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Dark fermentative biohydrogen production from organic waste and application of by-products in a biorefinery concept

Anish Ghimire

To cite this version:

Anish Ghimire. Dark fermentative biohydrogen production from organic waste and application of by-products in a biorefinery concept. Material chemistry. Université Paris-Est, 2015. English. NNT : 2015PESC1197 . tel-01404689

HAL Id: tel-01404689

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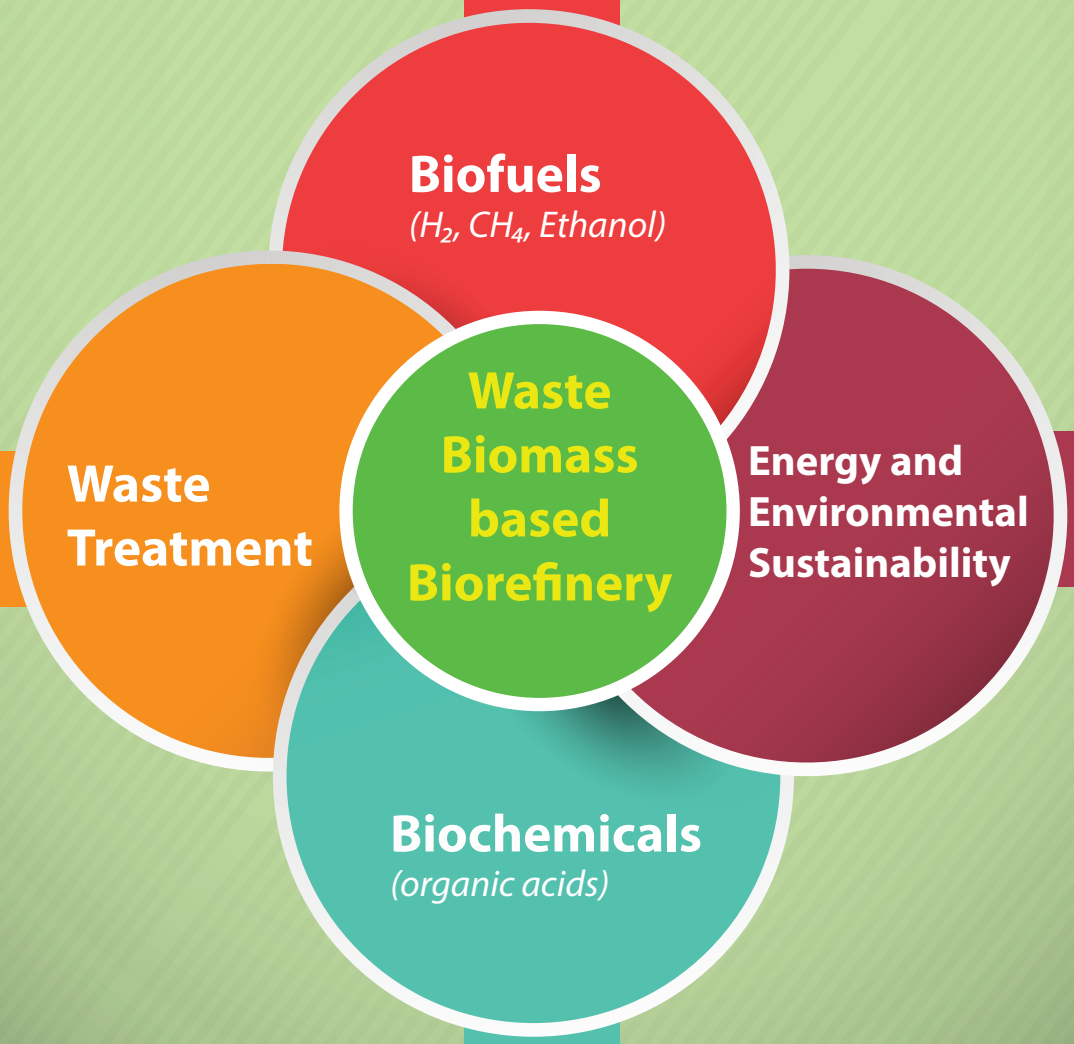
Dark Fermentative Biohydrogen Production from Organic Waste and Application of By-products in a Biorefinery Concept



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Spécialité : Science et Technique de l'Environnement

Dottore di Ricerca in Tecnologie Ambientali

Degree of Doctor in Environmental Technology

Thèse – Tesi di Dottorato – PhD thesis

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Dark fermentative biohydrogen production from organic waste and application of by-products in a biorefinery concept

Defended on December 17th, 2015

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ABSTRACT

Low biohydrogen (H_2) yields and limited use of process by-products from dark fermentation (DF) of waste biomass is limiting its scaled-up application. This study aims to investigate the effects of culture pH, substrate concentration, pre-treatment of substrate and inoculum adaptation on H_2 yields during the DF of three organic wastes biomass (i.e. food waste, rice straw and olive mill wastewater). The results showed that the biodegradability of the substrates is important for the selection and application of optimal operational parameters aimed at enhancing H_2 production.

Moreover, long-term operational feasibility and stability of dark fermentative H_2 production was demonstrated using food waste and cheese whey in two semi-continuous thermophilic DF reactors. The effect of Organic Loading Rates (OLRs), Hydraulic Retention Times (HRTs) and co-substrates (buffalo manure) addition, as a source of alkalinity, on culture pH and H_2 production stability was discussed. The results showed that combination of OLR, HRT and co-substrate addition could play a vital role in the culture pH and stability of H_2 production.

The by-products of DF process were utilized for H_2 production via photo fermentation (PF), while the waste stream generated from coupling of DF and PF processes was converted to methane in anaerobic digestion. The three-step conversion of food waste in a biorefinery concept increased the total energy yields. Moreover, PF also showed a good potential for concomitant production of H_2 and polyhydroxybutyrate (biopolymer). Likewise, dry fermentation of waste biomass could be promising for the production of bioenergy and biochemicals (organic acids and alcohols) in a biorefinery concept.

SINTESI

La produzione di Idrogeno mediante Dark-Fermentation (DF) rappresenta ad oggi uno dei processi biologici più promettenti nel campo della valorizzazione energetica delle biomasse di scarto. Sebbene, tale bio-tecnologia presenti un potenziale notevole, le basse rese in termini di produzione di idrogeno e l'assenza di metodologie che prevedano il riutilizzo dei sottoprodotti di pregio, rendono l'applicazione di tale processo non sostenibile a scala reale.

Il lavoro risulta articolato in quattro fasi distinte. Nella prima fase sono stati investigati gli effetti sulla produzione biologica di H_2 di specifici parametri operativi. In particolare, si è proceduto ad analizzare: i) l'effetto del pH, ii) l'effetto combinato del pH e della concentrazione iniziale di substrato, iii) l'effetto di pretrattamenti del substrato, iv) l'utilizzo di biomasse microbiche adattate. In fase di sperimentazione sono state utilizzate tre differenti tipologie di substrato. I risultati hanno mostrato che la biodegradabilità dei differenti substrati risulta fondamentale nella corretta definizione dei parametri di processo al fine di massimizzare la produzione di Bio-idrogeno.

La seconda parte dell'attività sperimentale è stata dedicata alla messa a punto e alla conduzione di due reattori di DF operanti in regime di termofilia e alimentati con frazione organica di rifiuto solido urbano e reflui caseari, mediante i quali viene dimostrata la fattibilità e la stabilità del processo di DF nel lungo periodo. Vengono, altresì, discussi gli effetti dovuti all'applicazione di differenti Carichi Organici (Organic Loading Rates – OLRs), differenti Tempi di Ritenzione Idraulica (Hydraulic Retention Times – HRTs) e dell'aggiunta di substrati ad elevato tenore di alcalinità. I risultati hanno dimostrato che la combinazione di OLR, HRT e l'aggiunta di co-substrato (refluo bufalino) possono giocare un ruolo fondamentale nella stabilità del processo di DF.

In un contesto di bio-raffineria, nella terza parte del lavoro viene proposto un sistema integrato costituito dall'abbinamento del processo di Dark Fermentation ai processi di Photo Fermentation (PF) e di digestione anaerobica (AD). Attraverso tale sistema a triplo stadio, è stato possibile non solo incrementare la resa energetica totale, ma, aspetto non trascurabile, ottenere mediante l'applicazione del processo di PF, la sintesi del biopolimero Poly-Hydroxy-Butyrate (PHB) che può essere utilizzato per la produzione di bioplastiche.

Infine, nella quarta ed ultima parte dello studio, è stato valutato il processo di DF in condizioni dry. Tale applicazione ha consentito la contestuale produzione di bio-energia

e di ulteriori bio-prodotti (e.g. alcoli, acidi organici etc.), amplificando ulteriormente il concetto di bio-raffineria.

RÉSUMÉ

La fermentation sombre est un procédé utilisant des déchets organiques dont le passage à l'échelle pilote est limité par les faibles rendements de production d'hydrogène ainsi que par l'utilisation des sous-produits métaboliques. Cette étude a pour premier objectif d'étudier des paramètres opératoires, par exemple, l'effet du pH, de la concentration en substrat, du prétraitement du substrat et de l'adaptation de l'inoculum microbien sur la fermentation sombre de trois types de déchets différents (i.e. déchets alimentaires, paille de riz et les eaux usées de pressoirs à d'olives). Il a été montré que la biodégradabilité des substrats jouait un rôle majeur dans le choix des paramètres opérationnels utilisés pour optimiser la production d'hydrogène.

De plus, la faisabilité et la stabilité à long terme de la production d'hydrogène par le procédé de fermentation sombre ont été observées en utilisant des déchets agroalimentaires et du petit lait dans deux réacteurs thermophiles fonctionnant en mode semi-continu. En particulier, il a été discuté de l'influence de la charge organique (OLR), du temps de rétention hydraulique (HRT) et de l'addition de co-substrats (fumier de buffle) comme source d'alcalinité. Ainsi, cette étude a permis de montrer que la combinaison de ces trois paramètres pouvait jouer un rôle important sur le pH et la stabilité de la production d'hydrogène.

Les sous-produits métaboliques de la fermentation sombre ont également été utilisés pour produire de l'hydrogène via la photo-fermentation, alors que les déchets générés par le couplage de la fermentation sombre et de la photo-fermentation ont été valorisés pour la production de méthane par digestion anaérobie. Ce concept de bioraffinerie basé sur la conversion en trois étapes des déchets agroalimentaires augmente le rendement énergétique global du procédé. Par ailleurs, le potentiel important du procédé de photo-fermentation pour la production concomitante de polyhydroxybutyrate (polymère) et de l'hydrogène a ainsi été démontré.

En conclusion, la fermentation par voie sèche de déchets organiques pour la production de bioénergie et de produits biochimiques (i.e. acides organiques et alcools) paraît prometteuse dans un contexte d'optimisation de la production d'énergies et de biomolécules au sein d'une bioraffinerie environnementale.

SAMENVATTING

Lage biowaterstof (H_2) produktierendementen en beperkt gebruik van bijproducten van in vergisting (dark fermentaiton, DF) van biomassa beperken de opschaling van dit process. Deze studie onderzocht het effect van pH, combinatie van substraat concentratie en cultuur pH, voorbehandeling van het substraat en entmateriaal op de H_2 opbrengst via DF van drie verschillende types afvalbiomassa, met name (i.e. keukenafval, rijststro en afvalwater van olijfolieproductie. Uit het onderzoek bleek dat de biologische afbreekbaarheid van de substraten een belangrijke rol speelde bij de selectie en toepassing van de optimale operationele parameters ter verbetering van de H_2-productie.

