycerol both oxidative and reductive [114]. Constructing and identifying the genes and enzymes evolved in metabolitic pathways is a very important step for the metabolic engineering and to understand its biochemistry.

Figure 2.9 shows general biochemical pathways for glycerol fermentation. During this process, glycerol is dehydrogenated to dihydroxyacetone which then can be converted (after phosphorylation) to pyruvate. This then enters to the glycolysis catabolism pathway. This process is regulated by GldA dehydrogenase and DHAK dihydroxyacetone kinase for obtaining ethanol, succinate, acetate, and formate (Figure 2.9) [67].
In general terms, the enzymes involved in the pathways for glycerol conversion to glycolytic intermediates (i.e., GlpK-GlpD and GldA- DHAK) and the enzyme involved in the pathway for D-lactic acid synthesis from pyruvic acid are i.e., D-lactate dehydrogenase [67].

In *Klebsiella*, *Citrobacter*, *Clostridium*, *Enterobacter*, and *E. coli* glycerol is metabolized both oxidatively and reductively [114]. In the oxidative pathway, the NAD+-dependent enzyme glycerol dehydrogenase (EC 1.1.1.6) catalyzes the conversion of glycerol to dihydroxyacetone and the glycolytic enzyme dihydroxyacetone kinase (EC 2.7.1.29) phosphorylates the latter product [104-106], which is then funneled, to glycolysis. The reducing pathway is catalyzed by coenzyme B12-dependent glycerol dehydratase (EC 4.2.1.30) and related diol dehydratases (EC 4.2.1.28) [115-117], converting glycerol to 3- hydroxypropionaldehyde [118-120], and by the $NADH^+$ H^+-dependent enzyme 1,3-propanediol dehydrogenase (1,3-propanediol-oxydoreductase, EC 1.1.1.202), reducing 3-hydroxypropionaldehyde to 1,3-propanediol and regenerating NAD+ [106, 110,111, 121, 122] (Figure. 2.9). The final 1,3-propanediol (1,3-PDO) product is highly specific for glycerol fermentation and cannot be obtained from any other anaerobic conversion [123,124].

In K. pneumoniae (Forage and Lin, 1982) and C. freundii, the genes encoding the functionally linked activities of glycerol dehydratase (dhaB), 1,3-PDO dehydrogenase (dhaT), glycerol dehydrogenase (dhaD), and dihydroxyacetone kinase (dhaK) are encompassed by the dha regulon [114] (Fig. 2.9). The 1,3-PDO operon of C. butyricum is composed of three genes, a different type of glycerol dehydratase (dhaB1), its activator protein (dhaB2) and dhaT [125]. In this bacterium, glycerol dehydratase is extremely oxygen sensitive, strongly associated with the cell membrane and vitamin-B_{12} independent [43, 69, 87, 125-127]. Fermentative production of 1,3-propanediol (PD) under anaerobiosis takes place in two parallel ways. First, a fraction of glycerol is oxidezed by glycerol-dehydrogenase (Glyc-DH) to dihydroxy-acetone (DHA), and then phosphorrylated by DHA kinase to enter glycollysis. The remaining glycerol is then dehydrated to 3-hydroxypropionaldehyde (3HPA) by glyceroldehydratase, where reduction continues by propanedioldehydrogenase (PPD-DH) and by a dependent NAD oxidorreductase to 1,3- propanediol [128,129].

Fermentation from glycerol to ethanol or butanol by *C. pasteurianum* does not depend on the formation of by-products [130], since hydrogen carriers are completely regenerated in the pathway [89]. Another example of a redox-balanced process is the conversion of glycerol into succinic acid. Although the pathways for ethanol and succinate are equivalent regarding the overall redox balance, the energetic contribution of the ethanologenic pathway is much higher, as 1 ATP is produced per each molecule of glycerol converted into ethanol, while production of energy in the succinate

pathway is limited to the potential generation of a proton motive force by fumarate reductase [57] (Figure. 2.8). Such a complication can be effectively overcome by the use of microaerobic conditions. ATP will be gained through oxidative phosphorylation resulting from the reducing equivalents generated during the utilization of glycerol, including those generated by the incorporation of glycerol into cell mass (i.e. cell mass is less reduced on average than glycerol) [131] (See Figure 2.8). However, inducing microaerobic can ultimately reduce the H_2 production.

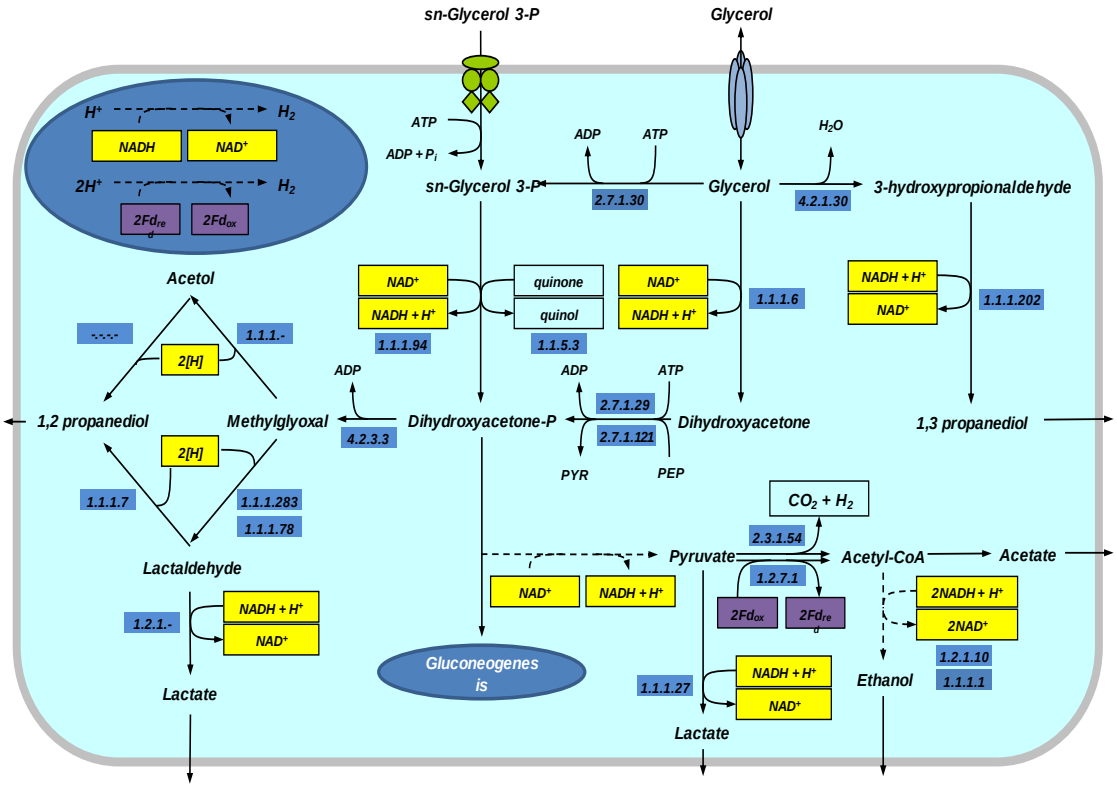


Figure 2.9. Glycerol conversion overview Figure 1 b) Metabolic pathways to 1.2- Propanediol (1,2-PD) and 1, 3-propanediol (1,3-PD) from dihydroxyacetone (DHAP), a common intermediate of sugar metabolism [15,70].

2.3 Conclusion and future perspectives

Glycerol containing waste from biodiesel manufacturing process is a potential feedstock for biohydrogen and biochemical production. Many researchers evaluated its performance as a cheap substrate for hydrogen production and indicated that its H_2 production potential is comparable to any other organic waste presently used for H_2 production. The most important advantage of using crude glycerol over other substrates for H_2 production is that it will increase the overall profit of biodiesel manufacturing plants. Such a situation may encourage the production and utilization of biofuels, which is environmentally beneficial. However, crude glycerol contains many impurities which are inhibitory to microbial growth and hydrogen production. Scarce literature reports are available on pretreatment of crude glycerol used for hydrogen production. Hence, further investigation is still required to optimize crude glycerol pretreatment for biohydrogen production. A collective removal method for different types of impurities and feasibility study of its industrial scale application may be helpful for crude glycerol bioconversion and large scale H_2 production in future. Accumulation of fermentation end products is known to have negative effect on overall H_2 yield. Hence, alternative strategy, such as further conversion of fermentation end product into CO_2 and H_2 by photo fermentation should be investigated in detail.

Similarly, most investigations on crude glycerol bioconversion have been carried out in serum bottle scale batch reactors. Only, a few studies carried out in continuous mode have given better yield of H_2 than batch experiments. Hence, further investigation of microbial H_2 production using continuous mode is recommended. Detailed study and optimization of fermentation parameters may play a vital role for large-scale hydrogen production in future. Alternatively, co-culture of two different strains can also be evaluated for crude glycerol bioconversion. Application of a co-culture, which is capable of reducing the accumulation of fermentation end products by simultaneously metabolizing them to H_2, is an interesting subject for future reserach.

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CHAPTER 3

3. BIOHYDROGEN PRODUCTION BY DARK FERMENTATION OF GLYCEROL USING *ENTEROBACTER* AND *CITROBACTER* SP.

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3.1 Abstract

Glycerol is an attractive substrate for biohydrogen production because in theory it can produce 3 mol of hydrogen per mol of glycerol. Moreover, glycerol is produced in substantial amounts as a byproduct of producing biodiesel, the demand for which has increased in recent years. Therefore, hydrogen production from glycerol was studied by dark fermentation using three strains of bacteria: namely, *Enterobacter* spH1*, Enterobacter* spH2*, Citrobacter freundii* H3 and a mixture thereof (1:1:1). It was found that when an initial concentration of 20 g/L (217.4 mM) of glycerol was used, all three strains and their mixture produced substantial amounts of hydrogen in the range 108.8 to 156.7 mmol/L (2400 to 3500 mL/L). The main non-gaseous fermentation products were ethanol and acetate, albeit in different ratios. For *Enterobacter* spH1, *Enterobacter* spH2*, C. freundii* H3, and the mixture (1:1:1) the ethanol yields (in mol EtOH /mol glycerol consumed) were 0.98, 0.67, 0.31, and 0.77, respectively. Compared to the individual strains, the mixture (1:1:1) did not show a significantly higher hydrogen level, indicating that there was no synergistic effect. *Enterobacter* spH1 was selected for further investigation because of its higher yield of hydrogen and ethanol.

Key-words: Enterobacter, Citrobacter freundii, biohydrogen production, glycerol, fermentation

3.2 Introduction

The global energy crisis and environmental concerns are stimulating the search for alternative energy sources. As a sustainable energy carrier, hydrogen (H_2) is an ideal alternative to fossil fuels because it is environmentally safe and the contribution to the greenhouse effect is lower [1]. It also has a high energy yield (122 kJ/g), which is about 2.75 times greater than that of hydrocarbon fuels, and can be directly used to produce electricity through fuel cells [2,3].

Biohydrogen can be produced from water, and also by microorganisms from renewable organic wastes, or biomass [4]. Several technologies are being used to produce H_2 from biomass economically [5]. One of these is anaerobic dark fermentation and it is particularly promising because it is simpler and cheaper than photofermentation; steam reforming or gasification, it adapts sources from a broad spectrum [6, 7] and it can generate energy at the same time as it treats biodegradable waste material [8]. Besides, unlike photofermentation, the process does not rely on the availability of light sources [3]. However, to make it more profitable and sustainable it is necessary to couple it to a photofermentative step or microbial electrolyisis for complete oxidation of acetate**.** Substrates, mainly carbohydrate sources such as glucose [9, 10] and xylose [11] have been tested in an attempt to maximize biohydrogen through dark fermentation. However, these carbon sources are very costly.

Recently, the feasibility of using organic wastes or waste-waters [12-16] has been widely studied for biohydrogen production. Glycerol waste from the biodiesel industry has emerged as a promising substrate for bioconversions [17]. The world biodiesel production is increasing. In 2010, the total annual production capacity in the US and EU was 6.9 million tones and it is expected to double in 2012 [18]. This rapid increase in biodiesel production will result in a considerable surplus of glycerol waste, because about 1 kg glycerol is generated for every 10 kg of biodiesel produced. In this regard, it is an attractive carbon source for dark fermentation. Although it has been known for decades that H_2 can be generated through glycerol fermentation, dark fermentative H_2 production from biodiesel-glycerol waste has been studied and used very little [17, 19-21].

Glycerol dissimilation is a dismutation process occurring through coupled oxidative and reductive pathways [22]. In the oxidative pathway, glycerol is first converted to dihydroxyacetone with the formation of $NADH_2$ [23]. This intermediate is further phosphorylated and channeled to pyruvate and may be metabolized to different end-products by different organisms [24]. Ethanol, butanol, 2,3-butanediol, acetate, butyrate and lactate are some of the possible metabolites of the oxidative metabolism of glycerol [25].

In the reductive pathway, glycerol is converted to 1,3-propanediol (1,3-PDO) via the production of an intermediate product 3-hydroxypropionaldehyde. For species unable to synthesize 1, 3-PDO, such as *E. coli*, the reductive pathway takes place through a respiratory pathway that requires an

external electron acceptor [26]. Alternatively, Gonzalez et al. [26] and Ko et al. [27] reported that 1,2-PDO can be synthesized from the glycolytic intermediate dihydroxyacetone-phosphate (DHAP) in *E. coli*.

In most of the glycerol bioconversion pathways, H_2 is also produced during oxidative metabolism along with other metabolites. A few microbial species belonging to the genera *Enterobacter*, *Escherichia, Klebsiella*, *Clostridium* and *Halanaerobium* are reported to produce H_2 through dark fermentation [19,28-31]. Various mixed micro-flora and co-cultures have also been examined for their ability to produce H_2 from glycerol [32,33].

From Equation (3.1), a theoretical maximum of 3 moles of H_2 can be produced per mole of glycerol when acetate is the fermentation end product. However, only 2 moles of H_2 or 1 mol of H_2 per mol glycerol can be generated during butyrate and ethanol production, respectively, and for reduced end-products such as diols and lactic acid H_2 generation can be even lower [4,34].

$$C_3H_8O_3 + H_2O \rightarrow CH_3COOH(\text{Acetic acid}) + CO_2 + 3H_2$$
$$C_3H_8O_3 \rightarrow C_2H_5OH(\text{Ethanol}) + CO_2 + H_2$$
$$2C_3H_8O_3 \rightarrow C_4H_8O_2(\text{Butyric acid}) + 2CO_2 + 4H_2$$
$$2C_3H_8O_3 \rightarrow C_4H_{10}O(\text{Butanol}) + 2CO_2 + H_2O + 2H_2$$

Equation (3.1)[35]

The use of glycerol as a carbon source for H_2 production using different organisms has yet to be thoroughly examined via dark fermentation. Therefore, identifying and isolating more H_2-producing strains and forming co-cultures for given media and different fermentation conditions, which could achieve the maximum hydrogen production (3 mol H_2/mol glycerol), is of great importance.

This study assesses the potential of glycerol for producing H_2 under anaerobic conditions (i.e. dark fermentation) using the newly isolated strains *Enterobacter* spH1*, Enterobacter* spH2*, Citrobacter freundii* H3 and their co-culture (1:1:1).

3.3 Material and Methods

3.3.1 Fermentable substrates

Pure glycerol (molecular biology, purity ≥ 99%) was obtained from Sigma Chemical Co., Madrid, Spain. All other chemicals used were of analytical grade. For this study a glycerol-limiting substrate concentration of 20 g/L was used as a carbon source. Lui et al.[36] also found that 20.4 g/L of initial glycerol was optimal for H_2 production by *Klebsiella pneumoniae.*

3.3.2 Microorganisms and media

Enterobacter spH1, *Enterobacter* spH2 and *Citrobacter freundii* H3, isolated from the Mediterranean Sea (San Carles de la Rapita, Spain), were tested for their ability to produce H_2 from glycerol. Standard microbiological and safety procedures were followed while the cultures were handled. During the isolation procedure, the marine water samples were filtered through an EZ-Pak membrane filter on a Microfil support (Millipore Co, USA). The membranes were removed from the support by sterile forceps, transferred to MacConkey's agar medium in Petri dishes, and incubated in nutrient medium at 37°C for 24 h. The colonies were re-isolated on MacConkey's agar medium and the pure cultures were identified using API 20E strips. PCR amplification and sequencing of the 16S rDNA amplicon was used to confirm the identities of the bacteria.

The growth medium, nutrient broth (NB), consisted of 5.0 g/L peptic digests of animal tissue, 5.0 g/L sodium chloride, 1.5 g/L beef extract and 1.5 g/L yeast extract [10]. The fermentation medium (MYG) was prepared with 10 g/L malt extract, 5 g/L yeast extract, 20 g/L glycerol and 10 g/L sodium chloride. The pH readings of the growth and the fermentation medium were adjusted to between 6.5–6.8 before sterilization at 121 $^{\circ}$C and 1.5 kg/cm^2 pressure for 20 minutes. Both media were selected because of their suitability for H_2 production [10]. The strains were aerobically precultured overnight in NB at 37°C in an incubator-shaker at 200 rpm. The cells were harvested at the end of the exponential phase, re-suspended in MYG and 10 % (v/v) was used as an inoculum for the batch experiment in the MYG medium (pH = 6.34) under anaerobic conditions.

3.3.3 Batch experiment

H_2 production by dark fermentation was investigated in a batch system. A 1.2 L bioreactor was used with a working volume of 500 mL, continuously agitated at 200 rpm. The bioreactor was water jacketed by a circulating water bath to maintain the reaction temperature at 37°C. At the top of the bioreactor, there were inlets for the medium and Ar, and outlets for gases. A total of 450 mL of MYG medium containing different concentrations of substrate was placed in the bioreactor and autoclaved (for 15 min at 1.5 kg/cm^2 pressure and 121°C). An anoxious atmosphere was created by continuous purging with 30 mL/min of Ar gas (99.99 %). The reactor was on-line connected to GC to directly analyze the gases generated. The liquid byproducts were analyzed by GC-MS. Unless stated otherwise, the duration of the batch fermentation was 72 h. Each experimental condition was studied in duplicate or triplicate.

3.3.4 Analytical methods

The composition of the gas was measured using a GC-14B gas chromatograph equipped with a thermal conductivity detector (TCD) and a 80/100 Porapak-Q column. Argon was used as the carrier gas at a flow of 30 mL/min. The H_2 from the fermentation was calculated by comparison

with standard pure gas. For each batch, the gas samples were continously analyzed by online GC connected to the bioreactor. The operational temperatures of the GC for the injection port, oven and detector were 150°C, 80°C and 200°C, respectively. The chromatogram was developed and analyzed using the Turbochrome Navigator (version 4.1) software from the Perkin Elmer Coorp.

The organic acids (formate, lactate, acetate, propionate, butyrate), alcohols (butanol, ethanol) and diols (1,2-propanediol, 1,3-propanediol, 2,3-butanediol) in the liquid phase were analyzed by GC-MS, which was equipped with an HP PLOT column (divinylbenzene/styrene polymer), 30 m long, 0.32 mm ID, 20 µm film thickness and operating at an inlet temperature of 200°C, a pressure of 6.1 psi and an oven temperature of 35°C for 5 min increasing to 150°C at 5°C/min. Prior to analysis by GC-MS, the liquid samples were centrifuged at 9800 rpm for 15 min and filtered through a 0.2 µm disposable filter. The injection volume of the sample was 5 µl.
Glycerol was analyzed using the K-GCROL enzymatic kit from Megazyme International. The samples for the kit analysis were also centrifuged at 9800 rpm and filtered through a 0.2 µm disposable filter before analysis.

The protein biomass estimations were measured using Peterson's protocol, a modified Lowry method [37]. The dry cell biomass was calculated taking into account that protein comprises about 60% of the cell content [38]. The carbon fraction represents 54% of dry weight [39].

For total organic carbon (TOC) analysis, 5 mL aliquots were filtered (0.22 µm pore filter size) and analyzed in TOC (Tekmar, Total Organic Carbon Analyzer) to determine soluble carbon.

3.3.5 Kinetic parameters

The cumulative H_2 production in anaerobic fermentation processes can be fitted to the data by the modified Gompertz equation, Equation (3.2) [33,40]. Cumulative H_2 production curves were obtained throughout the batch experiment. The total area was calculated at each point of the experiment.

$$H(t) = P_{\max,} .\exp\{-\exp\left[\frac{R_{\max,H2}.e}{P_{\max,H2}}\left(\lambda_{H2} - t\right)+1\right]\} \qquad \textbf{Equation (3.2)}.$$

Modified Gompertz equation, where *H(t)* is the cumulative H_2 production (mL), λ the lag-phase time (h), *P* the H_2 production potential (mL), R_m the maximum H_2 production rate (mL/h), t the incubation time (h), and e the exp(1) = 2.718. This equation was found to be suitable for describing the progress of cumulative gas production during the experiments [33,40].

3.4 Results and Discussion

3.4.1 Comparative H_2 production from glycerol using different strains

This section presents the results of a comparative study made of the production of H_2 and other usable byproducts from glycerol using *Enterobacter* spH1*, Enterobacter* spH2*, C. freundii* H3 and the mixed culture (1:1:1). Identifying the best microbial organism for the MYG medium and fermentation with glycerol as a substrate could be the first step in the process of optimizing H_2 production.

Figure 3.1 shows the total H_2 production for pure strains of *C. freundii* H3, *Enterobacter* spH1, *Enterobacter* spH2, and their co-culture from an initial glycerol concentration of 217.4 mmol/L (20 g/L) and their corresponding control (without glycerol), at 72 h of incubation. It can be seen that the highest H_2 production (3547 and 3506 mL /L) of *C. freundii* H3 and *Enterobacter* spH1 is almost the same. The overall productivity can be calculated from figure 3.1 over the incubation time of 72 h and it can be seen that *C. freundii* H3 (49.27 mL H_2/L h) and *Enterobacter* spH1 (48.71 mL H_2/L h) have the same magnitude.

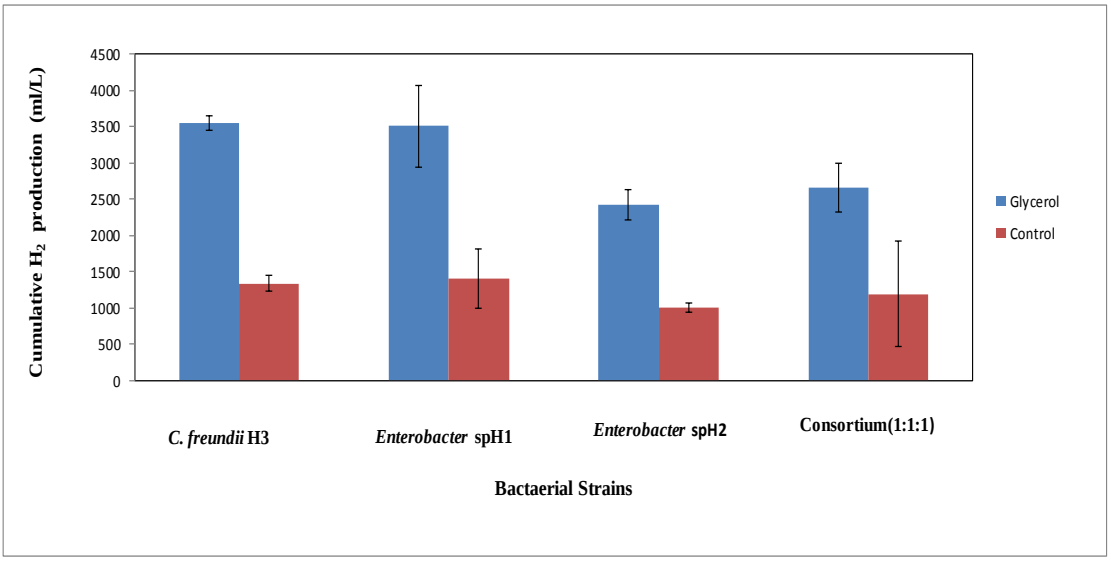

Figure 3.1. Comparative H_2 production by *C. freundii* H3, *Enterobacter* spH1, *Enterobacter* spH2, their mixed culture (1:1:1) and their control, at 72 h.

We also analyzed the end products of the fermentation and yield by *Enterobacter* spH1, *Enterobacter* spH2, *C. freundii* H3 and the mixed culture (Table 3.1). H_2 yield values of *C. freundii* and *Enterobacter* spH1 were on the maximum range of the ones obtained by other meshopllic bacteria [19,28, 31]. The difference in the maximal yield of H_2 between individual and mixed (1:1:1) strains may suggest that there is no synergistic effect on higher H_2 production in the MYG medium. However, a 10% increment was reported by Kotay et al. [41] using a consortium

consisting of *E. cloacae* IIT-BT 08, *C. freundii* IIT-BT L139 and *Bacillus coagulans* IIT-BT S1 with glucose as a substrate. This suggests that consortium production depends on the substrate, the microbial diversity and their ability to co-exist. It can be seen that although all strains produced mainly ethanol and acetate, the value of these products was significantly different. The ethanol yield (mol EtOH /mol glycerol consumed) was 0.98, 0.67, 0.31 and 0.77 for *Enterobacter* spH1, *Enterobacter* spH2*, C. freundii* H3, and the mixed culture, respectively. *Enterobacter* spH1 produced more ethanol (178.26 mM) than the other strains and the mixed culture showed intermediate values with respect to H_2 and ethanol. Although *C. freundii* H3 produced a high amount of H_2 from glycerol, the level of ethanol (45.65 mM) was relatively low. Using *Citrobacter* cultures, Homann et al. [42], Boenigk et al. [43] and Hao et al. [44] found that 1,3-propanediol (1,3PDO) and acetate were the predominant non-gaseous end product with small amounts of ethanol and lactic acid; but no H_2 production was discussed. In our study the amounts of acetic acid, ethanol and 1,3-propanediol produced by *C. freundii* were almost equivalent. The fact that less 1,3-propanediol was produced than in previous studies could be due to the medium MYG, which does not contain Vitamin B_{12} since the enzyme, 1,3-propanediol dehydrogenase, responsible for the production 1,3PDO, is Vitamin B_{12} dependent [24].Likewise, *Enterobacter* spH1 produced small amounts of 1,2-propanediol (17.11 mM), which is a useful product because of its many industrial applications. Small differences in the pH of the final medium corresponded with the type and amount of metabolite produced. The pH readings of the consortium (4.8) and *C. freundii* H3 (4.9) were lower than those of *Enterobacter* spH1 (5.2) and *Enterobacter* spH2 (5.0), probably because higher amounts of acetic acid were produced.

Table 3.1. Quantity, yield and productivity for all strains.

End products	*Citrobacter freundii* H3	*Enterobacter* spH1	*Enterobacter* spH2	*Mixed (1:1:1)*
	mmol of product per Liter of medium			
Ethanol	45.65	178.26	76.09	104.35
Lactic acid	15.52	20.69	15.52	18.97
Butyric acid	14.86	0.00	0.00	5.41
Formic Acid	4.35	10.87	21.74	13.04
Acetic acid	41.67	22.50	53.33	80.00
1,2-propanediol	0.00	21.84	9.47	0.00
1,3-propanediol	50.00	0.00	0.00	10.53
Carbon dioxide	43.18	73.86	36.36	53.41
Hydrogen	159.65	157.81	108.79	119.71
Residual glycerol	70.65	30.98	103.26	59.78
	Product yield and productivity			
Hydrogen Yield ,Y_{H2} (mol H_2/mol glycerol)	0.94	0.85	0.95	0.76
Ethanol Yield , Y_{EtOH} (mol EtoH/mol glycerol)	0.31	0.96	0.67	0.66
Specific H_2 production rate(mL H2/ mg protein h)	0.07	0.06	0.05	0.05

All analysis was done at the end of fermentation after 72 h of incubation.
Each value indicates average of three independent experiments.

Carbon material balance. We also calculated the carbon balance for each fermentation. Table 3.2 depicts the carbon-mass balance between the initial glycerol as a carbon source and the various end metabolites produced for all the isolated strains and their co-culture. The balance equations were based on the input-output determined by analyses of all the measurable metabolites and glycerol. The increment in the biomass growth was included in the carbon-balance and the initial carbon contained in the yeast and malt extract (TOC measurements) used in our MYG medium as well. The table also shows the fractional distributions of the glycerol carbons to the metabolites at the end of the fermentation. For all the strains and co-culture, carbon recovery was around 90 %, which was similar with the study done by Saka et al. [45].

Since *C. freundii* H3 mainly produced H_2, acetic acid, 1,3-propanediol and minimal amounts of other fermentation products, this microorganism can be used for high-yield H_2 production if it is coupled with a photo-bioreactor to convert the acetic acid [46]. What is more, *Enterobacter* spH1 produced a high amount of ethanol (178.26 mM), less 1,2-propanediol (21.84 mM) and very few other products. Therefore, it can be used to produce high yields of H_2 as well as useable fermentation products (ethanol and 1,2-propanediol) in dark fermentation and, for this reason, it was selected for further investigation. The result of this investigation is presented in the following sections.

Glycerol uptake efficiency by different strains. Glycerol uptake efficiency was the other parameter used to select the best strain for H_2 production. We calculated the glycerol uptake efficiencies for the experiments carried out in the section above. The initial concentration of glycerol was 2% w/v. All the fermentations took place under the conditions described above and were run for three days. The efficiency of glycerol uptake (E) by the strains was calculated using the following formula:

$$E = [(I-F)/I] \times 100\%, \qquad \textbf{Equation (3.3)}$$

Where I and F are the initial and final concentrations of glycerol, respectively. The glycerol uptake efficiencies were 85.75% for *Enterobacter* spH1, 67.5% for *C. freundii* H3, 62.5% for the mixed culture (1:1:1) and 52.5% for *Enterobacter* spH2. This shows that *Enterobacter* spH1 can utilize glycerol more efficiently than the other strains.

Table 3.2. Carbon balance and product distribution for *Citrobacter freundii* H3, *Enterobacter* spH1, *Enterobacter* spH2 and Mixed culture at initial glycerol concentration of 217,39 mM .[a]

Strains	*Citrobacter freundii* H3			*Enterobacter* spH1			*Enterobacter* spH2			Mixed culture (1:1:1)		
	Concentration (Conc.)		Total Carbon	Concentration (Conc.)		Total Carbon	Concentration (Conc.)		Total Carbon	Concentration (Conc.)		Total Carbon
Initial	**mmol/Liter**		**mmol/Liter[b]**	**mmol/Liter**		**mmol/Liter**	**mmol/Liter**		**mmol/Liter**	**mmol/Liter**		**mmol/Liter**
Glycerol	217.39		652.17	217.39		652.17	217.39		652.17	217.39		652.17
Malt and Yeast			167.67			167.67			167.67			167.67
	mg protein/L	dry weight g/L	Total Carbon mmol/Liter	mg protein/L	dry weight g/L	Total Carbon mmol/Liter	mg protein/L	Dry weight g/L	Total Carbon mmol/Liter	mg protein/L	Dry weight g/L	Total Carbon mmol/Liter
Biomass	177.94	0.30	13.35	198.00	0.33	14.85	165.43	0.28	12.41	182.52	0.30	13.69
	Conc.	**Total Carbon**	**Carbon Distribution[c]**	**Conc.**	**Total Carbon**	**Product Distribution**	**Conc.**	**Total Carbon**	**Product Distribution**	**Conc.**	**Total Carbon**	**Product Distribution**
End products	**mmol/Liter**	**mmol/Liter**	**%**	**mmol/Liter**	**mmol/Liter**	**%**	**mmol/Liter**	**mmol/Liter**	**%**	**mmol/Liter**	**mmol/Liter**	**%**
Ethanol	45.65	91.30	12.22	178.26	356.52	46.29	76.09	152.17	20.16	104.35	208.70	28.80
Lactate	15.52	46.55	6.23	20.69	62.07	8.06	15.52	46.55	6.17	18.97	56.90	7.85
Butyrate	14.86	59.46	7.96	0.00	0.00	0.00	0.00	0.00	0.00	5.41	21.62	2.98
Formate	4.35	4.35	0.58	10.87	10.87	1.41	21.74	21.74	2.88	13.04	13.04	1.80
Acetate	41.67	83.33	11.15	22.50	45.00	5.84	53.33	106.67	14.13	80.00	160.00	22.08
1,2-propanediol	0.00	0.00	0.00	21.84	65.53	8.51	9.47	28.42	3.77	0.00	0.00	0.00
1,3-propanediol	50.00	150.00	20.08	0.00	0.00	0.00	0.00	0.00	0.00	10.53	31.58	4.36
CO_2[d]	43.18	43.18	5.78	73.86	73.86	9.59	36.36	36.36	4.82	53.41	53.41	7.37
Residual Glycerol	70.65	211.96	28.37	30.98	92.93	12.07	103.26	309.78	41.05	59.78	179.35	24.75
Biomasas[g]		56.97	7.63		63.39	8.23		52.96	7.02		58.43	8.06
Total products		747.11			770.17			754.66			724.59	
Carbon Recovery %[e]	89.67			92.27			90.68			86.93		

[a] Calculated for a 1.2L bioreactor working volume of 500 mL. The reaction was started with 450mL of MYG (Malt 10g/L, Yeast 3g/L, and Glycerol 20g/L) medium and 50mL of inoculum. Each value was measured after 72h cultivation and was taken an average of triplicate experiments.

[b] Total carbon was determined by multiplying the number of carbons for each compound by moles of each compound; units are based on moles of carbon per liter

[c] Carbon distribution (%) calculated as d total carbon of each compound divided by the total product carbon multiplied by 100.

[d] CO_2 in the liquid phase was ignored.

[e] Carbon recovery (%) calculated as total product divided by total substrate carbon multiplied by 100

[f] TOC (2011 ppm) measured for the MY (malt 10 g/L and Yeast 3 g/L)

[f] Dry weight cell mass was calculated taking into account that protein comprises 60 % of the cell content [37-38] and the carbon fraction consumed for the growth was assumed of 54 % [38].

3.4.2 H_2 production from glycerol using *Enterobacter* spH1

A study was made of H_2 production and other useable byproducts using glycerol as a substrate and *Enterobacter* spH1. With glycerol, the theoretical maximum yield of H_2 is 3 mol per mol glycerol, and acetic acid is the main byproduct [32, 35]. Further conversion to H_2 is not possible without additional energy when acetic acid is the end product because of an overall endothermic reaction. The H_2 yields from pure glycerol fermentation are often substantially lower than this theoretical maximum value, mainly because 1,3-propanediol (PD) is formed as the result of a reaction that requires H_2 [32]. The formation of ethanol and other byproducts also requires H_2. Hence, if the glycerol fermentation leads to ethanol, it can produce a maximum amount of 1 mol H_2 and 1 mol ethanol per mol of glycerol consumed.

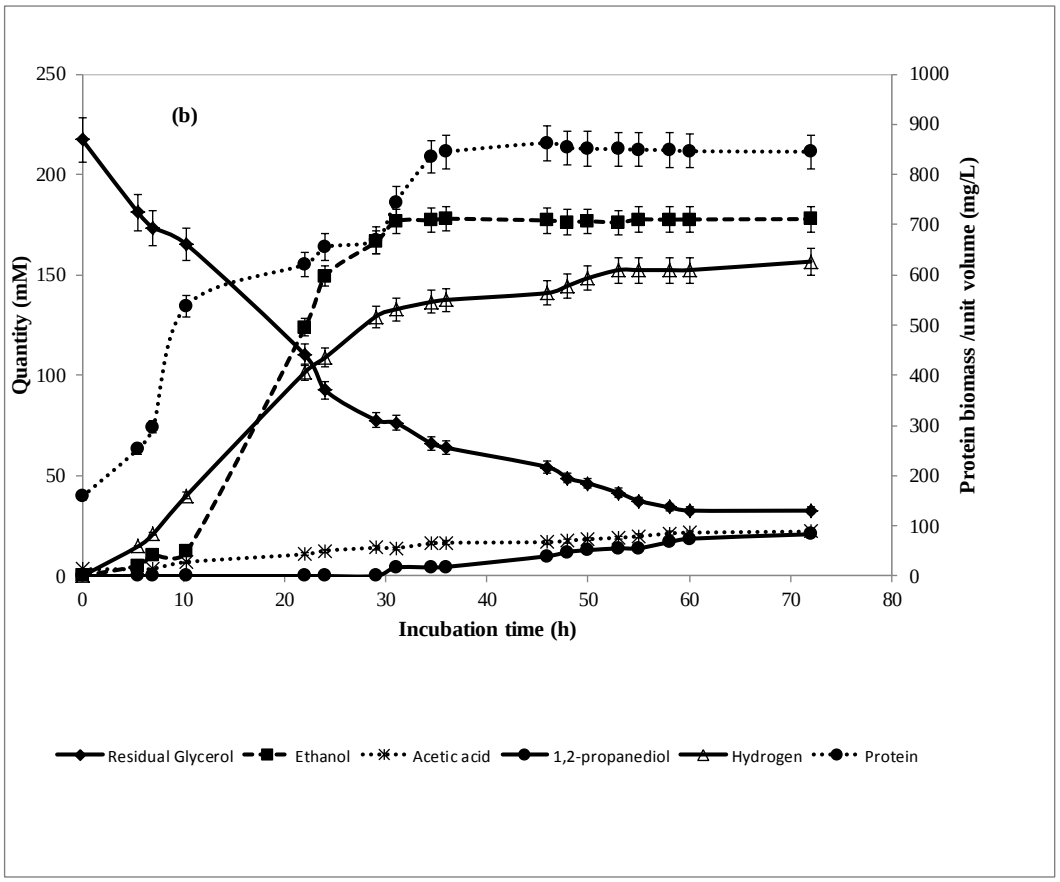

Figure 3.2. Quantity of end products, substrate residue and cumulative H_2 production by *Enterobacter* spH1 from glycerol.

Figure 3.2 illustrates the major fermentation products from glycerol (20 g/L) when *Enterobacter* spH1 is used. It can be observed that ethanol (0.96 mol ethanol/mol glycerol consumed) was the most dominant end product followed by 1,2-propanediol, acetate and small amounts of lactate (data not shown). From this figure, the H_2 yield can be calculated as an average value of 0.85 mol H_2/mol of glycerol consumed. This value is close to the theoretical yield of H_2 produced as can be seen from Equation (1) for glycerol fermentation to ethanol. This result agrees with previous data reported for *E. aerogenes* HU-101 (25 g/L of glycerol) where H_2 production was about 0.82 mol/mol glycerol [19]. Moreover, incubation time and biomass had an effect on the H_2 production. As shown in Figure 3.2, H_2 production also increased with cell biomass content to a maximum of 865 mg protein/L at 46 h with a corresponding H_2 production of 152.9 mM. After this increase, the H_2 production maintained almost constant. This may be due to the fact that, above the maximum value, the ethanol and acetate concentrations reach an inhibitory level. When the biomass of the reaction is between 2-10 % (v/v), Seifert et al. [33] and Kotay et al. [41] found that the H_2 concentration was increased. These studies also showed that substrate consumption increased as biomass increased.

3.4.3 Effect of gas purging

Argon was used to create an anaerobic environment. However, it was also necessary to see what other effects argon had on the production system. Figure 3.3 shows the effect of continuously purging the reactor with 30 mL/min of argon and purging with 30 mL/min for 5 minutes. It was concluded from Figure 3 that 30 mL/min continuous argon purging increased H_2 production by 53 mM and glycerol utilization by 15%. The yield also increased by 20.5%. This may be due to the reduction in total partial pressure in the forward reaction, which shifts the equilibrium of the reaction to the right if one or both of the gaseous products of the reaction is removed [47]. Hence, decreasing the H_2 partial pressure by reducing the total pressure of the system shifts the reaction equilibrium to the right and enhances H_2 productivity. Alshiyas et al. [48] reported that the partial pressure of a gas during H_2 production is one of the main factors that affects bacterial productivity. Levin et al. [4] have also reported that decreasing the H_2 partial pressure can improve H_2 productivity. In addition, Mizuno et al. [49] showed that gas spraying can also be useful for decreasing the H_2 partial pressure and enhancing yield. In their study, they observed that the specific H_2 production rate increased from 1.5 mL H_2/min g biomass to 3.1 mL/H_2/min g biomass under N_2 spraying conditions. And, more recently, Mandal et al. [47] showed that reducing H_2 partial pressure by lowering the operating pressure from 700 mmHg to 330 mmHg increased the maximum yield from 1.9 mol H_2/mol glucose to 3.9 mol H_2/mol glucose.

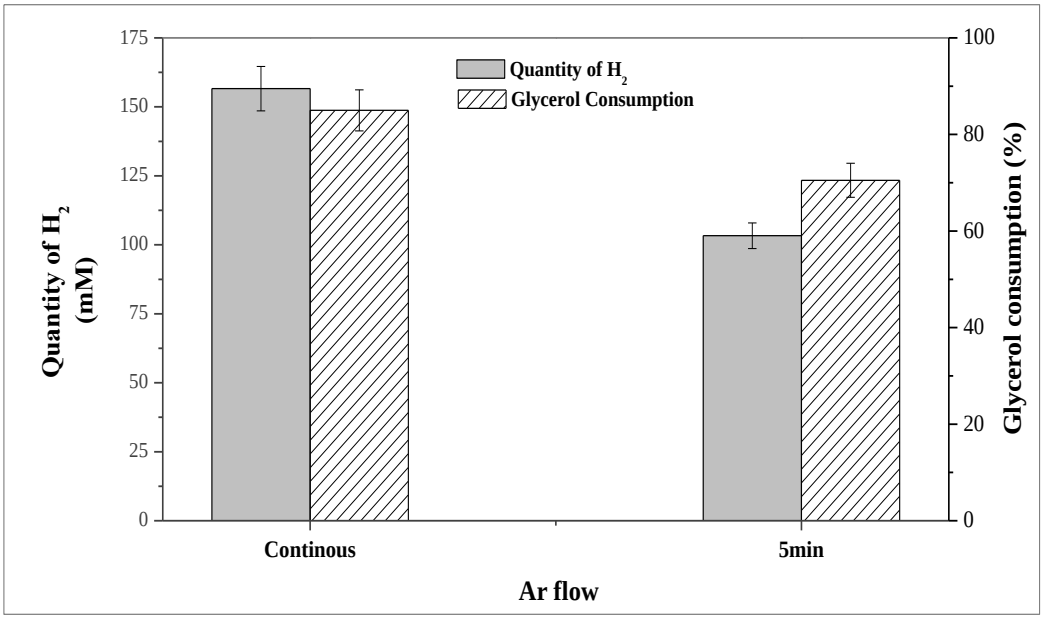

Figure 3.3. Effect of argon purging on glycerol consumption and H_2 production using *Enterobacter* spH1.