De operationele haalbaarheid en stabiliteit op lange termijn van H_2-productie via DF werd gedemostreerd met keukenafval en wei in twee semi-continue thermofiele DF reactoren. Het effect van de organische belasting OLRs), hydraulische retentietijd (HRT) en toevoeging van co-substraten (buffel mest als bron van alkaliteit) op de cultuur pH en H_2 productiestabiliteit zijn bestudeerd. Uit deze studie bleek dat een combinatie van OLR, HRT en co-substraat toediening een belangrijke rol kunnen spelen in de pH van de fermentor en de stabiliteit van de H_2-productie.

Bovendien werden de bijprodukten van het DF-proces gebruikt voor H_2 productie via photofermentatie (PF), terwijl de afvalstroom gegenereerd uit de koppeling van DF en PF processen omgezet werd naar methaan via anaërobe vergisting (AD). De drie-staps conversie van keukenafval in een bioraffinage concept verhoogde de totale energie opbrengst. Bovendien toonde PF een goede potentie voor de gelijktijdige productie van H_2 en polyhydroxybutyraat (biopolymeer). Ook droge fermentatie kan veel belovend zijn voor voor de productie van bio-energie en biochemicaliën (VFAs en alcoholen) in een bioraffinage concept op basis van afvalbiomassa.

ACKNOWLEDGEMENT

Persuing a doctoral degree was one of the aspirations of my life. I am grateful to all my friends, colleagues, mentors, supervisors and my family who have continuously inspired and supported during this journey. I would like to dedicate this Doctoral Thesis to all these people who did not gave up on me during the difficult times and shared joyful pleasant time together.

This desertation is a part of the Erasmus Mundus Joint Doctoral Program Environmental Technologies for Solids, Soils and Sediments (ETeCoS^3) developed in collaboration between the University of Cassino and Southern Lazio (Italy), University of Paris Est (France) and UNESCO-IHE (the Netherlands) and several other partner institutions. I am thankful for Erasmus Mundus comminssion for the funding support. Likewise, it has been a great opportunity to carry out my resaserch activites at the Department of Civil, Architectural and Environmental Engineering, University of Naples Federico II (Naples, Italy) and INRA, Laboratoire de Biotechnologie de l'Environnement (Narbonne, France).

First of all, I am thankful to my supervisor Prof. Ing. Giovanni Esposito for his support throughtout my doctoral studies. I am equally grateful to my co-supervisors Prof. Dr. Ir. P.N.L. Lens and Prof. Dr. Ing. Francesco Pirozzi for their constant support and guidance during the PhD desertation. I would like to thank Dr. R. Escudié and Dr. E. Trably for their guidance and care during my stay at INRA, LBE (Narbonne, France). Moreover, I do not have enough words to thank my mentor Dr. Ing. Luigi Frunzo for being supportive and who have become one of my closest friends at the end of my PhD.

Similarly, I would like to thank all my colleagues from the laboratories in Naples, Cassino and Narbonne with whom I have spent quality time at work and outside. I am thankful to Vincenzo, Alberto, Maria Rosaria, Marco, Fabio, Serena, Ester, Amelia and Martha from Naples for the joyful time in the laboratory in Naples (Italy). I am thankful to all the collegues from LBE, INRA, Narbonne (France) for their immense help and companionship during my stay there. Finally, I would like to thanks my parents Madav and Kalpana and my brothers Anup and Anil for their continuous inspirations and support throughout all these years.

CHAPTER 1

INTRODUCTION

1.1 Background and problem statement

The global reserves of fossil fuels are depleting due to their increasing consumption in energy and chemical sectors. In addition, environment is facing severe pollution problems due to the gaseous emissions (NO_x, SO_x, CO, CO_2, etc.) and waste generated from the production and use of fossil fuels. The scientific community has widely accepted the fact that the increasing CO_2 levels has impacted global warming phenomena, which is threatening the entire earth's ecosystem (Ciais et al., 2013). Therefore, a current need is to explore and invest in alternative ways to harness the energy and chemicals from the clean renewable sources that are carbon neutral and can reduce the global CO_2 emissions at the same time.

In this context, hydrogen gas (H_2) could represent a promising alternative energy carrier due to its social, economic and environmental credentials (Kotay & Das, 2008). The net energy content of the H_2 per unit mass is higher than other conventional fuels. The lower heating value (LHV) of hydrogen varies between 2.4-2.8 and is 4 times higher than that of methane, gasoline and coal respectively (Marbán & Valdés-Solís, 2007). H_2 is a carbon-free clean fuel as the ultimate by-products of combustion is only water. Thus, H_2 carries a long term potential to reduce consumption of fossil fuels that can be helpful in combating global warming and pollution problems.

A preliminary major challenge in the use of this promising source of energy carrier lies in the sustainable production of H_2. In commercial applications, H_2 have been produced from natural gas by steam reforming process, coal gasification and water electrolysis (Kotay & Das, 2008; Manish & Banerjee, 2008). At present, steam reforming of methane is the cheapest H_2 production method. However, for the equivalent amount of energy, it is four times more costly than gasoline (Crabtree et al., 2004). Bartels et al. (2010) reported an estimated cost of 0.36-1.83 $/kg and 2.48-3.17 $/kg for H_2 production from coal and natural gas, respectively. H_2 from conventional sources are economically convenient compared to biological routes for H_2 production (i.e. thermophilic dark fermentation process), which costs about $28.35/kg H_2 (€21/kg H_2 with €1=$1.35 in 2011) (HYVOLUTION, 2011). In a study, Das, (2009) reported a low production cost of energy as H_2 from dark fermentation of sewage sludge ($1.3/Million British Thermal unit, MBTU) compared to natural gas ($2–$7/MBTU, in 2007) and gasoline ($23.5/MBTU in 2008).

However, most of the physical and chemical H_2 production processes are highly energy intensive and/or dependent on fossil fuels. Moreover, the physical and chemical technologies do not reduce the consumption of fossil fuels or CO_2 emissions. This only shifts the point of CO_2 emissions to H_2 producing industries from vehicular emissions or emission from stationary hydrogen power stations, which makes them less attractive from environmental point of view. On the other hand, H_2 produced from biological processes, also known as biohydrogen is expected to be less energy intensive and can be produced from renewable sources (Das & Veziroglu, 2001; Hallenbeck & Ghosh, 2009).

H_2 can be produced biologically by autotrophic as well as heterotrophic microorganisms (Hallenbeck & Ghosh, 2009) (detailed in Chapter 2). Autotrophic conversions are mediated by microalgae utilizing inorganic carbondioxide as a carbon source whereas heterotrophs convert the organic carbon sources into simpler compounds producing molecular H_2. There are two types of heterotrophic conversions; one driven by light energy (photofermentation) and other that occurs in absence of light (dark fermentation).

Dark fermentation (DF) represents one of the most promising and cost-effective technologies for biohydrogen production due its faster conversion efficiencies. Moreover, DF process can utilize wide range of renewable complex waste biomass as feedstock and production of other valuable platform biochemicals of economic interest (Ghimire et al., 2015a). Currently, the major barriers in application of DF in scaled-up systems for H_2 production are: low H_2 yields and the high cost of production mainly due to the high cost of feedstock (Ren et al., 2011). Moreover, an inherent challenge of DF systems is to maximize the process conversion efficiencies, utilization and valorization of the by-products and minimize the ecological footprint of the process by reducing the water and energy input to the process.

In order to achieve a scaled-up development of dark fermentative processes, an immediate attention is required to improve H_2 yields utilizing the low cost materials like waste biomass such as agricultural residues, organic waste generated from municipalities and industries, that could also give competitive economic advantage (Chong et al., 2009; Kapdan & Kargi, 2006). The H_2 yields and production rates can be enhanced by optimizing the operational parameters such as culture pH and temperature and substrate concentration as well as by inoculum enrichment and substrate pre-treatment (Guo et al., 2010; Urbaniec & Bakker, 2015). Moreover, knowledge gaps in the long-term operational

feasibility of the DF process for continuous H_2 production needs to be filled for its development.

The by-products of DF process, which mostly includes volatile fatty acids (VFAs), lactic acid, alcohols and un-hydrolyzed residues, can be utilized in other biological systems for their valorization by energy recovery or can be used as a feedstock in production of platform chemicals of economic interests (Agler et al., 2011; Bastidas-Oyanedel et al., 2015; Ghimire et al., 2015a). The dark fermentation effluent (DFE) can be converted to H_2 photo fermentation (PF) process, which is mediated by purple non sulfur bacteria (PNSB). In addition to H_2 production, PNSB are known to synthesize polyhydroxybutyrate (PHB), a precursor for biopolymers (Hustede et al., 1993).

Likewise, un-utilized biomass residues as well as the waste streams generated from coupling of DF and PF process can be further converted to methane in anaerobic digestion process (Ghimire et al., 2015b). In this way, utilization of DF by-products can lead to realization of a biorefinery concept that could help in industrial development of DF technology. Moreover, Solid State Dark Fermentation (SSDF) process has been recently proposed for biorefinery concept due to its inherent characteristics such as higher process yields and less energy and water requirements (Motte et al., 2015; Elsamadony and Tawfik, 2015). Therefore, a study of major limitations in SSDF is necessary to exploit this technology. In this context, a general scheme of the present doctoral research is reported in Figure 1.1.

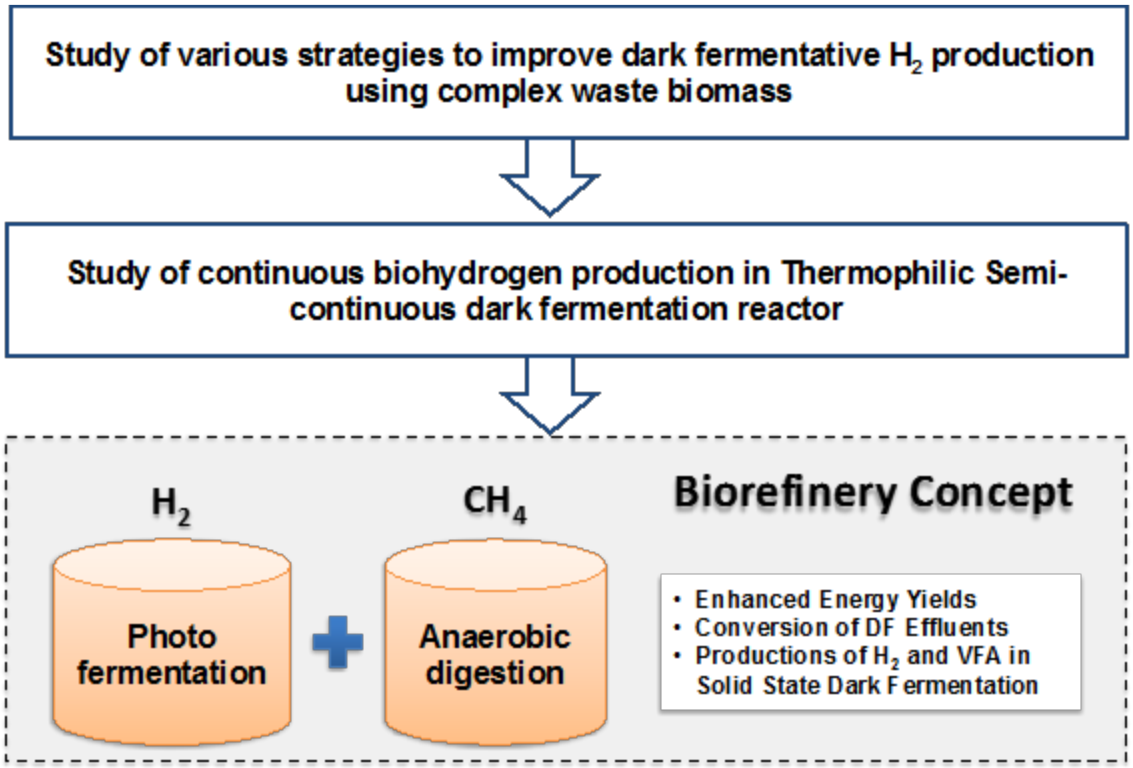

Figure 1.1 - A general schematic representation of the thesis study

1.2 Scope of the PhD thesis

The main objectives of this research were to study the process influencing parameters in the DF of complex organic waste and the valorization of the by-products in a biorefinery concept. The specific objectives were:

i. To assess the effect of different operational parameters on dark fermentative H_2 production from different complex waste biomass.
ii. To study long term continuous H_2 production from food and cheese whey waste with an emphasis on pH control.
iii. To investigate the integration of DF in a biorefinery concept coupling with photo fermentation and anaerobic digestion to maximize energy yields and valorize the by-products.
iv. To assess the limitations in the application of SSDF for H_2 and organic acids productions.

To achieve these four major aims, the research activities that were carried out are outlined as follows:

Chapter 1 explains the motivation, hypothesis and scheme of the doctoral research.

Chapter 2 provides the comprehensive state-of-art in parameters influencing the DF of complex waste biomass and use of by-products. Moreover, it also discusses the potential application of photofermentation processes to valorize the dark fermentation by-products by H_2 and biopolymer production.

Chapter 3 presents the influence of different operational parameters in DF of complex waste biomass. Various operational parameters such as inoculum sources and enrichment methods, pH, temperature and substrate concentration were studied.

Chapter 4 demonstrates the effects of different reactor operating conditions, such as organic loading rates (OLRs) and hydraulic retention times (HRTs), on long-term operational feasibility of H_2 production. This chapter also discusses the use of low OLRs and co-substrate addition as pH controlling strategies using food waste and cheese whey waste, respectively, in two separate studies.

Chapter 5 discusses the potential for the integration of DF process in a biorefinery concept. The coupling with photofermentation and anaerobic digestion by using DFE was studied to explore the potential for futher energy recovery. Similarly, this section presents the prospective of photofermentation process for maximizing the valorization of DFE via concomitant H_2 and biopolymer production. Moreover, possible limitations during the conversion of waste biomass in SSDF were studied.

Chapter 6 highlights the major findings and the implications of the research and provides future recommendations.

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CHAPTER 2

STATE OF THE ART IN DARK FERMENTATION OF COMPLEX WASTE BIOMASS BY MIXED CULTURE AND UTILIZATION OF DARK FERMENTATION EFFLUENTS IN PHOTO FERMENTATION

The section 2.1 of this chapter has been published as Ghimire, A., Frunzo, L., Pirozzi, F., Trably, E., Escudie, R., Lens, P.N.L., Esposito, G., (2015). A review on dark fermentative biohydrogen production from organic biomass: Process parameters and use of by-products. Applied Energy 144, 73–95

The section 2.2 of this chapter has been submitted as Ghimire, A., Luongo, V., Frunzo, L., Pirozzi, F., Lens, P.N.L., Esposito, G., 2016. Engineering strategies for enhancing photofermentative biohydrogen production by purple non-sulfur bacteria using dark fermentation effluents. In Microbial Fuels: Technologies and Applications. Taylor and Francis Group, CRC Press. Boca Raton, FL, USA.

2.1 Dark fermentative biohydrogen production from organic biomass

This section summarises the state of the art in the dark fermentative biohydrogen production from organic biomass such as agricultural residues, agro-industrial wastes and organic municipal waste. In spite of its potential, this technology needs further research and development to improve the biohydrogen yield by optimizing substrate utilization, microbial community enrichment and bioreactor operational parameters such as pH, temperature and H_2 partial pressure. On the other hand, the technical and economic viability of the processes need to be enhanced by the use of valuable by-products from dark fermentation, which mostly includes volatile fatty acids. This paper reviews a range of different organic biomasses and their biohydrogen potential from laboratory to pilot-scale systems. A review of the advances in H_2 yield and production rates through different seed inocula enrichment methods, bioreactor design modifications and operational conditions optimization inside the dark fermentation bioreactor is presented. The prospects of valorizing the co-produced volatile fatty acids in photofermentation and bioelectrochemical systems for further H_2 production, methane generation and other useful applications have been highlighted. A brief review on the simulation and modeling of the dark fermentation processes and their energy balance has been provided. Future prospects of solid state dark fermentation are discussed.

2.1.1 Introduction

Environmental friendly energy carriers and sources are the most highlighted topic in the energy and environmental sector. The current global energy demand is mostly dependent on reserves of fossil fuels, which are depleting, and the world is facing severe pollution problems from the by-products of fossil fuels uses (Marbán and Valdés-Solís, 2007). The scientific community has widely accepted the fact that the increasing CO_2 level due to the use of fossil resources is impacting the greenhouse gas effect and global warming. Therefore, different ways to harness the energy from clean renewable sources are being developed, but the search for reliable energy sources is still on.

In the past years, the research and development interests have been directed towards renewable energy technologies like the anaerobic digestion (AD) of organic biomass and waste. For alternative energy carriers, hydrogen could be the fuel of the future because of its high energy content, environmental friendliness of production, and also because it can give substantial social, economic and environmental credentials (Kotay and Das, 2008).

Hydrogen is a carbon-free clean fuel, as the only final by-product of its combustion is water (Kotay and Das, 2008). Hydrogen can also be helpful in addressing global warming and increasing pollution problems. Furthermore, it is preferred over methane owing to its wider industrial applications, i.e. H_2 is used in the synthesis of ammonia and hydrogenation of edible oil, petroleum, coal and shale oil (Kothari et al., 2012). Hydrogen can be directly used either in combustion engines because of its highest energy per unit weight, i.e. 143 GJ per ton (Kotay and Das, 2008) among known gaseous biofuels or to produce electricity via fuel cell technologies (Alves et al., 2013). Thus, the creation of a hydrogen economy which incorporates the production and use of hydrogen as an energy carrier could in the future lead to sustainable energy systems (Ekins and Hughes, 2009; Marbán and Valdés-Solís, 2007).

The major challenge in the use of this promising energy carrier lies in its sustainable production and storage. In commercial applications, hydrogen has been produced from natural gas (48%) and oil (30%) by steam reforming processes, and also by other industrial methods such as coal gasification (18%) and water electrolysis (4%) (Balat, 2008). However, these processes are highly energy intensive and use non-renewable sources of energy, which makes them less attractive from an environmental point of view. In order to produce a cleaner and more sustainable fuel, the hydrogen should come from processes that avoid or minimize CO_2 emissions.

Hydrogen can be produced from biological processes that are less energy intensive and more environmental friendly in terms of global reduction of CO_2. These renewable biohydrogen producing technologies have potential to become cost competitive as they can use low value waste biomass as feedstock (Kotay and Das, 2008), e.g. municipal, agricultural and industrial organic waste and wastewater. Biohydrogen can be produced by both autotrophic and heterotrophic microorganisms (Figure 2.1) (Das and Veziroglu, 2008; Kotay and Das, 2008). In autotrophic conversions (also known as direct or indirect biophotolysis), solar energy is directly converted to hydrogen via photosynthetic reactions mediated by photosynthetic microorganisms, i.e. microalgae, protists and photosynthetic bacteria. Under heterotrophic conditions, the organic substrates are transformed into simpler organic compounds with simultaneous production of molecular hydrogen (Das and Veziroglu, 2008; Li and Fang, 2007a). There are two types of heterotrophic conversions, photo-fermentation carried out by photosynthetic bacteria and

dark fermentation (DF) carried out by anaerobic bacteria that convert carbohydrates into biohydrogen.

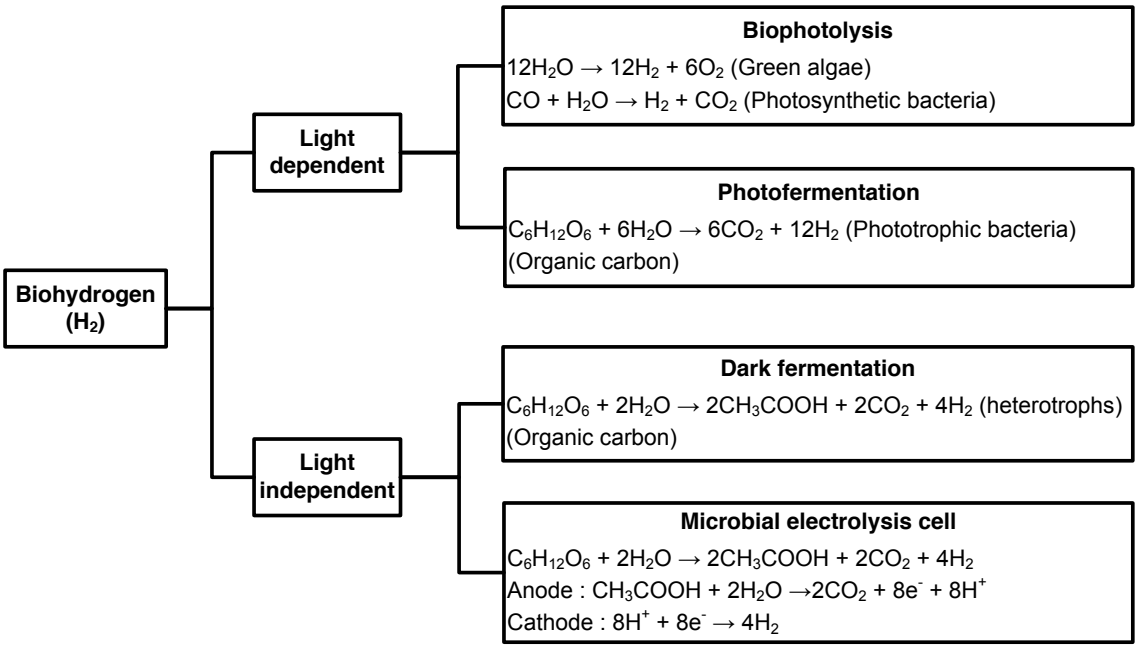


Figure 2.1 - Biological pathways to produce hydrogen

DF is the most studied and promising technology for biohydrogen production owing to its higher production rates and treatment capacity for organic wastes. Several substrates rich in carbohydrates are also usable, such as first generation fuel crops (e.g. sugar cane, wheat, corn, and sugar beets) as well as second generation biomass like agricultural residues as well as industrial waste and wastewater (Das and Veziroglu, 2008). In recent years, there are increasing research activities in this domain, as shown by the increasing number of peer-reviewed articles with “dark fermentation” in the title (Figure 2.2).