3.4.4 Kinetic parameters of hydrogen production

The cumulative H_2 production with 2% (m/v) glycerol for *Enterobacter* spH1 was fitted to the Gompertz equation (Equation 3.2). The values of *P,* R_m and λ were determined by the software program Sigma plot, which was used to fit the cumulative H_2 production data. In this software, the constraints were subjected to the equation category in the regression wizard of the fit curve. The data was iterated over 43 times. The values of the various parameters are given in Table 3.3. R_m, the maximum production rate, was 42.8 mL/h. This value was higher than those reported by Nath et al. [10] and Liu et al. [50]. The R_m and λ were in close agreement with the values obtained by Selembo et al.[32]. However, it is difficult to compare the values of these parameters because the conditions, micrograms and substrate are all different. In particular it was difficult to compare the values of the parameters for the glycerol substrate because few studies were conducted with *Enterobacter* genera.

Table 3.3. Kinetic parameters of cumulative H_2 production calculated from nonlinear regression of Gompertz equation for glycerol (20 g/L) substrates.

Kinetic parameters	Value
P (mL H_2)	3418.34
R_m (mL /h)	42.80
λ (h)	5.58
Correlation coefficient (R)	0.9987
R^2	0.9975
Adj. R^2	0.9972
Standard Error of Estimate	64.28
Residual sum of squares	15.00
D(Kolmogorov-Smirnov test)	0.13

3.5 Conclusions

This study confirms that glycerol can be used by these newly isolated bacteria to produce H_2.

This finding indicates that with glycerol as the substrate, *C. freundii* H3 mainly produced H_2 and acetic acid, and other byproducts. This is the first time that *Citrobacter* has been used to generate H_2 from glycerol. This microorganism, then, can be used for high-yield production if it is coupled with a photo bioreactor to convert acetic acid to H_2. This finding also shows that *Enterobacter* spH1 produces similar amounts of H_2, high amounts of ethanol, and other byproducts. *Enterobacte*r spH1 produced 0.85 mol H_2/mol glycerol, which is close to the theoretical yield of ethanol fermentation from glycerol (1 mol H_2/mol glycerol). Hence, it can be used for the high-yield production of H_2 and useable forms of ethanol in dark fermentation.

The combination (1:1:1) of the three cultures yielded less H_2. This suggests that there is no synergistic effect on H_2 production from pure glycerol. For all strains, the production of other metabolites such as, ethanol, 1,2-propanediol and 1,3-propanediol decreased H_2 production.
This study, therefore, has shown that *Enterobacter* spH1 and *C. freundii* H3 are effective producers of H_2 from glycerol. To optimize the yield further research needs to be carried out on the characterization of the strains and the operating conditions.

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4

CHAPTER

4. GLYCEROL FERMENTATION TO HYDROGEN BY *THERMOTOGA MARITIMA*: PROPOSED PATHWAY AND BIOENERGETIC CONSIDERATIONS

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4.1 Abstract

The production of biohydrogen from glycerol, by the hyperthermophilic bacterium *Thermotoga maritima* DSM 3109, was investigated in batch and chemostat systems. *T. maritima* converted glycerol to mainly acetate, CO_2 and H_2. Maximal hydrogen yields of 2.84 and 2.41 hydrogen per glycerol were observed for batch and chemostat cultivations, respectively. For batch cultivations: i) hydrogen production rates decreased with increasing initial glycerol concentration, ii) growth and hydrogen production was optimal in the pH range of 7-7.5, and iii) a yeast extract concentration of 2 g/l led to optimal hydrogen production. Stable growth could be maintained in a chemostat, however, when dilution rates exceeded 0.025 h^{-1} glycerol conversion was incomplete. A detailed overview of the catabolic pathway involved in glycerol fermentation to hydrogen is given. Based on comparative genomics the ability to grow on glycerol can be considered as a general trait of *Thermotoga* species. The exceptional bioenergetics of hydrogen formation from glycerol is discussed.

Key words: *Thermotoga maritima, biohydrogen, glycerol, carbon metabolism, glycerol 3-phosphate dehydrogenas*

4.2 Introduction

Hydrogen gas (H_2) is considered an attractive alternative to fossil fuels, as it burns cleanly, without emitting carbon dioxide (CO_2) or any other environmental pollutants [1]. H_2 possesses the highest energy content per unit of weight compared to other fuels, and it can be used in energy-efficient hydrogen fuels cells [2]. However, nearly 96% of the total current H_2 production, by catalytic steam reforming of natural gas, coal gasification or the partial oxidation of refinery oil, is still derived from fossil fuels. Since these processes are not based on renewable resources and still cause a net increase of atmospheric CO_2, they are not considered sustainable [3, 4]. To overcome the use of fossil hydrocarbons as sources for H_2 production, alternative methods, like electrolysis, thermal decomposition of water and biological methods, are preferred. The electro- and thermo-chemical means are very energy inefficient. Moreover, they still depend on fossil fuels for the generation of electricity and heat [5]. Biological hydrogen (biohydrogen) production by bacteria, on the other hand, is far more promising due to its potential as an inexhaustible, low-cost and environmentally friendly process, especially when it is generated from a variety of renewable resources [6, 7]. Biohydrogen is produced either by biophotolysis, microbial electrolysis, photo-fermentation, using light-dependent organisms, or by dark fermentation [8]. Biohydrogen production by dark fermentation is an anaerobic process, involving heterotrophic fermentative bacteria or archaea that convert biomass or biomass-derived hydrocarbons mainly to H_2 and acetate. To enhance the economy of H_2 production by dark fermentation it is important to explore suitable biomass substrates which can be utilized by a broad range of H_2 producing microorganisms.

Recently many research efforts have been devoted to microbial conversion of low-priced industrial and agricultural waste into bioenergy [9-11]. One of these industrial wastes concerns crude glycerol [9-11]. Crude glycerol is an inevitable by-product of the production of biodiesel; about 10 kg crude glycerol, containing 50-60% pure glycerol, is produced for every 100 kg of biodiesel [12]. In recent years, the accelerated growth of the biodiesel industry has generated a surplus of glycerol, that resulted in a 10-fold decrease in crude glycerol prices. Furthermore, this has generated environmental concern associated with glycerol disposals [11]. As a result, glycerol has gone from being a chemical commodity to a chemical waste in less than a decade. Its availability, low price and its potential to mitigate possible environmental hazards make glycerol an attractive carbon source for industrial microbiology including the dark fermentation processes.

Yet, another advantage is that fuels and reduced chemicals can be produced from glycerol at yields higher than those obtained from common sugars like glucose and xylose [11]. This is due to its highly reduced redox state of carbon, the degree of reduction per carbon for glucose and xylose is 4 compared to 4.67 for glycerol [13].

Until recently, the fermentative metabolism of glycerol had been reported in species of the genera *Klebsiella, Citrobacter*, *Enterobacter*, *Clostridium, Lactobacillus, Bacillus*, *and Anaerobiospirillum* [10, 11]. However, the potential for using these mesophilic organisms for H_2 production in dark fermentation is limited due to the low yield. In previous studies converting pure glycerol or glycerol-containing wastes [10, 14, 15] the maximum H_2 yield obtained was ~1 mol H_2 per mol of glycerol , concomitant with the production of ~1 mol of ethanol per mol of glycerol. Moreover, mesophilic microorganisms often produce reduced end-products such as diols and lactic acid, at the expense of H_2 [10, 16]. Therefore, for maximal H_2 production, oxidation of glycerol to acetic acid is required.

In light of yield optimization of H_2 from biomass, extreme thermophilic anaerobic bacteria are preferred. Their yields are reported to be approximately 83-100% of the maximum theoretical value of 4 mol hydrogen/mol glucose, in contrast to the mesophilic facultative anaerobes which show a H_2 yield of less than 2 [17]. Furthermore, thermophilic H_2 production benefits from some general advantages of performing processes at elevated temperatures, like a lower viscosity, better mixing, less risk of contamination, higher reaction rates and no need for reactor cooling [18]. Among the thermophiles, the order of the *Thermotogales* is characterized by the ability of its members to utilize a wide variety of carbohydrates [19] and to ferment sugars predominantly to acetate, CO_2, and H_2 [20, 21].

However, in literature some contradiction exists concerning the ability of *Thermotoga* species to convert glycerol. Previous studies reported that *T. maritima* contains the coding sequences for a complete pathway for both glycerol uptake and conversion [22]. A positive signal indicating oxidation of glycerol by *T. neapolitana* was found in a microplate assay [23]. Ngo et al. describes hydrogen production by *T. neapolitana* on biodiesel waste with a maximal yield of 2.73 mol H_2/mol glycerol consumed [24]. However, Eriksen et al. could not observe glycerol conversion by *T. maritima, T. neapolitana,* or *T. elfii* [25]. These, opposing results prompted us to reinvestigate the ability of *Thermotoga* species to use glycerol. Our preliminary data showed that *T. neapolitana*, but also *T. maritima* were able to form hydrogen from glycerol [26].

Here, biohydrogen production from glycerol by *T. maritima* was investigated in detail. Optimum growth parameters and cultivation conditions were determined. A putative glycerol catabolic pathway leading to hydrogen is presented, and the unusual thermodynamics and biochemistry of high yield hydrogen formation from glycerol are discussed.

4.3 Material and Methods

4.3.1 Organisms and growth conditions

Thermotoga maritima strain DSM 3109 [19] and *Thermotoga neapolitana* strain DSM 4359 [27] were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen and cultivated in M3 medium. M3 medium, which was based on M2 medium [28], consisted of (amounts are in grams per liter of deionized water): 1.5 g KH_2PO_4; 2.4 g $Na_2HPO_4 \cdot 2H_2O$; 0.5 g NH_4Cl; 0.2 g $MgCl_2 \cdot 6H_2O$; 2.0 mg $NiCl_2 \cdot 6H_2O$; NaCl, 2.7% (w/v) for *T. maritima* and 2.0% (w/v) for *T. neapolitana*; 11.9 g HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2 ethanesulphonic acid); 2 g yeast extract (YE); 15 mL trace element solution (DSM-TES, see DSMZ medium 141, complemented with Na_2WO_4 3.00 mg/L); 1.0 mL of vitamin solution (Biotin 2 mg, Nicotinamide 20 mg, *p*-Aminobenzoic acid 10 mg, Thiamine (Vit.B_1) 20 mg, Pantothenic acid 10 mg, Pyridoxamine 50 mg, Cyanocobalamin and Riboflavin 10 mg); 1.0 g/L of cysteine hydrochloride as reducing agent and 1 mg resazurin, which was used as a redox indicator. Anaerobic conditions were achieved by flushing the headspace of the serum bottles with N_2 gas. The starting pH of the medium was adjusted to pH 6.9 for *T. maritima* and pH 7.3 for *T. neapolitana* with 10 mM NaOH.

Batch cultivations were performed in 120- and 240-mL serum bottles with a working volume of 25 ml or 50 mL, at a constant temperature of 80^{o}C and shaking at 200 rpm. Cultures were inoculated with a 10% (v/v) pre-culture. The effect of the glycerol concentration (2.5 - 40 g/L) on the fermentation performance was tested for both *T. maritima* and *T. neapolitana*. Optimal growth parameters (pH, YE concentration) for glycerol (2.5 g/L) conversion by *T. maritima* were investigated for the pH range of 4.9-9.2 and YE concentration range of 0-4 g/L.

Chemostat cultivations of *Thermotoga maritima* were performed in a 2-l jacketed bioreactor (Applikon) with a working volume of 1 L. Fermentations were run at 80°C, using a stirring speed of 300 rpm and pH was controlled at 7.0 by automatic addition of 2 N NaOH. The broth was continuously sparged with N_2 gas (4 NL/h). To prevent the loss of volatile end products via the gas phase, off-gas was led through a water cooled condenser (4^{o}C). Cultivations were performed in the M3 medium without HEPES, using a glycerol concentration of 2.5 g/L and a YE concentration of 2 g/L. The medium was inoculated with an exponentially growing pre-culture (5% (v/v)). During the batch start-up phase the broth was not sparged and the gas outlet was closed, mimicking the closed bottle setup. Fermentation performance was investigated during growth at different dilution rates (0.017, 0.025, 0.036 and 0.050 h^{-1}). The system was assumed to be in steady state when H_2 and CO_2 concentrations in the off gas and fermentation profiles were constant, which in all cases occurred after ~1.5 volume change. For each dilution rate three samples at different time points were taken for further analysis.

4.3.2 Analytical methods

Substrate and fermentation end product concentrations were determined by HPLC, using a Shodex RSpak KC-811 ion exclusion chromatography column operating at 80^{o}C with a eluent of 3 mM H_2SO_4 (0.8 mL/min). Crotonic acid (10 mM) was added to the culture supernatant (16,000 × g, 10 min at 20^{o}C) as an internal standard in a 1:1 ratio to correct for differences in HPLC injection volumes. Concentrations were quantified using standard curves of the respective compounds.

During batch experiments the serum bottles headspaces were analysed for H_2 and CO_2 levels by GC, equipped with a Molsieve 13X column and a CP Poraplot Q column, respectively. For the chemostat cultivations, off-gas composition was continuously monitored using a Compact GC equipped with a Carboxen 1010 PLOT column and a Micro thermal conductivity detector using He as carrier gas.

Optical cell densities were determined at 600 nm (OD_{600}). Additionally, cell dry weight (CDW) was used to quantify the amount of biomass in the bioreactor during the continuous cultivations. CDWs were determined in technical duplicates. 2 x 15 mL culture was sampled and centrifuged (4800 g, 15 min at 4^{o}C). Each pellet was re-suspended in 2 mL ultrapure water. CDWs were determined after drying the samples for 2 days in an oven at 120^{o}C.

4.3.3 Data analysis

A modified Gompertz equation Eq. (4.1) [29] was used to estimate the maximum production rates and the production potentials of the fermentation end products acetate, lactate, H_2 and CO_2:

$$P_i(t) = P_{max,i} \exp\{-\exp[((R_{max,Pi} * e / P_{max,i})*(\lambda_i - t))+1]\} \qquad \textbf{Equation (4.1)}$$

Where: P_i – concentration of product i (mmol/L), t – fermentation time (h), $P_{max,i}$ – maximum concentration of product i formation (mmol/L), $R_{max,Pi}$ – maximum production rate of product i formation (mmol/L*h), λ_i – lag phase time. Accordingly, for the consumption of glycerol a modified Gompertz equation Eq. (4.2) [29] was used:

$$S_0 - S(t) = S_{max} \exp\{-\exp[((R_{max,s} * e / S_{max})*(\lambda_s - t))+1]\} \qquad \textbf{Equation (4.2)}$$

Where: S_0 – initial substrate concentration (mmol/L), S – substrate concentration (mmol/L), S_{max} – maximum concentration of consumed substrate (mmol/l), $R_{max,S}$ – maximum rate of substrate consumption (mmol/L*h). The fitting of the fermentation data was performed using Sigma plot application software version 12.3, where accuracy of the fit was given by correlation coefficients (R^2).

For batch cultivation yields of the fermentation end-products, expressed in mole product produced per mole glycerol consumed, were calculated using the values obtained from the data fits (Eq. (4.1) and Eq. (4.2)), according to Eq. (4.3):

$$Y_{Pmax,i} = P_{max,i} / (S_0 - S_{max}) \qquad \textbf{Equation (4.3)}$$

Where: $Y_{Pmax,i}$ – substrate yield for fermentation product *i*, S_0 – initial glycerol concentration (mol/l), S_{max} – maximum glycerol concentration (mol/L). For the chemostat cultivations molar yields were calculated using the biomass specific production and consumption rates (mmol/g*h).
Carbon balances (C-balance) and a balances of degree of reduction (γ-balance) were calculated according to Heijnen et al. [30] using the standard elemental biomass composition $CH_{1.8}O_{0.5}N_{0.2}$, which corresponds to a biomass carbon content of 48.8% and a degree of reduction of biomass of 4.2 electrons per C atom. For the batch cultivations biomass levels were estimated from OD_{max} using the relation (CDW (g/L) = 0.84 * OD_{max}, R^2 = 0.658), which was derived from the chemostat experiments.
When calculating the biomass yield in grams of CDW per mole of ATP produced (Y_{ATP}) four assumptions were made: (I) During the anaerobic oxidation of 1 mole of glycerol to 1 mole of acetate, 3 moles of ATP are produced, (II) glycerol enters the cell via passive transport, (III) 1 ATP is required for the phosphorylation of glycerol to glycerol-3-phosphate, and (IV) 1 ATP is required for the uphill formation of H_2 by proton reduction coupled to the oxidation of glycerol 3-phosphate to dihydroxyacetone phosphate. Overall this results in the formation of 1 mole ATP per mole of acetate.

4.3.4 Genomic neighborhood analysis of genes involved in glycerol conversion

The genomic neighborhoods of the *T. maritima* genes involved in glycerol metabolism were investigated using the Integrated Microbial Genomes (IMG) system (img.jgi.doe.gov).

4.4 Results and Discussions

4.4.1 Growth on glycerol

In contrast to earlier reports by Eriksen *et al.* [25] *T. maritima* was found to grow on glycerol as source of carbon and energy. Proper growth of *T. maritima* on glycerol required some adaptation time when the inoculum was pre-cultured on another substrate, like glucose. After several transfers on glycerol, the lag phase decreased and growth initiated immediately after inoculation in standard medium. Glycerol was fermented mainly to acetate, CO_2, H_2 and minor amounts of lactate (Fig. 4.1a; Table 4.1). The closely related *T. neapolitana,* that has been shown to grow on glycerol as well [24], was investigated here for comparison (Fig. 4.1b; Table 4.1). To be able to quantify and compare the different growth experiments, time courses of substrate consumption

and products formation were modelled using modified Gompertz equations (Eq. (4.1) and Eq. (4.2)). Fig. 4.1 shows a typical growth experiment for *T. maritima* and *T. neapolitana* with fitted curves for the main metabolites. The various growth parameters are summarized in Table 4.1. These data suggest that glycerol is fermented to acetate, CO_2 and H_2 in a ratio of 1:1:3. End products commonly found in mesophilic glycerol fermentation by enterobacteria [31] or clostridia [32], like ethanol, butanol, 1,3-propanediol, 1,2-propanediol, succinate, or formate, were never detected. The relatively constant C-balance near 100% also indicates that no major end product has been overlooked. In contrast to earlier data for *T. neapolitana* ([24]; ~5 mM lactate), almost no lactate was found. In accordance, the hydrogen yields of around 2.8 mol H_2/mol glycerol found here, were higher compared to the data of Ngo *et al*, who reported a value of 1.23 mol H_2/mol glycerol under non-N_2-sparged conditions [24]. This discrepancy could probably be a result of different culturing conditions, leading to variations in the dissolved H_2 concentration. For instance, Ngo et al. showed that N_2-sparging of the cultures led to H_2 yields (2.73 mol H_2/mol glycerol) [24], which are similar to the values found here. *T. neapolitana* is apparently able to adapt its metabolism from producing mainly H_2 to producing a mixture of H_2 and lactate, as reduced end products.

Both *Thermotoga* species showed a substantial decrease in optical density when the culture was approaching the stationary phase (Fig. 4.1a & 4.1b). A similar decrease in cell density has been reported by Ngo *et al.* for *T. neapolitana* [24]. The reason for the cell lysis is not known. However, this phenomenon did not affect the C-balance calculations, for which we used the maximum OD to estimate the carbon content of the biomass.

Our results clearly show that not only *T. neapolitana*, but also *T. maritima*, is perfectly able to grow on glycerol as source of carbon and energy. The inability of *T. neapolitana* and *T. maritima* to grow on glycerol, as reported by Eriksen *et al.* [25] is likely a result of differences in the growth medium. For instance, the medium used by Eriksen *et al.* [25], which was based on a medium by Van Ooteghem *et al.* [23] had an initial pH of 8.5, which is at the boundary of the optimal pH range found here. Moreover, Eriksen *et al.* used a lower NaCl concentration (10 versus 27 g/L) and added no additional Ni^{2+}, which is an essential metal in many hydrogenases.

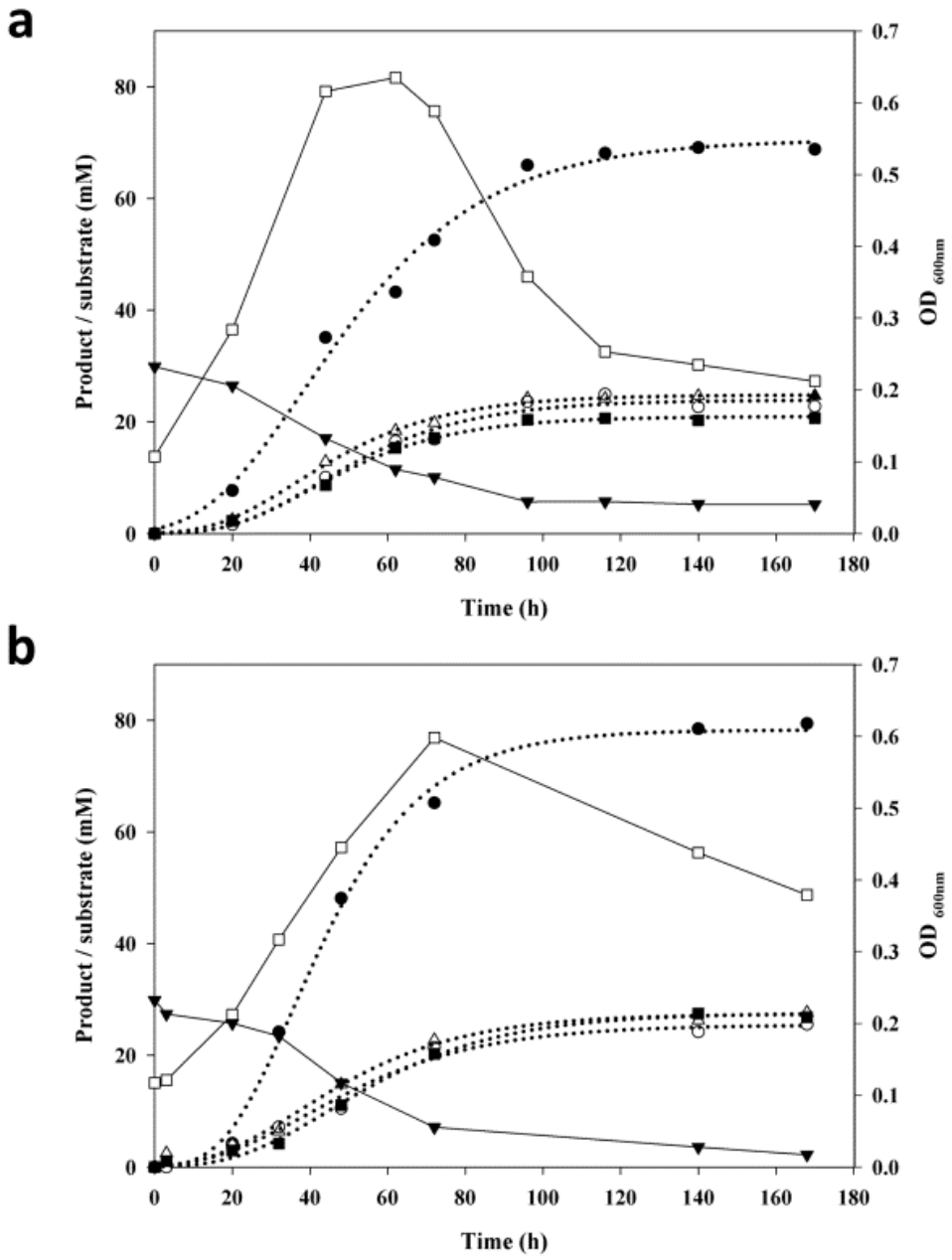

Figure 4.1. Fermentation profiles of batch cultivations on glycerol (2.5 g/L) of (a) *T. maritima* and (b) *T. neapolitana*. Residual glycerol (▼), glycerol consumed (Δ), acetate (■), H_2 (●), CO_2 (○) and OD (□). For glycerol consumed, acetate, H_2 and CO_2 data was fitted using the modified Gompertz equation (Eq. (1) and Eq. (2)) (dotted lines).

Table 4.1. Effect of different glycerol concentration on substrate consumption, end product production, H_2 productivities and yields for *T. maritima* and *T. neapolitana*

Initial glycerol conc.	Maximal consumption (S_{max}) and production ($P_{max,i}$)*					Maximal H_2 production rate	Molar yields			OD_{max}	C-balance
(mmol/L)	(mmol/L)					(mmol/L/h)	(mol/mol)				%
S_0	S_{max}	$P_{max,Act}$	$P_{max,Lact}$**	$P_{max,CO2}$	$P_{max,H2}$	$R_{max,H2}$	Y_{Act}	Y_{CO2}	Y_{H2}		
T. maritima											
29.9	24.8 (0.998)	21.0 (0.996)	0.1	23.9 (0.984)	70.5 (0.993)	1.01	0.84	0.96	2.84	0.64	105
71.4	22.4 (0.964)	17.1 (0.983)	0.0	19.2 (0.969)	62.6 (0.995)	0.63	0.76	0.85	2.79	0.43	97
164.2	17.0 (0.946)	16.0 (0.976)	0.1	16.9 (0.954)	48.2 (0.977)	0.38	0.94	0.99	2.84	0.37	101
210.8	22.0 (0.913)	18.9 (0.968)	0.1	17.4 (0.981)	46.7 (0.994)	0.38	0.86	0.79	2.12	0.45	100
T. neapolitana											
29.9	27.4 (0.990)	26.6 (0.996)	0.0	25.5 (0.982)	78.3 (0.997)	1.58	0.97	0.93	2.86	0.60	109
59.3	27.9 (0.999)	24.9 (0.995)	0.3	20.1 (0.994)	76.3 (0.999)	1.52	0.89	0.72	2.74	0.52	98
140.2	28.9 (0.985)	26.2 (0.997)	0.1	25.0 (0.997)	70.0 (0.998)	0.78	0.91	0.87	2.42	0.52	95
198.0	28.1 (0.970)	23.6 (0.995)	0.1	24.6 (0.996)	69.4 (0.999)	0.48	0.84	0.87	2.45	0.50	93

*Correlation coefficients (R^2) of the curve fits with the Gompertz equation (Eq. (4.1) or Eq. (4.2)) are given between brackets.
**Low lactate concentrations prevented proper curve fitting.

4.4.2 Effect of glycerol concentration

The type of carbon source and the initial substrate concentration usually play an important role on the bacterial growth and product yield [33]. Therefore, the effect of the initial glycerol concentration (29 mM – 210 mM) on hydrogen production by *T. maritima* and *T. neapolitana* was investigated (Table 4.1). No growth or H_2 formation was observed when glycerol was omitted from the medium. Maximal H_2 production rates ($R_{max,H2}$) decreased with increasing initial glycerol concentration. And it can be observed that irrespective of the initial glycerol concentration, total glycerol consumption is rather constant and amounts to a maximum value of approximately 25 mM and 29 mM for *T. maritima* and *T. neapolitana*, respectively. This observation suggests that glycerol conversion is not limited by the amount of glycerol present, but by some other environmental parameter. A possible reason could be the accumulation of fermentation end products, especially acetic acid, which has been shown before to cause end product inhibition [34]. The accumulation of acetate, being a weak acid, may impair the growth of bacteria by dissipation of the membrane potential [34]. Alternatively, growth inhibition may be a result of the lowering of the pH (*vide infra*).

4.4.3 Effect of pH on fermentative H_2 production

During glycerol fermentation, the pH value dropped from ~7 at the start to ~6 in the stationary phase. Therefore, we were interested in the effect of the initial pH on the growth, which was assessed here by measuring the H_2 production. Fig. 2a depicts the maximum H_2 concentration and the H_2 production rate for *T. maritima* as a function of the initial pH. Below pH 6 and above pH 8, H_2 production decreased considerably (Table 4.A). The results are in agreement with the cessation of growth around pH 6 as shown in Fig. 4.1. This might also explain the observation that higher initial glycerol concentrations do not lead to higher glycerol conversion. However, the investigation of the pH-dependence of *T. neapolitana* by Ngo *et al* [24], showed a broader pH range with growth even possible at pH 5 and pH 9.

4.4.4 Effect of YE concentration on fermentative H_2 production

Yeast extract (YE) is an important environmental determinant for the growth of many microorganisms. Here, different YE concentrations (0, 0.5, 1, 2 and 4 g/L) were tested on the glycerol conversion efficiency and the H_2 producing capacity. As seen from Fig. 2b glycerol conversion and H_2 production are low in the absence of YE. Addition of 0.5 g/L and 1 g/L results in a significantly better performance (Table 4.B). Above 2 g/L growth stimulation is limited. These results agree with the report of Schröder *et al*. [20], who found that yeast extract (0.5 g/L) was sufficient for growth and hydrogen production by *T. maritima* during growth on glucose.

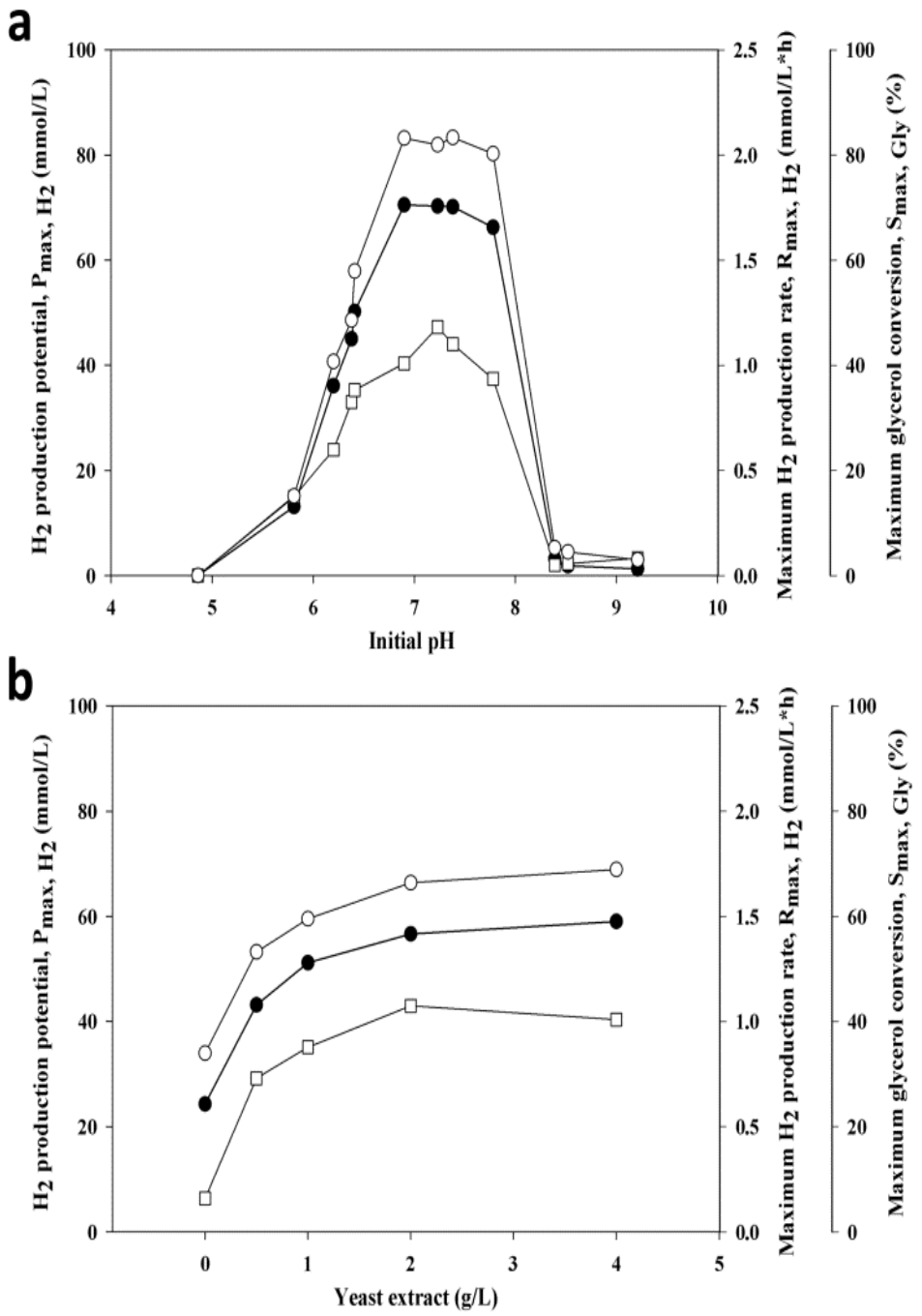

Figure 4.2. The effect of (a) initial pH and (b) yeast extract concentrations on batch fermentation of *T. maritima* grown on glycerol (2.5 g/L). Maximum glycerol conversion (○), maximum H_2 production rate (□) and H_2 production potential (●).

4.4.5 Glycerol fermentation by *T. maritima* in a continuous system

The possibility to grow *T. maritima* on glycerol in a chemostat setup was investigated. Our results (Table 4.2) show that *T. maritima*, grown on glycerol (2.5g/L), can be maintained in a continuous cultivation setup at different dilution rates (d, (h^{-1})). Similar to the batch cultivations, glycerol was mainly converted to acetate, H_2 and CO_2. Trace amounts of lactate were observed but no ethanol formation. Hydrogen yields ranged between 2.23-2.41 mol/mol glycerol consumed, which is somewhat lower than calculated for the batch cultivations as presented above. H_2 yields per acetate reach an average of 2.8 H_2/acetate, showing that almost all electrons released during acetate formation end up in H_2. Overall carbon recovery exceeded 100%, which probably reflects the consumption of YE for biomass formation. At higher dilution rates (d = 0.035 and 0.050), glycerol conversion was not complete. However, since no washout occurred, it is assumed that a factor other than glycerol was limiting growth. However, volumetric and specific H_2 production rates increased with increasing dilution rates. Interestingly, biomass yields (Y_{xs}) increased with increasing dilution rate, which may reflect changes in ATP usage for maintenance (Table 4.2).

Table 4.2. Fermentation details of *T. maritima* grown on glycerol (2.5 g/L) in a chemostat cultivation setup at different dilution rates.

Dilution rate	Substrate and product concentrations						Biomass			
	Medium	Effluent			Gas-phase*					Biomass/ATP
h^{-1}	mM							g/L	g/mol	g/mol
	Glycerol	Glycerol	Lactate	Acetate	H_2	CO_2	OD_{600}	CDW	Yxs	Y_{ATP}
0.017	27.01 ± 0.02	0.45 ± 0.02	0.44 ± 0.02	21.58 ± 0.06	64.07 ± 1.40	20.12 ± 0.39	0.47 ± 0.01	0.42 ± 0.01	15.73 ± 0.34	19.35 ± 0.45
0.025	27.01 ± 0.02	0.27 ± 0.01	0.20 ± 0.02	22.37 ± 0.22	60.66 ± 0.45	19.26 ± 0.44	0.63 ± 0.01	0.54 ± 0.03	20.22 ± 1.27	24.16 ± 1.45
0.035	27.01 ± 0.02	1.05 ± 0.04	0.17 ± 0.04	21.89 ± 0.19	57.93 ± 0.35	18.71 ± 0.05	0.70 ± 0.01	0.53 ± 0.03	20.98 ± 0.98	24.89 ± 1.27
0.050	27.01 ± 0.02	6.72 ± 0.12	0	16.71 ± 0.51	46.73 ± 0.71	14.89 ± 0.56	0.66 ± 0.02	0.50 ± 0.01	24.68 ± 0.48	30.20 ± 1.41

	Volumetric consumption/production rate					Specific consumption/production rate				
	mmol/L*h					mmol/g*h				
	q(Glycerol)	q(Lactate)	q(Acetate)	$q(H_2)$	$q(CO_2)$	q(Glycerol)	q(Lactate)	q(Acetate)	$q(H_2)$	$q(CO_2)$
0.017	-0.45 ± 0.01	0.01 ± 0.01	0.37 ± 0.01	1.09 ± 0.02	0.34 ± 0.01	-1.08 ± 0.02	0.02 ± 0.01	0.88 ± 0.02	2.61 ± 0.06	0.82 ± 0.02
0.025	-0.67 ± 0.01	0.01 ± 0.01	0.56 ± 0.01	1.52 ± 0.01	0.48 ± 0.01	-1.24 ± 0.08	0.01 ± 0.01	1.04 ± 0.06	2.82 ± 0.17	0.89 ± 0.07
0.035	-0.93 ± 0.01	0.01 ± 0.01	0.79 ± 0.01	2.09 ± 0.01	0.67 ± 0.01	-1.72 ± 0.08	0.01 ± 0.01	1.45 ± 0.08	3.84 ± 0.17	1.24 ± 0.06
0.050	-1.01 ± 0.01	0	0.84 ± 0.03	2.34 ± 0.04	0.73 ± 0.01	-2.03 ± 0.04	0	1.67 ± 0.09	4.67 ± 0.13	1.49 ± 0.05

	Molar yields						C-balance	γ-balance
	Per Glycerol consumed				Per Acetate produced		Recovery	
	mol/mol						%	
	Y(Lactate)	Y(Acetate)	$Y(H_2)$	$Y(CO_2)$	$Y(H_2)$	$Y(CO_2)$		
0.017	0.02 ± 0.01	0.81 ± 0.01	2.41 ± 0.05	0.76 ± 0.01	2.97 ± 0.06	0.93 ± 0.02	102.4 ± 0.8	101.5 ± 1.0
0.025	0.01 ± 0.01	0.84 ± 0.01	2.27 ± 0.02	0.72 ± 0.02	2.71 ± 0.01	0.86 ± 0.01	107.9 ± 2.1	105.5 ± 2.0
0.035	0.01 ± 0.01	0.84 ± 0.01	2.23 ± 0.01	0.72 ± 0.01	2.65 ± 0.04	0.85 ± 0.01	109.0 ± 1.4	106.0 ± 1.2
0.050	0	0.82 ± 0.01	2.30 ± 0.01	0.73 ± 0.02	2.80 ± 0.08	0.89 ± 0.05	109.6 ± 0.9	107.6 ± 0.9

*Values are expressed normalized to the liquid phase (1 L).

4.4.6 Glycerol degradation pathway in *Thermotoga maritima*

The proposed pathway for glycerol degradation in *T. maritima* is presented in Fig.4.3. *T. maritima* utilizes both the Embden-Meyerhof (EM) and Entner-Doudoroff (ED) pathways [40] when grown on glucose. The presence of all the conventional EM-pathway enzymes have been confirmed in cell extracts [20], and the corresponding genes have been identified in the *T. maritima* genome [22]. It was shown that the oxidation of glyceraldehyde-3-phosphate is catalysed by a NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenase [20] and the conversion of pyruvate to acetyl-CoA is catalysed by a pyruvate: ferredoxin oxidoreductase [41]. This indicates that, when glucose is converted to acetate, the reducing equivalents NADH and reduced ferredoxin are generated in a 1:1 ratio. The same reductant ratio is required in the H_2 formation step, which is catalysed by a bifurcating hydrogenase (TM1424-1426) [42]. However, the catabolism of glycerol via G3P requires an additional oxidation step. As discussed above, in *T. maritima* this is catalysed by a G3PDH, generating an additional reduced electron carrier. Based on the analogy with respiring glycerol utilizing microorganisms, and considering the redox potential of the DHAP/G3P couple of E_0 = -190 mV [43], FAD is a likely electron acceptor (FAD/$FADH_2$ couple of E_0 = -220 mV). The clustering on the genome with the pyridine nucleotide-disulphide oxidoreductase (FAD/NAD(P)-binding domain) suggests that $FADH_2$ might be used for the uphill formation of NADH. The formation of either $FADH_2$ or NADH disturbs the preferred 1:1 NADH/ferredoxin ratio, which is needed for H_2 formation via the bifurcating hydrogenase. Nevertheless, the observed H_2 yields of 2.5-3.0 mol/mol acetate, suggests that reductant derived from the oxidation of G3P, is also channelled to H_2. From a thermodynamic viewpoint the oxidation of G3P to DHAP and H_2 is not feasible ($\Delta G^{0'}$ +61 kJ/mol). Reverse electron flow, coupled to a Na^+ gradient, may enable the uphill electron transfer from NADH to the level of ferredoxin [44]. *T. maritima* contains a Rnf-cluster (TM0244-0249) that could accommodate this energy-dependent formation of reduced ferredoxin.

Based on these considerations it is expected that, the complete conversion of glycerol to acetate results in an ATP yield of 1 ATP per acetate. In *T. maritima,* glycerol enters the cell via passive transport either by diffusion or facilitated transport; one ATP is consumed when glycerol is phosphorylated; another ATP is required for the uphill formation of reduced ferredoxin from $FADH_2$ or NADH to restore the desired 1:1 NADH/ferredoxin ratio. Two ATP are generated during the conversion of 1,3-bisphosphoglycerate to 3-phosphoglycerate and the conversion phosphoenolpyruvate to pyruvate. The latter reaction could be catalysed either by a pyruvate kinase or a pyruvate, phosphate dikinase (PPDK). Based on the *T. maritima* genome, a catabolic role for PPDK seems very likely since the PPDK gene (TM0272) clusters with the fructose bis-phosphate aldolase (TM0273) and the acetate kinase (TM0274) genes as observed previously [45]. Finally, a third ATP is generated during the formation of acetate from acetyl phosphate. The ATP yield corresponds with the Gibbs energy of -73.2 kJ/mol that can be calculated for the oxidation of

glycerol to acetate and H_2 and the average amount of Gibbs energy required for the synthesis of 1 ATP (-70 kJ/mol, [46]).

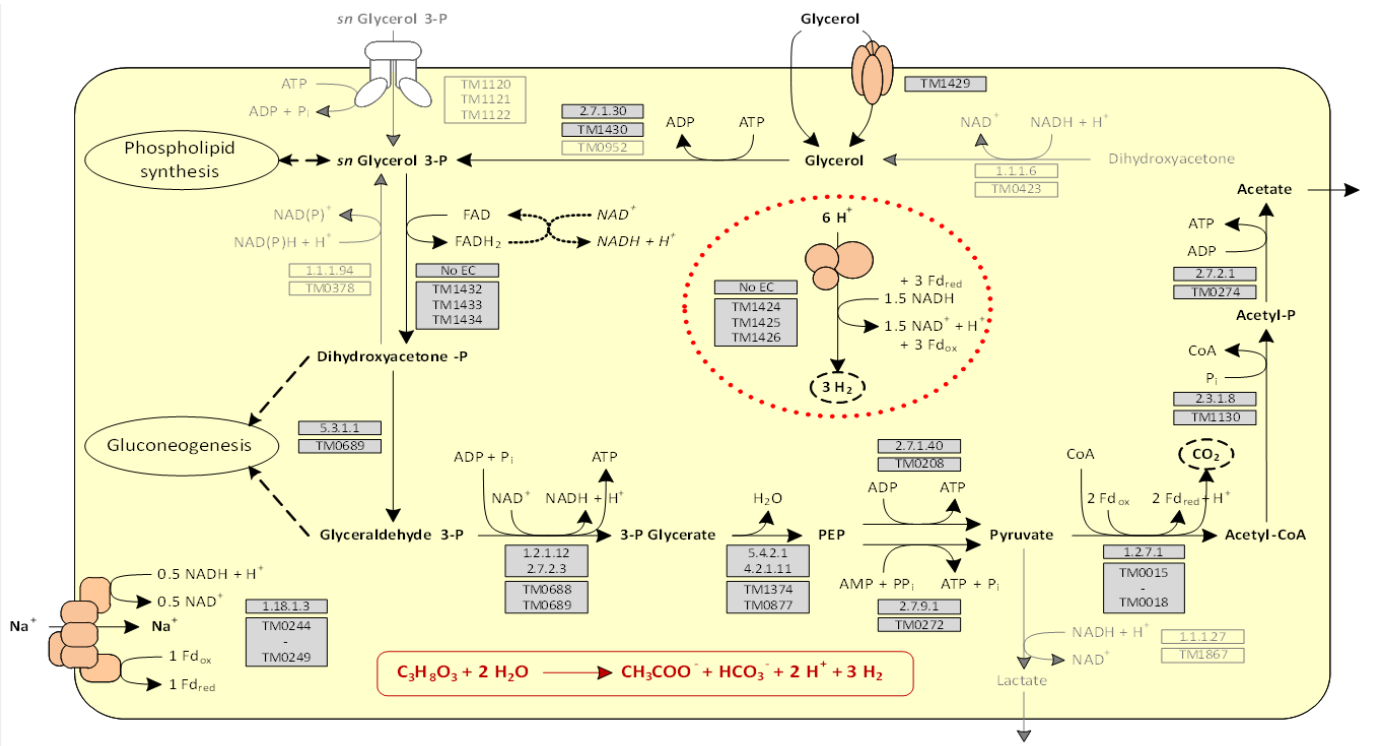

Figure 4.3. Proposed pathway for glycerol catabolism in *Thermotoga maritima*. For each reaction the locus tags of the genes coding for the respective enzymes and their EC numbers are given. Additional reactions involved in glycerol metabolism are indicated in light grey. Overall the complete conversion of glycerol to acetate yields 3 H_2 and a CO_2; lactate can be considered a minor side product of glycerol degradation. The membrane bound ion-translocating Fd:NADH oxidoreductase (EC 1.18.1.3) (Rnf) ensures the required NADH/Ferredoxin (Fd_{red}) ratio for the bifurcating hydrogenase reaction (encircled, no EC) at the expense of ion influx.

4.5 Conclusions

In this study, experimental evidence clearly shows that the hyperthermophile *Thermotoga maritima* DSM 3109 is able to grow on glycerol in both batch and chemostat cultivation setups. *T. maritima* converted glycerol to mainly acetate, CO_2 and H_2, with a maximal observed H_2 yield of 2.84 mol H_2 per mol glycerol consumed. The fermentation data suggest a stoichiometry of 1:1:3 ratio for acetate, CO_2, and H_2, respectively. The observed low diversity in fermentation end-products corresponds with the high H_2 yields, which are superior compared to those generally observed for mesophilic organism (~1 mol/mol). For batch cultivations optimal H_2 production was realised using an initial pH of 7-7.5 and a yeast extract concentration of 2 g/L. Fermentation performances of *T. maritima* on the different initial glycerol concentrations were comparable to those observed for *T. neapolitana*, with maximal observed H_2 production rates of 1.0 and 1.6 mmol/ L*h, respectively. The H_2 production rates decreased with increasing initial glycerol concentration, and substrate consumption was incomplete. Growth in controlled batch systems with fixed pH, might allow complete substrate conversion at higher glycerol loads, thus improving the cumulative H_2 production.

Stable growth on glycerol could be achieved for *T. maritima* in a chemostat system. H_2 was produced with yields ranging between 2.23 and 2.41 mol/mol. For the chemostat cultivations, the H_2 production rate increased with increasing dilution rate (from 1.1 till 2.34 mmol/L*h), however, at dilution rates exceeding 0.025 h^{-1}, glycerol (2.5 g/L) conversion was incomplete.
A pathway for glycerol fermentation by *T. maritima* is proposed. Based on comparative genomics, the ability to grow on glycerol can be considered as a general trait of *Thermotoga* species. In all probability, glycerol enters glycolysis via glycerol-3-phosphate. The co-localization of the genes coding for a glycerol kinase and an uncharacterized multimeric glycerol-3-phosphate dehydrogenase suggest their involvement in glycerol catabolism. The observed H_2 yields of 2.5-3.0 mol H_2 per mol acetate, indicated that reductant derived from the oxidation of glycerol-3-phosphate, is also channelled to H_2. However, the exact mechanism of how to overcome the endergonic electron transfer from glycerol-3-phosphate to H_2 requires further investigation.

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Supplementary materials

Table 4.A and Table 4.B

4.6 References

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Supplemental Table 4.4A . Effect of initial pH on fermentation performance of *Thermotoga maritima*

Initial pH.	Maximal consumption (S_{max}) and production ($P_{max,i}$)*					Maximal H_2 production rate	Molar yields			OD_{max}	C-balance
	(mmol/L)					(mmol/L/h)	(mol/mol)				%
	S_{max}	$P_{max,Act}$	$P_{max,Lact}$**	$P_{max,CO2}$	$P_{max,H2}$	$R_{max,H2}$	Y_{Act}	Y_{CO2}	Y_{H2}		
4.86	0.00 (0.000)	0.00 (0.000)	0.00	0.00 (0.000)	0.00 (0.000)	0.00	0.00	0.00	0.00	0.05	0.0
5.81	5.10 (0.984)	3.50 (0.953)	0.05	5.30 (0.975)	13.1 (0.976)	0.37	0.68	1.04	2.56	0.22	15.1
6.20	13.0 (0.998)	9.30 (0.994)	0.19	14.7 (0.995)	36.1 (0.941)	0.60	0.72	1.14	2.78	0.44	40.7
6.38	16.5 (0.982)	11.4 (0.994)	0.21	17.6 (0.996)	45.1 (0.991)	0.83	0.69	1.07	2.73	0.50	48.6
6.41	18.1 (0.998)	13.2 (0.995)	0.14	17.0 (0.969)	50.2 (0.992)	0.88	0.73	0.94	2.78	0.53	57.6
6.90	24.2 (0.997)	21.0 (0.996)	0.13	23.9 (0.985)	70.5 (0.993)	1.01	0.87	0.99	2.92	0.64	83.2
7.23	24.4 (0.997)	22.6 (0.982)	0.12	22.5 (0.994)	70.3 (0.978)	1.18	0.92	0.92	2.88	0.63	81.9
7.38	24.8 (0.999)	18.5 (0.999)	0.23	17.5 (0.977)	70.2 (0.969)	1.10	0.74	0.70	2.83	0.60	83.3
7.78	27.5 (0.982)	19.2 (0.986)	0.34	20.0 (0.997)	66.3 (0.997)	0.94	0.70	0.73	2.41	0.59	80.3
8.39	1.80 (0.922)	0.90 (0.751)	0.26	0.00 (0.000)	3.20 (0.883)	0.05	0.52	0.00	1.81	0.15	5.3
8.52	1.50 (0.810)	1.90 (0.857)	0.21	0.00 (0.000)	1.80 (0.976)	0.06	0.48	0.00	1.25	0.19	4.5
9.21	0.90 (0.764)	0.00 (0.000)	0.31	0.00 (0.000)	1.30 (0.930)	0.08	0.00	0.00	1.35	0.26	3.0

*Correlation coefficients (R^2) of the curve fits with the Gompertz equation (Eq. (4.1) or Eq. (4.2)) are given between brackets.
**Low lactate concentrations prevented proper curve fitting.

Supplemental Table 4.4B Effect of yeast extract (YE) concentration on fermentation performance of Thermotoga maritima

Yeast extract conc.	Maximal consumption (S_{max}) and production ($P_{max,i}$)*					Maximal H_2 production rate	Molar yields			OD_{max}	C-balance
(g/L)	(mmol/L)					(mmol/L/h)	(mol/mol)				%
	S_{max}	$P_{max,Act}$	$P_{max,Lact}$**	$P_{max,CO2}$	$P_{max,H2}$	$R_{max,H2}$	Y_{Act}	Y_{CO2}	Y_{H2}		
0.0	10.5 (0.997)	8.90 (0.983)	0.10	6.90 (0.961)	24.3 (0.992)	0.16	0.85	0.65	2.27	0.17	34.0
0.5	15.4 (0.965)	11.9 (0.995)	0.11	14.6 (0.958)	43.1 (0.993)	0.73	0.77	0.95	2.80	0.35	53.2
1.0	18.2 (0.992)	13.0 (0.983)	0.14	18.6 (0.991)	51.1 (0.980)	0.88	0.72	1.02	2.82	0.47	59.5
2.0	21.4 (0.983)	16.3 (0.994)	0.14	20.2 (0.991)	56.7 (0.979)	1.07	0.76	0.95	2.65	0.64	66.4
4.0	22.8 (0.970)	16.5 (0.995)	0.24	21.9 (0.995)	59.0 (0.976)	1.01	0.72	0.96	2.58	0.66	68.9

*Correlation coefficient R^2 of the fit is given between brackets.

**Low lactate concentrations prevented proper curve fitting.

5

CHAPTER

5. IMPROVEMENT OF BIOHYDROGEN AND USABLE CHEMICAL PRODUCTS FROM GLYCEROL BY CO-CULTURE OF *ENTEROBACTER* SPH1 AND *CITROBACTER FREUNDII* H3 USING DIFFERENT SUPPORTS AS SURFACE IMMOBILIZATION

Manuscript in preparation:

5.1 Abstract

Glycerol is a by-product of biodiesel production which is yielded at about 10% (w/w). In the present work an improvement of the dark fermentation of glycerol was proposed by surface immobilization of the microorganisms on supports. Four different supports were employed: maghemite (Fe_2O_3), activated carbon (AC), silica gel (SiO_2) and alumina (γ-Al_2O_3). A newly isolated co: culture of *Enterobacter* spH1 and *Citrobacter freundii* H3 was immobilized in these supports. Effect of iron species on the dark fermentation was also studied by its impregnation over AC and SiO_2.

The fermentative metabolites were mainly composed by ethanol, 1,3-propanediol, lactate, H_2 and CO_2. Modeling using Gompertz equation for the batch dark fermentation kinetics (maximum product formation ($P_{max,i}$), production rate ($R_{max,i}$) and product yield (Y_i)) were elucidate :

i) Maximum H_2 productivity (mmol/L) and yield(mol H_2/mol glycerol consumed) were higher in the order of: Fe/AC> AC> Fe/SiO_2>SiO_2 >Fe_2O_3> γ-Al_2O_3 > FC

ii) Maximum Ethanol productivity (mmol/L) were higher in the order of : AC > Fe/AC > Fe/SiO_2 >SiO_2 > γ-Al_2O_3 > Fe_2O_3> FC and yield (mol EtOH/mol glycerol consumed)were higher in order of: γ-Al_2O_3 > AC>SiO_2> Fe/SiO_2 Fe/AC>Fe_2O_3> FC (

iii) Maximum 1,3-propanediol productivity (mmol/L) and yield (mol 1,3PDO/mol glycerol consumed) were higher in the order of: FC > Fe/AC > Fe_2O_3> AC > Fe/SiO_2 >SiO_2 > γ-Al_2O_3

iv) Maximum Lactate productivity (mmol/ L) and yield (mol Lactate/mol glycerol consumed) were higher in the order of: FC > Fe_2O_3> Fe/AC > Fe/SiO_2> AC >SiO_2 > γ-Al_2O_3

Besides, the study shows, the glycerol conversion in all cases was higher than what obtained from the free culture. It is noted that the glycerol conversion and H_2 production was dependent on the specific surface area of the support. A progressive enhancement in the H_2 production is clearly observed comparing the Fe_2O_3, Al_2O_3, SiO_2 and AC supports. The H_2 production on iron impregnated AC and SiO_2 supports was enhanced comparing with the production achieved with the correspondent bare supports. These results indicate that support assisted carrier enhance the productivity of H_2 might be due to specific surface area attachment, biofilm formation of the microorganism and hydrogenase enzyme activation by iron species.

5.2 Introduction

Hydrogen has been accepted as an attractive, clean and renewable energy carrier. It represents an important area of bioenergy production and bioremediation [1,2]. At present most of the H_2 production however is generated by thermochemical processes from fossil fuels such as natural gas, thermal cracking and coal gasification [1]. However, these processes emit CO_2.

Lately, biological process such as biophotosynthesis, photodecomposition and anaerobic fermentation routes to harness H_2 from renewable sources such as water, waste organic matter and organic compounds have received considerable attention [3, 4]. Among this dark fermentation, that seems to be favorable not only stable but also more rapid and it can be carried out in the absence of light compared to the photo fermentation process [1, 5]. This process utilizes obligate and facultative anaerobic microorganisms to convert organic materials into H_2 from general anaerobic metabolism.

Dark fermentation for H_2 production can use various organic wastes as substrate for biohydrogen production. Glycerol is considered as an attractive cheap resource since it is produced inevitably as by-product from biodiesel production at yield about 10% (wt/wt). In addition to availability [6], low prices, and potential to mitigate possible environmental hazards and reducing greenhouse gas (GFG) emission. The main advantage of using glycerol in dark fermentation is the reduction of fuels and chemicals products at higher yield than common sugars such as glucose and xylose due to the highly reduced redox state of carbon in glycerol [7]. Henceforth, production of H_2 through dark fermentation is an alternative for the conventional fossil fuels. However, the main problem of the dark fermentation emanates from the lower yield [1]. In order to improve the performance of the dark fermentation using glycerol new biological activities on support immobilization need to be found. This can be taken as a step forward strategy for enhancing the yield of hydrogen production,

Cell immobilization technology has been applied to fermentation and enzymatic transformation [8]. Chen, 2006 [9] found that carrier supports were effective for stimulating cell growth and production of targeted metabolites. In Previous work by Lee K-S et al [10] showed that addition of an appropriate amount of solid carriers, such as activated carbon, into fermentation broth could markedly stimulate cell growth and H_2 production in dark fermentation. Other reports also showed that solid carriers were effective in stimulating cell growth and target metabolites production of (e.g., H_2 and biosurfactant) [11- 14,] It is thought that the carriers can provide more surface attachment sites, enhancing biofilm formation (Mason CA, 2000) and granular sludge formation [12,15]. The solid carriers may also provide buffer capacity for extreme conditions such as high organic loadings, pH shock, etc. [15, 16]. Similar to the function of immobilized cells, the carriers could enhance cell retention for continuous cultures, thereby avoiding wash-out of cells while operating at a high dilution rate (or a low hydraulic retention time) [17,18]. On the other

hand, the type and concentration of carbon substrate are critical factors affecting the fermentation kinetics of biohydrogen production [19]. The structure and morphology of the micro-beads strongly influence the physical properties and as a consequence the reactivity of the functional sites [20].

The glycerol bioconversion pathways to H_2 are based on a simple redox reaction: $2H^+ + 2e^- \leftrightarrow H_2$ [21]. This reaction is catalyzed by some hydrogen-producing enzymes namely [NiFe]-hydrogenases, and [FeFe]-hydrogenases which are mostly present in anaerobic bacteria [21-23]. This process takes place after glycerol enters the glycolysis pathways to produce pyruvate. Pyruvate then breaks down to acetyl-CoA via reduction of a ferredoxin (Fd) catalyzed by pyruvate ferredoxin oxidoreductase [24, 25]. Hydrogenase enzyme (E.C.1.12.7.1) oxidizes then the reduced ferredoxin (Fd) to produce molecular hydrogen [22].
Hydrogenase enzymes are clusters of FeFe or NiFe. Therefore, it can be assume that iron species can affect the activity of this enzyme. It has been reported that the in vivo activity of the hydrogenase decreases with iron depletion [25-27]. However these works on iron effect mainly focused on biochemical production mainly using glucose as a substrate and little work has been performed on H_2 production using glycerol as a substrate. In addition surface attachment immobilization techniques have not been widely adapted to H_2 production through dark fermentation from glycerol [28]. Hence, a study is needed on this field to enhance yield of hydrogen from glycerol using the promotion of iron species by surface attachment immobilization.

In this study we aimed to assess and study the effectiveness and feasibility of surface cell immobilization of mixed culture of *Enterobacter* and *Citrobacter* sp on four different porous solid supports: maghemite (Fe_2O_3), activated carbon (AC), silica gel (SiO_2) and alumina (γ-Al_2O_3) via batch dark fermentation. Effect of iron species on the dark fermentation H_2 production was also investigated by its dispersion over AC and SiO_2.

5.3 Material and methods

5.3.1 Fermentable substrates and chemicals

Pure glycerol (molecular biology, purity ≥ 99%), and all other chemicals of analytical grade used were purchased from Sigma Chemical Co., Madrid, Spain. Support materials were supplied by Merck: activated carbon ref. 2518 and silica gel ref. 2518.

5.3.2 Microorganism, medium and culture conditions

Microorganisms

Co-culture *of Enterobacter* spH1 and *Citrobacter freundii* H3 were used (isolated from San Carles de la Rapita, Spain), tested for their ability to produce H_2 from glycerol [29]. Standard microbiological and safety procedures were followed while the cultures were handled.

Culture Medium

The mixed co-culture was cultivated in synthetic medium consisted of (amounts are in grams per liter of deionized water): 7.0 g K_2HPO_4; 5.5 g KH_2PO_4; 1.0 g of $(NH_4)_2SO_4$; 0.021 g of $CaCl_2.2H_2O$; 0.25 g of $MgSO_4.7H_2O$; 0.25 g of $MgSO_4.7H_2O$; 0.021 g of $CaCl_2.2H_2O$; 0.12 g of $Na_2MoO_4.2H_2O$; 2.0 mg of nicotinic acid, 0.172 mg of Na_2SeO_3, 11.9 g HEPES (N-2-hydroxyethylpiperazine-N-2 ethanesulphonic acid); 0.5 g yeast extract (YE); 10 mL trace element solution containing 0.5 g of $MnCl_2.4H_2O$, 0.1 g of H_3BO_4, 0.01 g of $AlK(SO_4)_2.H_2O$, 0.001 g of $CuCl_2.2H_2O$ and 0.5 g of Na_2EDTA per liter; 0.5 g/L of cysteine hydrochloride as reducing agent and 1 mg resazurin, which was used as a redox indicator. Anaerobic conditions were achieved by flushing the headspace of the serum bottles with Ar gas. The starting pH of the medium was adjusted to pH 6.5 for both strains with 10 mM NaOH. The medium used was appropriate for the H_2-production since it contained the minimum nutrients required [Ito, 2005].

Support materials

The mixed co-culture of *Enterobacter* spH1 and *Citrobacter freundii* H3 was used to examine the effect of support on dark fermentation of glycerol to produce H_2. Four different supports were employed: maghemite (Fe_2O_3), activated carbon (AC), silica gel (SiO_2) and alumina (γ-Al_2O_3). Effect of iron species on the dark fermentation was also studied by its impregnation over AC and SiO_2. Iron supported over AC was prepared by incipient wetness impregnation method with aqueous solution using ferrous sulphate ($FeSO_4.7H_20$) as a precursor. The iron load was 10 wt %. After impregnation, the solids were left for 2 h at room temperature (RT), dried for 15 h at 60 °C and finally, calcinated at 200 °C for 4 h [30]. Before use each support was washed with distilled H_2O to remove all suspended fine colloidal particles and then autoclaved for 20 minutes at 121 °C to eliminate microbial contaminant.

5.3.3 Batch dark fermentation

Batch cultivations were performed in serum bottle of 100-ml fitted with gas-tight crimp-top rubber septa and flushed with Ar for 15 min and having working volume of 25 ml at a constant temperature of 37^{o}C and shaking at 200 rpm. Cultures were inoculated with 10% (v/v) pre-culture. The effect of the six different assisted carriers ((2 %(w/v)) on the fermentation was tested for mixed culture. A control batch experiment was done for each support (2 % (w/v)) without culture to measure the adsorption capacity (Q) of each support. The control and main experiment were prepared with the same synthetic medium containing glycerol nearly 25 g/L.

5.3.4 Analytical methods

5.3.4.1 Biomass growth

For the carrier assisted batch dark fermentation the biomass growth was determined using the sum of the cells which are grown freely in the liquid culture and cells attached to the support. A 0.8 mm filter was used to separate the attached cells from the freely suspended ones. After filtering, optical density (OD) at 600nm was measured for the filtrate, which corresponds to the cells which are not attached (free cells).

This residual on the filter was washed with 10 mL deionized water and centrifuged (600 g, 15 min at 4^{o}C) and the supernatant was discarded. Cells attached on support remains as residual on the filter and then re-suspended in 2 mL ultrapure water and dried for SEM and N_2 physisorption analysis. 1mg of this residue (contains attached cells and the support) was used for the scanning electron microscope (SEM) morphological studies. Cell attached on the support carrier was estimated by direct cell counting from the SEM pictures per m^2 using ImageJ 1.47i software. The total attached cells were related by multiplying cell counted per m^2 of the support by m^2 of the support per g.

Cell growth for the free cell experiment and the filtrate from the support assisted (unattached cells) was determined using an optical cell density at 600 nm (OD_{600}). Additionally, cell dry weight (CDW) was used to quantify the amount of biomass in the serum bottle. CDWs were determined in technical duplicates. 2 x 15 mL culture was sampled and centrifuged (600 g, 15 min at 4^{o}C). Each pellet was re-suspended in 2 mL ultrapure water. CDWs were determined after drying the samples for 1 day in an oven at 105^{o}C. Based on this, OD_{600} and CDW was correlated using CDW= 857.716 *OD, with R^2 =0.8782.

5.3.4.2 Analysis of gas production

During batch experiments the composition of the gas in the serum bottles headspaces were analysed for H_2 and CO_2 quantification by GC-14B GC, with a thermal conductivity detector (TCD) and a Carbosive column and a 80/100 Porapak-Q column with Argon (Ar) and Helium (He) as carrier gas with a flow rate of 30 mL/min, respectively. The operational temperatures for the injection port, oven and detector were 150°C, 80°C and 200°C, respectively.

A gastight syringe was used to sample out the gas produced from the processed anaerobic bottle. The syringe has a valve that can be closed to trap the gas within. 0.3 mL volume of gas was taken and equilibrated at an atmospheric pressure and it was calculated against a known calibrated standard of H_2 to get the volume gas in percent. In order to ensure the expansion, due to the pressure inside the bottle a permanent atmospheric pressure was taken as the reference. The H_2 gas was identified as a peak with a known pure H_2 in the computer software that is connected to the GC. The peak corresponds to a certain volume percent of H_2. The volume fraction of H_2 in the syringe is equivalent to the volume fraction of H_2 in the headspace at the time when the gas was sampled out of the processed serum bottle. The total volume of H_2 was calculated multiplying the volume percentage by headspace of the serum bottle, and thus ideal gas law can be used to calculate the number of moles of gas produced.

5.3.4.3 Liquid metabolites analysis

Fermentation products were identified by HPLC, using Transgenomic column, USA (ICSep ICE-COREGEL 87H3) equipped with Diode array (DAD) and a refractive index (RID) detectors. Aqueous sulfuric acid (H_2SO_4) adjusted at pH 2.2 was used as mobile phase. Operating conditions for the HPLC column were 50 °C with a mobile phase flow rate of 0.6 mL/min. Prior to analysis the liquid samples were centrifuged at 9800 rpm for 15 min and filtered through a 0.2 µm disposable filter. The injection volume of the sample was 20 µL. The complete sample elution was achieved within 40 min. Concentrations were determined using standard curves of the respective compounds. Concentrations of product fermentation metabolites were quantified using standard curves of the respective compounds.

GC-MS, was equipped with an HP PLOT column (divinylbenzene/styrene polymer, 30 m long, 0.32 mm ID, 20 µm film thickness) and operating at an inlet temperature of 200°C, a pressure of 6.1 psi and an oven temperature of 35°C for 5 min increasing to 150°C at 5°C/min. Prior the CG-MS analysis the liquid samples were centrifuged at 9800 rpm for 15 min and filtered through a 0.2 µm disposable filter. The injection volume of the sample was 5 µL Glycerol and product fermentation metabolites in the liquid phase were confirmed by GC-MS.

5.3.4.4 Support Characterization

Morphological studies of the mixed culture cells attached on each carrier were examined by scanning electron microscope (SEM, JEOL JSM-840, Japan) at 20 kV and working distance of 15 mm. Prior to SEM observation samples were fixed with 2% w/w glutaraldehyde and dehydrated in graded series of ethanol. Finally, the samples were dried by critical point drier (EMS-850, Japan), coated with gold [31].

N_2 physisorption adsorption-desorption isotherms at 77 K was measured using Micromeritics ASAP 2000 equipment for analysis of specific surface area (S_{BET}), average pore volume and pore size. Prior to the physisorption measurements, all the samples were degassed in vacuum (10^{-4} Pa) at 393K. N_2 physisorption was used to reveal information about the texture properties for each carrier before and after the batch fermentation.

X-ray Diffraction (XRD) analysis was used to determine for any change on the surface of the carrier before and after the fermentation. The XRD analysis of the carrier was recorded using a Siemens D5000 diffractometer (Bragg-Bentano for focusing geometry and vertical θ-θ goniometer) with an angular 2θ diffraction range between 3° and 90°. The samples were dispersed on a Si (510) sample holder. The data were collected with an angular step of 0.03° at 5 s per step and sample rotation. Cu Kα radiation (λ=1.54056 Å) was obtained from a copper X-ray tube operated at 40 kV and 30 mA. The crystalline phases were identified using the JCPDS powder diffraction files as a data references.

5.3.4.5 Total organic carbon analysis

The concentration of total organic carbon ***(TOC)*** before and after batch fermentation was measured using a Total Organic Carbon Analyzer (Analytik jena, Multi N/C 2100). Prior to analysis the liquid sample was filtered through a 0.2 µm disposable filter.

5.3.5 Data analysis

Data analysis and Kinetic parameters

The adsorption capacity of the adsorbents (support) was calculated based on the concentration change of glycerol in the solution according to Equation (1), where Q is the adsorption capacity (mg/g), M_s is the amount of adsorbent, and $Q_{Gly,i}$ and $Q_{Gly,f}$ are the glycerol concentration before and after fermentation, respectively. Glycerol concentration was determined by HPLC as mentioned above.

$$Q = \frac{Q_{Gly,i} - Q_{Gly,f}}{M_s} \qquad \textbf{Equation (5.1)}$$

A modified Gompertz equation Eq. (2) [32,33] was used to estimate the maximum production rates and the production potentials of the fermentation end products such as: ethanol, acetate, lactate, propionate, succinate, 2, 3 butanediol, CO_2 and H_2:

$$P_i(t) = P_{max,i} exp\left\{-exp\left[\frac{R_{max,P_i} * e}{P_{max,i}}(\lambda i - t) + 1\right]\right\} \qquad \textbf{Equation (5.2)}$$

Where $P_i(t)$ is the cumulative production (mmol/L), λ the lag-phase time (h), $P_{max,i}$ the production potential (mmol/L), $R_{max,i}$ the maximum production rate (mmol/L*h), t the incubation time (h), and e the exp(1) = 2.718. This equation was found to be suitable for describing the progress of cumulative production of compounds during the experiments.

Accordingly, for the consumption of glycerol a modified Gompertz equation Eq. (3) [33] was used:

$$S_0 - S(t) = S_{\max}.\exp\left\{-\exp\left[\frac{R_{\max,S}.e}{S_{\max}}(\lambda_S - t) + 1\right]\right\} \qquad \textbf{Equation (5.3)}$$

Where: S_0 – initial substrate concentration (mmol/L), S – substrate concentration (mmol/L) at time t, S_{max} – maximum concentration of consumed substrate (mmol/l), $R_{max,S}$ – maximum rate of substrate consumption (mmol/L*h). The fitting of the fermentation data was performed using Sigma plot application software version 12.3, where accuracy of the fit was given by correlation coefficients (R^2).

For batch cultivation yields of the fermentation end-products, expressed in mole product produced per mole of glycerol consumed, experimental data of substrate adsorbed determined in Equation (1) was considered in the yield calculation.

$$Y_{P\max,i} = \frac{P_{\max,i}}{S_0 - (S_{\max} - S_{ads})} \qquad \textbf{Equation (5.4)}$$

Where: $Y_{Pmax,i}$ – substrate yield for fermentation product i, S_0 – initial glycerol concentration (mol/l), S_{max} – maximum glycerol consumption(mol/L), S_{ads} maximum glycerol adsorbed by the support (mmol/L)

Maximum specific production or consumption ($q_{max,i}$) was calculated using the values obtained from the data fits (Eq. (2) and Eq. (3)), according to Eq. (5) and (6) respectively , and were

calculated using the ratio of the maximum production rate or substrate consumption rate to maximum dry cell weight (DCW_{max}).

$$q_{\max,i} = \frac{R_{\max,i}}{DCW_{\max}} \qquad \textbf{Equation (5.5)}$$

$$q_{\max,s} = \frac{S_{\max,i}}{DCW_{\max}} \qquad \textbf{Equation (5.6)}$$

Where: $q_{max,\ i}$ specific production or consumption rate i (mmol/l*h), $R_{max,i}$ – the maximum production rate i, maximum substrate consumption rate (mmol/L*h) and DCW_{max} maximum dry cell weight (g/L).

Carbon balances (C-balance) and a degree of reduction balances (ε-balance) were calculated according to Oh et al. and Converti et al. using the elemental biomass composition $CH_{1.74}O_{0.33}N_{0.23}$ [34,35]**.** This corresponds to a biomass carbon content of 53.6% and with a degree of reduction of 4.32 electrons per C atom. The reduction degree (*ε)* was calculated from the following equation (7) [34, 35].

$$\varepsilon = 4C + H - 2O - 3N \qquad \textbf{Equation (5.7)}$$

Where C, H, O, and N denote the atomic coefficient of the chemical formula of a compound.

Glycerol conversion (*E*) by the strains was calculated using the following equation (Eq 8):

$$E = \frac{S_0 - (S_{\max} - S_{ads})}{S_0} \times 100 \qquad \textbf{Equation (5.8)}$$

Determining hydrogen and CO_2 production. The number of moles of gas (n) injected into GC at a room temperature was calculated using ideal gas law in Equation 9.

$$PV = nRT \qquad \textbf{Equation (5.9)}$$

Where, P is atmospheric pressure, V is the volume determined by the injection, R is universal gas constant and T is RT.

5.4 Result and discussion

5.4.1 Textural characteristics of support materials

The textural characteristics of each support employed for the dark fermentation are summarized in Table 5.1. The N_2 physisorption of the carrier was performed before and after the dark fermentation.

Table 5.1 shows the surface area, pore volume and pores size for the different carriers before and after the dark fermentation. The introduction of Iron species on the SiO_2 and AC carriers produced a decrease in the surface area and pore volumes for both support, suggesting that the impregnation with iron species lead presumably to a pore blockage of the carriers. Maghemite (Fe_2O_3) silica gel (SiO_2) and alumina (γ-Al_2O_3) exhibited the typical mesoporous type IV isotherms (Figures 5.1 a, b, c) according to the Brunauer-Deming-Deming-Teller (BDDT) classification. Activated carbon (AC) (Figures 5.1 d) exhibited a microporous structure with type I isotherms characterized by a plateau that is nearly horizontal to the $P/P°$ axis.

After dark fermentation the total surface area, pore volume, and porous size of all materials decreased presumably by both the growth of the microorganisms and by the adsorption of organic metabolites.

Several studies employing AC as catalyst reveal a significant modification of both texture and surface group distribution of the original AC in the course of the experiment [36].

Table 5.1 Textural properties of the different *Citrobacter freundii* H3 support employed in the dark fermentation

Carrier	Before dark fermentation			After dark fermentation		
	$S_{BET}(m^2/g)$	Porous Volume (cm^3/g)	Porous Size (nm)	$S_{BET}(m^2/g)$	Porous Volume (cm^3/g)	Porous Size (nm)
Fe_2O_3	205	0.779	13.99	158	0.403	2.897
γ-Al_2O_3	253	0.450	4.54	105	0.192	2.271
SiO_2	685	0.822	3.32	202	0.347	1.872
Fe/SiO_2	440	0.540	2.84	150	0.251	1.871
AC	1195	0.675	1.69	462	0.269	1.614
Fe/AC	736	0.413	0.84	382	0.085	0.807

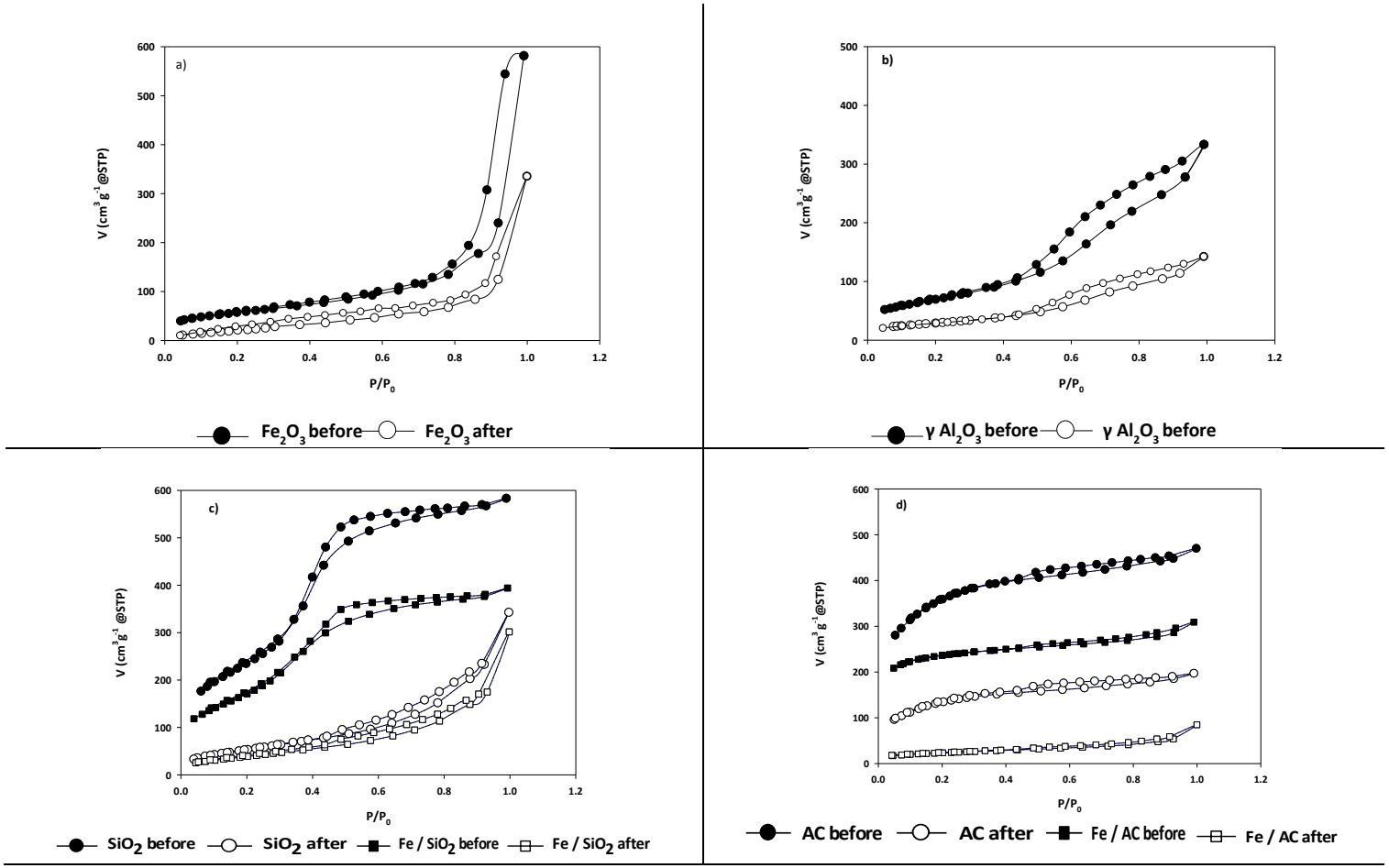
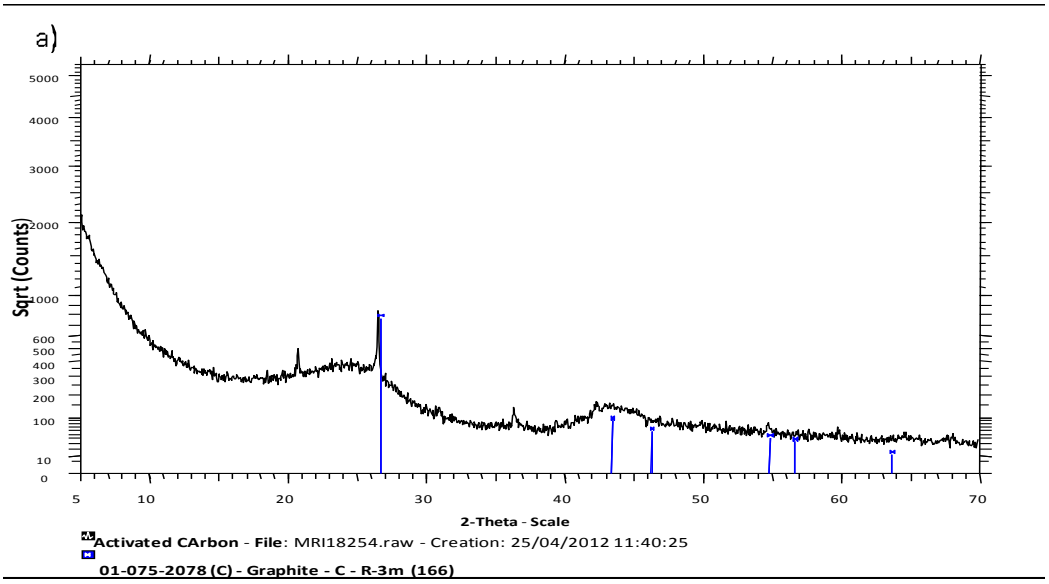


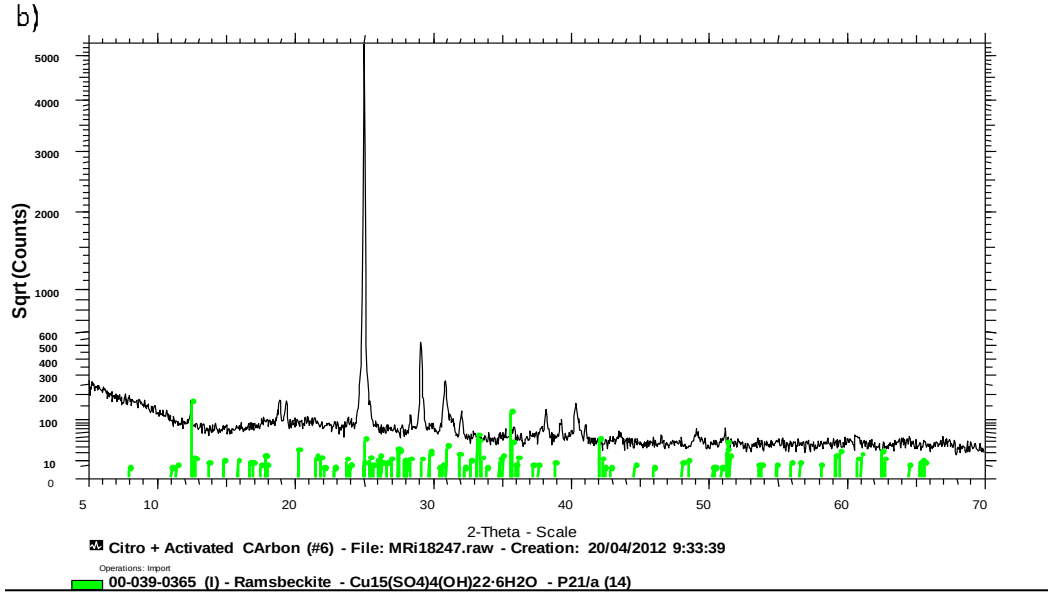
Figure 5.1. N_2 adsorption-desorption; isotherms for support before and after the batch dark fermentation (a) Fe_2O_3 (b) γ-Al_2O_3 (c) SiO_2 and Fe/SiO_2 (d) AC and Fe/AC

5.4.2 Assisted carrier characterization

Effect of support on adsorbing salt compounds



As it can be referred in Figure 5.2(a), no other specie other than carbon is observed before the fermentation.