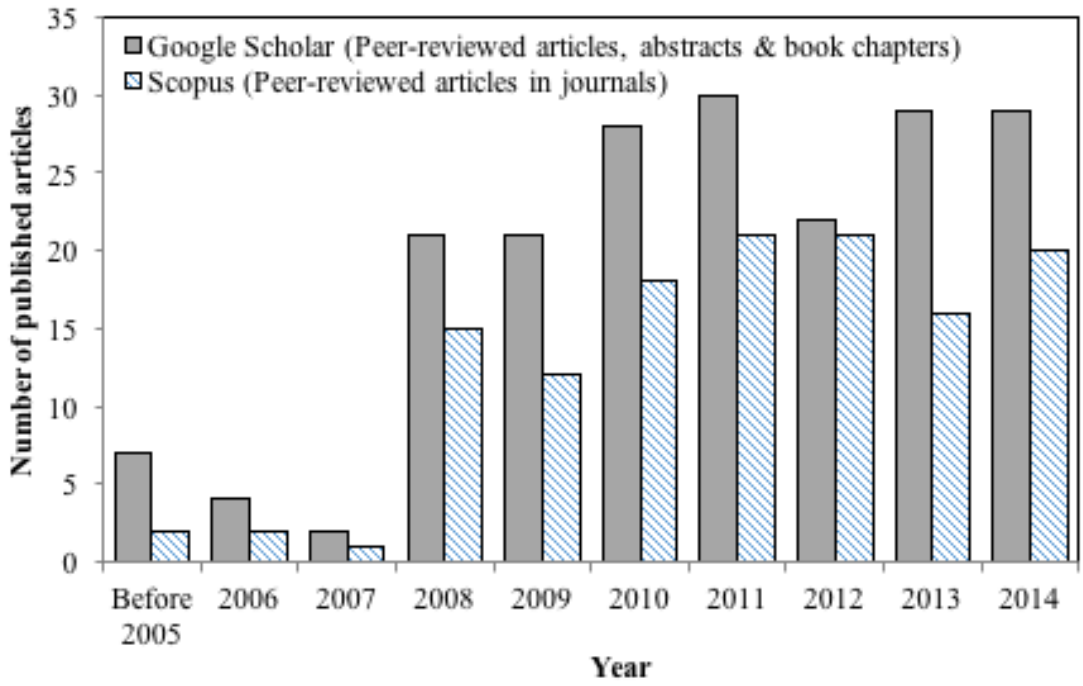


Figure 2.2 - Number of peer reviewed publications on DF published in the last decade (Google Scholar, 2014; Scopus, 2014)

At present, DF process development at industrial scale is limited by its lower hydrogen yield compared to its theoretical maximum yield of 4 moles of H_2 per moles of hexose, as well as the estimated costs associated with the H_2 production. There are areas for improvement to achieve higher H_2 yields and production rates by optimizing the design and operation of DF bioreactors (Show et al., 2011). The H_2 production cost in scaled-up systems can be minimized by using low cost renewable materials such as waste biomass as feedstock (Kapdan and Kargi, 2006; Ren et al., 2011). Inoculum enrichment methods (De Gioannis et al., 2013; Li and Fang, 2007a; Ntaikou et al., 2010; Show et al., 2012; Wong et al., 2014) can improve the H_2 yield, and pre-treatment of substrates can also enhance the biohydrogen production by improving the biodegradability of substrates (Ariunbaatar et al., 2014; Monlau et al., 2013b; Motte et al., 2014). Recently, there has been growing interest on coupled processes to obtain a higher H_2 yield by integrating DF with processes like photofermentation (PF) (Rai et al., 2014; Redwood et al., 2008) or bioelectrochemical systems (Chookaew et al., 2014; Guwy et al., 2011; Moreno et al., 2015). Because of the profitable production of biomethane, a coupled DF-methanogenic stage has also been a popular choice which increases the sustainability of the coupled-

process by improving the energy recovery from the DF residues (Elbeshbishy and Studies, 2011; Gómez et al., 2011; Gottardo et al., 2013).

The aim of this paper is to provide an updated overview of advancements in biohydrogen production via DF of organic biomass. Regardless of the increasing number of research articles and reviews published, there is a need to provide an extended overview of dark fermentative biohydrogen production with the utilization of by-products and the future challenges and prospects for its up-scaled development. This review provides an insight on the factors that influence the biochemical pathways in dark fermentative biohydrogen production to increase the H_2 yield and post-utilization of DF residues to realize its future sustainability. To summarize, this review provides an extended insight on a) possible feedstock or substrate sources and their biohydrogen potential (BHP), b) factors that influence the fermentative H_2 yield: (i) inoculum sources and enrichment methods, (ii) pre-treatment of substrates and (iii) bioreactor operation and design (culture pH, temperature and OLR, HRT, H_2 partial pressure, nutrients and elements addition), c) utilization of DF residues, d) pilot scale systems and e) challenges and future prospects: (i) modeling and simulation of DF process, (ii) energy balance and conversion of organic carbon, (iii) natural pH control and (iv) future prospects of solid state dark fermentation.

2.1.2 Microbiology and biochemical pathways of DF

In DF processes, carbohydrate-rich substrates are broken down anaerobically by hydrogen-producing microorganisms, such as facultative anaerobes and obligate anaerobes. Molecular hydrogen (H_2) is produced in the process of disposing the excess electrons through the activity of the hydrogenase enzyme (Das and Veziroglu, 2001; Li and Fang, 2007a). Under anaerobic environments, protons (H^+) can act as electron acceptors to neutralize the electrons generated by oxidation of organic substrates, consequently producing H_2. In contrast with aerobic respiration, where oxygen is reduced and water is the final product (Das and Veziroglu, 2008; Wang and Wan, 2009).

In the DF of glucose as the model substrate, H_2 -producing bacteria initially convert glucose to pyruvate through glycolytic pathways producing adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and the reduced form of nicotinamide adenine dinucleotide (NADH) (Li and Fang, 2007a). Pyruvate is further oxidized to acetyl coenzyme A (acetyl-CoA), carbon dioxide (CO_2) and H_2 by pyruvate ferredoxin oxidoreductase and hydrogenase. Depending on the type of microorganism and

environmental conditions, pyruvate may also be converted to acetyl-CoA and formate which may be further converted into H_2 and CO_2. Also, acetyl-CoA might be converted to acetate, butyrate, and ethanol (Li and Fang, 2007a). DF of complex carbohydrates by mixed anaerobic microbiota can result in a wide range of intermediates and by-products depending on the operational parameters, such as substrate type, substrate loading rate, pH, temperature and other operating and environmental conditions, as they also influence the microbial community structure in bioreactors. Figure 2.3 gives a schematic representation of the different steps and biochemical pathways involved in the DF of complex organic biomass.

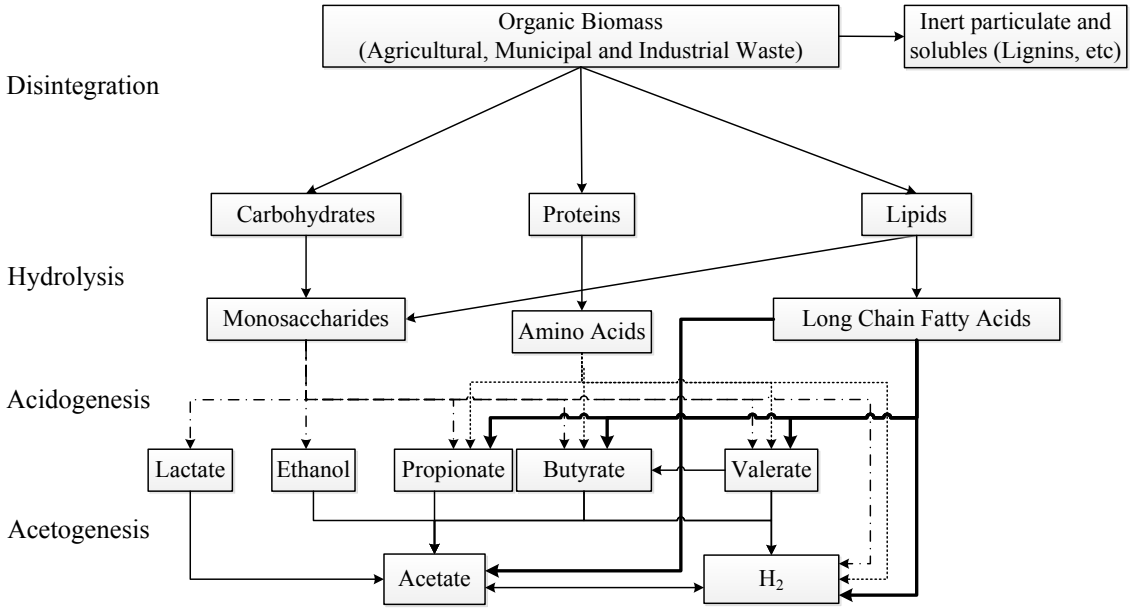


Figure 2.3 - Biodegradation and microbiological pathways involved in the fermentative breakdown of waste biomass (Adapted and modified from Peiris et al. (2006)

These biochemical pathways (Figure 2.3) can be mediated by strict anaerobes (*Clostridia*, methylotrophs, rumen bacteria, methanogenic bacteria, archea, etc.), facultative anaerobes (*Escherichia coli*, *Enterobacter*, *Citrobacter*), and even aerobes (*Alcaligenes*, *Bacillus*) (Li and Fang, 2007a). Acetate and butyrate are the most common products of DF (Hawkes et al., 2007). Common biochemical reactions during DF undertaken by facultative anaerobes are:

$$C_6H_{12}O_6 + 2H_2O \rightarrow \underset{\text{(Acetic acid)}}{2CH_3COOH} + 2CO_2 + 4H_2 \qquad (2.1)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (2.2)$$

(Butyric acid)

When the metabolic pathway is such that it favors the production of acetic acid, the stoichiometric yield of H_2 is 4 moles for each mole of glucose (i.e. 544 mL H_2/g hexose at 25 °C) as in equation 2.1, whereas the yield of H_2 is 2 moles for a mole of glucose (i.e. 272 mL H_2/g hexose at 25 °C) when the final product is butyric acid (equation 2.2) (Li and Fang, 2007a). However, the actual hydrogen yield is lower than the theoretical yield as part of the substrate is utilized for biomass production and the degradation of the substrates might follow other biochemical pathways without hydrogen production (Hallenbeck and Benemann, 2002; Nath and Das, 2004). Under some conditions, the metabolic pathways lead to ethanol and acetate production, lowering the stoichiometric hydrogen yield to 2 moles of H_2 for a mole of glucose (i.e. 272 mL H_2/g hexose at 25 °C) as represented in equation 2.3 (Li and Fang, 2007a):

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2OH + CH_3COOH + 2CO_2 + 2H_2 \quad (2.3)$$

A widely studied clostridia species, *Clostridium butyricum*, is responsible for the production of butyric acid as the major product of fermentation together with acetate and hydrogen (Hawkes et al., 2007). Another fermentation pathway is the production of propionate by *Clostridium articum* which is a hydrogen consuming pathway (equation 2.4). Similarly, metabolic pathways leading to only ethanol and lactic acid production by *Clostridium barkeri* yield no hydrogen (equations 2.5 and 6) (Khanal et al., 2003):

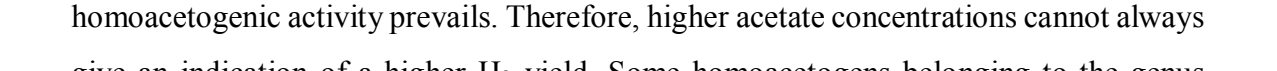
$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \quad (2.4)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \quad (2.5)$$

$$C_6H_{12}O_6 \rightarrow CH_3CHOHCOOH + 2CO_2 \quad (2.6)$$

Hawkes et al. (2007) and Kim et al. (2006) proposed the molar ratio of butyric to acetic acid (B/A ratio) as a quantitative indicator of the biohydrogen yield associated with microbial metabolic pathways. Kim et al., 2006) found that B/A ratios were directly proportional to H_2 yields (mol H_2/mol hexose) during DF of sucrose in CSTR reactors operated at an organic loading rate (OLR) of 10 - 60 g Chemical Oxygen Demand (COD)/L, pH 5.5 and 12 h hydraulic retention time (HRT). They also reported that a B/A ratio higher than 2.6 indicated an efficient H_2 production by anaerobic microbiota. In DF with mixed cultures, when a B/A ratio of 3:2 is generally observed, results in a H_2 yield

of 2.5 moles H_2 per mole of hexose fermented as given in equation 2.7 (Hawkes et al., 2007):



$$4C_6H_{12}O_6 + 2H_2O \rightarrow 3CH_3CH_2CH_2COOH + 2CH_3COOH + 8CO_2 + 10H_2 \quad (2.7)$$

In contrast, Guo et al. (2013) showed in their study performed with lignocellulosic substrates that this ratio might not give a good indication, particularly in batch tests where homoacetogenic activity prevails. Therefore, higher acetate concentrations cannot always give an indication of a higher H_2 yield. Some homoacetogens belonging to the genus *Clostridium* (e.g. *C. aceticum*) can lower the H_2 yield by converting H_2 and CO_2 to acetate or can convert hexose directly to acetate (Hawkes et al., 2007; Kim et al., 2006). However, analysis of soluble metabolites can give an indication of the fermentation pathways and thus the H_2 production performance.