From the XRD profile of the activated carbon support (Figure 5.2(b)), after the dark fermentation, it is visible crystallographic phases of the Ramsbeckite (JCDPS 39-0365). This copper species are probably coming from the adsorption of the metal species such as copper present in the culture medium.

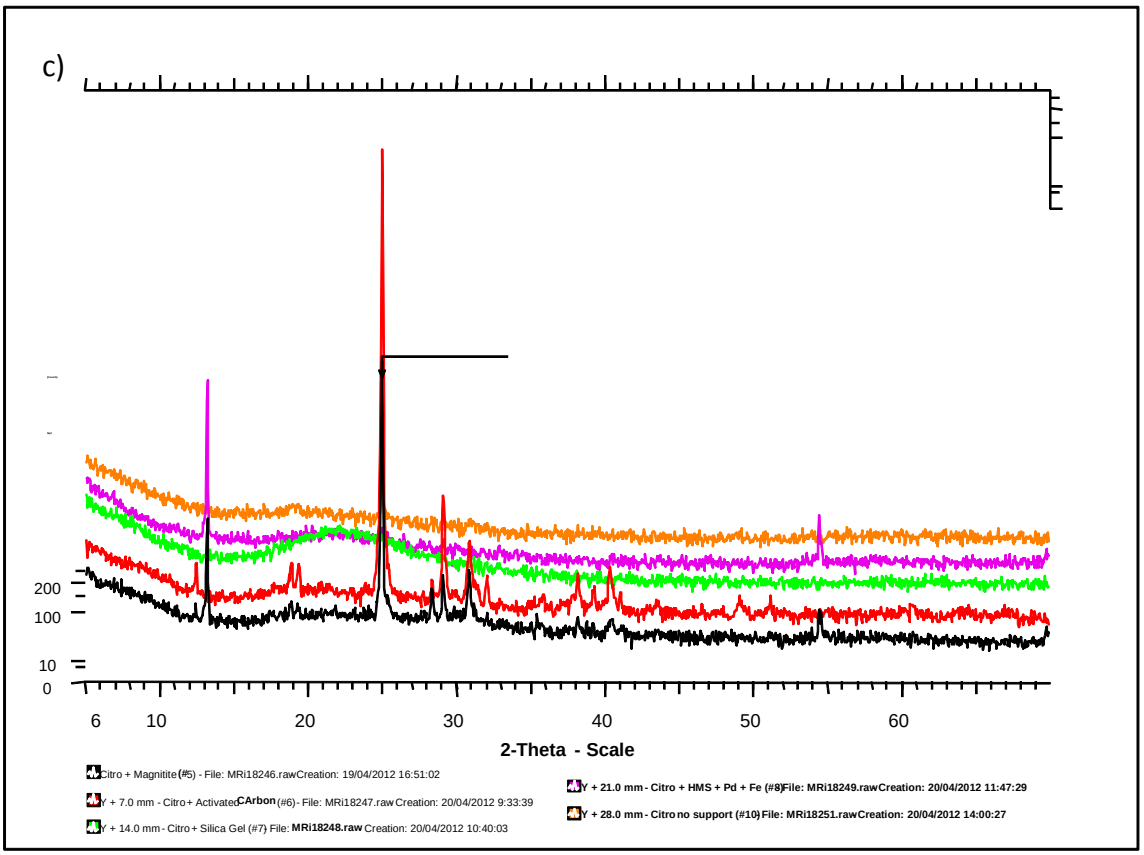
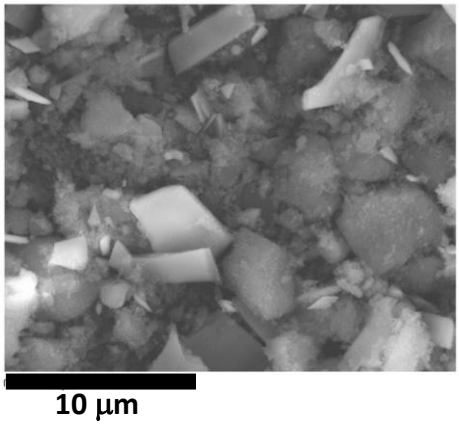
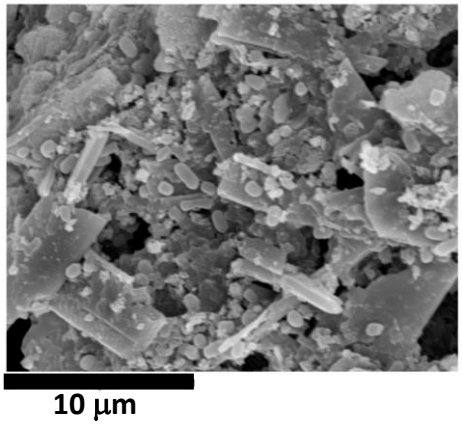
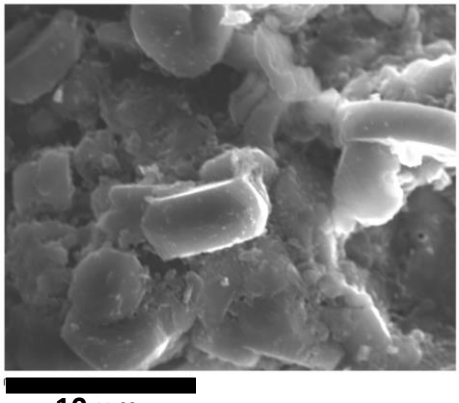
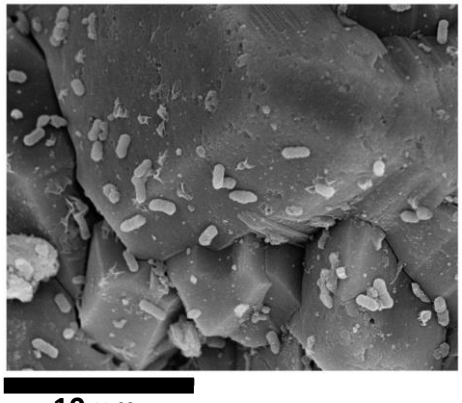
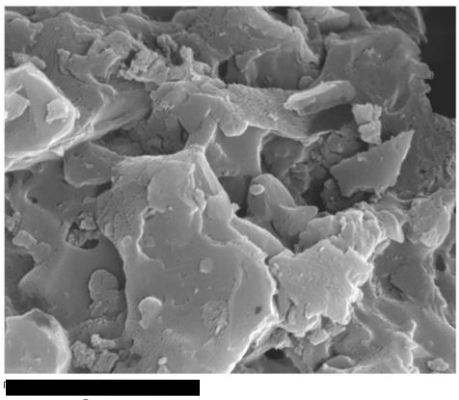
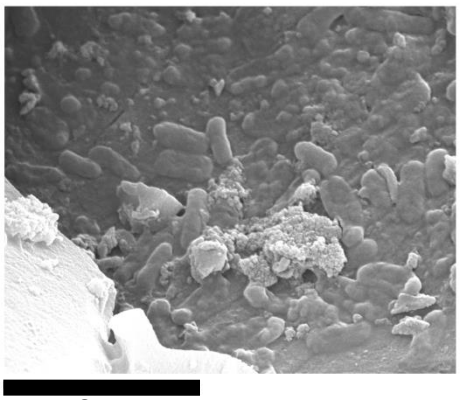


Figure 5.2. Powder XRD patterns a) AC before b) after the dark fermentation and c) all support after the dark fermentation.

From the XRD profile of the all the supports also (Figure 5.2(c)) after the dark fermentation it is visible crystallographic phases of the Ramsbeckite (JCDPS 39-0365). This copper species are probably coming from the adsorption of the metal species such as copper present in the culture medium.

5.4.3 Morphology cell attached and cell count on the support

The surface of immobilized cells was studied under ***scanning electron microscopy (SEM)***. The number of bacteria attached was counted using ImageJ 1.46r software. Figure 5.3 shows the random distribution of cells on each support. The bacterial attachments on the support were distinct. Figure 5.4 presents attached cell counts of mixed culture.

Support	Before fermentation	After fermentation
Fe_2O_3		
γAl_2O_3		
SiO_2		

Continued

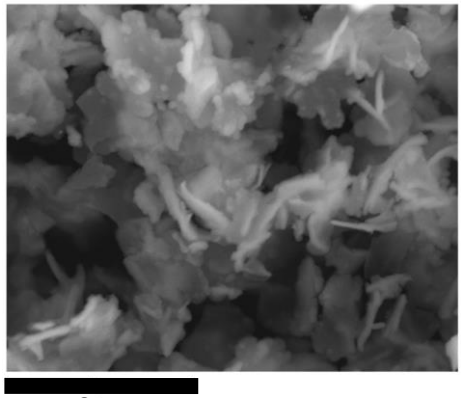
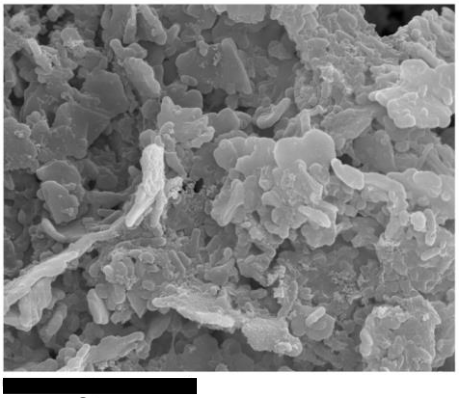
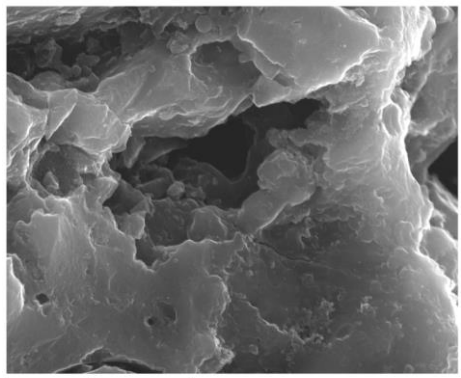
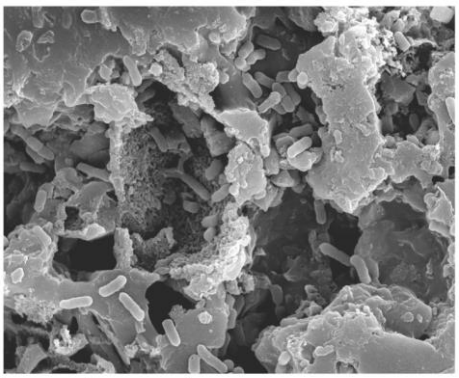
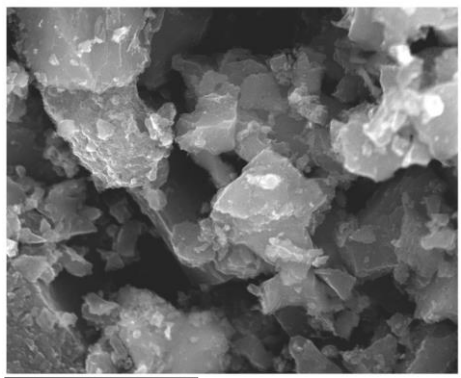
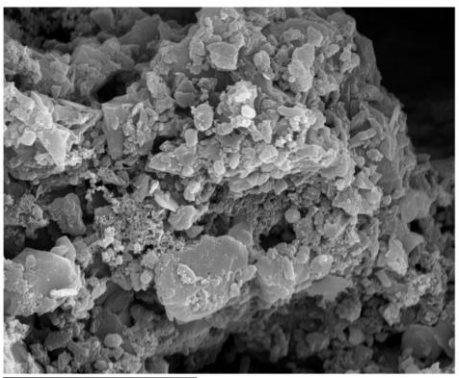
Support	Before fermentation	After fermentation
Fe / SiO_2	 10 μm	 10 μm
AC	 10 μm	 10 μm
Fe / AC	 10 μm	 10 μm

Figure 5.3. SEM images of bare support and attached cell before and after dark fermentation

5.4.3.1 Effect of bacterial surface attachment

Figure 5.4 shows improvement in cell population the order of AC>SiO_2 > Fe/AC >Fe/SiO_2 > γ-Al_2O_3 > Fe_2O_3 which might be due to the surface area and roughness. There was a qualitative increase in the number of attached cells on the AC than the other support. Attached cells or biofilms are defined as matrix-enclosed bacterial populations, which adhere to each other and with the support surfaces [37]. Biofilm attached bacteria predominates numerically and metabolically in virtually all ecosystems [38]. Costerton et al. [37] reported that the substratum structure promotes the attachment of bacteria to the surface, and there is considerable evidence of increased attachment with increasing surface roughness or rugosity of the support. Other factors, such as the coating of the substratum with biomolecules, e.g. proteins and polysaccharides, and the hydrodynamic flow velocity immediately adjacent to the substratum, also influence biofilm formation. In addition, certain cellular properties of bacteria, such as the presence of fimbriae and flagella, and the production of extracellular polymeric system increase bacteria attachment [39]. The concentrations of nutrients in the aqueous medium surrounding the attached cell also affect biofilm development.

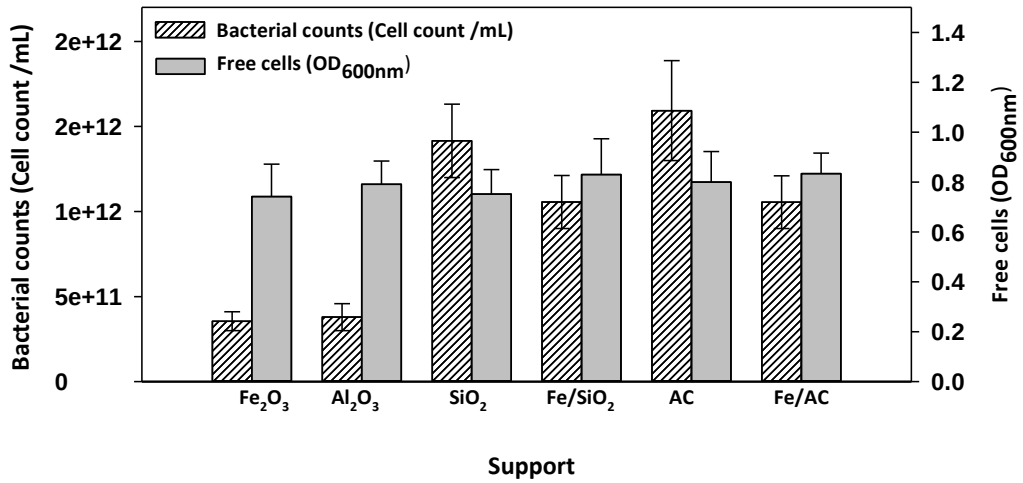


Figure 5.4. OD measurement for the free cells and cell counts of attached cell on the support.

Previous laboratory studies indicate the correlation of an increase in nutrient concentrations with increased numbers of attached bacterial cells [40]. Indeed, it has also been speculated that surface associations offer selective growth advantages for attached cells, particularly during the periods of nutrient limitation [41]. Overall, it was apparent that the mixed culture preferred to attach to the support. Of the entire assisted carrier (support) presented in this study, cell count was higher in AC. This could be attributed to its higher surface area comparing to the others. Furthermore, within the mixed culture cell attached context, comparing the end metabolites analysis's of all assisted carrier (Figure 5.5 (b)-(f)) with the FC (Figure 5.5 (a)) ethanol was observed

to be higher. This may suggested that *Enterobacter* spH1 benefited in the co-culture from the cell attachment. This is more consistent with our previous study [29] of *Enterobacter* spH1 which was found to be higher producer of Ethanol. Similar phenomenon has been noted in naturally occurring mixed-species biofilms containing among others, *Enterobacter* and *Citrobacter*, of water and food-environment origins, where proportions of *Citrobacter* spp. have been recorded as generally lower than *Enterobacter* spp. [38, 42,43].

Fermentative profile of glycerol metabolism by co-culture with and without support. Figure 6.5 (a)-(g) shows the fermentative growth of the mixed culture on each support and support free or free culture. The modeled kinetics of H_2 production performance of the carrier- supplemented cultures is shown in Table 6.5 (a)-(g). The use of carrier assisted in dark fermentation appeared to result in an enhancement in the H_2 evolution, H_2 production rate, H_2 yield, and glycerol conversion efficiency than those obtained from the control (support-free) culture. Even though the cell attached were lowered in the Fe/AC, Fe/SiO2 with their respective AC and SiO_2 - supplemented cultures, Fe/AC, Fe/SiO_2 showed significant improvement in the performance of H_2 production. This could be due to the iron evolvement in the metabolitic pathways. Iron-sulfur species has effect on protein functions primarily as an electron carrier. Iron could also induce metabolic change and be involved in Fe-S and non Fe-S proteins operating in hydrogenase [44]. The effect of each the assisted carrier and involvement of the iron species are discussed more detail below.

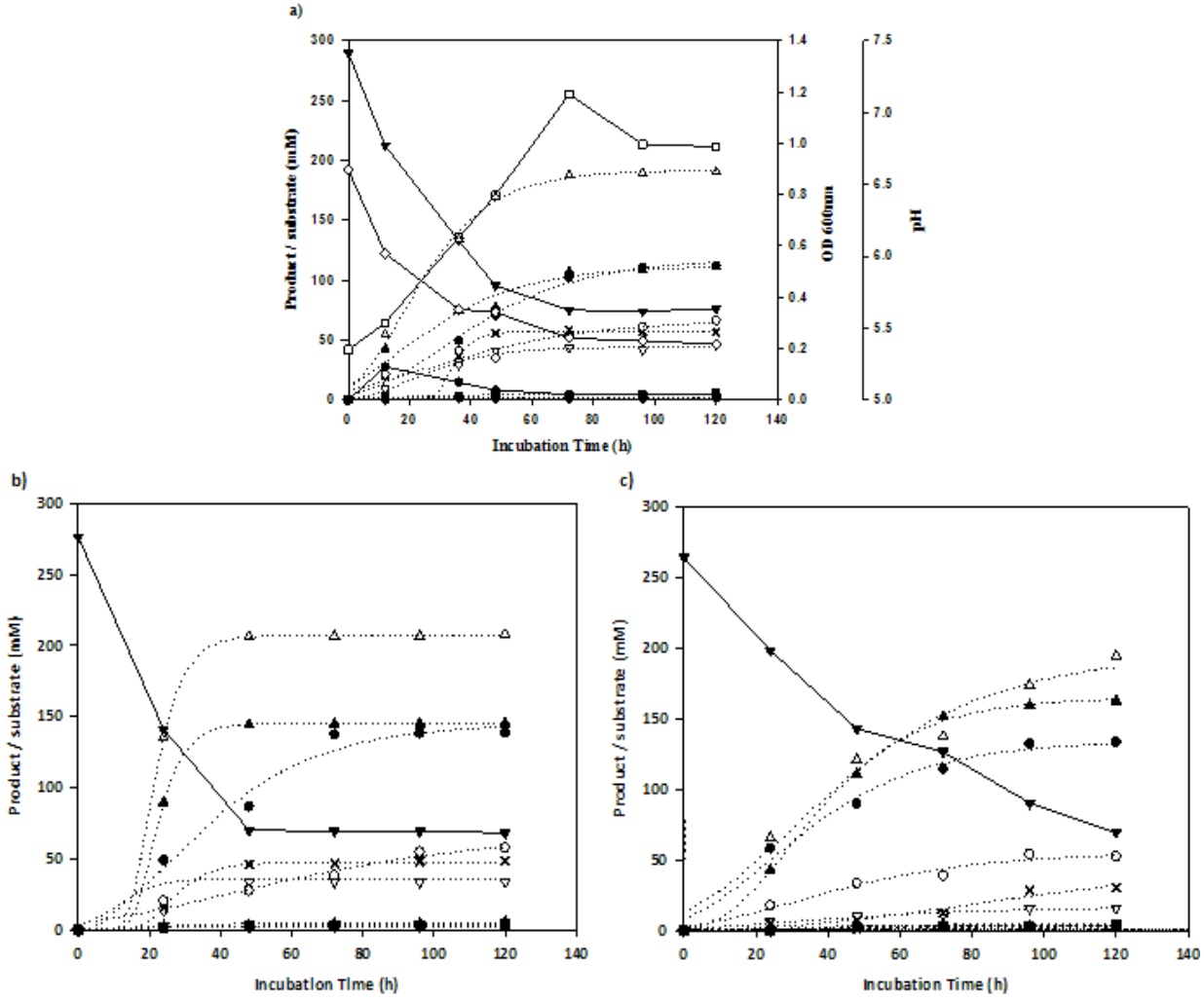
5.4.4 Effect of assisted carrier and iron on dark fermentative H_2 production

5.4.4.1 Effect of assisted carrier on H_2 production

According to the modified Gompertz equation (Eq. (5.2)), the R_{max} (representing the kinetic characteristics of H_2 production for the highest production rate), was found 7.8 mmol/L/h for Fe/AC slightly higher than AC (7.6 mmol/L/h), Fe/SiO_2 (7.3 mmol/L/h) and SiO_2 4.4 mmol/L/h All the carrier assisted (support) presented higher H_2 production than the carrier free cells (FC) which showed a rate of H_2 production 1.8 mmol/L/h. The similarities of H_2 production rate observed between Fe/AC and AC could be due to the fact that Fe/AC have an advantage for Fe species involvement in increasing the productivity however the count cell were lower probably due to the lower surface area and on the other hand AC supplemented the count cell density were higher due the surface area.

To explore possible explanations for the enhancement of the H_2 mechanism by the assisted support, the morphology of support surface was monitored before and after fermentation. Scanning electron microscopy (SEM) analysis shows that cells attached to the surface of the AC support forming biofilms on the surface of the reactor. This suggests that the solid support may provide extra surface area for attached cell growth and possibly increase the mass diffusion

transfer the substrate and somehow led to an increase in H_2 production. Similarly, recent studies showed that biofilm formation on carriers (e.g., activated carbon and silica gel) plays key roles in the enhancement of the biosurfactant production from *Bacillus subtilis* [45] and *Serratia marcescens* [46]. Also, the cell growth rate increased when solid carriers were added, especially in the case of using AC (Figure 5.4). This is consistent with previous reports which indicate that the solid carriers such as silica gel and b- cyclodextrin could be effective growth stimulants [46, 47]. The detailed mechanism of the carrier-induced promoting effects on dark fermentative H_2 production has not yet been clearly identified [28]. During the course of batch fermentation, the pH did not vary significantly changed compared with the FC (Table 5.2). Therefore, the two carriers (AC and SiO_2) were used for further investigation by impregnating iron to see its effect of on the H_2 production. These results indicate that addition of Fe/AC and Fe/SiO_2 could markedly enhance H_2 production performance in all categories (Table 5.2 and 5.3).



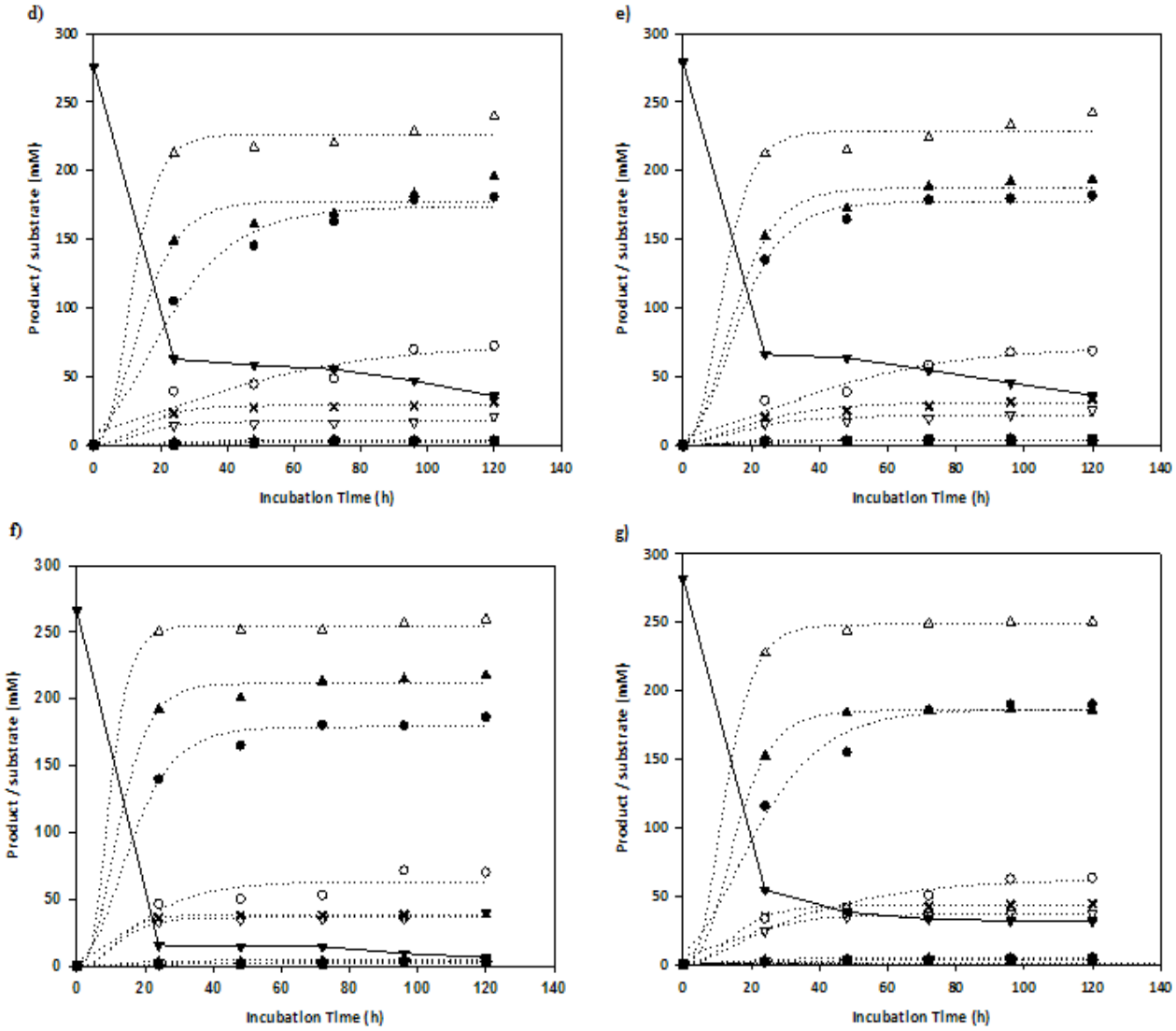


Figure 5.5. Glycerol fermentation profiles for a co-culture of *Enterobacter* spH1 and *Citrobacter freundii* H3 on support (a) Control without support (FC) , (b) Fe_2O_3, (c) γ-Al_2O_3,(d) SiO_2,(e) Fe / SiO_2, (f) AC,(g) Fe / AC . Residual glycerol (▼), glycerol consumed (Δ), lactate (✕) acetate (■), 1,3-propanediol (▽), ethanol (▲), butyrate (◆), succinate(★),formate (—●—), H2 (●), CO_2 (○), pH(—◇—) and mg protein (—□—). For glycerol consumed, lactate, 2,3-BDO, ethanol, propionate, acetate, H_2 and CO_2 data was fitted using the modified Gompertz equation (Eq. (5.1) and Eq. (5.2)) (dotted lines).

Table 5.2. Maximum consumption and production

Support	Initial substrate	Maximal consumption ($S_{max,i}$) and production ($P_{max,i}$)* (mmol/L)									Support	Glycerol conversion (%)	C-Balance (%)	ξ- Reduction (%)
		S_{max}	$P_{max,EtOH}$	$P_{max,Lact}$	$P_{max,1,3PDO}$	$P_{max,Suc}$	$P_{max,Act}$	$P_{max,But}$	$P_{max,CO2}$	$P_{max,H2}$	Spefic Area (m^2/g)			
FC	266.8	195.7	111.8	56.2	44.3	1.9	4.6	3.2	63.7	120.1		72.0	109.8	108.5
Fe2O3	276.2	213.0	143.1	49.0	35.1	5.2	3.2	2.4	67.2	156.0	205	78.4	107.7	107.6
Al_2O_3	264.7	196.6	165.3	28.2	17.1	3.3	3.8	3.8	55.9	135.0	253	72.3	104.0	107.2
SiO2	275.6	226.4	182.9	29.0	17.4	3.9	3.1	3.1	73.3	174.8	685	83.3	100.6	102.7
Fe/SiO2	278.8	228.8	182.6	38.1	21.8	4.2	3.9	3.3	71.9	183.5	440	84.2	104.1	106.2
AC	266.3	251.0	210.9	36.3	34.3	4.2	2.0	1.8	68.3	184.2	1195	92.4	107.8	112.9
Fe/AC	277.7	242.5	185.7	43.1	36.4	4.8	3.4	3.2	62.4	191.7	736	89.3	108.0	111.6

Support	Initial substrate	Maximal consumption ($R_{max,i}$) and production ($R_{max,i}$)* rate (mmol/L/h)									Support	Dry cell weight (DCW) (g/L)	Final pH
		$R_{max,S}$	$R_{max,EtOH}$	$R_{max,Lact}$	$R_{max,1,3PDO}$	$R_{max,Suc}$	R_{maxAct}	$R_{max,But}$	$R_{max,CO2}$	$R_{max,H2}$	Spefic Area (m^2/g)		
FC	266.8	4.5	1.9	4.8	1.1	0.1	0.1	0.1	0.8	1.8		1.25	5.39
Fe2O3	276.2	14.1	10.5	2.5	2.2	0.1	0.1	0.1	0.6	2.4	205	1.58	5.61
Al_2O_3	264.7	2.4	3.2	0.4	0.2	0.1	0.1	0.3	0.7	2.1	253	1.65	5.81
SiO2	275.6	15.9	8.6	1.3	0.8	0.1	0.0	0.1	0.8	4.4	685	1.88	5.82
Fe/SiO2	278.8	15.2	9.1	0.8	0.6	0.2	0.1	0.1	0.9	7.3	440	2.05	5.72
AC	266.3	24.1	13.1	2.7	2.2	0.2	0.1	0.1	1.8	7.6	1195	2.22	5.87
Fe/AC	277.7	16.2	9.7	2.1	1.3	0.1	0.1	0.1	0.9	7.8	736	2.19	5.66

Table 5.3. Maximum specific productivity and production

Support	Spefic Area (m^2/g)	Maximal specific (qmax,i) and production (qmax, i)* rate (mmol/gDCW*h)								
		$q_{max,S}$	$q_{max,EtOH}$	$q_{max,Lact}$	$q_{max,1,3PDO}$	$q_{max,Suc}$	q_{maxAct}	q_{maxBut}	$q_{max,CO2}$	$q_{max,H2}$
FC		3.6	1.5	3.8	0.9	0.1	0.1	0.0	0.6	1.4
Fe2O3	205	8.9	6.6	1.6	1.4	0.1	0.1	0.1	0.4	1.5
Al_2O_3	253	1.4	2.0	0.2	0.1	0.0	0.1	0.2	0.4	1.3
SiO2	685	8.4	4.5	0.7	0.4	0.1	0.0	0.0	0.4	2.4
Fe/SiO2	440	7.4	4.2	0.4	0.3	0.1	0.1	0.1	0.4	3.6
AC	1195	10.8	5.9	1.2	1.0	0.1	0.0	0.0	0.8	3.4
Fe/AC	736	7.4	4.4	1.0	0.6	0.1	0.0	0.0	0.4	3.6

Support	Spefic Area (m^2/g)	Molar yields (mol/mol)								Biomass yield $gDCW_{max}$/mol
		Y_{EtoH}	Y_{Lact}	$Y_{1,3PDO}$	Y_{suc}	Y_{Act}	Y_{but}	Y_{CO2}	Y_{H2}	
FC		0.57	0.29	0.23	0.01	0.02	0.02	0.33	0.61	6.40
Fe2O3	205	0.67	0.23	0.17	0.02	0.01	0.01	0.32	0.73	7.40
Al_2O_3	253	0.84	0.14	0.09	0.02	0.02	0.02	0.28	0.69	8.40
SiO2	685	0.81	0.13	0.08	0.02	0.01	0.01	0.32	0.77	8.30
Fe/SiO2	440	0.80	0.15	0.10	0.02	0.02	0.01	0.31	0.80	9.00
AC	1195	0.84	0.14	0.14	0.02	0.01	0.01	0.27	0.74	8.80
Fe/AC	736	0.77	0.18	0.15	0.02	0.01	0.01	0.26	0.79	9.00

5.4.4.2 Effect of iron

End liquid fermentative metabolites were of the same type and these are mainly 1,3-propanediol, ethanol and lactate with gaseous H_2 and CO_2. Effect of support assisted carrier due to surface area attachment and iron involvement can be seen from Table 5.2, 5.3 and Figure 5.6.

i) Maximum H_2 productivity (mmol/L) and yield(mol H_2/mol glycerol consumed) were higher in the order of: Fe/AC> AC> Fe/SiO_2>SiO_2 >Fe_2O_3> γ-Al_2O_3 > FC

ii) Maximum Ethanol productivity (mmol/L) were higher in the order of : AC > Fe/AC > Fe/SiO_2 >SiO_2 > γ-Al_2O_3 > Fe_2O_3> FC and yield (mol EtOH/mol glycerol consumed)were higher in order of: γ-Al_2O_3 > AC>SiO_2> Fe/SiO_2 Fe/AC>Fe_2O_3> FC.

iii) Maximum 1,3-propanediol productivity (mmol/L) and yield (mol 1,3PDO/mol glycerol consumed) were higher in the order of: FC > Fe/AC > Fe_2O_3> AC > Fe/SiO_2 >SiO_2 > γ-Al_2O_3

iv) Maximum Lactate productivity (mmol/ L) and yield (mol Lactate/mol glycerol consumed) were higher in the order of: FC > Fe_2O_3> Fe/AC > Fe/SiO_2> AC >SiO_2 > γ-Al_2O_3

It was reported that iron- sulfur has effect on protein functions primarily as an electron carrier and it is involved in pyruvate oxidation to acetyl-CoA, CO_2 and H_2. Iron could induce metabolic change and be involved in Fe-S and non Fe-S proteins operating in hydrogenase [41].

In our previous work, we observed that the addition of Fe^{2+} (Fe_5O_4) at 20–30 mg also showed enhancing effect on the total hydrogen production. Other previous works also reported that iron-sulfur species has effect on protein functions primarily as an electron carrier and it is involved in pyruvate oxidation to acetyl-CoA, CO_2 and H_2. Iron could also induce metabolic change and be involved in Fe-S and non Fe-S proteins operating in hydrogenase.

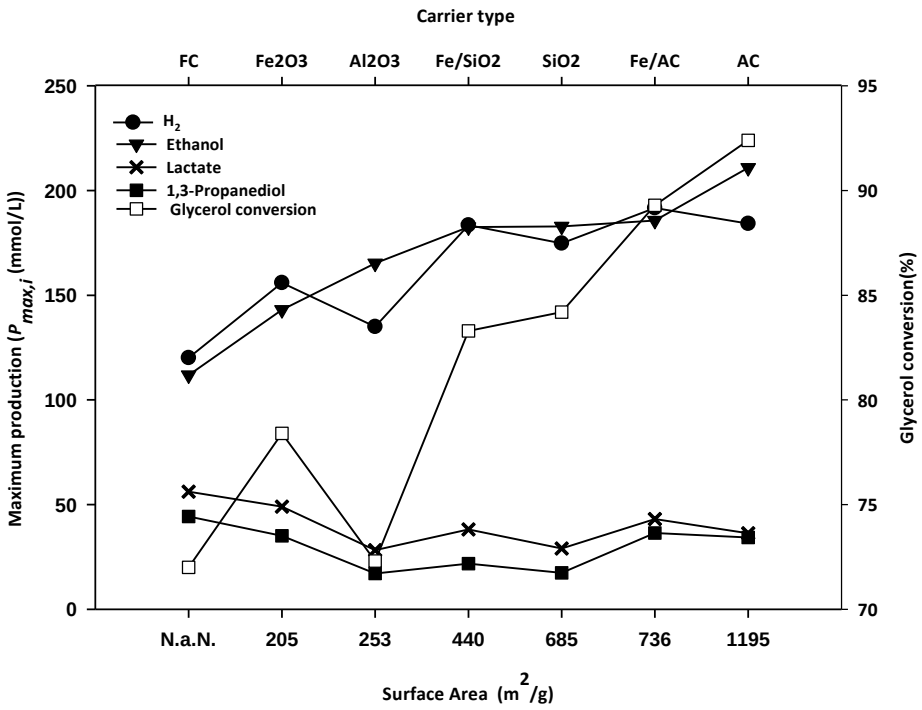


Figure 5.6. Effect of area and iron species on H_2 production, Ethanol production, 1,3-PDO production and lactate production

5.4.5 Effect of support on glycerol Adsorption

In our reference experiments (only support), it was observed that there was no H_2 and other metabolite production without the culture. Due to the adsorption, however, there was a decline in glycerol through time. Figure 5.7 shows the capacity of each support to adsorb glycerol. The adsorption capacity of the adsorbents was calculated based on the concentration change of glycerol in the solution according to Equation (5.1).

As shown in Figure 5.7, the maximum adsorbed glycerol amount is 150 mg/ g of AC. Some reports on phenol adsorption tests also show a maximum capacity of 370 mg $_{ph}/g_{AC}$ at 20 ^{o}C for the same active carbon support employed [36].

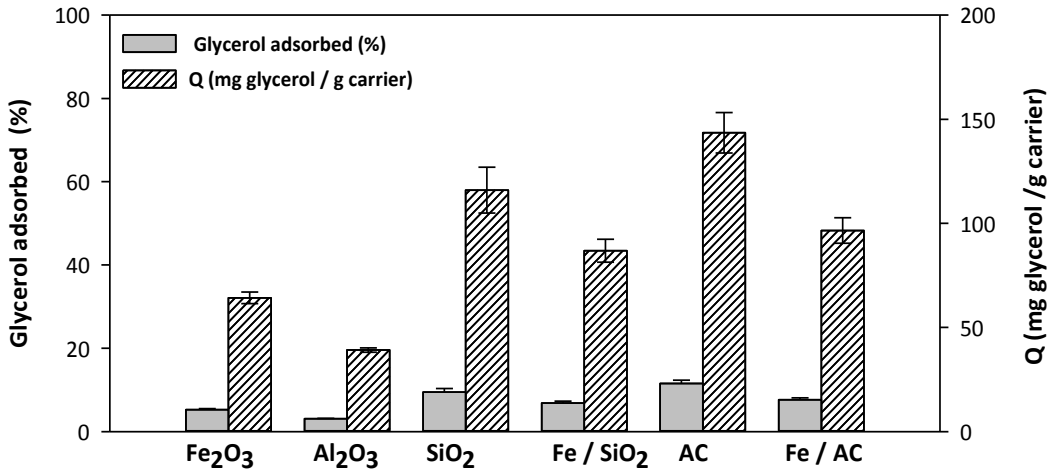


Figure 5.7. Effect of support on glycerol adsorption,

5.5 Conclusions

This study demonstrates that both support supplementation and iron played crucial roles in affecting the performance of dark fermentation for H_2 production from glycerol. Addition of iron species to the supports enhanced the H_2 production rate and yield by 333.3 and 32.2%, respectively when compared to the carrier-free culture.

Effect of support assisted carrier due to surface area attachment and iron involvement were showed:

i) Maximum H_2 productivity (mmol/L) were higher in the order of: Fe/AC (192) > AC (184) > Fe/SiO_2 (183) >SiO_2 (174) >Fe_2O_3(156) > γ-Al_2O_3(135) > FC (120)

ii) Maximum Ethanol productivity (mmol/L) were higher in the order of : AC (211) > Fe/AC (186) > Fe/SiO_2 (183) >SiO_2 (182) > γ-Al_2O_3 (165) > Fe_2O_3 (143) > FC(112)

iii) Maximum 1,3-propanediol productivity (mmol/L) were higher in the order of: FC (45) > Fe/AC (36) > Fe_2O_3(35) > AC (34) > Fe/SiO_2 (22) >SiO_2 (17) > γ-Al_2O_3 (16)

iv) Maximum Lactate productivity (mmol/ L) were higher in the order of: FC (56) > Fe_2O_3(49) > Fe/AC (36) > Fe/SiO_2 (38) > AC (36) >SiO_2 (29) > γ-Al_2O_3(28)

The H_2 yield (mol H_2 /mol glycerol consumed) was observed to be higher for Fe/AC.

Assisted carriers have induced different pathways. The influence of the support on the enzymes participated in the metabolitic activity has to be to be studied in more detail.

The detailed mechanism of the support -induced promoting effects on dark fermentative H_2 production has not yet been clearly identified and will be the focus of our future studies.

5.6 References

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CHAPTER 6

6. HYDROGEN PRODUCTION FROM BIODIESEL WASTE GLYCEROL USING CO-CULTURE OF *ESCHERICHIA COLI* AND *ENTEROBACTER* SP, AND *THERMOTOGA MARITIMA* VIA DARK FERMENTATION

Manuscript in preparation:

6.1 Abstract

Waste glycerol from biodiesel (crude glycerol) was used as a substrate for H_2 production using a mixed culture of *Enterobacter* spH1 and *Escherichia* coli CECT432. In the previous comparative studies, *Enterobacter* spH1 was selected as the best hydrogen and ethanol producer (chapter 3). The same procedure as in chapter 3 was followed for making a selection between the strains of *E. coli* CECT432, *E. coli* CECT434 and *Enterobacter cloacae* MCM2/1. *E. coli* CECT432 was selected due to its higher productivity of H_2 (1307 mL/L). The co-culture of *Enterobacter* spH1 and *E. coli* CECT432 was expected to have a higher productivity of H_2: i) similarity of fermentation end product formation such as ethanol and especially small amount of 1,2-propanediol, ii) co-culture of these strains may simultaneously metabolize the impurities present in crude glycerol. Indeed a microbial co-culture (1:1) of *Enterobacter* spH1 and *E.coli* CECT432 showed a higher H_2 productivity (4767 mL/L) from pure glycerol (220.1 mM). This synergistic effect of the co-culture was also tested for H_2 production using waste glycerol from biodiesel. The composition of the crude glycerol was investigated and found to consist of (w/v): glycerol 47.5%, water 40.5%, ash content 4.8% and Material Organic Non-Glycerol (MONG) 7.2%. The amount of total soluble organic carbon (TOC) in the crude glycerol was 316.6 g/L.

A maximum H_2 yield and ethanol yield of 1.21 and 1.53 mol/mol glycerol was obtained on the waste glycerol, respectively. These yields are the highest reported to date using mesophilic strains. This indicates that the co-culture has a strong synergistic effect.

The use of crude glycerol was also tested for *Thermotoga maritima* strain DSM 3109. It showed growth on crude glycerol. The yield observed was 3.21 mol H_2 /mol Gly and the rate of H_2 was 2.38 mmol/L*h. The yield and rate were higher than the pure glycerol.

The ability to produce H_2 production without prior purification of the waste glycerol is attractive because it avoids extra costs.

Keywords: *Hydrogen; Dark fermentation; E. coli; Enterobacter, Biodiesel, Glycerol*

6.2 Introduction

The dramatic increase in the demand for transport fuels coupled with diminishing crude oil reserves and the increase in environmental concerns have increased the interest in renewable energy. Unlike fossil fuels, hydrogen gas (H_2) burns cleanly, without emitting any environmental pollutants [1]. In addition, H_2 possesses the highest energy content per unit of weight (i.e. 142 kJ/g) which is about 2.75 times greater than that of hydrocarbon fuels. H_2 is considered to be one of the energy carrier of the future [2] and could have an important role in reducing environmental emissions. However nearly 96% of the H_2 used in fuel cells or as raw material in the petrochemical industry is produced from fossil fuels by methods such as: catalytic steam reforming, refinery oil partial oxidation and gasification. These processes however, these processes, release carbon dioxide (CO_2) are not sustainable [3, 4].

To overcome the use of petroleum-derived hydrocarbons as sources for H_2 production, the use of electrolysis of water, thermal decomposition of biomass and biological methods are preferred. The thermo-chemical and electro-chemical processes means are energy inefficient because they require large amounts of energy and may still depend on fossil fuels for the electricity and heat generation [5]. On the other hand, biological H_2 (biohydrogen) production using bacteria is a promising alternative and has attracted worldwide attention for its potential as an inexhaustible source and low-cost. The process does not require additional input of energy when operating under moderate temperatures and is environmentally advantageous especially when it is derived from renewable resources [4,6,7]. Biohydrogen is produced either by photo-biological production or dark fermentation. Dark fermentation is a reduced pathway of anaerobic digestion but with different organisms and optimized reactor operating conditions can offer an excellent potential for practical application such as treatment of organic wastes [8].

The production of H_2 through dark fermentation offers significant advantages over other forms of biohydrogen production because it requires lower investment and simpler operational conditions compared to more sophisticated technologies. This makes it ideal for local applications [9]. A variety of biomass resources can be used to convert to H_2 and some of these are: energy crops, agricultural residues and other kinds of organic waste (forestry waste, industrial and municipal wastes [10]. In order to enhance the yield and economy of H_2 production by dark fermentation, it is important to explore suitable cheap substrates, which can be utilized by a broad range of H_2 producing microorganisms [11].

Recently, many research efforts have been devoted to microbial conversion of low-priced industrial and agricultural wastes into bioenergy [11-13]. The use of the unpurified side product of biodiesel production, viz. crude glycerol, could be an ideal source for industrial fermentation. Biodiesel, one of the promising alternative and renewable fuels, has been viewed with increasing interest and its production capacity has been well developed in recent years [14]. Although

biodiesel represents a secure, renewable and environmentally safe alternative to fossil fuels, its economic viability is a major concern. At the same time, the increased production of biodiesel influenced remarkably the glycerol market due to the generation of a surplus of crude glycerol which is yielded as by-product at about 10% (w/w) of the biodiesel production [15]. The global biodiesel market is estimated to reach 37 billion gallons by 2016 with an average annual growth of 42%, which means about 4 billion gallons of crude glycerol will be produced each year [16].

Biodiesel can be produced using a variety of feedstocks like pure plant oil, waste vegetable oil, waste animal fat and algae oil [17-21]. Apart from the feedstock (fat or oil), several other chemicals are needed to make biodiesel. The esterification and transesterification process requires an alcohol (methanol or ethanol), a catalyst (KOH or NaOH) and a neutralizing agent (HCl, H_2SO_4 or H_3PO_4) [19-21].

At the end of the transesterification reaction, the heavier and polar glycerol-alcohol-catalyst mixture, called crude glycerol, is drained from the bottom of the batch reactor leaving the desired biodiesel fuel. This crude glycerol is a mixture of glycerol, alcohol, catalyst (inorganic salts), water, unreacted mono-, di-, and triglycerides, free fatty acids from lower grade feed stocks, unrecovered esters, and MONG (a miscellaneous catch all group for other “matter organic non-glycerol”). A typical crude glycerol mixture contains approximately 50 to 60% of pure glycerol, 12 to 16% alakalies, 15 to 18% methyl esters, 8 to 12% methanol and 2 to 3 % water. In addition, elements such as Ca, Mg, P, or S can be present [22-24] and which might be useful for the microorganisms. The glycerol produced in the transesterification is not pure and thus of low value. There are different approaches to its utilization. Small producers usually limit the glycerol treatment to dehydration and either sell it to the refiners or burn it onsite for steam production. On the other hand, refined glycerol can influence economically, therefore the large production plants refine it at least to a technical grade.
However, the composition in organic matter and basic elements is one of the reasons why glycerol has been identified as a promising carbon source for industrial microbiology in the future [25]. Besides, it can improve the economic viability of the biodiesel industry.

In addition to availability, low prices and potential to mitigate possible environmental hazards and reducing greenhouse gas emission (GHG) [26], another advantage of using crude glycerol in dark fermentations is that the highly reduced redox state of carbon in glycerol [27] compared to common sugars like glucose and xylose. This enables it to have a higher yield of reduced products such as H_2 [27-29].

Until recently, the fermentative metabolism of glycerol has been reported in species of the genera *Klebsiella*, *Citrobacter, Enterobacter, Clostridium*, *Lactobacillus*, *Bacillus, and Anaerobiospirillum* [11, 13, 29-32, 55]. In addition, various mixed microflora and co-cultures have also been examined for their ability to produce H_2 from glycerol [11, 33, and 34].

In this chapter, first we evaluated different strains like *E.coli* CECT432, *E.coli* CECT434 and *E. cloacae* MCM2/1, for their capacity to perform dark fermentative H_2 production and other useable side products with pure glycerol (PG) as substrate.

Secondly, the strain that performed best (*E. coli* CECT432) was mixed (ratio1:1) with a previously isolated strain of *Enterobacter* spH1 [11] to study the effect of co-culturing on the H_2 production using pure glycerol and crude glycerol. The kinetics of the H_2 production, end products the fermentation and the carbon balance were determined. In addition, the composition of the crude glycerol was thoroughly investigated to see the effect of the impurities on the production of biohydrogen and other value-added products. The use of crude glycerol was also tested for *Thermotoga maritima* strain DSM 3109.

6.3 Materials and Methods

6.3.1 Fermentable substrates

Pure glycerol (purity ≥ 99%), glucose (D-glucose, anhydrous) and all other chemicals were purchased from Sigma Chemical Co., Madrid, Spain. Crude glycerol was obtained from a local biodiesel production plant (Stocks del Vallés BDP S.A., Barcelona, Spain) that utilizes waste vegetable oil (WVO) and waste animal fats (WAF) as the raw material for biodiesel production via the alkali mediated transesterification process.

6.3.2 Microorganisms and media

Escherichia coli CECT432 and *E. coli* CECT434 were obtained from the Spanish culture collection (CECT, Valencia). *Enterobacter cloacae* MCM2/1 was previously isolated in our laboratory from a gasoline contaminated soil [35]), and *Enterobacter* spH1 was previously isolated from San Carles de la Rapita, Spain [11]. The latter strain (*Enterobacter* spH1), which showed highest H_2 production among other strains, was used for mixed cultures *with E.coli* CECT432. The first three stains were tested for their ability to produce H_2 from pure glycerol and mixed culture was tested on both pure and crude glycerol.

The growth medium, nutrient broth (NB), consisted of 5.0 g/L peptic digests of animal tissue, 5.0 g/L sodium chloride, 1.5 g/L beef extract and 1.5 g/L yeast extract. [11]. The fermentation medium (MYG) was prepared with 10 g/L malt extract, 5 g/L yeast extract, 10 g/L sodium chloride and 20.2 g/L pure glycerol or 5.1 % (v/v) crude glycerol. The pH of the growth and the fermentation media was adjusted to 6.7 before sterilization at 121C for 20 minutes. Both media were selected because of their suitability for H_2 production [11]. The strains were aerobically precultured overnight in NB at 37 °C in an incubator-shaker at 200 rpm. The cells were harvested at the end of the exponential phase, and 10 % (v/v) this inoculum was inoculated to the MYG

medium for the batch experiments. The MYG medium was maintained at (under anaerobic conditions and pH ~ 6.34).

Thermotoga maritima strain DSM 3109 was obtained from DSMZ (the Deutsche Sammlung von Mikroorganismen und Zellkulturen) and cultivated in M3 medium. M3 preparation and was the same in chapter 4 except here the crude glycerol of 0.25 % (v/v) instead of pure glycerol. The initial amount of glycerol in the crude glycerol was around 33 mM.

6.3.3 Batch experiment

H_2 production by dark fermentation for the co-culture was investigated in a batch system. A 1.2 L bioreactor was used with a working volume of 500 mL at continuously stirred at 200 rpm. The bioreactor was water jacketed by a circulating water bath to maintain the reactor temperature at 37°C. At the top of the bioreactor, there were inlets for the medium and argon and outlets for gases. A total of 450 mL of MYG medium, containing different concentrations of substrate, was placed in the bioreactor and autoclaved (for 15 min at 121°C). An anoxic atmosphere was created by continuous purging with 30 mL/min of argon gas (99.99 %). The reactor was on-line connected to a GC to directly analyze the gases generated. The liquid fermentation products were analyzed by GC-MS and HPLC. Unless stated otherwise, the duration of the batch fermentation was 72 h. Each experimental condition was studied in duplicate or triplicate.

The batch experiment for *Thermotoga maritima* strain DSM 3109 was done as described in chapter 4 using a serum bottles.

6.3.4 Analytical procedures

6.3.4.1 Biogas analysis

The composition of the gas phase for the co-culture experiments was measured every 30 minutes throughout the fermentation using a GC-14B gas chromatograph (Shimazdu, Japan) equipped with a thermal conductivity detector (TCD) and a 80/100 Porapak-Q column. The operational temperatures of the GC for the injection port, oven and detector were 150°C, 80°C and 200°C, respectively. Argon and helium were used as the carrier gas at a flow of 30 mL/min for measuring H_2 and CO_2 respectively. The H_2 from the fermentation was quantified by comparison with pure gas standards. For each batch, gas samples were analyzed continuously by online GC connected to the bioreactor. The chromatogram was developed and analysed using the Turbochrome Navigator (version 4.1) software from the Perkin Elmer Corperation. The concentration of hydrogen in the gas outlet connected to the chromatograph and computer to treat the data.

The biogas analysis for *Thermotoga maritima* strain DSM 3109 experiment was done as described in chapter 4.the

6.3.4.2 Liquid analysis

The concentration of substrates (glycerol) and fermentation products such as organic acids (formate, lactate, acetate, propionate, butyrate), alcohols (butanol, ethanol and methanol), diols (1,2-propanediol, 1,3-propanediol, 2,3-butanediol) and alcohol sugars, were determined and quantified by HPLC (Agilent 1100 Series) furnished with HP Chemstation software (Agilent, Waldbron, Germany) for data acquisition. The column was a Transgenomic ICSepICE COREGEL-87H3. The method used has been previously published by Garcia-LLobodanin et al 2007 [36]. Prior to the analysis the liquid samples were centrifuged at 9800 rpm for 15 min and filtered through a 0.2 µm disposable filter. The injection volume of the sample was 20 µl.

The liquid analysis for *Thermotoga maritima* strain DSM 3109 experiments was done as described in chapter 4.

6.3.4.3 Biomass analysis

Biomass was estimated by protein measurements using Peterson's protocol, a modified Lowry method [37]. The dry cell biomass was calculated taking into account that protein comprises about 60 % of the cell content [38]. The carbon fraction of biomass dry cell weight content was estimated to be 54 % with an average chemical composition of $CH_{1.74}O_{0.33}N_{0.23}$ [39,40].

The biomass analysis for *Thermotoga maritima* strain DSM 3109 experiment was done as described in chapter 4.

6.3.4.4 Analytical procedures for biodiesel waste (crude glycerol)

The glycerol content of crude glycerol was determined after appropriate dilution and filtration by HPLC, as described above.

The water content was measured following the standard method (ISO 2097-1972) by using the volumetric Karl Fisher titration. Ash content was analyzed according to the Standard method (ISO 2098-1972) by burning 1 g crude glycerol in a muffle furnace at 750 °C for 3 h. The MONG levels were calculated from the previous three compositions according to the following equation: (100−(% glycerol content+ % water content+ % ash content)) [41,42].

The composition of other compounds present in the biodiesel waste (mainly MONG), were determined by gas chromatography mass spectrometry (GC/MS). The equipment used was a

GC/MS (6890N, Agilent of GC/Pegosees III, Lego of MS) equipped with a capillary column HP-FFAP (Agilent 19091F-433, 0.25mm × 30m × 0.25um). The column temperature was initially kept at 35 °C for 4 min , then raised at 10 °C/min to 150 °C, kept at 150 °C for 10 min, then raised to 250 °C at 15 ° C/min and maintained at this temperature for 10 min. The temperatures of the injector and detector were set at 245 and 250 °C, respectively.

The concentration of total organic compound (TOC) was measured using a Total Organic Carbon Analyzer (Analytik jena, Multi N/C 2100). Pure glycerol was used as a reference.

X-ray Diffraction (XRD) analysis of ash was used to identify the salts. The XRD analysis of the ash was recorded using a Siemens D5000 diffractometer (Bragg-Bentano for focusing geometry and vertical θ-θ goniometer) with an angular 2θ diffraction range between 3° and 90°. The samples were dispersed on a Si (510) sample holder. The data were collected with an angular step of 0.03° at 5 s per step and sample rotation. Cu Kα radiation (λ=1.54056 Å) was obtained from a copper X-ray tube operated at 40 kV and 30 mA. The crystalline phases were identified using the JCPDS files.

The elemental composition of the ash was determined by Scanning electron microscope (SEM) with a JEOL JSM-35C scanning microscope operated at an acceleration voltage of 15 KV. A small portion of each sample powder was coated on a metallic disk holder and covered with a thin carbon layer before SEM analysis.

Fourier transformed infrared (FT-IR) spectra were recorded on a Bruker IFS 28 instrument with a resolution of 4cm^{-1}. It was used to analyze the characteristics of the pure glycerol and the crude glycerol in the biodiesel waste.

6.3.5 Data analysis and Kinetic parameters

We modelled the fermentation data using the Gompertz equation (6.1) [43,44,] to estimate the maximum production rate and the production potentials of the fermentation end products. Cumulative production curves were obtained throughout the batch experiment [44]

$$P_i(t) = P_{max,i} exp\left\{-exp\left[\frac{R_{max,P_i}{}^{*}e}{P_{max,i}}(\lambda i - t) + 1\right]\right\} \qquad \textbf{Equation (6.1)}$$

Where $P_i(t)$ is the cumulative production (mmol/L), λ the lag-phase time (h), $P_{max,i}$ the production potential (mmol/L), $R_{max,i}$ the maximum production rate (mmol/L*h), t the incubation time (h), and e the exp(1) = 2.718. This equation was found to be suitable for describing the progress of cumulative production of compounds during the experiments.
Accordingly, for the consumption of glycerol a modified Gompertz equation Eq. (6.2) [45] was used:

$$S_0 - S(t) = S_{\max}.\exp\left\{-\exp\left[\frac{R_{\max,S}.e}{S_{\max}}(\lambda_S - t)+1\right]\right\} \qquad \textbf{Equation (6.2)}$$

Where: S_0 – initial substrate concentration (mmol/L), S – substrate concentration (mmol/L) at time t , S_{max} – maximum concentration of consumed substrate (mmol/l), $R_{max,S}$ – maximum rate of substrate consumption (mmol/L*h). The fitting of the fermentation data was performed using Sigma plot application software version 12.5, where accuracy of the fit was given by correlation coefficients (R^2).

For batch cultivation, yields of the fermentation end-products Eq. (6.3), were determined using the values obtained from the data fittings (Eq. (6.1) and Eq. (6.2)), and expressed in mole product produced per mole glycerol consumed..

$$Y_{P\max,i} = \frac{P_{\max,i}}{S_0 - (S_{\max} - S_{ads})} \qquad \textbf{Equation (6.3)}$$

Where: $Y_{Pmax,i}$ – substrate yield for fermentation product i, S_0 – initial glycerol concentration (mol/L), S_{max} – maximum glycerol concentration (mol/L).

Likewise, maximum specific production or consumption ($q_{max,i}$) were calculated using the values obtained from the data fittings (Eq. (6.1) and Eq. (6.2)) , according to Eq. (6.4) It was the ratio of the maximum production rate or maximum production rate to maximum dry cell weight (DCW_{max}).

$$q_{\max,p_i} = \frac{R_{\max,i}}{DCW_{\max}} \text{ or } q_{\max,s_i} = \frac{S_{\max,i}}{DCW_{\max}} \qquad \textbf{Equation (6.4)}$$

Where: $q_{max,\ i}$ specific production or consumption rate i (mmol/L*h), $R_{max,i}$ – the maximum production rate i , maximum substrate consumption rate (mmol/L*h) and DCW_{max} maximum dry cell weight (g/L).

Carbon balances (C-balance) and degree of reduction balances (*ε*-balance) were calculated according to Oh et al. and Converti et al. using the elemental biomass composition (molecular formula for the mixed culture) $CH_{1.74}O_{0.33}N_{0.23}$ [38-40].
This corresponds to biomass carbon content of 53.6% and with a degree of reduction of 4.32 electrons per C atom. The degree of reduction (*ε)* was calculated from the following equation (6.5) [39, 40].

$$\varepsilon = 4C + H - 2O - 3N \qquad \textbf{Equation (6.5)}$$

Where C, H, O, and N denote the atomic coefficient of the chemical formula of a compound.

Glycerol uptake efficiency

The efficiency of glycerol uptake (E) by the strains was calculated using the following equation:

$$E = \frac{I - F}{I} 100\% \qquad \textbf{Equation (6.6)}$$

Where I and F are the initial and final concentrations of glycerol, respectively.

6.4 Results and Discussion

6.4.1 Characteristics of crude glycerol from a WVO and WAF origin.

The crude glycerol (CG) obtained was a dark brown liquid with a neutral pH of around 6.8. The chemical characterization of the crude glycerol used in this work is presented in Table 6.1. The glycerol content was around 47.5 % (w/w), which was lower than what was reported by other authors with values between 65 % to 85 % (w/w) [44, 46].

The ash content (4.8±0.5 % w/w) was comparable to the average values reported in a study done by Manosak et al. [47] 4.31±0.27% w/w). In contrast, the MONG reported by Manosak et al. [47] was by far the largest contaminant present (44±0.44 % (w/w)) as compared the MONG content in our crude glycerol (7.2 ±2.5 % (w/w)). However, the amount of water (40.5±2 % (w/w)) in our CG was higher than that reported by the same authors (14.7±.9 % (w/w)). However, the composition of the crude glycerol is much more similar to the data of Saenge et al. 2011 [48] who reported a glycerol content of 50% with impurities composed mainly of potassium and sodium salts (4–5%), methanol (1-3%), non-glycerol organic matter (1.6–7.5%) and water (36%).

The differences in composition of CG can be attributed to different glycerol purification methods used by the biodiesel producers and the different feedstocks used in biodiesel production. It is important to note that the feedstock used by the company, from which we obtained the crude glycerol is composed exclusively of WVO and WAF which may explain the low content of glycerol.

Table 6.1. Physicochemical parameter characteristics of crude glycerol

	parameter	Value	
	Glycerol % (w/w)	47.5±2.5	
	Water % (w/w)	40.5±2.5	
	Ash content % (w/w)	4.8±0.85	
	MONG[a] % (w/w)	7.2±2.5	
	Density (g/cm^3)	1.12	
	Colour	Dark brown	
	Odor	unpleasent	

[a]MONG: matter organic non-glycerol. Defined as 100 – [glycerol content (%) + water content (%) +ash content]

6.4.2 GC-MS

The composition of CG derived from the WVO and WAF methyl ester plant was analyzed by a GC/MS. The list of the compounds that were detected and their abundance using this technique is shown in figure 6.1. Besides, glycerol (92.5%), the CG contains various compounds, including fatty acids and other derivatives, such as 2-piperidinone (1.56%), butanoic acid, 2,3-dihydroxypropyl ester (0.69%), tripropylene glycol monomethyl (0.60%), acetic acid (0.49%), 5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a;1',2'-d]pyrazine(0.43%), butanoic acid (0.31 %), 1,2,3-propanetriol, monoacetate (0.27%), 1,3-propanediol (0.27%), phenol (0.25%), dodecanoic acid (0.18%), hexanedioic acid, bis(2-ethylhexyl) ester (0.18%), propanoic acid (0.17%), glycolaldehyde dimethyl acetal (0.16%), phosphoric acid, trimethyl ester (0.14%) amongst others are the main components of CG. As shown in Table 6.2 and Figure 6.1, the compounds determined by GC/MS are diverse, which could be related to the type of feed stock (WVO and WAF), as observed by Shengjun Hu [49].

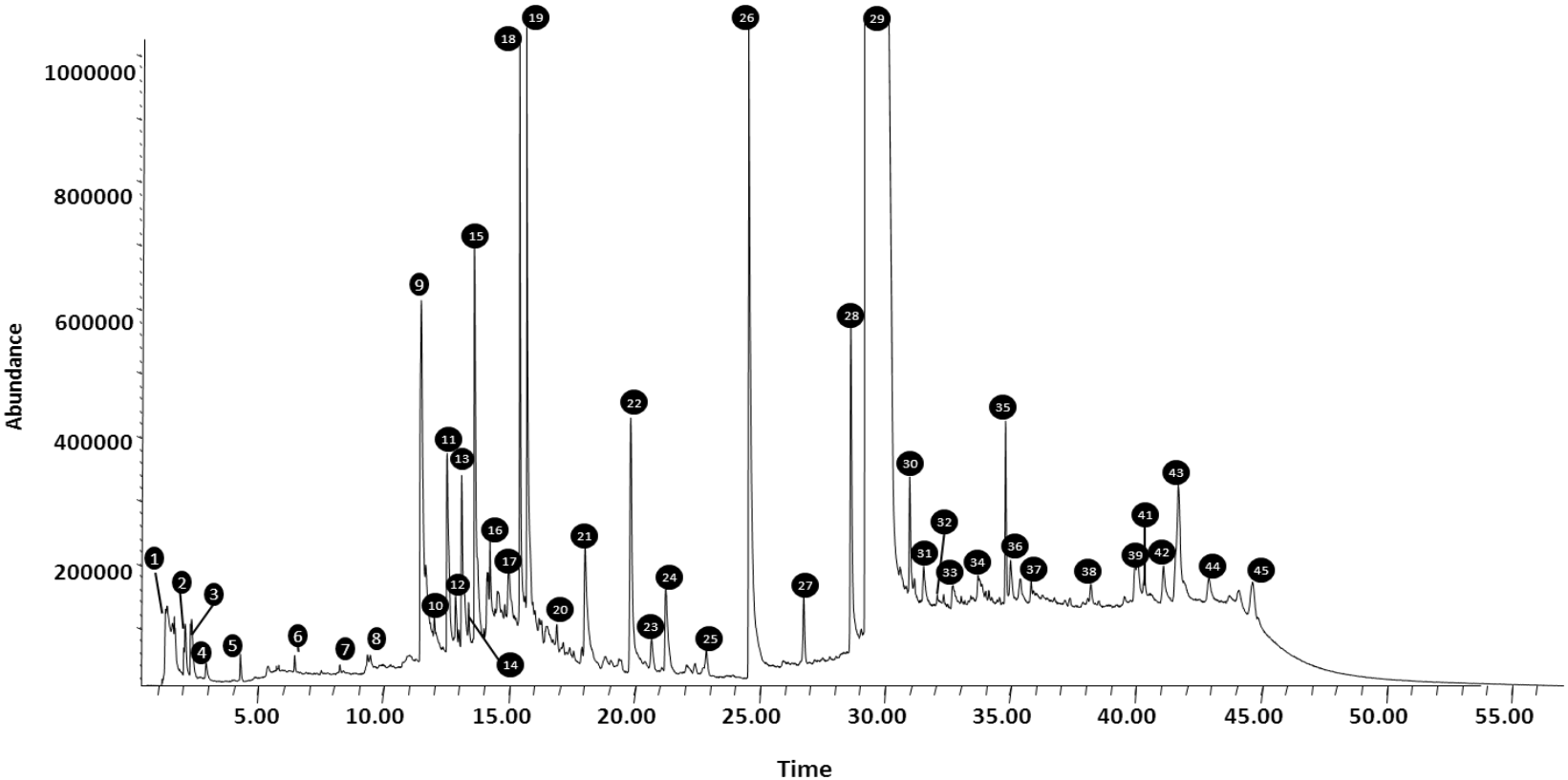


Figure 6.1. GC/MS analysis of CG from biodiesel waste

Numbers refer to identified components as lsited in Table 6.2

Table 6.2. GCMS analysis result for the crude glycerol.

peak #	Retention time (tR)	% area	Compound
1	1.24	0.235	Methane, chloro-
2	1.99	0.031	Acetic acid, chloro-
3	2.26	0.047	Isopropyl Alcohol
4	2.87	0.012	Butanoic acid, methyl ester
5	4.30	0.012	Cyclotetrasiloxane, octamethyl-
6	6.56	0.010	L-Alanine, 3-sulfo-
7	8.44	0.004	Propanoic acid, 2-hydroxy-, methyl ester, (.+/-.)-
8	9.59	0.022	Boronic acid, ethyl-
9	11.83	0.492	Acetic acid
10	12.37	0.025	Hydrazine, 1,2-dimethyl-
11	12.90	0.168	Propanoic acid
12	13.25	0.027	Propanoic acid, 2-methyl-
13	13.51	0.136	Phosphoric acid, trimethyl ester
14	13.80	0.015	Butane(dithioic) acid, methyl ester
15	14.04	0.315	Butanoic acid
16	14.57	0.029	Octanoic acid, 2-methyl-
17	15.47	0.097	Pentanoic acid
18	15.94	0.268	1,3-Propanediol
19	16.25	0.601	Tripropylene glycol monomethyl ether
20	17.31	0.015	Acetic acid
21	18.29	0.157	Glycolaldehyde dimethyl acetal
22	19.87	0.246	Phenol
23	20.59	0.024	Succinic acid, dodecyl tetrahydrofurfuryl ester
24	21.08	0.089	2-Pyrrolidinone
25	22.48	0.022	Octanoic acid, methyl ester
26	23.99	1.558	2-Piperidinone
27	26.55	0.054	Octanoic acid, 8-hydroxy-, methyl ester
28	28.67	0.273	1,2,3-Propanetriol, monoacetate
29	30.66	92.600	Glycerin
30	31.32	0.692	Butanoic acid, 2,3-dihydroxypropyl ester
31	32.02	0.182	Dodecanoic acid
32	32.56	0.075	L-Arabinitol
33	33.33	0.043	Diglycerol
34	33.69	0.069	dl-Threitol
35	34.65	0.103	2,4-Imidazolidinedione, 5-methyl-
36	35.71	0.175	Hexanedioic acid, bis(2-ethylhexyl) ester
37	36.28	0.047	Hexanedioic acid, bis(2-ethylhexyl) ester
38	36.70	0.040	15-Crown-5
39	38.98	0.047	Octaethylene glycol monododecyl ether
40	40.78	0.135	1,4,7,10,13,16-Hexaoxacyclooctadecane
41	41.04	0.034	Probarbital
42	41.78	0.087	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-
43	42.35	0.430	5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a;1',2'-d]pyrazine
44	43.52	0.071	Hexaethylene glycol monododecyl ether
45	44.68	0.056	1,4,7,10,13,16-Hexaoxacyclooctadecane
46	45.21	0.130	Octaethylene glycol monododecyl ether

6.4.3 FTIR

Figure 6.2 shows the FTIR spectra (400-4,000 cm^{-1}) of CG in comparison with the spectra of pure glycerol. The FTIR analysis shows the presence of functional groups of –COO-, -OH, C=O, CH [50, 51].

The entire functional group spectrum of the crude glycerol was almost similar to that of pure glycerol but with a broader absorption band at 3300 cm^{-1} and two well-define bands at 1644 and 1216 cm^{-1}. The glycerol moiety of the pure compound is evidenced by the absorption band at 1500-1200 cm^{-1} assigned to overlapping of the C-H in-planes and O-H bending in the glycerol molecule. The presence of the OH group in both samples (pure glycerol and CG) was evidenced by the fundamental mode of OH stretching at 3600-3000 cm^{-1} [52]. The broader band at 3300 cm^{-1} was probably due to the –OH groups from water and in addition to the -OH from the glycerol molecules as the crude glycerol still contained about 40% (w/w) water [53]. The C-H stretching band was seen at around at 2862.6 cm^{-1} to 2929.6 cm^{-1}, while the bands for C-H scissoring and bending appeared at the region of 1410 – 1450 cm^{-1}. The bands around 1000– 1300 cm^{-1} were contributed by the C-O group stretching in the sample. The 2970, 1235, and 1220 cm-1 bands fit in the ranges that indicate –OH bonds. C-O-H bending was found at 1403.5 cm^{-1} to 1486 cm^{-1}, C-O stretching at 1454.5 cm^{-1} and 1113.6 cm^{-1} represented the primary alcohol and secondary alcohol. The presence of some impurities (MONG 7.2% w/w) was shown by the absorption band at 1644 cm-1 (C=O group) which was similar to the research findings of Yong et al. [53]. Yong et al., [53] observed a band around 1649 cm-1 and suggested that this peak corresponded to the oxidation products of glycerol such as glyceraldehydes, dihydroxyacetone and free fatty acids.

Another study by Hidawit et al [54] also suggested that in soap formation when the carboxyl groups (COO^-) of fatty acids are attached to the metal ions, the COO stretch band is usually seen at 1650-1540 cm^- [55]. The presence of COO- functionality was indicated by the absorption frequency at ~1640 cm-1 which was absent in pure glycerol [54]. The other band around 1216 could be C–O stretches (strong absorptions; asymmetrical coupled vibrations) of saturated aliphatic esters from the MONG [56].

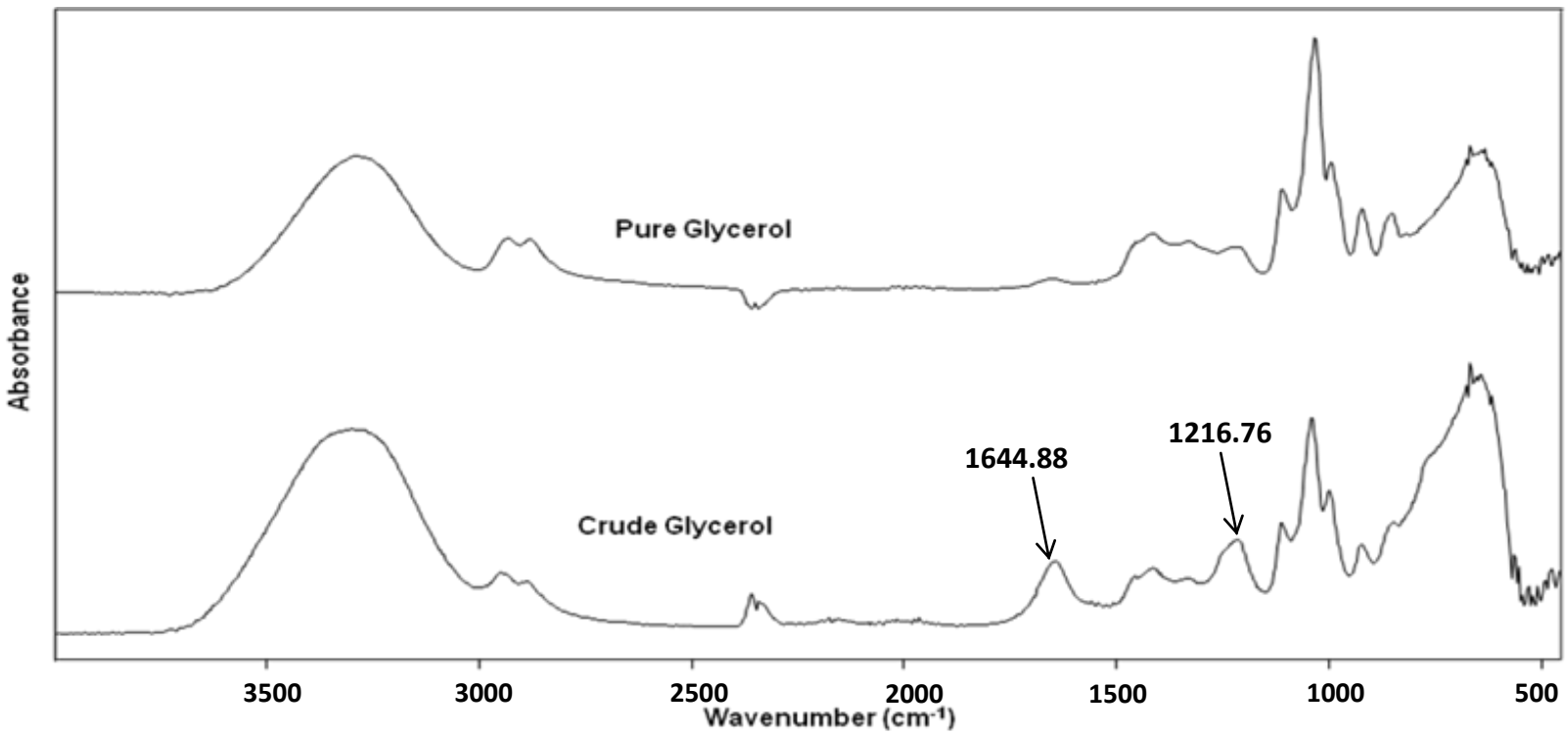


Figure 6.2. FTIR of pure glycerol and crude glycerol

6.4.4 XRD

The XRD analysis showed (see Fig. 4) that the CG contains a large amount of salt crystals of K_2SO_4 and lower amounts of $Na_2Mg(SO_4)_2(H_2O)_4$. These salts are a result of the KOH, used as a catalyst in the transesterification, and sulphuric acid (H_2SO_4) used for neutralization or/and esterification processes. Different authors also reported these facts [22, 57, 58].

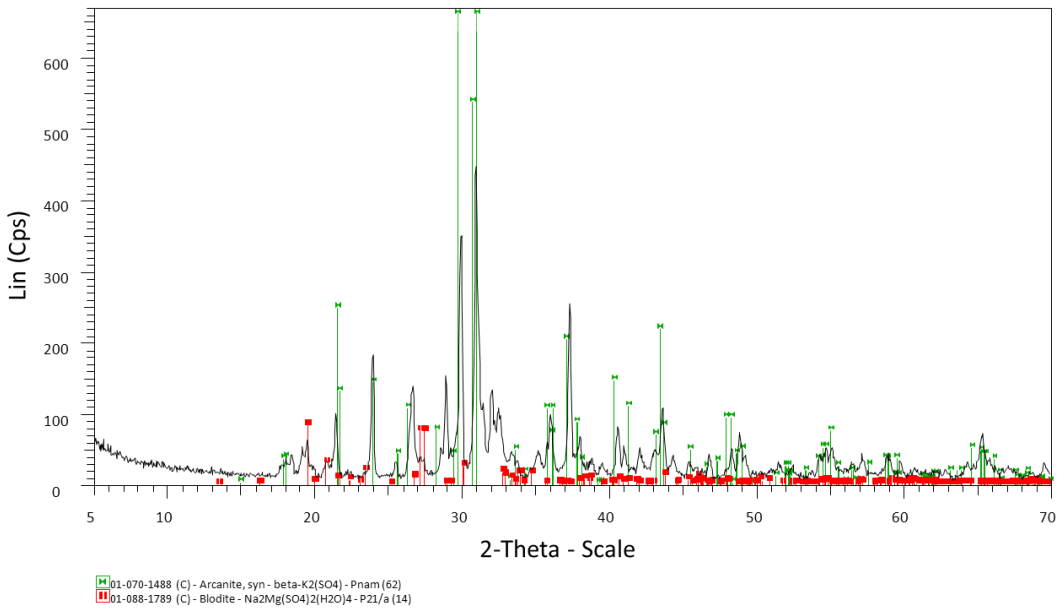


Figure 6.3. XRD of salt in the CG after 750°C calcinations

6.4.5 SEM

The Elemental analysis using SEM for the biodiesel ash (5.6 % (w/w) is shown in Table 6.3. As can be referred in Table 6.3 the CG has a very large amount of potassium and phosphorus. Thompson and He [22] also investigated the elemental composition of crude glycerol from different feedstocks (such as mustard seeds, canola, soybean, and waste vegetable oil. In their finding, they showed the ranges of 10-20 ppm calcium, 3-7 ppm magnesium, 10-60 ppm phosphorous, and 14-21 ppm sulfur. Schröder and Südekum [59] have also reported the elemental composition of crude glycerol from rapeseed oil feedstock. The weight percentile (% (w/w)) of elements in the crude glycerol were: 1.05% -2.36 phosphorous, 2.20% -2.33% potassium and 0.09%- 0.11% sodium. Cadmium, mercury, and arsenic were all below detectable limits.

Table 6.3. Elemental analysis of crude glycerol.

Elemental Spectrum	C	O	Na	Mg	Al	Si	P	S	K	Ca	Zr	Total
Average w/w (%)	5.2±1.4	36.19±1.3	5.43±1.7	0.1±0.05	0.26±.01	0.27±.3	10.48±5.4	9.39±.3.69	29.42±.6.42	0.47±.01	3.133±1.1	100
Average w/w (%)[a]	0.232±0.14	2.03±.13	0.3±.16	0.01±.01	0.02±.001	0.02±.02	0.59±.37	0.53±.26	1.65±.46	0.03±.001	0.18±.06	5.6±.31

[a] The % elemental composition was calculated from the ash composition to estimate in the CG. All values of average and standard deviation.

6.4.6 TOC

The total carbon composition (%) of the biodiesel revealed that 66.48± 3.99 % TOC was due glycerol and 33.52 ± 3.99 % due to MONG and others.

6.4.7 Comparative biohydrogen production *E. coli* and *Enterobacter* strains using pure glycerol as a carbon source.

Figure 6.4 shows the comparison of H_2 production, biomass growth and ability of glycerol conversion by *E. coli* CECT432, *E.coli* CECT434 and *E. cloacae* MCM2/1 using pure glycerol (20 g/L) as substrate. It can be seen that *E. coli* CECT432 strain shows the highest H_2 production, followed by *E. coli* CECT434 and, finally, *E. cloacae* MCM2/1. Over a period of 72 h productivity values of 21.17, 12.17 and 10.37 mL H_2/L*h were found for *E. coli* CECT432, *E. coli* CECT434 and *Enterobacter* MCM2/1, respectively. The increase inH_2 production is correlated with an increase in dry cell mass. The specific H_2 productivity was 38.65, 58.04, 31.12 mL/ gDCW*h for *E. coli* CECT 432, *E. coli* CECT434 and *E. cloacae* MCM2/1, respectively. However, it is important to note that there is also a growth and H_2 production for all the three strains in control conditions (without glycerol carbon source). This is mainly due to the yeast and malt extract (Figure 6.4). It is reported by Ito et al. [56] that addition of both yeast extract (5 g/L) and tryptone (5 g/L) to synthetic medium, effectively increased the rate of H_2 and ethanol production and glycerol consumption. They suggested that some nutrients, such as specific amino acids and vitamins are still needed for the better growth of *Enterobacter* sp.

The H_2 yield (mol H_2/mol glycerol consumed) was 0.56, 0.43 and 0.34 for *E. coli* CECT 432, *E.coli* CECT434 and *E. cloacae* MCM2/1, respectively. These yields are lower compared to a previous study with newly isolated *Enterobacter* and *Citrobacter* sp [11] and similar studies done by other researchers [27,60].

E. coli ferments glycerol anaerobically [27, 61]; but its specific growth rate on glycerol is very low [27]. Moreover, as in indicated in Figure 6.4 the maximum dry cell mass growth was small. Nonetheless, *E. coli* is very promising for glycerol utilization because it is one of the most commonly used host organisms for metabolic engineering and industrial applications. Besides, it is easy to manipulate genetically, can produce a wide variety of anaerobic fermentation products, and it is the best-characterized bacterium [29].

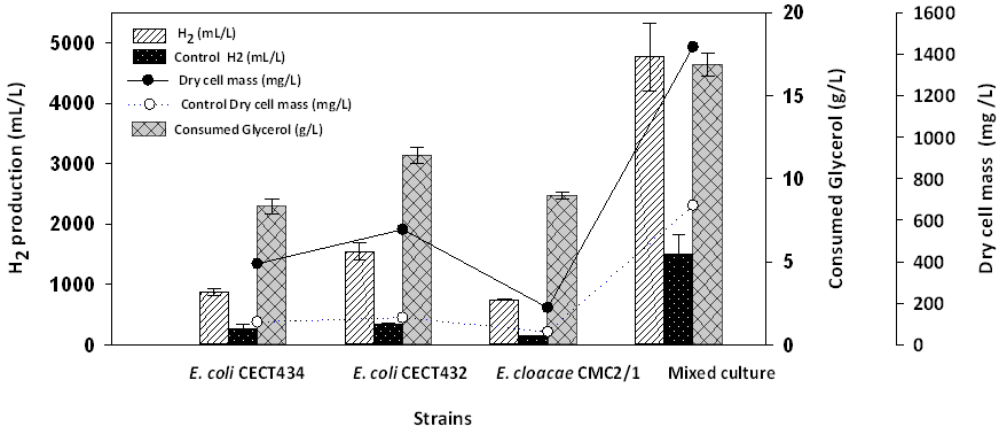


Figure 6.4. Comparison of H2 production using, *E. coli* CECT432 a, *E. coli* CECT434, *E. cloacae* MCM2/1 and mixed culture (*E. coli* CECT432 and *Enterobacter* spH1) from pure glycerol.