Clostridia have been identified as the dominant hydrogen producing microorganisms in DF operated with mesophilic mixed cultures at a pH of 5.5 (Fang et al., 2002). Fang and Zhang (Fang et al., 2002) identified that 64.6% of all the microorganisms were affiliated with three *Clostridium* species (*Clostridiaceae*), 18.8% with *Enterobacteriaceae*, and 3.1% with *Streptococcus bovis* (*Streptococcaceae*) based on the phylogenetic analysis of the rDNA sequences. Interestingly, Rafrafi et al. (Rafrafi et al., 2013) reported recently that sub-dominant species, in spite of their low abundance, can also have substantial impact on the hydrogen production performance. The presence of some species like *E. coli* can aid in increasing the H_2 yield by diverting the metabolic pathways to the acetate and butyrate hydrogen producing pathways (equation 2.7), while other species communities such as *Bacillus spp*. and *Lactobacillus spp*. can lower the H_2 yield by diverting the pathway to lactate accumulation (equation 2.6).

Other results of the identification of the microbial diversity by community fingerprinting techniques in the thermophilic DF of rice straw showed that hydrolytic and fermentative bacteria such as *Clostridium pasteurianum, Clostridium stercorarium and Thermoanaerobacterium saccharolyticum* dominated in the sludge of a repeated fed-batch reactor (Chen et al., 2012).Shin et al. (Shin et al., 2004) detected the hydrogen producing microorganisms *Thermoanaerobacterium thermosaccharolytium* and *Desulfotomaculum geothermicum* in a thermophilic acidogenic culture, while *Thermotogales* strains and *Bacillus* species were detected in a mesophilic acidogenic culture by Polymerase Chain Reaction (PCR)-Denaturing Gradient Gel Electrophoresis

(DDGE) analysis during DF of food waste. In another study, Quemeneur et al. (Quéméneur et al., 2011) investigated the potential of a molecular capillary electrophoresis-single strand conformation polymorphism (CE-SSCP) fingerprinting method based on the *hydA* functional genes to better describe the bacterial community dynamics in a mixed dark fermentative culture at different pH conditions.

Some undesirable microorganisms which lower the total H_2 yield might be present in mixed cultures of fermentative microorganisms, either by consuming the H_2 produced or by altering the biochemical pathways of the H_2 synthesis (Li and Fang, 2007a). The main H_2 consumers include methanogens, homoacetogenic bacteria and sulfate reducing bacteria (SRB). The activity of these hydrogen consumers can be controlled by inoculum pre-treatment methods or bioreactor operating conditions (Guo et al., 2010; Wang and Wan, 2009). The activity of methanogens and SRB can be significantly reduced by operating at a pH below 6 along with the control of the HRT and OLR. Therefore, hydrogen production via a mixed dark fermentative culture is a complex microbial system, influenced by a number of parameters such as substrate types, substrate pretreatment, inoculum type, inoculum enrichment method, bioreactor design and operation.

2.1.3 Potential sources of organic biomass for fermentative biohydrogen production

The substrate plays an important role in the H_2 yield, H_2 production rate and the overall economy of the process. These are mainly dependent on the substrate´s carbohydrate content, bioavailability and biodegradation rate (Chong et al., 2009; Guo et al., 2010; Kapdan and Kargi, 2006; Ntaikou et al., 2010; Ren et al., 2011). Carbohydrate rich substrates have been extensively used in DF studies, in particular pure glucose, sucrose and starch mixtures (Wang and Wan, 2009). But renewable biohydrogen production requires the substrate or feedstock to come from renewable resources (Hawkes et al., 2007; Ren et al., 2011). Second generation biomass sources, such as waste biomass, are abundant and can thus support the supply of renewable substrates for DF (Guo et al., 2010; Kapdan and Kargi, 2006; Show et al., 2012). Besides biohydrogen and volatile fatty acids as valuable by-products, DF also offers biological treatment of the organic waste.

In more recent dark fermentative studies, complex substrates have been considered, such as the organic fraction of municipal solid waste (OFMSW) (Chen et al., 2012; Nissilä et

al., 2011; Tawfik and El-Qelish, 2012; Valdez-vazquez et al., 2005; Zhang et al., 2007), agricultural residues like lignocellulosic biomasses (e.g. rice straw, wheat straw and corn stalks), agro-industrial wastes like those from food processing industries (e.g. olive mill wastewater and cheese whey), effluents from livestock farms and aquatic plants (Kapdan and Kargi, 2006; Show et al., 2012). With the integration of DF within a biorefinery concept, the waste generated from biofuel production such as crude glycerol (Chookaew et al., 2014; Varrone et al., 2012), de-oiled algal cake (Venkata Subhash and Venkata Mohan, 2014) or cotton seed cake (Panagiotopoulos et al., 2013) can be utilized as a substrate, while dark fermentative metabolites can be utilized in the production of micro-algal biomass (Liu et al., 2013; Lo et al., 2010; Turon et al., 2015) and biodiesel (Fei et al., 2011), which in turn can serve as feedstock for DF processes.

Table 2.1 - Dark fermentative biohydrogen potential of different waste biomass under varying operating conditions

Substrate type	Microbial inoculum source	Reactor type	Temperature (°C)	pH	Maximum H_2 yield (mL H_2/g VS_{added})	Maximum H_2 production rate	H_2 in biogas (%)	Reference
Food waste	Heat shock treated anaerobic sludge	Leaching Bed Reactor	37	5.5 - 7	310	151.25 mL H_2/L/h	10—55	(Han and Shin, 2004)
Food waste	Thermophilic acidogenic culture	Batch	55	4.5	46.3	3 mL H_2/g VSS/h	23	(Shin et al., 2004)
Vegetable kitchen waste	Kitchen waste compost	Intermittent-CSTR	55	6.0	38[a]	1.0 L H_2 /L/d	40	(Z.-K. Lee et al., 2010)
Food waste and sewage sludge	Anaerobic digester sludge	Batch	35	5.0–6.0	122.9[a]	111.2 mL H_2/g VSS/h	-	(Kim et al., 2004)
OFMSW	Anaerobic digestate	Semi-continuous CSTR	55	6.4	360[b]	-	58	(Valdez-vazquez et al., 2005)
OFMSW	Non-anaerobic inocula (soil, pig excreta)	Packed bed reactor	38	5.6	99[b]	-	47	(Alzate-Gaviria et al., 2007)
Wheat straw	Cow dung compost	Batch	36	6.5	68.1	10.14 ml H_2/g VS/h	52	(Fan et al., 2006)
Rice straw	Wastewater treatment plant sludge	Batch CSTR	55	6.5 (initial)	24.8[c]	-	-	(Chen et al., 2012)
Corn stalk wastes with acidification pre-treatment	Enriched cow dung composts	Batch CSTR	50	7 (initial)	149.69	7.6 mL H_2/h	45–56	(Zhang et al., 2007)
Rice slurry	Anaerobic digester sludge	Batch	37	4.5 (initial)	346[d]	2.1 L/g VSS/d	45–56	(Fang et al., 2006)
Cheese whey	Adapted anaerobic sludge	Batch	55	7 (initial)	111[e]	3.46 mL H_2/ L/h	-	(Kargi et al., 2012a)

Pig slurry	Mesophilic methanogenic sludge	CSTR	70	6.7 (feed)	3.65	-	-	(Kotsopoulos et al., 2009)
Untreatedde-oiled algae cake	Anaerobic digester sludge	Batch	29	6 (initial)	66[f]	0.08 ml/h	-	(Venkata Subhash and Venkata Mohan, 2014)
Potato and pumpkin mixture	BESA treated anaerobic sludge	Batch	35	7.4 (initial)	171.1	-	-	(Ghimire et al., 2015b)

[a] mL H_2/g COD, [b]mL H_2/g $VS_{removed}$, [c]mL/g TS, [d]mL H_2/g carbohydrate, [e]mL H_2/g total sugar, [f]mL H_2/g algal biomass

Based on their availability, novel low-cost substrate sources need to be explored and assessed for their biohydrogen potential (BHP). Table 2.1 presents the biohydrogen production potential of different organic biomasses by dark fermentative process. The fermentation pathways depend on the substrates and the microbial metabolism (Li and Fang, 2007a). It has been well established that the type of substrate influences the biohydrogen yields (Choi and Ahn, 2013; Guo et al., 2013). Monlau et al. (2012) and Guo et al. (2013) in their studies reported that the soluble and readily accessible sugars represent the main fraction of biomass that can be converted into hydrogen. However, the biohydrogen production also depends on a number of parameters such as inoculum type and enrichment methods, bioreactor design and operation conditions. The latter are covered in the sections below.

Agricultural residues

Agricultural residues, which mainly include lignocellulosic wastes, are an economically viable and renewable source of second generation carbon neutral biofuels (Mtui, 2009). These include plant biomass waste, which generally contains cellulose, hemicellulose and lignin formed by of photosynthesis. Agricultural residues are produced when economically valuable products of the crops are harvested and the residues such as straw, stover, peelings, cobs stalks, bagasse and others are left over (Mtui, 2009). The 2010 global annual production of agricultural residues was around 5.1 billion dry tonnes (Eisentraut, 2010). The waste generated by the agricultural, forestry and aquaculture sectors is increasing with the increasing population and thus the waste from this sector will be increasing further in the future. Guo et al. (Guo et al., 2010) have reported the potentials and challenges of agricultural wastes as substrates for biohydrogen production. Examples of agricultural residues as a potential feedstock sources for DF processes and recent advancements in their application are discussed below.