Glycerol uptake efficiency The other parameter used for the comparison was the glycerol uptake efficiency. The glycerol uptake efficiency for *E. coli* CECT432 (56.98 %) was higher than that of *E. coli* CECT434 (41.68%) and *E. cloacae* MCM2/1 (44.95 %). However, these data are significantly lower than those reported earlier for the strains *Enterobacter* spH1 (85.75%) and *Citrobacter freundii* H3 (62.5 %) [11]. Nevertheless the uptake of *Enterobacter* MCM2/1 was higher than the *E.coli* CECT434, the H_2 production of *Enterobacter* MCM2/1 was lower than the *E.coli* CECT434 this may suggested that the metabolic pathway of this strain was directed to produce other products such as 2,3 butanediol, lactate and succinate, which consume $NADH_2$.

6.4.8 Optimization of H2 production using mixed culture (1:1) of *Enterobacter* spH1 and *E.coli* CECT432

The aim of this study was to investigate whether a co-culture (*Enterobacter* spH1 and *E.coli* CECT432) would give higher amounts of H_2 using pure glycerol and CG. In our previous study *Enterobacter* spH1 [11] had produced 3750 mL/L of H_2. Kotay et al [62] had reported a 10% increment using a consortium consisting of *E. cloacae* IIT-BT 08, *C. freundii* IIT-BT L139 and *Bacillus coagulans* IIT-BT S1 with glucose as a substrate. It was suggested by the same authors that at the primary level it was found necessary to explore the phylogenetic relationship of these bacteria to understand their microbial diversity and ability to co-exist within a consortium. It is well known

that *Enterobacter* and *E. coli* are closely related belonging both to the family of *Enterobacteriaceae*. The members of this group have been known from earlier works for their potential high H_2 yields. These bacteria are metabolically versatile, and utilize a wide range of carbon sources. Of all the fermentative hydrogen producers, *Enterobacter* sp. have attracted much attention due to their high growth rate, easiness of culture similar to *E. coli,* and wide substrate range [63].

The significance of biological diversity for ecosystem function is highly debated; however, greater species diversity is generally associated with improved community function and stability. The effect of diversity on ecosystem function may be due to the following reasons [64]: (i) more efficient utilization of resources due to increased competition, niche differentiation, and resource exploitation; and (ii) presence of individual species with crucial functional characteristics. Community structural and functional characteristics may, however, be as important as overall diversity. Defined communities allow the researcher to manipulate specific factors such as the degree of functional redundancy among individuals, and provide greater confidence that potentially deleterious organisms are not being introduced into the system.

6.4.9 Pure glycerol and crude glycerol consumption of *E.coli* CECT432 and *Enterobacter* spH1

E.coli CECT432 *pathways similarities with Enterobacter* spH1

Figure 6.5 gives an overview of the metabolic pathways involved in glycerol fermentation by *E. coli* CECT432 (data not shown) and *Enterobacter* spH1 [11] based on the identified metabolites. The dashed boxes show that *E. coli* CECT432 and *Enterobacter* spH1 use similar reductive pathways to 1,2–propanediol and ethanol (Figure 6.5).
It was reported by Hu et al. [29], that chemically mutated strains of *E. coli* can produce more ethanol. In our previous study [11] with *Enterobacter* spH1 the production of ethanol was high and there was only a small amount of 1,2-propanediol produced. These similarities in metabolic pathways prompted us to create a mixed culture of these two strains to improve the production of H_2 and other usable end products. HU et al. [29] also clearly demonstrated that the ability of *E. coli* to ferment glycerol in to the 1,2-propanediol pathway with the supplement of tryptone.

Interestingly, the constructed microbial co-culture (1:1) of *Enterobacter* spH1 and *E. coli* CECT432 resulted in a higher H_2 production of 4750 mL/L from pure glycerol (Figure 4). This suggests that mixed co-culture (1:1) has the ability to co-exist. Therefore, energy and carbon balances, kinetic parameters over the H_2 production and the usable products were studied in more detail using the pure glycerol and the crude glycerol.

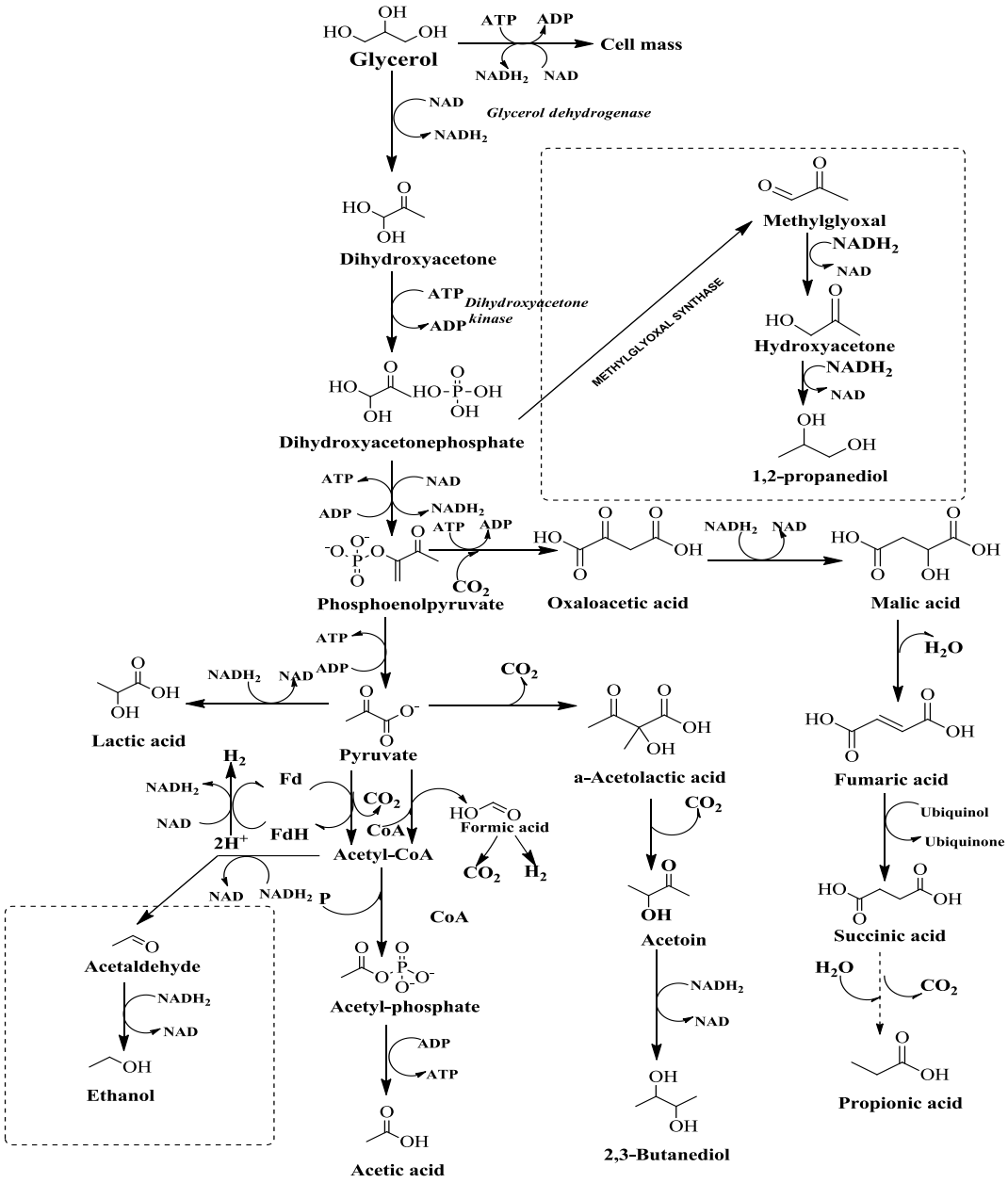


Figure 6.5. Biochemical pathways of glycerol fermentation for the *Enterobacteriaceae* (adapted from da Silva et al [64] and Hu et al 2010 [29]).

6.4.10 Growth on pure glycerol (PG) and crude glycerol (CG)

Figure 6.6 (a) and (b) show the time course of a batch fermentation by the mixed culture (1:1) of *Enterobacter* spH1 and *E.coli* CECT432 using PG and CG, respectively. Our results clearly show that the mixed culture is perfectly able to grow on pure glycerol and CG as source of carbon and

energy. The type of carbon source and the initial substrate concentration usually play an important role in the bacterial growth and product yield [65]. It has been discussed by Choi et al [66] that one of the primary issues when using CG for bioconversions is to acquire a microbial host which is able to tolerate batch-to-batch variations and the impurities found in this co-product [66]. These inconsistencies are largely due to differences in the quality of the initial oil feedstock used and the presence of contaminants as seen in Table 6.3 similar to the investigation by Thompson et al. [22]. Finding organisms that produce H_2 and that can tolerate the impurities found in the CG is the main challenge [66]. Here, we show that the mixed culture of *Enterobacter* spH1 and *E.coli* CECT432 has the ability to produce H_2 without any complex pre-treatment of the CG. This utilization by the mixed culture is attractive in reducing the cost and scaling up of the process. Therefore, the improve bioconversion of crude glycerol to hydrogen by co-culture of *Enterobacter* spH1 and *E.coli* CECT432 may be due their ability to producing hydrogen bacteria and degrading crude glycerol impurities. This can be consolidate with the study done by Amund 1997 et al [67] that some species of *Klebsiella*, *Escherichia*, *Enterobacter* are known to have soap degradation potential [67] one of the impurities found in the CG.

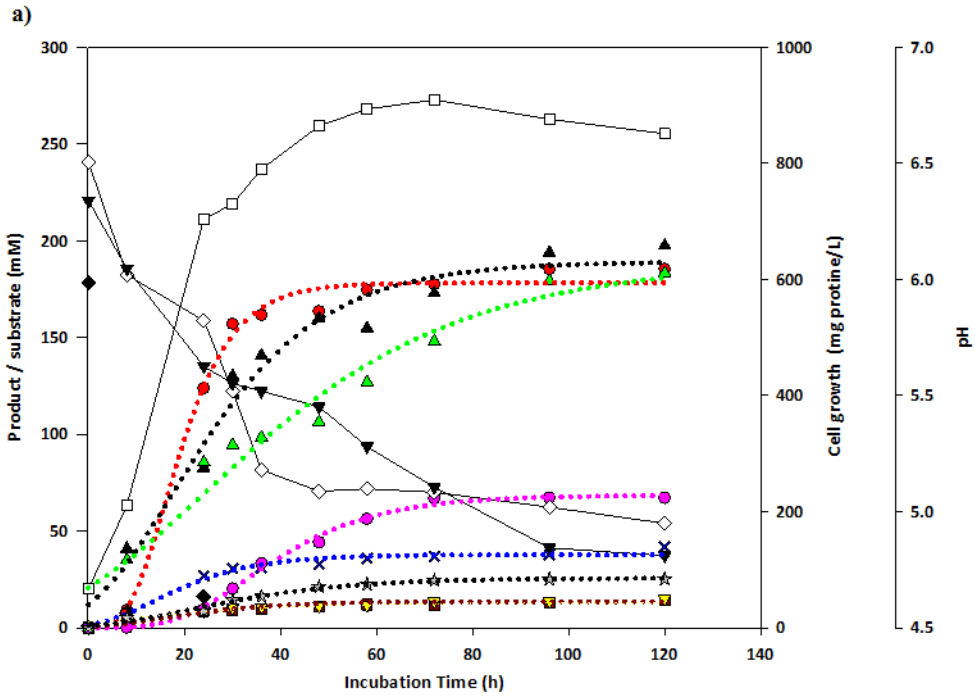
From Fig. 6.6 (a) & (b), it can be observed that PG and CG were mainly fermented to ethanol, lactate, 2,3-butanediol, acetate, CO_2, H_2 and minor amounts of succinate and 1,2-propanediol (data not shown in figures 6.6 (a) & (b)). However, 1,3-propanediol and formate were not detected. These end products are commonly found in mesophilic glycerol fermentation by *enterobacteria* [68] or *clostridia* [69]. The only difference we observed using the two carbon sources (PG and CG) was small amount of propionate production for the GC. On both PG and CG the highest soluble metabolite concentration was found for ethanol with an amount of 170 and 222 mM, respectively. This suggests that the mixed culture of *Enterobacter* spH1 and *E.coli* CECT432 is a good producer of ethanol. Ito et al. [60] have also proposed to produce hydrogen gas and ethanol from glycerol-containing wastes of a biodiesel manufacturing process using *Enterobacter aerogenes* HU-101. Alternatively, according to Dharmadi et al [70], the anaerobic fermentation of glycerol by *E. coli* can also generate ethanol, lactate, succinate, and hydrogen.

Figure 6.6 (a) also shows that total H_2 production from pure glycerol was lower than crude glycerol. This could be due to the higher succinate formation (23.6 mM) using the PG, higher than what was obtained with the crude glycerol (3.5mM). This could be due to growth of *E. coli* on glycerol as carbon source a particulate system catalyzing the reduction of fumarate a precursor of succinate at the expense of molecular hydrogen [71]. It is known that the production of succinate under anaerobic conditions is consistent with the existence of the anaplerotic reaction catalyzed by phosphoenolpyruvate carboxylase, which is necessary to satisfy the redox balance of the main reductive metabolism of most *Enterobacteriaceae* [72]. Both increased formation of ethanol and decreased lactate formation would be responsible for increased production of reducing equivalents, which could be regenerated by increased production of succinate. According to these results, for both substrates almost the same amount of 2,3-butanediol (13.32 mM) was produced.

Substrate consumption and product formation for the mixed culture (1:1) of *Enterobacter* spH1 and *E.coli* CECT432 has been fitted (dotting line in Figure 6.6 (a) & (b)) using modified Gompertz equations (Equation (1) and Equation (2)). These Figure 6.6(a) & (b) show the typical curves of product formation and substrate consumption. Tables 6.4 and 6.5 show the kinetic parameters determined from these modeling such as: the maximum production potential ($P_{max,i}$) and substrate consumption (S_{max}) ,maximum rate of production ($R_{max,i}$)and the lag time phase (λ_s) and with the their respective R^2. Additionally these tables show the maximum molar yields ($Y_{Pmax,i}$), maximum specific production or consumption ($q_{max,\ i}$) and maximum glycerol conversion (%). It can be seen from these Tables 6.4 and 6.5 that the overall magnitude of the regression coefficients (R^2) were higher than 0.993, except for acetic acid. This indicates a good correlation between the experimental data and the model. The maximum rates of H_2 production ($R_{max,\ H2}$) (Table 6.4 and 6.5) were 8.5 and 15.8 mmol/L*h and the corresponding yields (Y_{H2}) were 0.87 and 1.53 mol H_2 / mol glycerol consumed for PG and CG respectively. The $R_{max,\ EtOH}$ were 4.0 and 10.5 mmol/L*h with a corresponding (Y_{EtOH}) of 0.88 and 1.21 mol ethanol / mol glycerol consumed for PG and CG respectively. The higher yield above the theoretical values of ethanol and the H_2 for the CG suggested the contribution of the unknown carbon sources or electron sources present in the CG. The maximum rate production of H_2 was less than the value of Ito et al. [60] (30 mmol/L*h). This difference could be due to the type of medium used (synthetic and MYG). The same authors have reported that addition of a porous ceramic material supports to fix cells in the reactor, increased the H_2 production rate to 63 mmol /L*h with a corresponding ethanol yield of 0.85 mol/mol-glycerol. This indicates that the ethanol yield of 1.21 mol/mol-CG obtained in our work is much higher.

It has been also reported by Ito et al. [60] that the yield of H_2 and ethanol decreases with an increase in the concentrations of biodiesel waste and commercially available glycerol. Moreover, they pointed out that due to a high salt content in biodiesel waste, the rates of H_2 and ethanol production were much lower than those found at the same concentration of pure glycerol. However, in this study the 5% (w/w) CG, which contains 279 mM of glycerol, did not inhibit the growth and H_2 production. Rather it was shown that the H_2 cumulative production of 281 mmol/L on CG is higher than that on PG, which contains only 220 mM of glycerol (Figures 6 6(a) & (b)). The two main reasons for the presence of inhibition by CG as suggested by Ito et al. [60], were the high amount of salt (ash 8% (w/w)) and the high amount of methanol (25 % (w/w)) present in CG from biodiesel waste. Since our biodiesel contained a very low the amount of methanol, of about 1% (w/w). In this study, however, about 0.05% (w/w) of methanol was contained whereas in the study by Ito et al.'s [60] study it was it about 1.5% (w/w)and they have observed some inhibition. Therefore, the lower amount present in study could be one of the reason we do not see any inhibition on utilizing the 5 % (w/w) CG over the PG.

The other interesting fact we observed is the total amount of the salt that the medium contains. In this study the medium MYG contained only 10 g/L NaCl while the medium of Ito et al. [56] was a synthetic medium with 7 g/L K_2HPO_4 and 5.5 g/L $KHPO_4$. It can be concluded that in their study the amount of K was initially high in the medium. In their study the ash contained was about 0.2 to 0.5 % (w/w) whereas in this study we used 0.25 % (w/w) (see materials and methods). On average the salt amount present in the ash was similar in the two studies. If the resulting ash is considered to be mostly salt and the type depending on the transesterification of alkali addition in the biodiesel processes, mostly either Na or K salt. In this study it is confirmed from the XRD and SEM analysis (Table 6.3 and Figure 6.3) that the salt we have in the ash is mostly K_2SO_4 and $Na_2\ Mg(SO_4)_2(H_2O)_4$. Therefore, the total K_2SO_4 in this study is about 2.5g/L which is much less than the 7 g/L K_2HPO_4 and 5.5 g/L $KHPO_4$ present in the synthetic medium used in Ito et al.'s [60] study. Henceforth the total amount salt present in this study due to NaCl in the MYG medium and the CG, is not likely to inhibit the H_2 production.



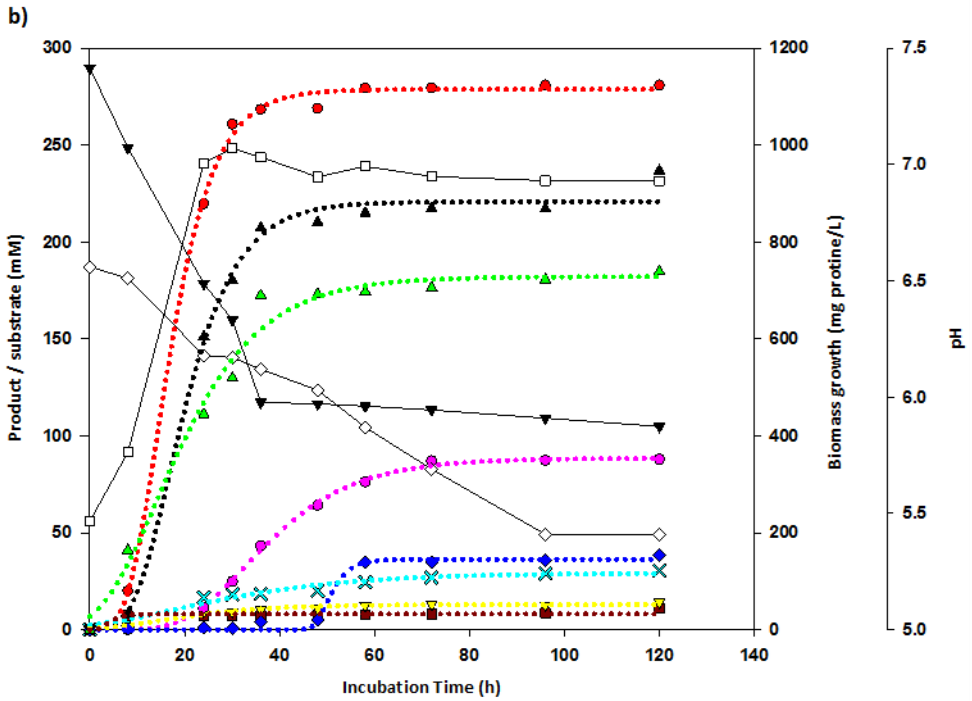


Figure 6.6. Fermentation profiles for a mixed culture (1:1) of *Enterobacter* spH1 and *E. coli* CECT432 on of (a) pure glycerol and (b) crude glycerol from biodiesel waste. Residual glycerol (—▼—), glycerol consumed (Δ), lactate (×) acetate (■), 2,3-BDO (▽), ethanol (▲), propionate (◆), succinate(☆), H_2 (●), CO_2 (●), pH(—◇—) and mg protein (—□—). For glycerol consumed, lactate, 2,3-BDO, ethanol, propionate, acetate, succinate, H_2 and CO_2 data was fitted using the modified Gompertz equation (Eq. (1) and Eq. (2)) (dotted lines).

Table 6.4. Kinetics parameters for mixed culture using pure and b) crude glycerol

Substrate	Maximal consumption ($S_{max,i}$) and production ($P_{max,i}$)* (mmol/L)								Dry cell mass (g/L)	Max. glycerol conversion (%)
	S_{max}	$P_{max,EtOH}$	$P_{max,Lact}$	$P_{max,Act}$	$P_{max,Suc}$	$P_{max,2,3BDO}$	$P_{max,CO2}$	$P_{max,H2}$	DCM_{max}	
Pure Glycerol	192.6	169.2	27.8	13.0	23.6	13.5	64.5	168.3	1.5	85.5

Maximal consumption ($R_{max,i}$) and production ($R_{max,i}$)* rate (mmol/L/h)							
$R_{max,S}$	$R_{max,EtOH}$	$R_{max,Lact}$	R_{maxAct}	$R_{max,Suc}$	$R_{max,2,3BDO}$	$R_{max,CO2}$	$R_{max,H2}$
2.3	4.0	1.2	0.2	0.5	0.4	1.7	8.5

Consumption (R^2_{Si}) and production correlation cofficient (R^2_{Pi})							
R^2_S	R^2_{EtOH}	R^2_{Lact}	R^2_{Act}	R^2_{Suc}	$R^2_{2,3BDO}$	R^2_{CO2}	R^2_{H2}
0.9611	0.9726	0.9729	0.8921	0.9866	0.9706	0.9948	0.9933

Consumption (λ_{Si}) and production Lag time phase(λ_{Pi}) (h)							
λ_S	λ_{EtOH}	λ_{Lact}	λ_{Act}	λ_{Suc}	$\lambda_{2,3BDO}$	λ_{CO2}	λ_{H2}
3.7	0.2	2.3	6.6	3.2	2.4	18.3	8.4

Maximal specific (qmax,i) and production (qmax, i)* rate (mmol/gDCW*h)							
$q_{max,S}$	$q_{max,EtOH}$	$q_{max,Lact}$	q_{maxAct}	$q_{max,Suc}$	$q_{max,2,3BDO}$	$q_{max,CO2}$	$q_{max,H2}$
1.5	2.7	0.8	0.1	0.3	0.2	1.1	5.6

Molar yields (mol/mol)							Biomass yield $gDCW_{max}$/mol
Y_{EtoH}	Y_{Lact}	Y_{Act}	Y_{suc}	$Y_{2,3BDO}$	Y_{CO2}	Y_{H2}	Yxs_{max}
0.88	0.14	0.07	0.12	0.07	0.33	0.87	7.9

Table 6.5 Kinetics parameters for mixed culture using crude glycerol

Substrate	Maximal consumption ($S_{max,i}$) and production ($P_{max,i}$)* (mmol/L)								Dry cell weight (g/L)	Max. glycerol conversion (%)
	S_{max}	$P_{max,EtOH}$	$P_{max,Lact}$	$P_{max,Act}$	$P_{max,Pro}$	$P_{max,2,3BDO}$	$P_{max,CO2}$	$P_{max,H2}$	DCM_{max}	
Crude Glycerol	182.1	220.8	29.3	8.1	36.3	13.0	88.5	278.7	1.7	62.9

Maximal consumption ($R_{max,i}$) and production ($R_{max,i}$)* rate (mmol/L/h)							
$R_{max,S}$	$R_{max,EtOH}$	$R_{max,Lact}$	R_{maxAct}	$R_{max,Pro}$	$R_{max,2,3BDO}$	$R_{max,CO2}$	$R_{max,H2}$
5.6	10.5	0.5	1.6	5.1	0.4	2.6	15.8

Consumption (R^2_{Si}) and production correlation cofficient (R^2_{Pi})							
R^2_S	R^2_{EtOH}	R^2_{Lact}	R^2_{Act}	R^2_{Act}	$R^2_{2,3BDO}$	R^2_{CO2}	R^2_{H2}
0.9851	0.9945	0.9581	0.8523	0.9905	0.9727	0.9987	0.9989

Consumption (λ_{Si}) and production Lag time phase(λ_{Pi}) (h)							
λ_S	λ_{EtOH}	λ_{Lact}	λ_{Act}	λ_{Pro}	$\lambda_{2,3BDO}$	λ_{CO2}	λ_{H2}
2.2	9.1	1.6	1.8	47.2	2.2	20.1	7.8

Maximal specific (qmax,i) and production (qmax, i)* rate (mmol/gDCW*h)							
$q_{max,S}$	$q_{max,EtOH}$	$q_{max,Lact}$	q_{maxAct}	$q_{max,Suc}$	$q_{max,2,3BDO}$	$q_{max,CO2}$	$q_{max,H2}$
3.4	6.4	0.3	1.0	3.1	0.2	1.6	9.6

Molar yields (mol/mol)							Biomass yield $gDCW_{max}$/mol
Y_{EtoH}	Y_{Lact}	Y_{Act}	Y_{Pro}	$Y_{2,3BDO}$	Y_{CO2}	Y_{H2}	Yxs_{max}
1.21	0.16	0.04	0.20	0.07	0.49	1.53	9.1

6.4.11 Carbon balance and carbon distribution for CG and PG

It is important in any microbial fermentation process that the mass balances of substrates and end products are fitting; otherwise, some important microbial metabolite might be overlooked. Table 6.6 presents the balances of carbon (C-balance) and degree of reduction (ε-balance), using PG and CG as substrate. These were carried out using the experimental final concentrations of products (ethanol, lactate, acetate, succinate, 2,3- butanediol, 1,2-propanediol, carbon dioxide, hydrogen and biomass). The C-balance calculation was based on the input-output determined by analyses of all the measurable metabolites and the TOC analysis. The degree of reduction balance values were calculated based on equation (6.5). The increment in the biomass growth was included in the C-balance and ε-balance as well as the initial carbon contained in the yeast and malt extract (TOC measurements) used in the MYG medium. The validity of the present approach was confirmed by checking the carbon material balance and degree of reduction balance Table 6.6), for which carbon and the degree of reduction recoveries for PG and CG were around 97.5%, 103.9% and 102.7, 104.3, respectively.

The carbon recovery for CG was slightly more than 100% indicating that there might be some carbon sources other than glycerol that might be involved in the fermentation, whereas the carbon recovery of 97% for the PG was similar to the values reported earlier [39, 40]. The minimal deviation of C-balance from 100% also indicates that end product has been consistently analyzed by HPLC and TOC. As Table 6.6 indicates, the carbon distributions of the PG to the metabolites were: biomass (3.81%), ethanol (20.20%), lactate (4.97%), acetate (1.55%), 2,3-butanediol (3.23%), succinate (5.65%), 1,2-propanediol (0.05%), carbon dioxide (3.85%) and residual glycerol (5.03%).While the fractional carbon distributions in various metabolites of crude glycerol were (Table 6.6): biomass (4.15%), ethanol (26.36%), lactate (5.24%), acetate (0.96%), propionate (6.51%), succinate (0.63 %), 2,3-butanediol (3.1%), 1,2-propanediol (0.12%), carbon dioxide (5.28%), MONG and others (23.02%) and residual glycerol (19.27 %).

Table 6.6. Carbon and reduction degree balance for a PG and CG using mixed culture (1:1) *Enterobacter* spH1 and *E.coli* 432[a].

Substrate	**Pure Glycerol**				**Crude glycerol**			
Initial	**Concentration (Conc.) mmol/Liter**		**Total Carbon[b] mmol/Liter**	**Reduction degree[j] (mmol e⁻)**	**Concentration (Conc.) mmol/Liter**		**Total Carbon mmol/Liter**	**Reduction degree (mmol e⁻)**
Crude glycerol							1372.63	6405.61
Glycerol , S_0	220.65		661.96	3089.146667	289.7		869.1	4055.80
MONG and others							503.53	2349.81
Malt and yeast[c]			167.67	838.35			167.67	838.35
	mg protein/L	**dry weight g/L**	**Total Carbon mmol/Liter**	**Reduction degree (mmol e-)**	**mg protein/L**	**dry weight g/L**	**Total Carbon mmol/Liter**	**Reduction degree (mmol e-)**
Biomass[d]	66.85	0.11	5.01	21.64	224	0.37	16.8	72.58
End products	**Conc. mmol/Liter**	**Total Carbon mmol/Liter**	**Carbon Distribution %**	**Reduction degree (mmol e⁻)**	**Conc. mmol/Liter**	**Total Carbon mmol/Liter**	**Product Distribution %**	**Reduction degree (mmol e⁻)**
Ethanol (Pmax,EtOH)	169.22	338.44	20.2	2030.64	220.77		26.36	2649.30
Lactate (Pmax,Hlac)	27.78	83.33	4.97	333.32	29.28		5.24	351.36
Acetate (Pmax,HAct)	12.98	25.96	1.55	103.84	8.08		0.96	64.64
Propionate(Pmax,Hpro)					36.33		6.51	
2,3-butanediol (Pmax,2,3BDO)	13.54	54.16	3.23	297.88	12.97		3.1	285.40
Succinate	23.65	94.59	5.65	331.06	3.5		0.63	42.00
1,2-propanediol	0.3	0.91	0.05	4.85	0.66		0.12	10.56
Residual glycerol (S_0 -$S_{max,Gly}$)	28.07	84.2	5.03	392.93	107.59		19.27	1506.31
CO_2[f] (Pmax,CO2)	64.51	64.51	3.85		88.47		5.28	
H2 (Pmax,H2)	168.3			336.6	278.7			557.40
MONG and others[g]					340.81		23.02	1862.65
Biomass[d]	63.83	63.83	3.81	275.75	69.44		4.15	299.98
Total products		809.92		4106.88		1540.42		7629.60
Carbon[h] and degree reduction[i] recocovery (%)	97.04			103.99	102.68			104.28

[a]Calculated for a 1.2Lbioreactor working volume of 500 mL.bioreactor working volume of 500 mL. The reaction was started with 450mL of MYG (Malt 10g/L, Yeast 3g/L, and PG 20g/L or CG 5 % (v/v)) medium and 50mL of inoculum. Each value was calculated using modeling equation (1) and (2) and others are measured 72h and was taken an average of duplicate experiments and fitting value

[b]Total carbon was determined by multiplying the number of carbons for each compound by moles of each compound; units are based on moles of carbon per liter

[c] TOC (2011 ppm) measured for the MY (malt 10 g/L and Yeast 3 g/L)

[d]Dry weight cell mass was calculated taking into account that protein comprises 60 % of the cell content [38] and the carbon fraction composition of the dry cell mass was assumed of 54 % [39,40].

[e] Carbon distribution (%) calculated as d total carbon of each compound divided by the total product carbon multiplied by 100.

[f] CO2 in the liquid phase was ignored.

[g] MONG and other was calculated by subtracting the sum of calculated and measured end metabolites from the final measured reaction of TOC (TOC $_{Final}$=1382.5g/L)

[h] Carbon recovery (%) calculated as total product divided by total substrate carbon multiplied by 100

[i]Degree reduction recovery (%) calculated as total product divided by total substrate degree reduction multiplied by 100

6.4.12 *T. maritima* grown in Crude glycerol

Figure 6.7 shows *T. maritima* growth on crude curve. In our previous study [34] *T. maritima* had shown a growth on pure glycerol and we have proposed the path way. The metabolites showed using the crude glycerol are almost the same with the pure glycerol. The yield (3.21 mol H_2 /mol glycerol) and the rate (2.38 mmol/L*h) (Table 6.7) were higher than the pure glycerol.

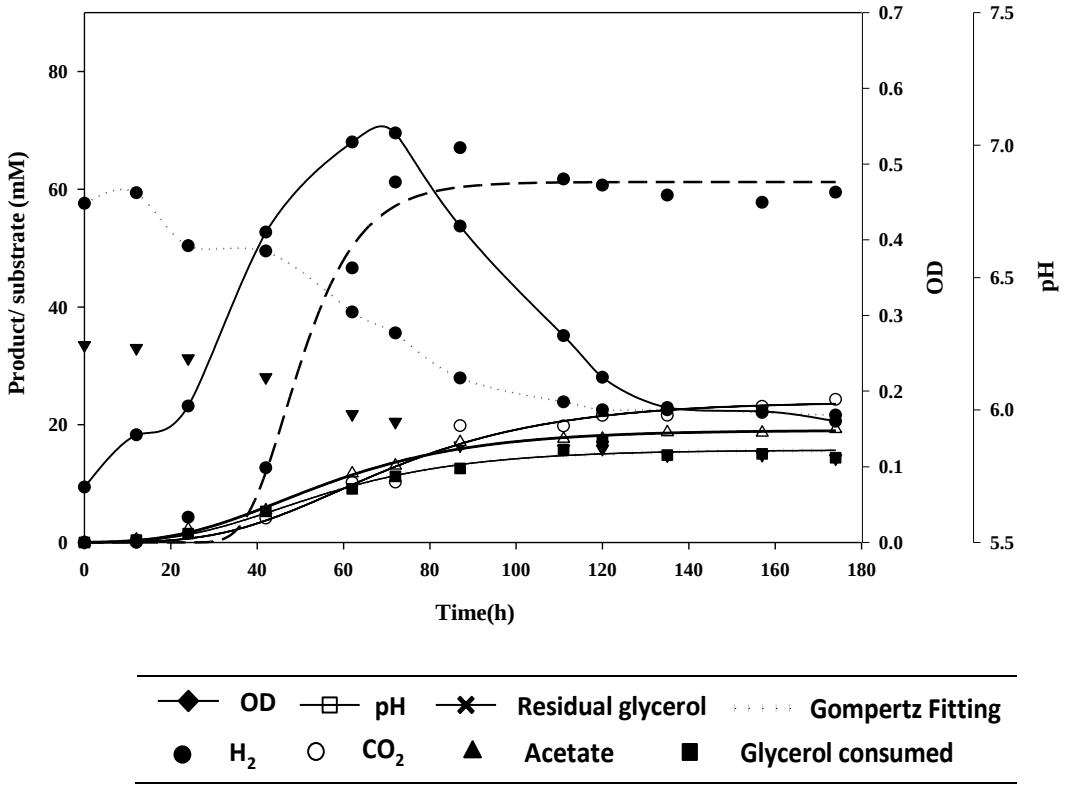


Figure 6.7. Fermentation profile of *T. maritima* using crude glycerol

Table 6.7. Carbon and reduction degree balance for *T. maritima* using crude glycerol

Substrate	Substrate	Maximal consumption ($S_{max,i}$) and production ($P_{max,i}$)* (mmol/L)				Dry cell mass (g/L)	Max. glycerol conversion (%)	Carbon balance (%)	Reduction balance (%)
		S_{max}	$P_{max,Act}$	$P_{max,CO2}$	$P_{max,H2}$	DCM_{max}			
T. maritima	**Crude Glycerol**	19.05	15.70	23.90	61.25	0.5	56.9	109.2	96.0

Initial Glycerol conc. S_0, Gly (mmol/L)
33.51

Maximal consumption ($R_{max,i}$) and production ($R_{max,i}$)* rate (mmol/L/h)			
$R_{max,S}$	R_{maxAct}	$R_{max,CO2}$	$R_{max,H2}$
0.24	0.26	0.32	2.38

Consumption (R^2_{Si}) and production correlation cofficient (R^2_{Pi})			
R^2_S	R^2_{Act}	R^2_{CO2}	R^2_{H2}
0.9956	0.9807	0.9784	0.9865

Consumption (λ_{Si}) and production Lag time phase(λ_{Pi}) (h)			
λ_S	λ_{Act}	λ_{CO2}	λ_{H2}
21.60	80.21	31.64	37.21

Maximal specific (qmax,i) and production (qmax, i)* rate (mmol/gDCW*h)			
$q_{max,S}$	q_{maxAct}	$q_{max,CO2}$	$q_{max,H2}$
0.5	0.6	0.7	5.2

Molar yields (mol/mol)			Biomass yield $gDCW_{max}$/mol
Y_{acet}	Y_{CO2}	Y_{H2}	Yxs_{max}
0.82	1.25	3.21	23.8

6.5 Conclusions

Crude glycerol diluted to 20x can be used as substrate for anaerobic fermentation by *Enterobacter* spH1 and *E. coli* CECT432, alone or as mixed culture.

A mixed culture of *Enterobacter* spH1 and *E. coli* CECT432 has a synergistic effect on the fermentation of PG and CG

Glycerol conversion by the mixed culture shows a mixed-acid type of fermentation, with ethanol, lactate, acetate, succinate, 2,3-butanediol, H_2 and CO_2 as main end products.

A highest H_2 yield (Y_{H2}) and ethanol yield (Y_{EtOH}) of 1.21 and 1.53 mol / mol glycerol was obtained from the crude glycerol, respectively. The yields achieved are the highest obtained using a mesophilic strains that has been reported to date. This depicts that the co-culture has a stronger synergetic effect to coexist. Further study has to be conducted to verify the mechanism of this synergistic effect.

Considering the kinetics of the study the best fitting were obtained using the modified Gompertz equation for both the product formation , gaseous product (R^2 > 0.9976) and liquid ferment (R^2> 0.9775 except for acetic acid ,R^2> 0.8575) and substrate consumption (R^2> 0.9875).

Thermotoga maritima strain DSM 3109 showed growth on crude glycerol (0.25 % v/v) at higher diluted. The yield observed was 3.21 mol H_2 /mol glycerol and the rate of H_2 was 2.38 mmol/L*h. These yield and rate were higher than the pure glycerol.

The optimization with higher amount of crude glycerol (> 5% v/v) has to be studied for the dark fermentation in the future, since it will reduce the cost due to the dilution process used in the medium.

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CHAPTER 7

7. HETEROGENEOUS CATALYSIS AND DARK FERMENTATION INTEGRATED SYSTEM FOR THE CONVERSION OF CELLULOSE INTO BIOHYDROGEN

Manuscript in preparation:

7.1 Abstract

A two-step integrated system consisting of heterogeneous catalysis followed by dark fermentation was investigated for the production of biohydrogen. Hydrolysis of cellulose in the aqueous phase was carried out in an autoclave reactor with ZrO_2 catalysts modulated by three different promoters: sulfate, fluoride, and phosphate. The resultant water-soluble fractions (WSFs) derived from the catalytic cellulose hydrolysis were then submitted to dark fermentation without any additional treatment. The dark fermentation step tested three different microorganisms, *Enterobacter* spH1, *Citrobacter freundii* H3 and *Ruminococcus albus* DMS 20455, for their ability to produce H_2 from cellulose and glucose and the liquid product derived from cellulose hydrolysis. The two enteric bacteria (*Citrobacter freundii* H3 and *Enterobacter* spH1) effectively fermented the WSFs, producing H_2 and other organic compounds as metabolites. For the WSFs derived from cellulose hydrolysis with ZrO_2-P and ZrO_2-S catalysts, *Enterobacter* spH1 exhibited values of 1.40 and 1.09 mol H_2/mol hexose, respectively.

Keywords*: hydrolysis, Zirconium catalyst, HMF, Enterobacter, Citrobacter, Ruminococcus, biohydrogen*

7.2 Introduction

Fossil fuels are currently the most widely used global energy source, contributing about 85% of the planet's total energy usage [1]. This exaggerated use is rapidly depleting the earth's petroleum reserves, leading to pollution and the energy crisis that human society is facing. The development of clean and sustainable alternative sources of energy is therefore a global priority.

Biomass is organic material which stores sunlight in the form of chemical energy. The rate of energy capture by photosynthesis in the Earth is approximately 100 terawatts per day [2], about six times the energy consumption of human civilization [3]. This makes organic biomass a clear source for renewable energy, maintaining a closed carbon cycle with no net increase in atmospheric CO2 levels. Biomass can also be transformed into the same or similar compounds as those derived from fossil fuels. The energy-carrying solids, liquids, and gases produced from biomass are called biofuels [6]. In order to be used for the production of biofuels, biomass is generally transformed into sugar monomers. Once sugar monomers are formed, they can be processed by micro-organisms [7].

H2 is a biofuel believed to have strong potential for use with future technologies. It has been reported that biological H2 can be produced from a wide spectrum of carbohydrates. Molecular hydrogen has the highest calorific value per unit mass, at 143 GJ/ton, among known gaseous fuels [8]. The maximum H2 yields obtained from these pure carbohydrates vary from 2.40 mol H2/mol hexose using cellulose [9, 10] to 3.33 mol H2/mol hexose from starch [11] and glucose [9], indicating that these carbohydrates are indeed suitable as feedstocks for dark fermentation.

Lignocellulosic biomass is a particularly low-impact source of carbohydrates to be used for the production of fuels, chemicals, power and heat, since, unlike other sources such as corn, its usage does not interfere with the food industry [12]. Lignocellulose is composed of cellulose, hemicellulose, and lignin. Cellulose and hemicellulose are two carbohydrate polymers tightly bound to lignin forming a recalcitrant matrix, making this material difficult to transform [12-13]. Cellulose is the most present component in lignocellulosic materials, and consists of a chain of glucose units bonded by □-1,4-glycosidic linkages protected by a tight packing of diverse strands via hydrogen bonds. This structure confers upon cellulose a highly recalcitrant nature, making it resistant to attack and deconstruction [14]. To break down lignocellulosic biomass, three steps are required: first, separation of the long-chain polysaccharides, cellulose, and hemicellulose; second, the hydrolysis of these polymers into their structural units of five- and six-carbon sugars; and finally the conversion of these sugars into biofuels or other value-added compounds. Commercial applications of these steps are still in early development and need to be improved upon to be efficient enough to become economically viable [15].