Lignocellulosic waste

Rice straw is an example of a typical agricultural residue. It is the world's third largest agricultural residue, after maize and wheat, with a reported global yearly production of approximately 916 million tons in 2009 (Mussoline et al., 2012). Thus, the use of this abundant biomass as a feedstock in dark fermentative hydrogen production might hold future potential for feedstock supply. Similarly, wheat straw, barley straw, corn stalk, corncobs and others could be potential DF feedstock. The cellulose and hemicellulose

part of these wastes can be hydrolyzed into carbohydrates which are further biologically converted to organic acids and biohydrogen in DF processes (Table 2.2). The composition of typical lignocellulosic crop residues in terms of cellulose, hemicellulose and lignin content is presented in Table 2.2

Table 2.2 - Composition of typical agricultural waste (% of dry matter)

Component	Rice straw[a] (%)	Wheat straw[b] (%)	Barley straw[c] (%)	Corn stalk[c] (%)	Corn cob[d] (%)
Cellulose	38.6	44.1	37.2	36.7	35.3
Hemicellulose	19.7	36.0	24.4	26.2	37.1
Lignin	13.6	6.9	16.1[e]	16.9[e]	16.4[e]
Ash	-	6.1	6.4	4.9	1.5

[a]Data obtained from (Zhu et al., 2005) on wet basis; [b]Data obtained from (Motte et al., 2013); [c]Data obtained from (Panagiotopoulos et al., 2009); [d]Data obtained from (Panagiotopoulos et al., 2011); [e]Acid-insoluble lignin

The main limitation in the utilization of these valuable resources lies in the complex structure of lignocellulosic materials: a cross-linking between polysaccharides (cellulose and hemicellulose) and lignin via ester and ether linkages, which decreases their biodegradability (Hendriks and Zeeman, 2009; Mtui, 2009; Quéméneur et al., 2012; Zheng et al., 2014). Therefore, prior to DF, these biomasses are often subjected to physical, chemical and biological pre-treatment to increase their digestibility (Brodeur et al., 2011; Harmsen and Huijgen, 2010; Hendriks and Zeeman, 2009; Mtui, 2009; Quéméneur et al., 2012; Saritha et al., 2012; Taherzadeh and Karimi, 2008; Zheng et al., 2014).

Livestock waste (manure)

Livestock wastes include solid animal manure waste, fodder waste (which generally contains a lignocellulosic fraction) and wastewater, which include urine and feces. A large quantity of livestock manure comes from cattle feedlots, poultry and swine buildings, identified as pollution sources, which pose threats to the atmospheric and water environment (Cantrell et al., 2008). The current practices of management of livestock waste include its application in agricultural fields as well as biological stabilization or treatment such as composting and AD. The former management practice contributes in uncontrolled greenhouse gas emissions (mainly CH_4) from land applications. Manure

management practices can reduce direct and indirect greenhouse gas emissions by generating energy in the form of biogas from the manure prior to its land application (Cantrell et al., 2008; Wu et al., 2009; Xing et al., 2010).

However, manure substrates need physical and chemical treatment to inhibit the methanogenic activity that consumes H_2 (Cheong and Hansen, 2006; Wu et al., 2009). Another, problem that might occur during the use of this feedstock type is the inhibition of the biohydrogen production by ammonia as its high nitrogen content might cause failure of the bioreactor: swine, poultry and dairy manure have a low C/N ratio (C/N ratio of swine manure: 12.8) (Yin et al., 2014) and high levels of ammoniacal nitrogen (cattle slurry: 1.04 –1.9 g/L and chicken manure 7.0 – 12.8g/L) (Callaghan et al., 2002). Salerno et al. (2006) reported that hydrogen production is possible at high concentrations up to 7.8 g N/L in continuous flow systems if the microbial culture is initially acclimated to a lower ammonia concentration of 0.8 g N/L. total ammonia) However, the biohydrogen production decreases when the total ammonia concentration increases to above 2 g N/L (Cavinato et al., 2012). Also, high sulfate concentrations in swine manure can inhibit the biohydrogen production due to the presence of hydrogen consuming sulfate reducers (Guo et al., 2010).

Because of the high nitrogen content of animal manure, it can be used as a co-digestion substrate for nitrogen supplementation of other agricultural residues to maintain a suitable carbon to nitrogen ratio. Wu et al. (2009) reported a H_2 yield between 1.18 and 1.63 mol H_2/mol glucose in a fermentation of swine manure supplemented with glucose. Xing et al. (2010) achieved an enhanced H_2 yield of 31.5 mL/g Volatile Solids (VS) with acidification pretreated dairy manures while treating 70 g VS/L of substrate at operating pH 5.0.

Industrial waste

Agro-industries waste such as palm oil mill wastewater (Mohammadi et al., 2011; O-Thong et al., 2008, 2007; Tabatabaei et al., 2009) and olive mill wastewater (OMWW) (Eroglu et al., 2006; Ntaikou et al., 2009), tapioca industries and food industries such as brewery and dairy industries (Castelló et al., 2009; Gadhe et al., 2013; Kargi et al., 2012b; S Venkata Mohan et al., 2008) produce large quantities of carbohydrate rich non-toxic waste in the form of solid waste and wastewater. It can be potential substrates for dark fermentative biohydrogen production. Ren et al. (2006) demonstrated that waste molasses

are an excellent substrate in a pilot scale system operated under mesophilic conditions (35 °C) where very good results were obtained in terms of H_2 production rate (232 mL H_2/L/h) and yield (26.13 mol/kgCOD$_{removed}$). The production of large quantities of this type of waste biomass supports its utilization in up-scaled DF systems for continuous biohydrogen production. Similarly, cheese whey, a waste by-product generated by cheese manufacturing industries and characterized by high organic loads, comprising mainly carbohydrates (lactose), protein and lipids, is a very good potential substrate for biohydrogen production (Moreno et al., 2015; Teli et al., 2014; Venetsaneas et al., 2009).

Organic fraction of municipal waste

Organic fraction of municipal waste (OFMSW) generally constitutes food waste which contains a high biodegradable carbohydrates fraction with 85-95% volatile solids and 75-85% moisture content making it a good substrate for DF (Guo et al., 2010). Food waste present in municipal waste is mainly responsible for methane emissions and leachate production from landfills (Jiang et al., 2013). AD has been proposed as the most suitable treatment option for OFMSW or food waste with energy recovery and other environmental credentials (Esposito et al., 2012). Thus, food waste has been used extensively in DF experiments (Cavinato et al., 2012; Kim et al., 2008; Lee and Chung, 2010; Pan et al., 2008; Shin et al., 2004). Gioannis et al. (De Gioannis et al., 2013) have reviewed the studies of DF processes utilizing OFMW or food waste for dark fermentative biohydrogen production.

Large quantities of waste biosolids or sludge are generated from municipal wastewater treatment plants which generally contain carbohydrates or polysaccharides and proteins (Wang et al., 2003). Several researchers have used the available carbohydrates present in these biosolids in fermentative hydrogen production (Cai et al., 2004; Kim et al., 2004). However, the sludge needs pre-treatment, such as ultrasonication, acidification, sterilization, freezing-thawing or alkaline pre-treatment, to facilitate the fermentative process (Cai et al., 2004; Wang et al., 2003). Besides, Kim et al. (2004) demonstrated the usefulness of sewage sludge as co-substrate in the DF of food waste.

2.1.4 Factors affecting DF pathways and H_2 yield

DF via mixed cultures is a complex system where environmental factors and bioreactor operation conditions such as temperature, pH and H_2 partial pressure regulate metabolic

pathways of hydrogen producing microorganisms (Guo et al., 2010; Li and Fang, 2007a; Liu et al., 2006; Wang and Wan, 2009). In addition, substrate types and their pre-treatment methods, bioreactors configurations, inoculum sources and enrichments also influence the biohydrogen production. Three categories of parameters that influence the DF pathways, and thus the yield of biohydrogen, can be distinguished (Figure 2.4). These parameters are reviewed below and compared in relation to H_2 yield and production rate.

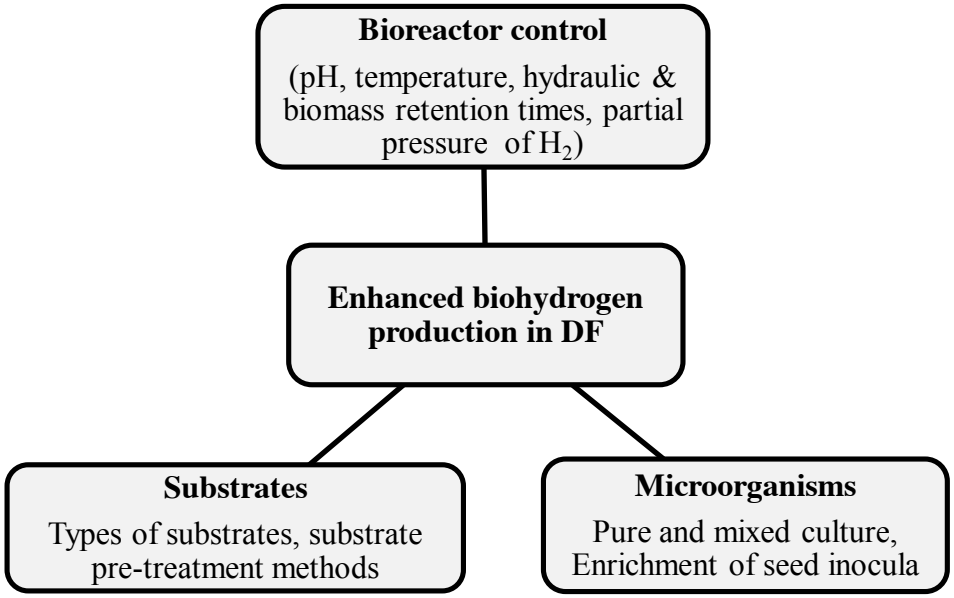


Figure 2.4 - Strategies to enhance the biohydrogen yield in DF of organic biomass

2.1.5 Inoculum and enrichment methods

The hydrogen producing seed inoculum or culture is very important for the startup of the hydrogen production process. Several studies using pure cultures have been done using a range of substrates (Table 2.3). Various species of *Clostridia* and *Enterobacter* are widely used in pure cultures (Table 2.3). Lee et al. (2011) and Elsharnouby et al. (Elsharnouby et al., 2013) have reviewed the studies of DF performed with pure cultures. Table 2.3 presents some of the dark fermentative biohydrogen studies done with pure cultures.

Table 2.3 - Biohydrogen production studies using pure culture

Culture	Substrate	Culture type	Temp. (°C)	pH	Optimum H_2 yield (mol H_2/mol glucose eqv.)[a]	Reference
Enterobacter cloacae IIT-BT08	Glucose	Batch	36	6	2.2	(Kumar and Das, 2000)
Clostridium thermolacticum DSM 2910	Lactose	Continuous	58	7	1.5	(Collet et al., 2004)
Enterobacter cloacae DM 11	Malt, yeast extract & glucose	Continuous	37	6	3.9	(Mandal et al., 2006)
Caldicellulosiruptor saccharolyticus DSM 8903	Hydrolyzed potato steam peels	Batch	70	6.9	3.4	(Mars et al., 2010)
Thermotoga neapolitana DSM 4349	Hydrolyzed potato steam peels	Batch	80	6.9	3.3	(Mars et al., 2010)
C. thermocellum DSM 1237and C. thermopalmarium DSM 5974	Cellulose	Batch	55	7	1.36	(Geng et al., 2010)
Clostridium thermocellum 7072	Corn stalk	Continuous (100 Liters)	55	7.2	1.2	(Cheng and Liu, 2011)

[a] mol H_2/mol glucose equivalent was calculated based on the information provided from references at Standard Temperature and Pressure (STP) (0°C and Pressure 1 atm)

H_2 synthesizing bacteria exist commonly in environments such as soil, wastewater sludge and compost. All these materials can thus be used as an inoculum for fermentative H_2 production (Li and Fang, 2007a). Indeed, cow dung, anaerobic sludge, municipal solid waste, soil and compost are some of the common sources of mixed cultures. A mixed culture of hydrogen producers is generally preferred over a pure culture due to its practicability for environmental engineering applications, economic benefits in operation (as it can economize asepsis costs), easiness in control based on differential kinetics of microbial subgroups and broader feedstock choice (Li and Fang, 2007a; Valdez-vazquez et al., 2005; Wang and Wan, 2009). However, enrichment of mixed cultures becomes necessary to enhance the biohydrogen production on the one hand and inhibit hydrogen consumers such as methanogens and homoacetogens, often present in these mixed inocula (Wang and Wan, 2009), on the other hand. Table 2.4 summarizes the common pre-treatment measures adopted for enrichment of hydrogen producers.

Pre-treatment of the inoculum to obtain an enrichment of hydrogen producers often relies on the spore forming characteristics of H_2 producers such as *Clostridium*, which are ubiquitous in anaerobic sludge and sediments (Faloye et al., 2013; Li and Fang, 2007a; S Venkata Mohan et al., 2008; Wang and Wan, 2008). These organisms have a better chance to survive the harsh conditions during the pre-treatment of the inoculum than the non-spore forming bacteria such as methanogens, as the spores can germinate again under favorable conditions (Li and Fang, 2007a; Wong et al., 2014). Heat treatment of mixed cultures for the enrichment of H_2 producers is a simple, inexpensive and effective method (Li and Fang, 2007a; Wang and Wan, 2009). However, the effect of heat treatment might be different depending on the inoculum source such as activated sludge or anaerobic sludge (Wang and Wan, 2009). Some studies (O-Thong et al., 2009; Zhu and Beland, 2006) reported a lower hydrogen yield by a heat shock treated seed inoculum than obtained by other pre-treatment methods. This could be due to the inhibition of other non-spore forming hydrogen producing bacteria which might destabilize the main hydrogen production pathways. Similarly, acid or base treatment is based on the notion that the activity of methanogens drops sharply at a pH below 6.3 or above 7.8 (Li and Fang, 2007a), while the activity of *Clostridia sp*. and other hydrogen producers is not affected by an acidic pH (below pH 6).

Other pre-treatment methods such as chemical pretreatment and aeration are directed towards the selective inhibition of methanogens present in anaerobic sludge, which are very sensitive to changes in environmental conditions. Besides being strict anaerobes, methanogens are sensitive to many chemicals (Li and Fang, 2007a). Thus, oxygen can inhibit their activity during aeration (Wang and Wan, 2008; Zhu and Beland, 2006). Wang and Wan (2008) aerated the inoculum sludge with air for 24 hours to inhibit the activity of methanogens. Likewise, chemical inhibitors like sodium 2-bromoethasulfonic acid (BESA), iodopropane, chloroform and acetylene are used to inhibit methanogens (Li and Fang, 2007a; O-Thong et al., 2009; Venkata Mohan et al., 2008; Wang et al., 2011; Zhu and Beland, 2006). Thus, selective inhibitors like chloroform or BESA selectively inhibit the activity of H_2 consumer methanogens. In methanogens, BESA functions by inhibiting the activity of co-enzyme M reductase complex, which is a key co-enzyme of methanogenesis (Venkata Mohan et al., 2008; Zhu and Beland, 2006).

Table 2.4 - Pre-treatment methods used to enriching hydrogen producing microorganisms in anaerobic sludge

Treatment	Description	Inoculum source	Reference
Heat	100 °C for 15 min	Anaerobic digested sludge	(Wang and Wan, 2008)
Heat	80°C, 90°C and 100°C for 15-30 min	Anaerobic sludge	(Wang et al., 2011)
Heat	Heating in boiling water bath for 10-30 min	Anaerobic granular sludge	(Mohammadi et al., 2011)
Heat	105 °C for 4 hour	Anaerobic granular sludge	(Giordano et al., 2011)
Heat	Incubation at 90 °C for 1 hour	Anaerobic granular sludge	(Luo et al., 2010a)
Heat	100–105 °C in oven for 2 hour	Cow dung compost	(Fan et al., 2004)
Acid	pH to 2 for 24 h and increasing pH to 5.5 by adding a 2 N NaOH solution	Anaerobic digested sludge	(Lee et al., 2009) (Mohammadi et al., 2011)
Acid	pH 3 with 2 N HCl for 24 hours	Anaerobic digested sludge	(Luo et al., 2011)
Acid	pH to 3 with 1 N HCl for 30 min	Anaerobic digested sludge	(Zhu and Beland, 2006)
Acid	pH 3 with 0.1 N HCl solution for 24 hours and adjusting back to pH 7	Anaerobic granular sludge	(Hu and Chen, 2007)
Base	pH of the sludge to 3 with 1 mol/L of NaOH for 24 hours	Anaerobic digested sludge	(Wang and Wan, 2008)
Base	pH 8, 9 and 10 with 1 mol/L of NaOH for 3 hours	Anaerobic sludge	(Wang et al., 2011)

Base	pH 12 with 1 M NaOH for 24 hours and adjusting back to pH 7 using 1 M HCl	Anaerobic digested sludge	(O-Thong et al., 2009)
Load shock	Sludge (50 ml) spiked with 40 g of sucrose and acidification for 2 d	Anaerobic granular sludge	(Luo et al., 2010a)
Load shock	Sludge (50 ml) spiked with 500 mL of sucrose (50 g/L) and acidification for 2 d	Anaerobic digested sludge	(O-Thong et al., 2009)
Chemical inhibition	10 mmol of BESA for 30 min and gravity separation for 2 h	Anaerobic digested sludge	(Zhu and Beland, 2006)
Chemical inhibition	0.2 g/l BESA for 24 h	Anaerobic granular sludge	(Venkata Mohan et al., 2008)
Chemical inhibition	0.1% (v/v) chloroform for 24 h	Anaerobic digested sludge	(Mohammadi et al., 2011)
Aeration	Aerate with air for 24 hours	Anaerobic sludge	(Wang and Wan, 2008)
Aeration	Flushing with air for 30 min	Anaerobic sludge	(Zhu and Beland, 2006)
Microwave irradiation	Microwave radiation for 1.5 min	Cow dung compost	(Song et al., 2012)

Table 2.5 - Comparison of various inoculum pre-treatment methods for enriching hydrogen producing inocula

Inoculum source	Inoculum treatment methods	Substrate	Culture Temperature (°C)	Culture pH	Optimal pre-treatment method	Maximum H_2 Yield (mol H_2/mol glucose eqv.)[a]	Maximum H_2 Production Rate (mL H_2/L/h)	Reference
Anaerobic digested sludge	HS[b], aeration, acid, base, BESA[c] and iodopropane	Sucrose	35	-	Base treatment	3.06	-	(Zhu and Beland, 2006)
Anaerobic granular sludge	HS, acid and base	Glucose	35	-	Chloroform	1.55	-	(Hu and Chen, 2007)
Anaerobic sludge (UASB)	Acid, BESA, HS and their four combination	Dairy wastewater	29	-	BESA	0.0317[d]	-	(S Venkata Mohan et al., 2008)
Anaerobic digested sludge	Acid, base, HS, aeration and chloroform	Glucose	36	7	Heat shock treatment	1.9	120.4 mL H_2/h	(Wang and Wan, 2008)
Anaerobic digested sludge	Acid, base, LS[e], HS and BESA	Sucrose	60	5.5	Load shock treatment	1.96	11.2 mmol H_2/L/h	(O-Thong et al., 2009)
Suspended & granular anaerobic sludge mixture	HS, chloroform and combination of both	Ground wheat solution	37	7	Repeated heat shock treatment	25.7[f]	-	(Argun and Kargi, 2009)
Anaerobic sludge	HS, acid and base	Glucose	35	6.2	Heat treatment at 80°C for 30 min	3.84	-	(Wang et al., 2011)

[a] Calculated based on the information provided from references at Standard Temperature (0°C and Pressure 1 atm), [b]HS :Heat shock, [c]BESA:2-bromoethanesulfonic acid, [d]mmol H_2/g COD, [e]LS : Load shock, [f]mL H_2/g cells/h

Table 2.6 - Evaluation of inoculum pre-treatment methods to enhance the DF capacity of the inoculum sludge

Pretreatment Method	Energy Requirement	Chemical Requirement	Economic cost	Scale-up application
Heat shock treatment	+ + +	+	+ + +	+ +
Acid treatment	+	+ + +	+ +	+ + +
Chemical Treatment	+	+ + +	+ + +	+ +
Aeration	+ + +	+	+ +	+ + +
Load shock treatment	+ +	+ +	+	+ + +

+ Less intensive; ++ Moderately intensive; +++ Very intensive

The effect of inoculum enrichment methods on H_2 production is different based on the source of inoculum (Table 2.5). However, in order to select an inoculum pre-treatment method for scaled-up systems, several parameters needs to be considered, such as operational costs, feasibility or complexity of the methods, time for the enrichment of the hydrogen producing seed, use of the DF residues in the post treatment processes. Table 2.6 gives a simple assessment of the commonly applied inoculum pre-treatment methods based on the authors' information from the literature. The selection of a chemical treatment method such as using BESA inhibits the methanogens, which will give problems when the DF residues are to be used in AD. In addition, BESA is not environmental friendly and expensive to use a large industrial scale (Li and Fang, 2007a). Likewise, heat shock treatment requires large energy inputs, which makes it less attractive for large-scale applications. Acid and shock load pre-treatment can be applied at large scale to select the hydrogen producing inocula without net energy concerns.

There have been some dark fermentative studies done without the addition of seed inoculum, utilizing the microorganisms present in the waste itself (Favaro et al., 2013; Kim et al., 2009). The fermentative hydrogen production took longer than in the tests with inoculum supply. Nonetheless, inoculum pre-treatment is important in batch tests or at process start-up. A high rate hydrogen producing microbial community can be develop in the fermentative bioreactors when applying appropriate reactor operating conditions (Castelló et al., 2009; Fang and Liu, 2002; Lee et al., 2008; Zahedi et al., 2014).

2.1.6 Design and operation of bioreactors

The process design for dark fermentation depends mostly on substrates which limits the operational conditions of bioreactors such as culture temperature (mesophilic or thermophilic), reactor configurations (reactor types, wet, semi-dry or dry conditions) and

feeding modes (mono substrate or co-substrates) (Motte et al., 2013). Weiland (Weiland, 2006) reported the several types of bioreactors used for the conversion of agricultural biomass to energy through upscaled AD systems. Although these bioreactors are designed for biomethanation by AD, these can be used for biohydrogen production after modification of some operational parameters (Guo et al., 2010).

Bioreactor configuration

Different DF bioreactor configurations have been used in laboratory studies for a wide range of substrates (see Tables 2.2, 2.7 and 2.8). Most of the dark fermentative hydrogen production studies are carried out in a batch CSTR under wet conditions (<10 % total solids, TS). Besides CSTR, many studies have been carried out in anaerobic fluidized bed reactor (AFBR), anaerobic sequencing batch reactors (ASBR), fixed or packed bed reactors, UASB reactor, leaching bed reactor, anaerobic baffled reactors, plug flow reactors or membrane bioreactors (MBR) and with an objective to enhance the biohydrogen yield and production rate. Recent research (Motte et al., 2014, 2013; Robledo-Narváez et al., 2013; Valdez-Vazquez and Poggi-Varaldo, 2009) has focused on the application of high solids processes such as semi-dry (10–20 % TS) and dry (>20 % TS) DF processes for biohydrogen production, as the interests in the conversion of second generation lignocellulosic biomass (mostly agro-industrial residues) is growing.

Studies have correlated the biohydrogen production with the size of the microbial population and therefore different cell retention strategies have been investigated (Show et al., 2011, 2010; Zhang et al., 2008). The latter include sludge granulation and biofilm systems to increase the bacterial concentration in the reactor. The results of these studies showed that the volumetric hydrogen production rate of a bioreactor depends on the ability to maintain a high microbial density. Gavala et al. (2006) showed higher hydrogen production rates in a UASB (which has a granular biomass retention) than in a CSTR at low retention times (19.05 and 8.42 mmol H_2/h/l, respectively at 2 h HRT), while the CSTR reactor gave higher hydrogen yields (mmol H_2/mol glucose) at all HRTs tested. This suggests a compromise should be sought between technical efficiency (based on H_2 yields) and economic efficiency (based on H_2 production rate), when one of these two systems is selected.