The most often-utilized method is hydrolysis with mineral acids, but this process carries problems, such as the generation of acid wastes as well as corrosion of equipment, which make this

technology difficult to manage [16]. Hydrothermal liquefaction, catalytic and physical treatments, enzymatic digestion, and bacterial hydrolysis/fermentation have all been proposed as new processes for biomass transformation [17]. Enzymatic digestion is an approach that merits interest, but it still requires new developments to economize on the production of enzymes, which make this technology too expensive for the time being [14]. Heterogeneous catalysis has been demonstrated to be one of the most effective methods [15, 17-21]. Sulfated zirconium dioxide (ZrO2) has been successfully applied to catalyze hydrolysis reactions over cellulose [22, 23]. Onda et al. [22] have shown highly selective hydrolysis of cellulose into glucose under hydrothermal conditions at 423 K in the presence of sulfonated active carbon (AC-SO3H).

In this study, two methods are integrated into an integrated system using heterogeneous catalysis and batch dark fermentation. This integrated system is designed to provide a new route to convert cellulose into biohydrogen in a light-independent process.

The first step of the integrated systems is catalytic hydrolysis of cellulose using an acid catalyst (ZrO2) to break down the complex structure of the cellulose, producing easily fermentable sugars; the so-called “water-soluble fraction” (WSF). The second step of the system is dark fermentation of the WSF, without any additional pretreatment. The WSF is mainly composed of sugars and other organic compounds derived from the cellulose hydrolysis, including furfural, hydroxymethylfurfural (HMF), and acetic acid. However, these compounds can have inhibitory effects on the growth and metabolic ability of microorganisms [24-28]. Consequently, it is necessary to remove or neutralize these compounds from the WSF before fermentation. This step increases the cost of the process. An important issue in the present work is therefore direct fermentation using the WSF resultant from the hydrolysis step without any further treatment, by employing microbes with the ability to survive and metabolize under these conditions. Here, two enteric bacteria, Enterobacter spH1 and Citrobacter freundii H3, were studied. These strains had previously been isolated and shown to effectively convert glucose and glycerol into H2 and other value-added products such as ethanol and 1,3-propanediol [29]. In addition to these strains, a cellulolytic bacteria, Ruminococcus albus DSM 20455, was used to hydrolyze the cellulose and for the fermentation of the remaining WSF. In continuous culture, it can yield 2.4 mol H2/mol glucose and is known for cellulosic degradation [30].

7.3 Materials and Methods

7.3.1 Preparation of the supports and catalysts

Zirconium dioxide (ZrO2) (commercial sample from Degussa) was prepared for use as a catalyst for cellulose hydrolysis with three different promoters: sulfate, phosphate, and fluoride. Calcined ZrO2 at 673 K was impregnated with 5% (w/w) of aqueous solutions of H2SO4, H3PO4, and HF,

respectively. The resultant solids were dried at 373 K for 12 h and calcined at 673 K for 4 h in a muffle. The catalysts obtained were then labeled as ZrO2-S, ZrO2-P and ZrO2-F, respectively.

7.3.2 Textural and structural characterization of ZrO_2

Textural properties were obtained by N_2 adsorption-desorption isotherms at 77 K using a Micromeritics ASAP 2000 equipment. Before analysis, all the samples were degassed in a vacuum chamber at 393 K for 12 h.

X-ray diffraction (XRD) was recorded using a Siemens D5000 diffractometer (Bragg Bentano for focusing geometry and vertical θ-θ goniometer) with an angular 2θ -diffraction range from 9.5 to 70°. The samples were dispersed on a Si (510) sample container with a cavity of 0.1 mm depth. The cavity was filled with the same amount of sample to ensure the same baseline for all analysis and sample packaging. The diffraction data were collected with an angular step of 0.03° at 5 s per step and sample rotation. Cu Kα radiation (λ = 1.54056 Å) was obtained from a Cu X-ray tube operated at 40 kV and 30 mA. The crystalline phases were identified using the ICDD files (International Centre for Diffraction Data, release 2007). The crystallinity index (CrI) of cellulose was calculated according to the modified Segal's method [31] (Eq. 7.1):

$$CrI = \left[\left(I_{cel} - I_{am}\right)\right] / I_{cel} \qquad \textbf{Equation (7.1)}$$

Where Icel corresponds to the sum of intensities of peaks from cellulose that appear in the range 10-27° 2θ and Iam is the intensity of the amorphous peak (18° 2θ). It must be noted that this CrI refers only to a ratio between diffracted intensities and does not refer to a mass ratio.

All diffractograms were fitted with the TOPAS software (TOPAS, 2009). This software uses the Rietveld method [32] and the Fundamental Parameters Approach [33], which consists in calculating the instrumental contribution to the peak width by describing the different components of the diffractometer.

The crystallite-size contribution to the peak width (τ) was calculated by fitting a Lorentzian and Gaussian function (double-Voigt approach) and applying the modified Scherrer equation Eq. (7.2) [34] from the peak width:

$$\tau = \frac{\lambda}{\beta \sin\theta} \qquad \textbf{Equation (7.2)}$$

Where β is the mean integral breadth and λ is the wavelength used.

The background was considered as a straight line with constant slope. The amorphous part of the sample was assigned to a pseudo-Voigt peak at 2θ = 18° with refinable peak width. The cell parameters for each phase present were refined for each sample. From the same fitting, the relative weight fraction of each crystalline phase, Wi, was calculated from the equation Eq. (7.3) [35].

$$W_i = \frac{S_i \rho_i V_i^2}{\sum_{j=1}^{n} S_j \rho_j V_j^2} \qquad \textbf{Equation (7.3)}$$

Where ρi is the crystal density, Vi is the unit cell volume and Si is the refinable scale factor of the crystal structure for phase i. The previous equation is applicable when all phases considered in the sample are crystalline. As in the present case this is not true, we must consider Wi as a relative rather than an absolute weight fraction.

7.3.3 NH_3-Temperature Programmed Desorption (TPD)

The acid properties of the materials were characterized by NH3-TPD using a ThermoFinnigan (TPRDO 110) apparatus equipped with a programmable temperature furnace and a TCD detector, calibrated using pulses of NH3 of differing concentrations. For each sample, 20 mg of catalyst was placed between plugs of quartz wool in a quartz reactor. The sample was first purged using pure He (flowing at 20 cm3/min) at 393 K for 30 min. After the temperature was cooled to room temperature, the sample was treated with an NH3/He (3/97, v/v %) mixture flowing at 20 cm3/min. The temperature was raised to 353 K at a rate of 20 K/min and then held at 353K for 60 min. The weakly adsorbed NH3 was then purged using He at 353 K for 30 min. The NH3 desorption was then started by heating the sample from 300 to 1123 K at 10 K/min in He flow (20 cm3/min). Magnesium perchlorate was used as a trap for water. The number of acid sites was calculated using the integral of the desorption of NH3.

7.3.4 Characterization of the cellulose

The surface morphology of the cellulose, before and after the catalytic treatment, was analyzed with a scanning electron microscope (SEM-JEOL JSM-35C), operated at an acceleration voltage of 15 kV. A small portion of each sample powder was coated onto a metallic disk holder and covered with a thin layer of gold to facilitate SEM analysis.

7.3.5 Hydrolysis of cellulose

The bulk cellulose hydrolysis (molecular biology, ≥99 %) was performed in an autoclave reactor (Parker Autoclave Engineers, 100 ml) under anaerobic conditions, using 0.2 g of catalyst at 453 K and 30 bar for 15 h. The solution was continuously stirred at 400 rpm while the reactions were

carried out. Prior to the reactions, the autoclave was fed with 50 ml of water, 0.8 g of cellulose and 0.2 g of catalyst and then purged with Argon gas (Ar). The reactor was then heated to 453 K and pressurized to 30 bar with Ar.

7.3.6 Dark Fermentation

7.3.6.1 Fermentable substrates

Pure cellulose (molecular biology, ≥99 %) and glucose (D-glucose, anhydrous) were obtained from Sigma Chemical Co., Madrid, Spain. The WSFs derived from the hydrolysis reactions were used in dark fermentation without filtration or any further treatment.

7.3.6.2 Microorganisms and media

Three different strains, two of which are facultative anaerobic strains (*Enterobacter* spH1 and *Citrobacter freundii* H3), and one of which, *Ruminoccus albus* DSM 20455, is a strict anaerobe, were tested for their ability to produce H2 from a combination of cellulose and glucose and the liquid product derived from cellulose hydrolysis.

Enterobacter spH1 and *Citrobacter freundii* H3 were aerobically pre-cultured in a synthetic medium at 310 K in an incubator-shaker overnight at 200 rpm. The synthetic medium used contained, per liter: 7.0 g of K_2HPO_4, 5.5 g of KH_2PO_4, 1.0 g of $(NH_4)_2SO_4$, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.021 g of $CaCl_2 \cdot 2H_2O$, 0.12 g of $Na_2MoO_4 \cdot 2H_2O$, 2.0 mg of nicotinic acid, 0.172 mg of Na_2SeO_3, 0.02 mg of $NiCl_2$ and 10 ml of trace element solution containing 0.5 g of $MnCl_2 \cdot 4H_2O$, 0.1 g of H_3BO_4, 0.01 g of $AlK(SO_4)_2 \cdot H_2O$, 0.001 g of $CuCl_2 \cdot 2H_2O$ and 0.5 g of Na_2EDTA per liter. A complex medium was prepared by adding 0.5 g/l of yeast extract to the synthetic medium. To study the batch dark fermentation, the reaction medium was prepared by adding different carbon sources, at around 5g/L.

The *Ruminococcus albus* DSM20455 medium contained, in g per 920.0 mL of distilled water: 5.0 Tryptone, 2.0 Yeast extract, 3.0 Glucose, 2.0 Cellobiose, 40.0 Mineral solution 1, 40.0 Mineral solution 2 and 1.0 Resazurin. After bioiling and cooling under CO_2, the mixture was amended with 4.0 g Na_2CO_3, 1.0 mL Fatty acid mixture and 500.0 mg Cysteine-HCl $\times$ H_2O. It was adjusted to pH 7.0 and distributed under Ar. Mineral solution 1 contained 0.6 % (w/w) of K_2HPO_4. Mineral solution 2 contained, in % (w/w): 0.6 KH_2PO_4.2 $(NH_4)_2SO_4$, 1.2 NaCl, 0.25 $MgSO_4 \times 7$ H_2O, 0.16 $CaCl_2 \times 7$ H_2O. The Fatty acid mixture contained 10 mL Isobutyric acid, 10 mL Isovaleric acid, 10 mL 2-Methylbutyric acid in 70.0 mL distilled water. Inoculation was performed under strictly anaerobic conditions, using Ar to purge, and incubated at 310 K. The cells were harvested at the end of exponential growth phase and 10% (v/v) were used as inoculum for the main batch experiments.

7.3.6.3 Dark fermentation

H_2 production by dark fermentation was investigated in a batch system, using 100 mL bioreactors of serum bottles sealed with rubber butyl stoppers and aluminium caps with a working volume of 50 mL, and continuously agitated in a shaker at 200 rpm at a constant temperature of 310 K. Initially, an anaerobic atmosphere was created in each bottle by purging with 30 mL/min of Ar (99.99 %) for 15 min. Before inoculation all reactors were autoclaved (for 20 min, 393 K and 1.5 Kg/cm^2 of pressure). Each experiment was performed in duplicate for each of the three individual strain and each of the three different carbon sources. Synthetic and *Ruminococus albu*s (without glucose and cellulose) media were used. The carbon sources used in the experiments were: (i) a WSF generated from the catalytic hydrolysis of the cellulose; (ii) glucose as a positive control; (iii) cellulose as a positive control to create similar conditions to the product of the hydrolysis. The initial amount of carbon in the reference test batch of glucose was 166.67 C-mmol/L. An overnight culture (10% (v/v)) was used as inoculum and then batch fermentation was run for 120 h.

7.3.7 Analytical methods

The WSFs remaining in the autoclave reactor were filtered and analyzed in a TOC (Tekmar, Total Organic Carbon Analyzer) to determine the total concentration of soluble carbon, directly related to cellulose solubilization from the hydrolysis. The composition of glucose and other by-products of the liquid phase after both the hydrolysis and dark fermentation steps was analyzed with a High Performance Liquid Chromatograph (HPLC) (Agilent technologies 1100 series), equipped with an ICSep ICE-COREGEL 87H3 Column, serial no. 12525124, a diode-array (DAD), and refractive index (RID) detectors. A mobile phase of H_2SO_4 (2.2%) was employed at a constant flow of 0.6 ml/min, the temperature of the column was maintained at 323 K, and each sample was analyzed for 40 min. The chromatograms were developed using Chem station for LC 3D. The total soluble carbon was then compared to the initial carbon present in the cellulose to determine its solubilization, or its conversion into any soluble chemical. With this, the cellulose conversion capacity of each catalyst was calculated. Calculations were made analytically from TOC results as follows Eq. (7.4) and Eq. (7.5)

$$Reacted\ Cellulose = 100 \times \frac{mg\ C_{liquid\ phase}}{mg\ C_{initial\ cellulose}} \qquad \textbf{Equation (7.4)}$$

$$Glucose\ Selectivity = \frac{Carbon\ moles\ in\ glucose}{Reacted\ carbon\ moles\ from\ cellulose} \qquad \textbf{Equation (7.5)}$$

The composition of the gas was measured using a GC-14B gas chromatograph equipped with a thermal conductivity detector (TCD) and a 80/100 Porapak-Q column. Argon was used as the carrier gas at a flow of 30 mL/min. The hydrogen from the fermentation was calculated by comparison with standard pure gas. Hydrogen was measured using a tight syringe, after an

incubation of 96 h, in a gas chromatograph GC-14B. The operational temperatures of the GC for the injection port, oven, and detector were 423 K, 353 K, and 473 K, respectively. The chromatogram was developed and analyzed using the Turbochrome Navigator (version 4.1) software from Perkin Elmer.

A modified Gompertz equation Eq. (7.6) [36] was used to estimate the maximum H_2 production rates.

$$H(t) = H_{\max,H2}.\exp\{-\exp\left[\frac{R_{\max,H2}.e}{H_{\max,H2}}(\lambda_{H2}-t)+1\right]\} \qquad \textbf{Equation (7.6)}$$

Where $H_2(t)$ is the cumulative H_2 production (mmol/L), λ the lag-phase time (h), $H_{max,H2}$ the maximum H_2 production (mmol/L), $R_{max,H2}$ the maximum H_2 rate (mmol/L*h), t the incubation time (h), and e the exp(1) = 2.718. This equation was found to be suitable for modelling the progress of H_2 production experimental data [37].

7.4 Results and Discussion

7.4.1 Characterization of the ZrO_2 materials

The XRD results of both the non promoted ZrO_2 and the promoted ZrO_2 materials are shown in Fig. 7.1. The introduction of phosphate and sulfate species to the ZrO_2 did not induce any appreciable phase modification. XRD patterns of ZrO_2, ZrO_2-P and ZrO_2-S samples revealed the major phase to be tetragonal and the minor one to be monoclinic (Table 7.1), with the peaks at 2θ = 30.26°, 35.10°, 50.42°, and 60.04° attributed to the tetragonal phase of ZrO_2, corresponding to the planes (101), (110), (112) and (211) (ICDD 80-2155), respectively. With the fluoride-species impregnated ZrO_2-F, a shift from a tetragonal to monoclinic (Baddeleyite) structure (ICDD, 7-3430) was observed, presenting values of 4.78% and 95.22 %, respectively, for the two phases (Table 7.1). The degree of tetragonal to monoclinic ZrO_2 transformation can be seen to have been affected by the type of the promoter. The average crystallite size of all ZrO_2 materials ranged from 6.2 to 10.48 nm (Table 7.1).

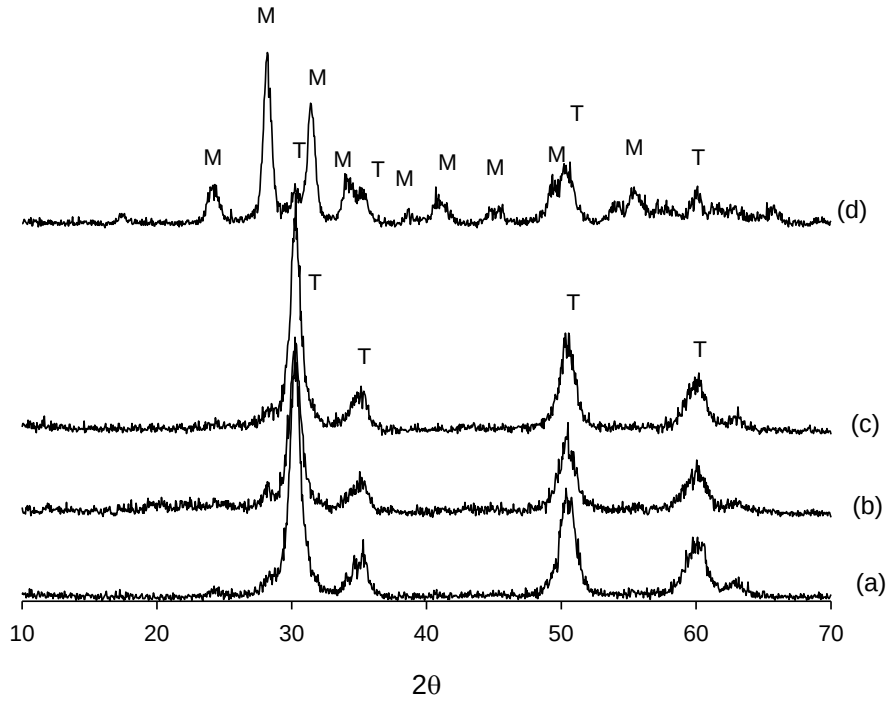
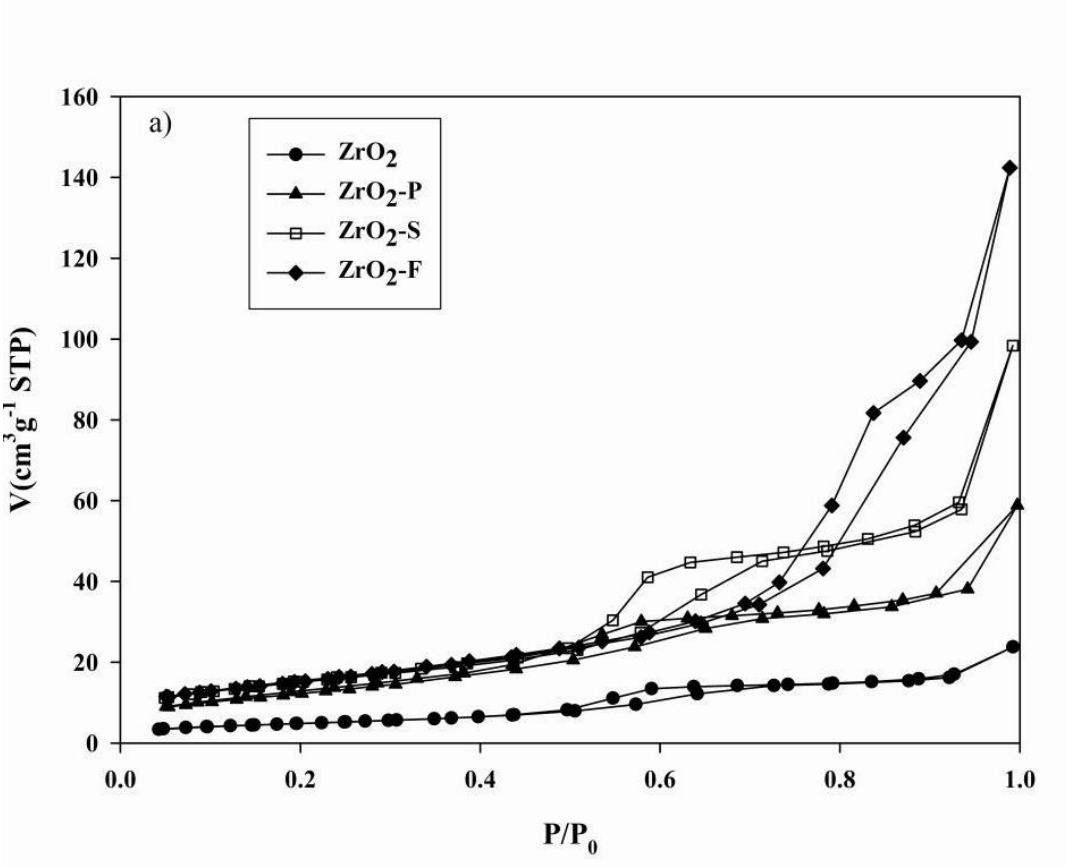


Figure 7.1. Powder XRD patterns of ZrO_2 materials employed for the cellulose hydrolysis. (a) ZrO_2, (b) ZrO_2-P, (c) ZrO_2-S and (e) ZrO_2-F. T= tetragonal phase; M = monoclinic phase.

Table 7.1 lists textural parameters calculated from the adsorption-desorption isotherm of nitrogen for each sample. The promotion of ZrO_2 by impregnation of phosphate and sulfate species resulted in a decrease of its surface area and pore volume. In the case of ZrO_2 promoted by the fluoride species, an increase in porous volume was observed, possibly attributable to the phase transition from tetragonal to monoclinic. The N_2 adsorption-desorption isotherms of the ZrO_2 materials are shown in Fig. 7.2a. The pure ZrO_2 sample presented typical type-IV isotherms according to the Brunauer-Deming-Deming-Teller (BDDT) classification, common for mesoporous materials. The same type-IV isotherms were observed for the ZrO_2-S and ZrO_2-P samples. All isotherms of these two samples at low equilibrium pressures are reversible, whereas at higher equilibrium pressures they exhibit a hysteresis loop of the H_2 type [38]. This type of hysteresis loop indicates the presence of tubular or ink-bottle pores. Conversely, the ZrO_2-F sample has a hysteresis loop of type H3, consisting of aggregates of plate-like particles [38]. This sample possesses a comparatively broader pore-size distribution. The presence of fluoride species leads to the collapse of the porous structure of the ZrO_2, generating less pore structure and a broader size distribution, as shown in Fig. 7.2b.

The surface acidity was calculated from total acidity and expressed per μmol of NH_3 desorbed per gram of sample as shown in Table 7.1. The total amount of acid was estimated from the peak area of TPD plots in association with calibration data. The amount of acid present in each sample followed the hierarchical order of ZrO_2-S > ZrO_2-P> ZrO_2 > ZrO_2-F, with the ZrO_2-P and ZrO_2-S samples exhibiting the highest total acidity with values of 15.9 and 19.3 of μmol of NH_3 desorbed per gram of catalyst, respectively. It is worth noting that the acid sites are correlated to the Zr^{+4} cation and that the acid strength is enhanced due to several factors such as the induction effect of the promoter species S=O in the sulfate species as well as the valence, the electronegativity and coordination number of Zr^{+4} cation [39]. The electronegativity of the ions used to promote ZrO_2 proportionally affected the acid capacity of the resulting catalysts.



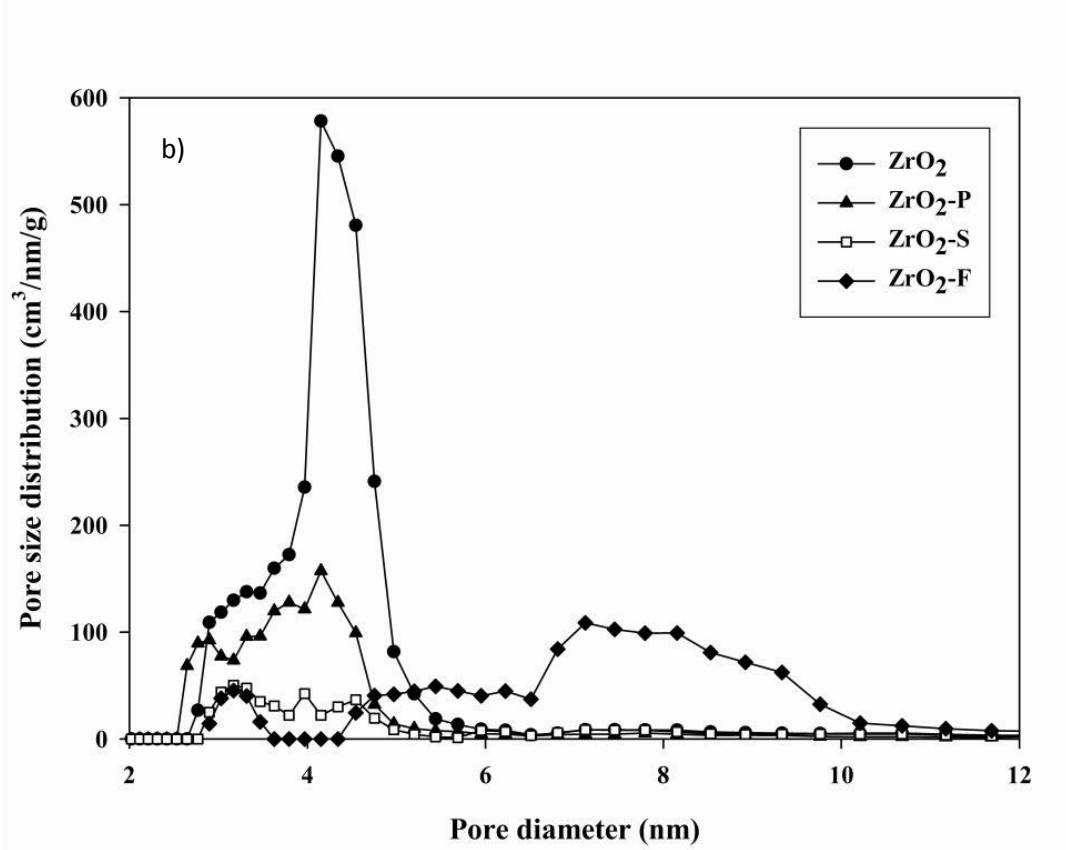


Figure 7.2. N_2 adsorption-desorption; (a) isotherms, and (b) the corresponding pore size distributions of the ZrO_2 materials.

HPLC analysis of the hydrolysis products revealed compounds such as cellobiose glucose, HMF and ethanol as shown in Fig. 7.3. In addition, 2-furaldehyde (2-FA), lactic acid (LA), acetic acid, oxalic acid, glycolic acid, formic acid, pyruvic acid, malic acid, maleic acid, were also identified. Unknown compounds were also present. Generally, it is assumed that molecular hydroxyacids lower than the parent sugar are formed by hydrolytic β-dicarbonyl cleavage without intermediation of the oxidizing agent via oxidative α-dicarbonyl cleavage induced by the oxidizing species [40]. The carbon balance based on TOC analysis of the liquid products showed negligible gas-phase production. The results of cellulose conversion and selectivity towards glucose over the different catalysts are summarized in the same figure.

The cellulose conversion was enhanced in all promoted ZrO_2 materials relative to the pure ZrO_2. Indeed, the pure ZrO_2 showed the lowest cellulose conversion, with a value of only 9 %. The highest selectivity to glucose (14.6%) was observed for the ZrO_2-P sample, with cellulose conversion of 13 %. The ZrO_2-P sample also displayed the highest selectivity to HMF (26.9%), which may stem from glucose dehydration [14]. On the other hand, ZrO_2-S material exhibited the highest percentage of cellulose conversion (22%) and lowest selectivity values of glucose and

HMF, at 8.9 % and 3.2 %, respectively. In this case, the selectivity to other compounds was the highest. ZrO_2 promotion by sulfate species conferred higher acidity, which may deliver higher hydrolyzing capacity, leading to other byproducts as shown in Fig 7.3.

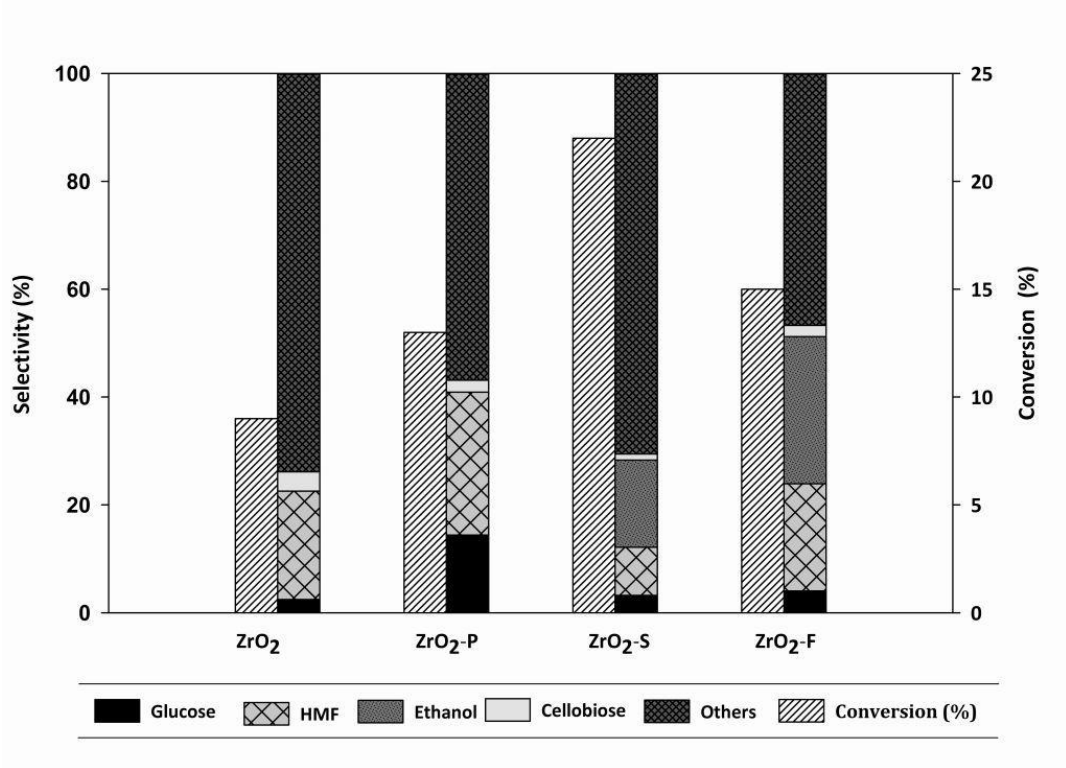


Figure 7.3. Cellulose conversion and products selectivity by the different ZrO_2 materials during the hydrolysis at 453 K and 30 bar.

Table 7.1. Parameters of textural properties and crystallite size of ZrO_2 materials

Sample	Surface area (m^2/g)	Pore diameter (nm)	Pore volume (cm^3/g)	Total acidity ($\mu molNH_3/g_{cat}$)	ZrO_2 Crystallite size (nm)		ZrO_2 (% Phase)	
					Tetragonal	Monoclinic	Tetragonal	Monoclinic
ZrO_2	113	4.15	0.161	6.89	7.18 (19)	8.6 (1.7)	88.32	11.68
ZrO_2-P	52	4.15	0.062	15.96	6.7 (3)	6.2 (1.4)	84.07	15.93
ZrO_2-S	59	4.53	0.095	19.34	7.38 (23)	5.94 (1.1)	84.78	15.22
ZrO_2-F	63	8.15	0.173	1.95	9.58 (1.75)	10.48 (27)	4.78	95.22

Table 7.2. X-ray diffraction of starting and hydrolyzed cellulose

Sample	Crystallinity (%)	Remaining cellulose together ZrO_2 catalysts after hydrolysis test		
		Cellulose (%)	ZrO_2 Phase (%)	
			Tetragonal	Monoclinic
Starting Cellulose	74.01	100	-	-
Cellulose after hydrolysis with ZrO_2	73.05	91.83	8.17	-
Cellulose after hydrolysis with ZrO_2-P catalyst	64.57	76.59	22.05	0.89
Cellulose after hydrolysis with ZrO_2-S catalyst	69.61	89.32	10.13	0.55
Cellulose after hydrolysis with ZrO_2-F catalyst	71.56	90.91	0.27	8.82

7.4.2 Cellulose characterization

The cellulose which remained after catalytic hydrolysis was characterized by scanning electron microscope (SEM) and X-ray diffraction (XRD) to investigate structural changes in its surface due to the hydrolytic property of the catalysts.

Characterization by SEM. The SEM image of the pure cellulose (Fig. 7.4a) exhibits a notably smooth surface. Figures 7.4b-e show images of cellulose after catalytic hydrolysis with ZrO_2 materials. The apparent shortening and cracking of the cellulose fibers is thought to have occurred due to fractionation of the cellulose during the hydrolysis. As the images show, the fibers of the cellulose surface samples have become markedly more exposed. This morphology change is marked by the intrinsically acidic character of the acid promoter.

Characterization by XRD. The XRD patterns of the starting cellulose and of the corresponding cellulose after catalytic hydrolysis using different ZrO_2 materials are depicted in Fig. 7.5. The diffractogram of the cellulose showed two peaks with 2θ range 21°-23°, corresponding to the crystallographic phase of cellulose, and broad peaks with 2θ range of 15°-19°. The cellulose polymorph that was present in all samples was identified as Cellulose-Iβ. The crystalline structure was taken from [41] (P21, a: 7.784(8) Å, b: 8.201(8) Å, c: 10.380(10) Å, γ: 96.5°). Near the peak thought to correspond to the "amorphous portion" at 2θ =18°, there is the reflection (111) of cellulose in such a way that if the crystalline structure is not considered, one could estimate more amorphous content than actually occurs. The two ZrO_2 structures identified were monoclinic (Baddeleyite) [42] (P21/c, a: 5.120Å, b: 5.216 Å, c: 5.281 Å, β: 99.01°) and ZrO_2 tetragonal [43] (P42/nmc, a: 3.5957 Å, c: 5.185 Å).

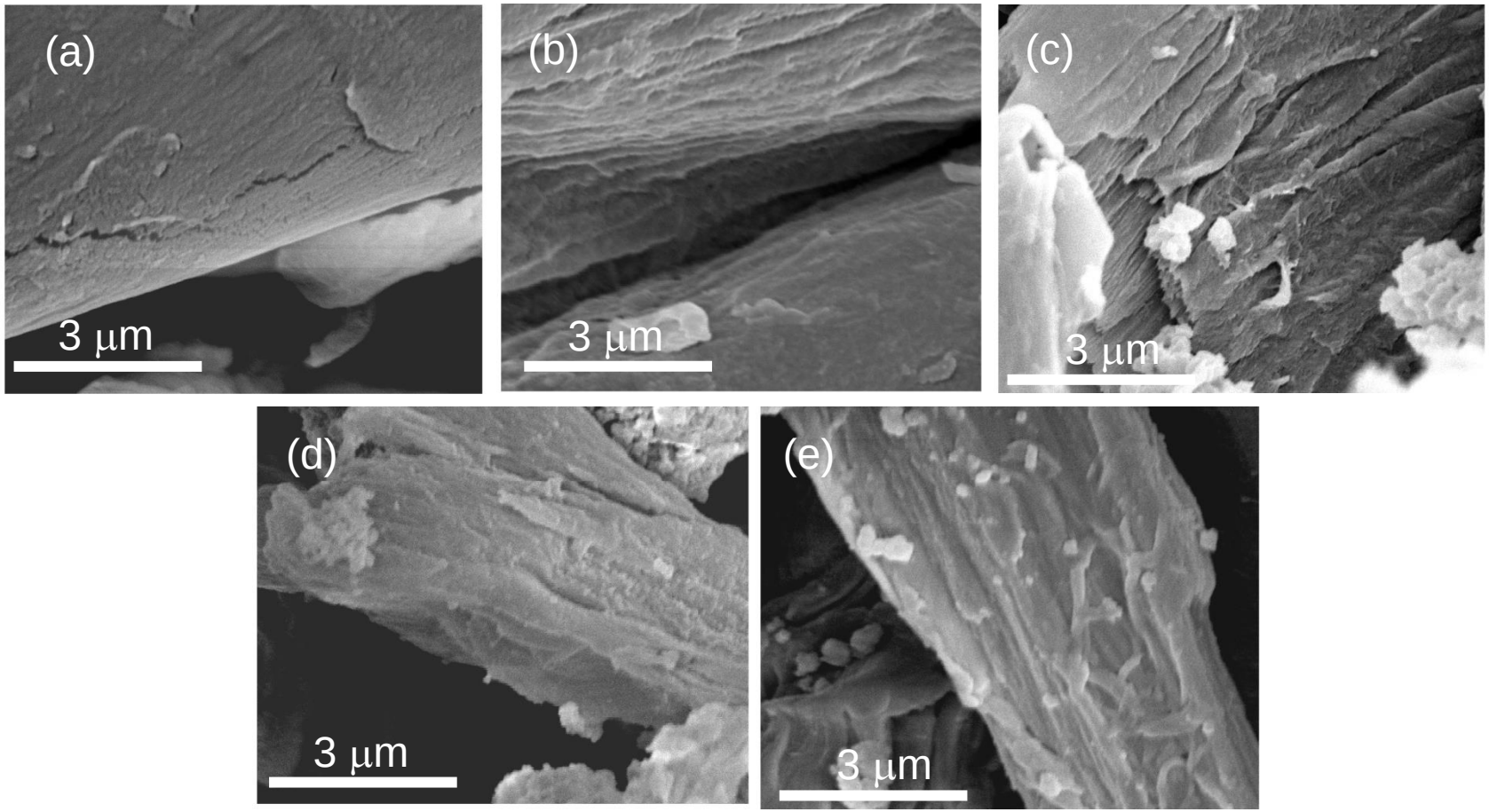


Figure 7.4. SEM images of the cellulose material: (a) starting cellulose; and cellulose surface after catalytic hydrolysis by ZrO_2 materials, (b) pure ZrO_2, (c) ZrO_2-P, (d) ZrO_2-S and. (e) ZrO_2-

The crystallinity of the cellulose after hydrolysis with different ZrO_2 materials is presented in Table 7.2. From this Table 7.2, the hydrolyzed cellulose had a lower crystalline index than the starting cellulose. The pure ZrO_2 had a slight effect on the decrystallization of the cellulose, but decrystallization was higher when the ZrO_2 included a promoter, as shown particularly for the ZrO_2-P and ZrO_2-S samples. Table 2 also exhibits information about the composition of the cellulose and ZrO_2 mixtures after hydrolysis.

This study rejected the possibility of estimating the amorphous content of each sample by adding an internal standard and applying the Rietveld method. In this case, it would have been difficult to find a suitable standard due to the differences between the calculated linear absorption coefficients for the phases involved [44] (25.24, 596.37 and 634.0 cm^{-1} for Cellulose-Iβ, monoclinic ZrO_2 Baddeleyite and tetragonal ZrO_2, respectively). The addition of an internal standard would add more uncertainty to the quantitative analysis by the Rietveld method. For all these reasons, the quantitative values presented must be taken as relative values and not as absolute ones.

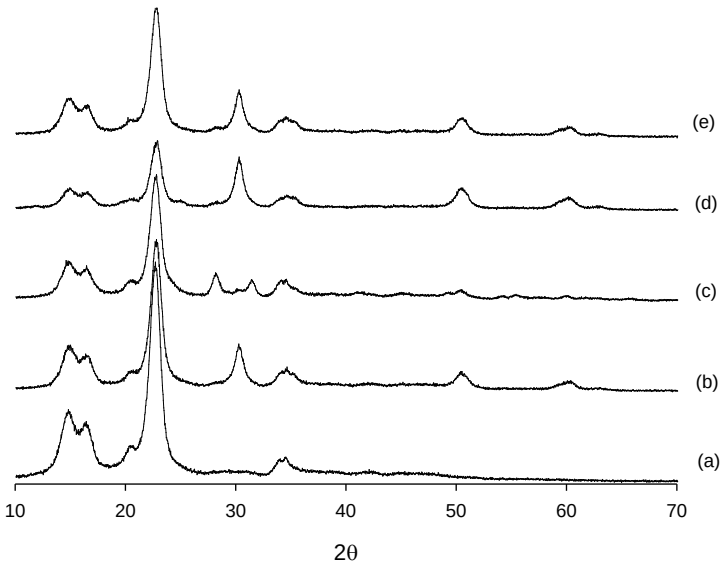


Figure 7.5. XRD patterns. (a) Cellulose material before the hydrolysis and after catalytic hydrolysis ZrO_2 materials, (b) pure ZrO_2, (c) ZrO_2-F, (d) ZrO_2-P and (e) ZrO_2-S.

7.4.3 Dark Fermentation of the water-soluble fractions (WSFs).

Higher monosaccharide yields, minimization of carbohydrates losses, and treatment for low levels of inhibitory substances are important issues for the feasible use of the WSF in dark fermentation [45]. Here, the cellulose hydrolysis pathway was established as the step for isolation of sugars monomers, such as cellobiose and glucose, for dark fermentation. The resultant WSF, without

filtration or any pretreatment was then used as the carbon source for batch dark fermentation by *Enterobacter* spH1, *C. freundii* H3 and *R. albus* DSM20455.

Analysis of the WSF revealed the presence of cellobiose, glucose, furfural, and HMF among other products obtained in the hydrolysis of cellulose with ZrO_2-P and ZrO_2-S catalysts are presented in Fig.7.3.

Glucose is the most easily fermentable compound by most microorganisms. Some by-products such as furfural, HMF, phenols, aromatic substances, and certain organic acids can also inhibit bacterial growth [24]. Previous works have shown that relatively low concentrations of furfural and HMF, between 1.0 to 2.0 g/L, can act as inhibitors [27]. In addition, other works have reported that furfural is inhibitory to microorganisms even at low concentrations, such as 1-12 mM [16]. On the other hand, Boopathy, et al. [28] employed a survey methodology using seventeen enteric bacterial strains and showed that furfural and, with lesser certainty, HMF can be reduced to their corresponding alcohols by enteric bacteria.

7.4.3.1 Biohydrogen production

The biohydrogen production, defined as cumulative H_2 production and yield, by all strains employed with the different substrates (WSF, glucose, cellulose and mixture of glucose and cellulose) is shown in Table 7.3. The H_2 yield is expressed in terms of moles of H_2 produced per mole of carbon (mol H_2/mol Carbon), determined by TOC and in terms of moles of H_2 produced per mole of hexose (mol H_2/mol Hexose).

The cumulative H_2 production for each strain using the WSF, derived from ZrO_2-P and ZrO_2-S samples, was lower than that obtained in the reference batch control with glucose as substrate. The lower cumulative H_2 production observed in the dark fermentation for each WSF from hydrolysis with ZrO_2-P (37.25 C-mmol/L) and ZrO_2-S (44.80 C-mmol/L) samples may be due to the initial carbon amount, which is lower than that of the reference batch test of glucose (166.67 C-mmol/L), as shown in Table 7.4.

Enterobacter spH1 presented the highest H_2 yield in the dark fermentation for both WSFs, derived from cellulose hydrolysis with ZrO_2-P and ZrO_2-S catalysts, showing values of 1.40 and 1.09 mol H_2/mol hexose, respectively (Table 7.3). These values are lower than the reference batch using glucose as substrate, which had a value of 1.60 mol H_2/mol hexose. This was also noted in the yield values expressed in terms of mol H_2/mol of carbon. In addition, the H_2 yield for *Citrobacter freundii* H3 is 1.19 and 0.99 mol H_2/mol hexose for both the WSF derived from the hydrolysis step with ZrO_2-P and ZrO_2-S catalysts, respectively. *R. albus* exhibited negligible H_2 yield in the dark fermentation of both WSFs. The enteric bacteria (*Enterobacter* and *Citrobacter*) presented higher

cumulative H_2 production than *R. albus*, likely due to their ability to adapt to the inhibitory environment found in the WSF [29].

Table 7.3. Hydrogen production and yield in the dark fermentations by the microorganisms with the different substrates

Strain	Liquid fraction						Controls					
	Catalyst											
	ZrO_2-P			ZrO_2-S			Glucose			Cellulose		
	C_{H2}	$Y_{H2/C\ mol}$	$Y_{H2/hexose}$	C_{H2}	$Y_{H2/C\ mol}$	$Y_{H2/hexose}$	C_{H2}	$Y_{H2/C\ mol}$	$Y_{H2/hexose}$	C_{H2}	$Y_{H2/C\ mol}$	$Y_{H2/hexose}$
C. freundii H3	7.42	0.20	1.19	7.38	0.16	0.99	35.42	0.21	1.28	1.42	0.01	0.04
E Enterobacter spH1	8.71	0.23	1.40	8.12	0.18	1.09	44.28	0.27	1.60	1.52	0.01	0.04
R. albus DSM20455	4.62	0.12	0.74	2.38	0.05	0.32	22.52	0.14	0.81	10.91	0.05	0.35

[a] C_{H2} denotes the cumulative hydrogen production mmol/L

[b] $Y_{H2//C\ mol}$ denotes hydrogen yield calculated as mmol of hydrogen per mmol of initial total carbon (mol H_2/mol of carbon).

[c] $Y_{H2/hexose}$ denotes hydrogen yield calculated as mmol of hydrogen per mmol of initial total hexose(mol H_2/mol Hexose).

The amount of cumulative H_2 production using glucose as substrate was in the following order: *Enterobacter* spH1 (44.28 mmol/L) > *C. freundii* H3 (35.42 mmol/L) > *R. albus* DSM 20455 (22.52 mmol/L) (Table 7.3). The higher yield in the reference batch containing glucose (*Entrobacter* spH1 ($Y_{H2/hexose}$ = 1.60) and *C. freundii* H3 ($Y_{H2/hexose}$ = 1.28)) compared with the WSF fermentation could be due the higher amount and purity of the substrate in the reference batch. The maximum theoretical production is 4 mol of H_2 per mol of glucose consumed [30]. However, thermodynamically this yield could not be achieved by mesophilic organisms. The maximum possible yield that could be achieved without any additional adjustments, such as lowering the partial pressure by purging inert gases, is about 2 mol H_2/mol of glucose [9]. Therefore, the yield obtained in this study via dark fermentation, especially for the *Enterobacter* spH1, is within the range of feasibility.

The batch control test for dark fermentation employing pure cellulose as substrate revealed negligible cumulative H_2 production for both of the enteric bacteria, neither of which are able to degrade the pure cellulose. The negligible cumulative H_2 amount (shown in Table 7.3) using pure cellulose as carbon source for the enteric bacteria is thought to actually be a byproduct of the synthetic medium, which contains yeast extract (0.5g/L). This was investigated and confirmed using a batch test without any carbon sources as a control (data not shown).

On the other hand, *R. albus* showed significant cumulative H_2 production, at 10.91 mmol/L (Table 7.3). This suggests that *R .albus* could be suitable for H_2 production using the remaining cellulose after the catalytic hydrolysis step as long as inhibitory compounds such as HMF are removed before the fermentation step.

Fig. 7.6 displays how the fermentative profile of the WSF looks like by selecting out *C. freundii* H3 as a representative of the three strains. Dark fermentation of the WSF derived from the ZrO_2-P sample (Fig. 7.6a), showed constant cumulative H_2 production after 50 h with a value of about 6.80 mmol/L with the formation of other metabolite products such as acetic acid (8.43 mM), lactic acid (2.21 mM), formic acid (0.4 mM), and propionic acid (3.72 mM). In addition, the cumulative H_2, produced in the dark fermentation of the WSF derived from the ZrO_2-S test (Fig. 7.6b), after 50 h was still increasing until the end of the incubation (120 h), forming major end metabolites such as acetic acid (9.03 mM), ethanol (2.11 mM), 2,3-butanediol (1.33 mM), and propionic acid (3.89 mM).

Two main conclusive points must be mentioned here: (i) The H_2 production from the WSF derived from cellulose hydrolysis was higher for the *Enterobacter* and *Citrobacter* strains. This may indicate that these microorganisms adapt better to the compounds present in this WSF; (ii) On the other hand, *R. albus* presented lower H_2 production. Based on this behavior, the *R. albus* strain seems to have been inhibited by the presence of compounds such as HMF in the WSF.

The H_2 production rate (mmol/L×h) of ZrO_2-P was modeled using equation 6 for each strain, which can also be seen in Table 7.4. The H_2 production rate was highest for *Enterobacter* spH1, at 0.36 mmol/L×h and *C. freundii* H3 at 0.32 mmol/L×h. The lowest production rate was observed for *R. albus*, at 0.06 mmol/L×h. The R^2 values of the linear regression were 0.977, 0.939, and 0.975 for *Enterobacter* spH1, *C. freundii* H3, and *R. albus,* respectively.

The H_2 production rate (mmol/L×h) of ZrO_2-S was also modeled using Equation 6 for each strain and can be seen in Table 7.4. The H_2 production rate was higher for *Enterobacter* spH1, at 0.19 mmol/L×h, and for *C. freundii* H3, at 0.17 mmol/L×h. The lowest observed rate was for *R. albus* at 0.02 mmol/L×h. The R^2 values for the linear regression were 0.945, 0.953, and 0.937 for *Enterobacter* spH1, *C. freundii* H3, and *R. albus*, respectively.

7.4.3.2 Degradation of HMF

The initial concentrations of HMF present in the WSF produced in the cellulose hydrolysis with ZrO_2-P and ZrO_2-S samples were 0.8 mmol/L and 0.4 mmol/L, respectively. Figs. 7.6a and 7.6b show that HMF was indeed degraded during dark fermentation, producing H_2 and usable chemicals using *Citrobacter freundii* H3 for the WSF derived from cellulose hydrolysis with ZrO_2-P and ZrO_2-S samples, respectively. Similar results were observed for *Enterobacter* spH1 (data not shown). These two enteric bacteria were not inhibited by the presence of HMF (0.8 mmol/L) in the WSF. It has been proposed that HMF can be fermented to corresponding alcohols by enteric bacteria [28]. However, it is not yet known which organisms can mineralize 5-HMF [28]. On the other hand, when furfural is present at sufficiently low concentrations (5-10 mM), it can be completely converted to acetic acid, as the sole carbon source, by sulfate-reducing bacteria. [26, 46, 47]

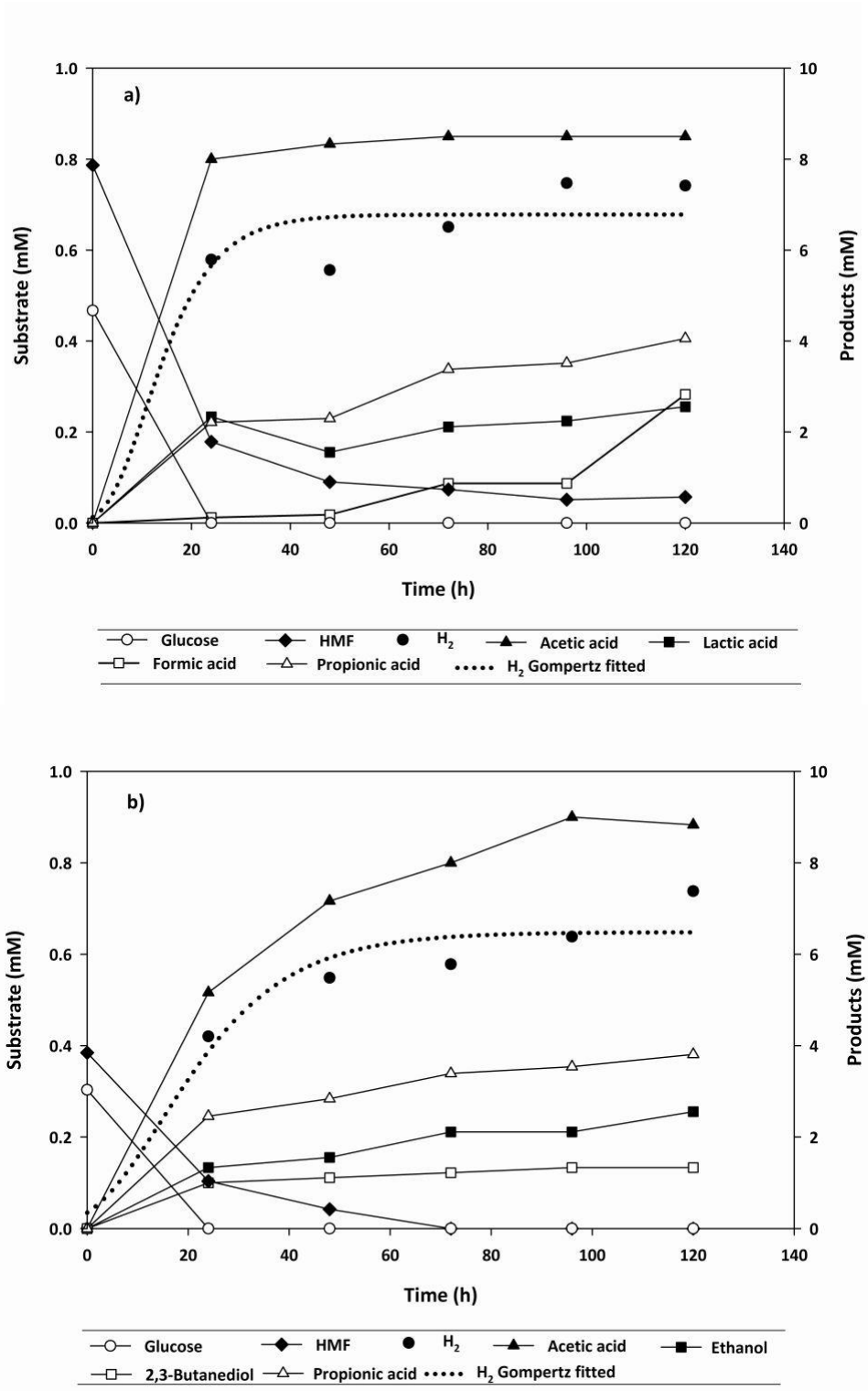


Figure 7.6. Dark fermentation profile using *Citrobacter freundii* H3 in the water soluble fraction resultant from the hydrolysis tests with a) ZrO_2-P and b) ZrO_2-S.

7.4.3.3 Liquid metabolites

Table 7.4 summarizes the end metabolites for each strain, maximum H_2 production rate ($R_{max,H2}$), incremental cell counts, and carbon recovery from the dark fermentation of the WSF. H_2 production and formation of other organic compounds such as acetic acid, lactic acid, propionic acid, succinic acid, formic acid, and 2,3 butanediol can also be observed. Intermediates of such metabolisms are used as electron acceptors resulting in branched pathways leading to other organic compounds [48]. This fact suggests that these enteric strains (*Enterobacter* spH1, *C. freundii* H3) perform a similar mixed acid fermentation, comparable to that of *Escherichia coli, Enterobacter aerogenes* and *Citrobacter amalonaticus* Y19, observed in previous reports [49 -52].

7.4.3.3.1 Metabolites derived from cellulose hydrolysis with ZrO_2-P sample

The initial amount of carbon in the medium measured by TOC was 37.25 mmol/L (Table 7.4). The composition of the WSF carbon source was composed of HMF 0.8 mM, glucose 0.32 mM, and others.

A larger quantity of acetic acid (11.67 mM) was found as the end metabolite of dark fermentation by *R. albus* of the WSF derived from the ZrO_2-P-catalyzed cellulose hydrolysis, as shown in Table 7.4. The CO_2 formation was observed to increase with an increase in acetic acid formation. In most cases of dark fermentation, higher production of H_2 is followed by higher acetic acid production since it does not consume NADH [48]. The *Enterobacter* spH1 strain produced the highest quantity of formed ethanol (5.22 mM). The *C. freundii* H3 strain produced the highest quantity of lactic acid (2.56 mM). No propionic acid was detected for *R. albus* however, it was observed for *C. freundii* H3 (4.05 mM) and for *Enterobacter* spH1 (2.91mM). No succinic acid was observed for any strains. There was increased formic acid formation, at 2.83 mM, using *C. freundii* and for *R. albus*, at 1.96 mM.

The growth of *C. freundii* H3 was the highest of the three strains, at 5.05E+09 cell/mL, followed by *Enterobacter* spH1, at 3.91E+09 cell /mL, as shown in Table 7.4. This growth can indicate that the enteric bacteria are more capable of adapting to the HMF medium than the *R. albus*. In addition, after incubation, the pH was almost constant across all strains. This could be due the buffering effect of the HEPPES in the medium and the lower quantity of the organic acids formed. The carbon recovery for *C. freundii* H3, *Enterobacter* spH1, and *R. albus* was measured to be 101.6 %, 102.6 %, and 85.6 %, respectively.

7.4.3.3.2 Liquid fraction derived from cellulose hydrolysis with ZrO_2-S sample

The initial amount of carbon in the medium measured by TOC was 44.80 mmol/L, as shown in Table 7.4. The carbon source of the WSF was composed of HMF 0.4 mM, glucose 0.32 mM and others.

The acetic acid formation using the WSF derived from the hydrolysis with ZrO_2-S sample was higher for *C. freundii* H3 at 7.17 mM than for either *Enterobacter* spH1, at 5.83 mM, or for *R. albus* at 4.75 mM. Ethanol formation was observed in dark fermentation with *Enterobacter* spH1, at 2.28 mM, and *C. freundii* H3 at 1.09 mM. Lactic acid formation was highest for *R. albus* at 5.22 mM, followed by *C. freundii* H3 at 3.0 mM, and then *Enterobacter* spH1 at 2.18 mM. Propionic acid formation also occurred in all the three strains, with the highest value observed for *Enterobacter* spH1 at 6.35 mM, followed by *R. albus* at 4.66 mM, and *C. freundii* H3 at 1.15 mM. A small amount of succinic acid (0.25mM) was observed only for *R. albus*. No formic acid or 2,3-Butanediol were observed for *R. albus*, however small amounts were detected for *Enterobacter* spH1, with values of 1.30 mM and 0.28 mM, respectively, and also for *C. freundii* H3, with values of 0.65mM and 1.11mM, respectively.

The presence of formic acid indicates incomplete transformation of cellulose to H_2 and CO_2. The gaseous CO_2 level was lower for *Enterobacter* spH1, with a value of just 1.93 mmol/L, possibly indicating higher propionic acid formation, a process which consumes CO_2.

With the sulfate-promoted ZrO_2 sample, microbial growth was highest for the *C. freundii* H3 strain, at 4.89E+09 cell /mL, followed by the *Enterobacter* spH1 and *R. albus* strains , at 3.35E+09 cell /mL and 2.63E+09 cell counts/mL, respectively. The pH remained essentially constant, as in the case of the ZrO_2-P WSF, for all strains used. The carbon recovery values for the strains *C. freundii* H3, *Enterobacter* spH1, and *R. albus* were 74.1 %, 93.7 %, and 88.2 %, respectively.

Table 7.4. End metabolites of dark fermentation with *Citrobacter freundii* H3, *Enterobacter* spH1 and *Ruminococcus albus* DSM 20455 strains using the water soluble fraction resultant from the hydrolysis tests with ZrO_2-P and ZrO_2-S.

	ZrO_2-P														
	mmol/L									Cell counts/L	C-mmol/L	Carbon Recovery %	pH	$R_{max\ H2}$	
Strains	H_2	Acetic acid	Lactic acid	Ethanol	2,3-Butanediol	Propionic acid	Succinic acid	Formic acid	CO_2		Initial TOC			mmol/L× h	R^2
C. freundii	7.42	8.50	2.56	0.00	0.00	4.05	0.00	2.83	2.53	5.05E+09	37.25	101.61	6.35	0.321	0.939
Enterobacter spH1	8.71	8.50	0.00	5.22	1.11	2.91	0.00	0.00	1.93	3.91E+09	37.25	102.56	6.37	0.361	0.977
R.albus	4.63	11.67	1.89	0.89	0.00	0.00	0.00	1.96	4.74	2.16E+09	37.25	85.65	6.38	0.059	0.975
	ZrO_2-S														
	mmol/L									Cell counts/L	C-mmol/L	Carbon Recovery %	pH	mmol/L* h	
Strains	H_2	Acetic acid	Lactic acid	Ethanol	2,3-Butanediol	Propionic acid	Succinic acid	Formic acid	CO_2		Initial TOC			$R_{max\ H2}$	R^2
C. freundii	7.38	7.17	3.00	1.09	1.11	1.15	0.00	0.65	2.43	4.89E+09	44.80	74.12	6.19	0.173	0.953
Enterobacter spH1	8.12	5.83	2.17	2.28	0.28	6.35	0.00	1.30	1.93	3.35E+09	44.80	93.70	6.19	0.189	0.945
R.albus DSM 20455	2.38	4.75	5.22	0.00	0.00	4.66	0.25	0.00	4.89	2.63E+09	44.80	88.17	6.77	0.024	0.937

* the initial carbon amount of the reference batch test of glucose is 166.67 C-mmol/L

7.5 Conclusions

A integrated system consisting of heterogeneous catalysis and dark fermentation to convert cellulose into biohydrogen was proposed. ZrO_2 catalysts were used to foment cellulose hydrolysis to its structural fraction sugar units. ZrO_2 was promoted by phosfate, sulfate, and fluoride species. The resultant WSF contained glucose, HMF and furfural among the products.

The WSF derived from the cellulose hydrolysis with ZrO_2-P andZrO_2-S catalysts was further processed without filtration or any further pretreatment via dark fermentation. The two enteric bacteria (*Citrobacter freundii* H3 and *Enterobacter* spH1) effectively fermented it, producing H_2 and other organic compounds as metabolites. The use of *C. freundii* H3 in the dark fermentation to produce hydrogen has been studied in less extent. The highest H_2 yield and production rate in the dark fermentation was observed for *Enterobacter* spH1 and *C. freundii* H3.

The cellulolytic bacteria *Ruminococcus albus* was not able to process the WSF. However, *R. albus* did show an amount of cumulative H_2 production from the reference batch with cellulose. This suggests that *R .albus* could also be used for H_2 production from the degraded cellulose after the catalytic hydrolysis step, if inhibitory compounds such as HMF and furfural were first removed.

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CHAPTER 8

8. GENERAL CONCLUSIONS, AND FURTHER RESEARCHES

General conclusions

The total world energy requirement is increasing due to population growth, which is estimated to reach to 8.5 billion by 2035 [1]. Consequently, the total world energy consumption is expected to increase in absolute terms to 700-810 EJ by 2035 [1,2]. Fossil fuels are the main principal sources of energy worldwide and are responsible for the supply of up to 80-85% of current demand [3,4].However, it has been estimated that fossil fuel will be depleted by the year 2100 [5].Additionally, use of fossil fuels releases carbon dioxide and other greenhouse gases into the atmosphere thereby, negatively impacting the environment and society through anthropogenic climate change. In 2010 worldwide GHG increased by 31% against the 1990 levels [6]. These effects are stimulating a rapid growth in search for alternative energy sources to complement or possibly substitute the conventional fossil fuels.

Biohydrogen holds the promise for a substantial contribution to the future renewable energy demands. Hydrogen is often cited as the clean, 'green' fuel of the future. It has a high energy yield (122 kJ/g), which is about 2.75 times greater than that of hydrocarbon fuels [7].

Currently almost 96% of the total production of H_2 comes from steam reforming (SR) of natural gas (48%) on partial oxidation of refinery oil (about 30%) and coal gasification (18%) [8,9]. However, this H2 production process is energy intensive. It is also not environmentally friendly and un-sustainable due to cost and high level of CO_2 emission. An alternative process to produce hydrogen in sustainable and profitable way is biological hydrogen production.

Biological H_2 production delivers clean H_2 in sustainable manner with simple technology and more attractive potential than the current chemical? Production of H_2 since it is suited for the conversion of a wide spectrum of substrate utilization such as organic wastes, industrial manufacturing process byproducts and biomass as raw material.

Consequently, the major focus of this thesis is on biological and catalytic process of H_2 and useable chemicals from biodiesel waste crude glycerol, which is vastly abundant, cheap and renewable inevitable byproduct of biodiesel manufacturing process, and cellulose also abundant and cheap renewable resource from agricultural and wood industries. In general, the use of renewable waste substrates is an environmental-friendly choice that also contributes to the reduction of waste treatment costs and increases the economic value of by-products.

The overall aim of this thesis is to contribute to new understanding on the production of H_2 for energy from crude glycerol and catalytically degraded cellulose using highly productive microorganism for optimal H_2 production and amendment to dark fermentation for environmentally benign H_2 production processes. The following is a summary of the main findings of this research study presented in detail in the seven chapters that form this thesis.

1. Based on an extensive literature review presented in **Chapter 2,** it was concluded glycerol is an attractive and versatile, carbon and energy for biohydrogen and biochemicals. Glycerol

containing waste from biodiesel manufacturing process is a potential feedstock for biohydrogen and biochemical production. Different researchers have evaluated its performance as a cheap substrate for hydrogen production and indicated that its H_2 production potential is comparable to any other organic waste presently used for H_2 production. The most important advantage of using crude glycerol over other substrates for H_2 production is that it will increase the overall profit of biodiesel manufacturing plants. Such a situation may encourage the production and utilization of biofuels, which is environmentally beneficial. However, crude glycerol contains many impurities which might be inhibitory to microbial growth and hydrogen production and, there is only a scant literature available on pretreatment of crude glycerol used for hydrogen production. We characterized crude glycerol from BPD Biodiesel Company, in Barcelona, Spain as a case study for use in dark fermentation. This characterization which identified the impurities in the crude glycerol, will contribute in filling this gap in the literature.

2. Some chemical commodities currently produced from petroleum can be, in principle, produced biotechnologically from glycerol using microorganisms. This bioconversion would directly benefit the environment by obtaining biodegradable polymers, promoting the use of biodiesel, and reducing petroleum dependency. The development of processes for converting inexpensive glycerol into higher value products is expected to make biodiesel production more economical and will, thus, help establish more bio-refineries This will also have an important social impact, as small farmers cultivate oleaginous plants which in turn are the basis for biodiesel production.

3. The study reported in **chapter 3,** confirms that glycerol can be used by the newly isolated bacteria *Enterobacter* spH1 and *C. freundii* H3 and which also found to be effective producers of H_2. The finding in this chapter indicates that with glycerol as the substrate, *C. freundii* H3 mainly produces H_2 and acetic acid, and other by-products. This is the first time that *Citrobacter* has been used to generate H_2 from glycerol. This microorganism, then, can be used for high-yield production if it is coupled with a photo bioreactor to convert acetic acid to H_2. This finding also shows that *Enterobacter* spH1 produces similar amounts of H_2, high amounts of ethanol, and other by-products. *Enterobacte*r spH1 produced 0.85 mol H_2/mol glycerol, which is close to the theoretical yield of ethanol fermentation from glycerol (1 mol H_2/mol glycerol). Hence, it can be used for the high-yield production of H_2 and useable forms of ethanol in dark fermentation. The combination (1:1:1) of the three cultures yielded less H_2. This suggests that there is no synergistic effect on H_2 production from pure glycerol. For all strains, the production of other metabolites such as ethanol, 1,2-propanediol and 1,3-propanediol decreased H_2 production.

4. **Chapter 4** presents experimental evidence which clearly shows the hyperthermophile *Thermotoga maritima* DSM 3109 is able to grow on glycerol in both batch and chemostat cultivation setups. *T. maritima* converted glycerol to mainly acetate, CO_2 and H_2, with a maximal observed H_2 yield of 2.84 mol H_2 per mol glycerol consumed. The fermentation data suggest a stoichiometry of 1:1:3 ratio for acetate, CO_2, and H_2, respectively. The observed low diversity in fermentation end-products corresponds with the high H_2 yields, which are superior compared to those generally observed for mesophilic organism (~1 mol/mol).

5. For batch cultivations *of T. maritima* optimal H_2 production was realized using an initial pH of 7-7.5 and a yeast extract concentration of 2 g/L. Fermentation performances of *T. maritima* on the different initial glycerol concentrations were comparable to those observed for *T. neapolitana*, with maximal observed H_2 production rates of 1.0 and 1.6 mmol/ L*h, respectively. The H_2 production rates decreased with increasing initial glycerol concentration and substrate consumption was incomplete. Growth in controlled batch systems with fixed pH, might allow complete substrate conversion at higher glycerol loads, thus improving the cumulative H2 production.

6. Stable growth on glycerol could be achieved for *T. maritima* in a chemostat system. H_2 was produced with yields ranging between 2.23 and 2.41 mol/mol. For the chemostat cultivations, the H_2 production rate increased with increasing dilution rate (from 1.1 till 2.34 mmol/L*h). However, at dilution rates exceeding 0.025 h-1, glycerol (2.5 g/L) conversion was incomplete. A pathway for glycerol fermentation by *T. maritima* is proposed. Based on comparative genomics, the ability to grow on glycerol can be considered as a general trait of *Thermotoga* species. In all probability, glycerol enters glycolysis via glycerol-3-phosphate. The observed H_2 yields of 2.5-3.0 mol H_2 per mol acetate, indicated that reductant derived from the oxidation of glycerol-3-phosphate, is also channelled to H_2.

7. **Chapter 5** demonstrates that support supplementation, and iron species all played crucial roles in affecting the performance of dark fermentation H_2 production from glycerol. Addition of Fe/AC carriers enhanced H_2 production rate and yield by 333.3 and 32.2%, respectively when compared to the carrier-free culture. The Effects of support assisted carrier due to surface area attachment and iron involvement were:

i) Maximum H2 productivity (mmol/L) and yield (mol H_2/mol glycerol consumed) in the following descending order: Fe/AC> AC> Fe/SiO_2 Fe_2O_3>SiO_2 >Fe_2O_3> γ-Al_2O_3 > FC.

ii) Maximum Ethanol productivity (mmol/L) in the following descending order: Fe/AC > AC > Fe/SiO_2 >SiO_2 > Fe_2O_3> γ-Al_2O_3 > FC and yield (mol EtOH/mol glycerol consumed) were higher in order of: γ-Al_2O_3 > AC>SiO_2> Fe/SiO_2 Fe/AC>Fe_2O_3> FC.

iii) Maximum 1,3-propanediol productivity (mmol/L) and yield (mol 1,3PDO/mol glycerol consumed) in the following descending order: FC > Fe/AC > Fe_2O_3> AC > Fe/SiO_2 >SiO_2 > γ-Al_2O_3

iv) Maximum Lactate productivity (mmol/ L) and yield (mol Lactate/mol glycerol consumed) in the following descending order: FC > Fe/AC > Fe_2O_3> AC > Fe/SiO_2 >SiO_2 > γ-Al_2O_3

8. **Chapter 6** presents a clear research evidence that a significant amount H_2 can be produced from crude glycerol. Using mixed co-culture (1:1) of *Enterobacter* spH1 and *E. coli* CECT432 crude glycerol containing MONG of around 7.2 % (w/w) and 4.8 % ash content derived from biodiesel production plant that used waste vegetable oil and waste animal as a feed stock, is able to produce hydrogen and ethanol and other reduced chemicals.

9. The highest H_2 yield (Y_{H2}) and ethanol yield (Y_{EtOH}) of 1.21 and 1.53 mol / mol glycerol was obtained from the crude glycerol, respectively. Of those reported to date in the literature, the yields achieved in this study are the highest obtained using a mesophilic strains. This depicts that the co-culture enables coexistence with stronger synergetic effect than mon-culture. Considering the kinetics of the study the best fitting was obtained using the modified Gompertz equation for both the product formation , gaseous product ($R^2 > 0.9976$) and liquid ferment ($R^2 > 0.9775$ except for acetic acid ,$R^2 > 0.8575$) and substrate consumption ($R^2 > 0.9875$).

10. An integrated system between heterogeneous catalysis and dark fermentation to convert cellulose into biohydrogen was proposed **(chapter 7)**. ZrO_2 catalysts were used to foment cellulose hydrolysis to its structural fraction sugar units. ZrO_2 was promoted by phosphate, sulphate and fluoride species. The resultant WSF contained glucose, HMF and furfural among the products. The WSF derived from the cellulose hydrolysis with ZrO_2-P and ZrO_2-S without filtration or any further pretreatment via dark fermentation. The two enteric bacteria (*Citrobacter freundii* H3 *and Enterobacter* spH1) effectively fermented it, producing H_2 and other organic compounds as metabolites. The use of *C. freundii* H3 in the dark fermentation to produce hydrogen has been studied in less extent. The highest H2 yield and production rate in the dark fermentation was observed for *Enterobacter* spH1 and *C. freundii* H3.

11. The cellulolytic bacteria *Ruminococcus albus* DSM20455 was not able to process the WSF. However, *R. albus* DSM20455 did show an amount of 10.91 mmol/L cumulative H_2 production from reference batch with cellulose. This suggests that *R .albus* could also be used for H_2 production from the degraded cellulose after the catalytic hydrolysis step, if inhibitory compounds such as HMF and furfural were first removed.

12. The work presented in this thesis provides a "proof-of-concept" that crude glycerol from the biodiesel industry and catalytically degraded cellulose can be used for producing high value biohydrogen and biochemical by mesophilic and thermophilic organism. Because glycerol is the major byproduct of the biodiesel manufacturing process, the disposal of crude glycerol has been a major issue faced by biodiesel producers. The biological methods that have been investigated in this thesis are as possible value-added outlets for this currently under-utilized and under-valued byproduct. Glycerol had been largely neglected as substrate for microbial fermentations, the main reason being glycerol's high cost. Recent developments as it is seen in this thesis, however, reversed this situation and nowadays glycerol is becoming one of the preferred fermentation substrates. This reversal was due to the thriving biodiesel industry, which produces large amounts of glycerol as a byproduct. Thus, glycerol's cost became almost zero making glycerol an attractive feedstock.

Further Research

The thesis has also identified promising research areas needing further investigation. These are outlined as follows. .

1. **In Chapter 2** a need for further investigation is identified that optimizes crude glycerol pretreatment for biohydrogen production. Accumulation of fermentation end products is known to have negative effect on overall H_2 yield. Hence, alternative strategy, such as further conversion of fermentation end product into CO_2 and H_2 by photo fermentation should be investigated in detail.

2. Similarly, most investigations on crude glycerol bioconversion have been carried out in serum bottle scale batch reactors. Only, a few studies carried out in continuous mode have given better yield of H_2 than batch experiments. Hence, further investigation of microbial H_2 production using continuous mode is recommended. Detailed study and optimization of fermentation parameters may play a vital role large scale hydrogen production in future. Alternatively, co-culture of two different strains can also be evaluated for crude glycerol bioconversion. Application of a co-culture, which is capable of reducing the accumulation of fermentation end products by simultaneously metabolizing it to H_2, is an interesting subject for future investigation.

3. Glycerol containing waste from biodiesel manufacturing process is a potential feedstock for biohydrogen and biochemical production. Similarly, most investigations on crude glycerol bioconversion have been carried out in serum bottle scale batch reactors. Only few studies carried out in continuous mode have given better yield of H_2 than batch experiments. Hence, further investigation of microbial H_2 production using continuous mode is recommended. Detailed study and optimization of fermentation parameters may play a vital role large scale hydrogen production in future.

4. In **Chapter 3,** *Enterobacter* spH1 and *C. freundii* H3 are identified as effective producers of H_2 from glycerol. To optimize the yield further research needs to be carried out on the characterization of the strains and the operating conditions.

5. In **Chapter 4** the need for further investigation on the exact mechanism of how to overcome the endergonic electron transfer from glycerol-3-phosphate to H_2 is required.

6. **Chapter 5** presented that both support supplementation and iron played crucial roles in affecting the performance of dark fermentation H_2 production from glycerol using the co-culture of *Enterobacter* spH1 and *C. freundii* H3. The detailed mechanism of the support-induced promoting effects on dark fermentative H_2 production has to be clearly identified. Biofilm formation study has to be done.

7. The study presented in **Chapter 6** also identifies that the optimization with higher amount of crude glycerol (> 5% w/w) has to be studied for the dark fermentation in the future, because it will reduce the cost due to the dilution process used in the medium.

8. **Chapter 7** presents that the effect of HMF and furfural on *R. albus* viability should be performed. *R .albus* could also be used for H_2 production from the degraded cellulose after the catalytic hydrolysis step if inhibitory compounds such as HMF and furfural are removed. Further study on the attached cellulose by the catalytic process has been done. However, the conversion efficiency of the catalytic process from cellulose to monomer sugar has to be improved by different acid hydrolysis process.

In concluding the recommendations for further study we note that future research should focus on the characterization of microorganisms of the newly isolated *Enterobacter* spH1, *C. freundii* H3 and *T. maritima* that can use glycerol as a carbon source and generate valuable molecules with unusual properties, thereby broadening the potential applications of this cheap by-product of transesterification, crude glycerol.
Integrated two stage fermentation (dark fermentation with photo fermentation) system that would use waste glycerol (from biodiesel production) to produce a suite of fuels, including additional biodiesel, H_2, and ethanol or any other usable products.

Further work on optimization of process parameters has to be done for each experiment in this thesis and extrapolation from experimental results will allow further insight to the feasibility of process and play an important role in the final selection of a promising process route. Accordingly a work has to be done which compares the use of mesophilic and thermophilic bacteria in the dark fermentation step, analyzing the effects on the overall process. Based on experimental results, simulation models can be developed with Aspen Plus V7.1® which will be used to calculate the mass- and energy balances of the process. Based the material and energy balance the environmental impact and economical advantage can be calculated using Life Cycle Assessment (LCA) using SimaPro 8 software and economic analysis. The results of the thesis can in principle easily be used in future programs to simulate chemical and biochemical processes with SuperPro Designer software package, (www.intelligen.com), as well as to perform economic analyses. This would allow a shortening of time required to achieve technology transfer to interested economic agents.

Moreover, further advancements in the fields of metabolic engineering and synthetic biology should increase the number of products that can be synthesized from glycerol and help to determine how many of these process can be commercially successful.

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Improvement of biohydrogen and usable chemical products from glycerol by co: culture of *Enterobacter* spH1 and *Citrobacter* freundii H3 using support assisted surface immobilization

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ANNEX I

CASE STUDY: Production process of Stocks del Valles: use of technical grade glycerol containing biodiesel waste for hydrogen production

An application on use of technical glycerol after the post reaction process was studied for the mixture of selected strains for hydrogen production. In this study we have been summarized how the technical glycerol was produced this will help for the analysis of the constituents in the technical grade glycerol.

Stocks del Valles, S.A. is a Catalan company that owns a biodiesel plant near Barcelona, with the capacity of 31,000 tons/yr. The feedstock is composed exclusively of WVO and WAF. It is a pioneer plant, the first one of this type in Spain and second in the world. Its construction was inspired by the fact that in the year 2000 the use of waste animal fat and waste vegetable oil as animal feed was prohibited in Spain.

The plant can process feedstocks with FFA content from about 5% up to 20-30%. It has been in operation since 2002, with a production of 6,000 tons/yr. In 2006 there was a major technology upgrade and additionally the production capacity was significantly increased to its present level. A year later they started the production of transparent biodiesel, which exceeds the requirements of the norm EN 14214 for several parameters. There is a special emphasis on low sulphur content (2mg/kg vs. max. 10mg/kg required by EN 14214), low water content (50mg/kg vs. max.500mg/kg required by EN 14214), low total contamination (1mg/kg vs. max. 24mg/kg required by EN 14214) and ester content (99.8% vs. min. 96.5% required by EN 14214). The norm EN 14214 does not specify CFPP (it is defined by each country); therefore legally the quality of the fuel does not depend on this parameter. It only concerns blends, and in Spain it is 0°C for summer and -10°C for winter. The neat biodiesel, B100, does not have any restrictions regarding CFPP. Today, the biodiesel blends used in Spain are usually between B10 and B30. There are also certain bus companies that run on B100. In order to comply with the EU biofuel directive, the mandatory blends will be as follows: min. 3.4% in 2009 and 5.75% in 2010. The plant does not receive any subsidies (neither the whole region of Catalonia), however they have a 'zero tax' guaranteed until 2012 (the tax for fuels is 270 EUR/m3). The price of the fuel product depends on the market price of fossil diesel. It is sold at the cost of diesel minus a certain percentage because the biofuel has a lower energy density. The biodiesel price at the moment is around 650 EUR/m^3.

The production, due to uncertainty of the feedstock content, is carried out in a batch system (although a continuous system is less expensive). Due to traceability regulations, there are several tanks destined for feedstock storage. After filtration and dehydration of the feedstock by centrifuge, the first step of the production process is esterification. The company does not reveal the information about the catalyst used. This process takes 9-10 hours and only one tank is used for it. For transesterification (after FFA content goes down to 2%), KOH is used. There are 3 tanks

designed for this process, since this part takes more time, approximately 15 hours. Then the whole mixture is decanted. Solvent recovery is carried out separately from both phases: biodiesel and glycerin, through centrifugation.

Subsequently, the biodiesel is distilled in vacuum at 220°C. At the bottom of a distillation column a low quality part of biodiesel (3-4%) is collected, which is sold as heating oil; it is dark, dense and it contains unreacted compounds, impurities, trace amounts of glycerin and water and methanol, and its quantity depends on feedstock quality.
Before the upgrade of the installation, the biodiesel treatment only included stripping; therefore the final product had a light yellowish color, whereas at present it is completely transparent.

The whole production process takes around 40 hours/batch. Its block diagram is presented in Figure A.
After each step of the process the product is analyzed to make sure that everything is working correctly. Glycerin (with 50% water content) is sold to a refinery, since it is not profitable to build a separate unit for this purpose in a plant this size.

Every batch of delivered feedstock is analyzed in the laboratory before accepting it. The tests for water content (usually about 0.3%), total contamination (usually 0.1-0.15%) and the FFA content are carried out. The whole analysis takes 10-15 minutes.

As it has already been mentioned, both WVO and WAF feedstocks are used. The oil is obtained at the cost of transport only; there is a company contracted that collects it from restaurants. Animal fat, on the other hand, is purchased (about 50% of the total feedstock used). The plant is adjusted to run on a wide range of FFA content, therefore the selection of feedstocks is flexible and depends on the market prices. In the occasions of particularly favorable purchase costs, bulk amounts are acquired and stored in paid facilities. The storage space at the plant is only for 1,000 tons of feedstock and 1,000 tons of biodiesel.

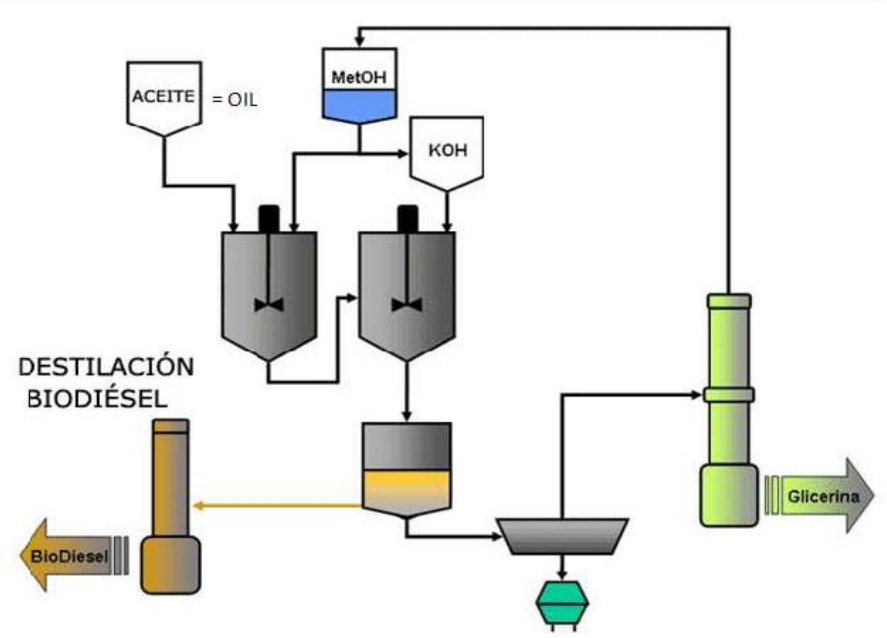

Figure A. Production process in stocks del vallles (BDP)