Show et al. (2010) compared the performance of a CSTR and an AFBR for biohydrogen production using different biomass growth strategies with glucose as the substrate. The

different bioreactor configurations used in their research were: suspended sludge CSTR system, granular sludge CSTR system, granular sludge AFBR system and biofilm AFBR system. The maximum H_2 yield of their suspended sludge CSTR system, granule reactor and biofilm amounted to, respectively, 1.92 mol H_2/mol glucose at a HRT of 6-12 hours, 1.83±0.09 mol H_2/mol glucose at a HRT of 0.5 hours and 1.81 ± 0.08 mol H_2/mol glucose at a HRT of 0.5 hours.

Besides the specific advantages of these different bioreactor systems, the major drawbacks are the washout of hydrogen-producing bacteria at short HRT in CSTR systems, low conversion rates in granular reactor systems and rapid biofilm development leading to fragmentation and separation from the supporting media in biofilm systems (Show et al., 2010). In another study by Zhang et al. (2008), their biohydrogen production potential of biofilm based and granule based reactors were compared. They concluded that the granule based system was advantageous as it gave better results in terms of biomass retention without being subjected to washout of the biomass support carriers.

The incompatibility of the use of high organic loading rates and rapid microbial growth in biofilm systems makes them thus less attractive than granular systems. Show et al. (2010) recommended the column-shaped granular reactor for fermentative biohydrogen production from wastewater though the system is not suitable for digestion of substrates with a high solids content or for a longer retention time in which anaerobic granules may disaggregate. High rate bioreactors are necessary to convert complex organic biomass like OFMSW and agricultural waste.

Hydraulic retention time (HRT)

The HRT can affect substrate hydrolysis and thus the production of intermediates and products, thus affecting fermentative H_2 production. Besides hydrolysis, the HRT can also be used as control parameter of the methanogenic activity. Some studies have demonstrated the effect of the HRT on the biohydrogen production in DF processes (dos Reis and Silva, 2011; Kim et al., 2006; Liu et al., 2008; Pakarinen et al., 2011). The different growth rates of hydrogen producers and consumers make it possible to use the HRT as a controlling parameter to inhibit the activity of H_2 consumers in the DF. It has been reported that low HRTs favor hydrogen production as the methanogens are washed out, and hydrogen production increases as the HRT decreases (Kim et al., 2006; Liu et al., 2008; Oh et al., 2004; Pakarinen et al., 2011). However, the optimum HRT for

biohydrogen production in DF depends on the type of substrates used as the hydrolysis rate depends on the biodegradability of the substrates (Tables 2.2, 2.6 and 2.7).

However, the HRT alone is not sufficient to fully suppress the methanogenic activity (Liu et al., 2008). Liu et al. (2008) investigated the effects of pH and HRT on hydrogen production using household solid waste as a substrate in a hyperthermophilic (70 °C) CSTR. The effect of the HRT (1, 2, 3, 4 and 6 days) at a constant pH of 7 and the effect of pH (5, 5.5, 6, 6.5 and 7) at a constant HRT of 3 days was investigated. The results of the experiments at different HRTs and constant pH 7 showed unstable H_2 production with subsequent methanogenic activities at the end. However, a combination of pH 5.5 and HRT of 3 days gave the optimum biohydrogen production conditions.

pH and temperature

The operational pH and temperature are the most crucial parameters that determine the optimum metabolic pathways of hydrogen synthesis as well as the inhibition of the hydrogen consuming processes which may occur simultaneously (Hu et al., 2005; Khanal et al., 2003). An acidic operational pH (below 6) mainly inhibits the methanogenic activity under both mesophilic and thermophilic conditions, but the inhibition of hydrogen consuming homoacetogenic activity can only be achieved under thermophilic conditions at the initial pH of 5.5 (Luo et al., 2011). Thus, the control of the process pH and temperature plays an important role in achieving high biohydrogen conversion rates by minimizing the activity of the hydrogen consumers.

The pH is one of the key parameters that can influence the metabolic pathways as it may directly affect the hydrogenase activity, an iron containing enzyme which plays a major role in DF (Dabrock et al., 1992). An acidic pH affects the activity of the hydrogenase enzyme while it is one of the important parameters for the inhibition of methanogenic activities in a mixed culture system (Khanal et al., 2003; Li and Fang, 2007a).

The optimum pH range for biohydrogen production varies from pH 4.5 (Khanal et al., 2003) to 9 (Lee et al., 2002) in DF of sucrose. Table 2.8 provides optimum operating pH ranges in different studies. The possible explanations for the disagreements in optimum pH in the various studies can be differences in inoculum sources, inoculum enrichment methods, substrate types and applied OLR (Wang and Wan, 2009).

The operational pH influences the metabolic by-products and biohydrogen yields. In most of the studies, acetate and butyrate are the major end products of favorable hydrogen synthesis (equations 2. 1, 2 and 7). Table 2.8 shows that a neutral operational pH favors the acetate pathways, while acidic pH conditions favor the butyrate pathways. However, Khanal et al. (2003) concluded the independence of the acetate and butyrate levels from different initial pH ranges studied (4.5-7.5). Similarly, Luo et al. (2010b) reported butyrate as a major VFA in the DF of cassava stillage in both BHP tests carried at the initial pH 5 and 7. Luo et al. (2011) found acetate as a major metabolic product when the operational pH was 7, while butyrate dominated at an initial pH 5.5 in the BHP tests carried under mesophilic (37 °C) conditions using an acid pre-treated inoculum. Luo et al. (2011) further reported the inhibition of homoacetogenesis can be achieved at pH 5.5 and thermophilic temperatures (55 °C).In a recent study of the DF of cheese whey from mozzarella production at different pH ranges (5.5-7.7) and a temperature of 39 °C, De Gioannis et al. (2014) reported pH 6 as the optimal pH and acetate levels were higher in all the tests except at pH 6.5 where butyrate and propionate levels exceeded those of acetate.

A lower pH (≤ 4.5) favors the solvent production (Van Ginkel and Logan, 2005). In the DF of glucose by *Clostridium pasteurianum*, a pH below 5 favors the butanol and acetone production (Dabrock et al., 1992). Selection of the operational pH is also substrate type and OLR dependent, which determines the VFA concentrations and thus the pH of the solution. The optimum temperature for DF processes varies with the substrates type and operational pH (Table 2.8). The optimum pH for organic food waste varies from 4.5 to 7, for lignocellulosic waste it varies from 6.5-7, whereas a neutral pH is optimal for animal manure (Guo et al., 2010). However, Tang et al. (2008) reported an optimum pH of 5.5 at 45 °C for the DF of cattle wastewater. Thus, it is important to determine the optimum pH conditions for DF of a selected substrate type at a particular loading rate and operational temperature.

A range of operational temperatures, i.e. mesophilic (35°C), thermophilic (55°C) and extreme thermophilic (>65°C) has been studied to determine its effect on the biohydrogen production (Kongjan and Angelidaki, 2010; Shin et al., 2004; Valdez-vazquez et al., 2005). These studies have shown that the temperature can affect the metabolic pathways, thus shifting the composition of the by-products of DF (Table 2.8). Valdez-vazquez et al. (2005) reported higher H_2 yields for thermophilic fermentation than in the mesophilic

temperature range. Also acetic acid was a dominant by-product in thermophilic digestion, whereas butyrate was in formed in a higher proportion during mesophilic digestion. Similarly, results of the extreme thermophilic (70 °C) DF of household organic waste also showed acetic acid as the dominant by-product in DF tests conducted at pH 7 (Liu et al., 2008). In contrast, Shin et al. (2004) showed acetate as major end-product at mesophilic culture while butyrate levels and hydrogen production was higher by the thermophilic culture, obtained in DF of food waste carried at pH 5.5. In another study, Wang and Wan (2011) found the maximum substrate degradation efficiency, maximum H_2 yield and production rate at 37.8 °C in DF of glucose. These studies suggest temperature influences biochemical pathways, although other factors such as culture pH, substrate types and loading rates are equally important.

The H_2 yields depend on temperature as it affects the hydrolysis rate (Kim et al., 2006; Shin et al., 2004; Valdez-vazquez et al., 2005). Biomass such as agricultural residues require a high temperature to achieve a higher H_2 yield because a better hydrolysis of lignocellulosic compounds is needed (Guo et al., 2010). Kongjan and Angelidaki, (2010) demonstrated biohydrogen production from extreme thermophilic DF of wheat straw hydrolysate. Similarly, thermophilic temperatures are favored in the DF of food waste (Shin et al., 2004). In contrast, easily biodegradable substrates prefer mesophilic temperatures for an optimal H_2 yield. The difference between the optimum operational temperatures is due to the difference in the fraction of easily biodegradable compounds present in the feed substrate and the different inocula used. Table 2.8 reports ranges of optimum temperatures, which vary depending on the type of substrate and inoculum used.

There are some techno-economic studies done, which compare the mesophilic and thermophilic operation of DF processes. A thermophilic process seems to be more economical because of its higher yield and lower requirement of feedstock in comparison to mesophilic DF processes (Foglia et al., 2006). Foglia et al. (2006) reported a better economic performance for thermophilic DF in comparison to a two-step mesophilic process, converting sugars to hydrogen, CO_2 and organic acids followed by a photo-heterotrophic fermentation.

H_2 partial pressure

The partial pressure of hydrogen inside a biohydrogen reactor can influence the dark fermentative biohydrogen production as a lower partial pressure in the head space of the reactors facilitates the mass transfer of hydrogen from the liquid to gas phase (Bastidas-Oyanedel et al., 2012; Mandal et al., 2006). During the fermentation process, the hydrogenase is involved in reversibly oxidizing and reducing ferredoxin. If the hydrogen concentration in the liquid phase increases, the oxidation of ferredoxin becomes less favorable and the reduction of ferredoxin takes place (Chong et al., 2009), thus reducing the H_2 production.

Lee et al. (2012) studied the effect of the reduced partial pressure on the hydrogen production in a CSTR reactor. Reduction in the partial pressure during the DF could lead to an improvement in H_2 production. At a HRT of 6 h, they found an optimum hydrogen yield and hydrogen production efficiency of 4.50 mol H_2/mol sucrose and 56.2% respectively. Similarly, the reduced pressure of 380 mm Hg gave a higher yield than the partial pressure of 760 mm Hg in another study done by Mandal et al. (2006).

In the AD process, the H_2 and CO_2 partial pressure is reduced by methanogens by their conversion into CH_4. Jung et al. (2011) reported strategies to remove dissolved H_2 from the mixed liquor, including avoiding supersaturation by strong mixing, sparging with N_2 and CO_2 and application of a H_2-permeable membrane to withdraw dissolved H_2 from the mixed liquor. Similarly, the partial pressure of H_2 could be reduced directly by decreasing the operating pressure in the reactor using a vacuum pump (Lee et al., 2012). Mandal et al. (2006) reduced the partial pressure of H_2 in a methanogenic reactor by adjusting the saline level of the gas collector using a peristaltic pump. However, the use of vacuum pumps increases the cost of the process, while the sparging with N_2 and CO_2 might render the recovery of H_2 difficult due to the dilution of the H_2 stream. An effective way to reduce the H_2 partial pressure would be to continuously collect the produced gas phase from the reactor.

Table 2.7 - Examples of innovative continuous DF bioreactors

Major substrate	Biomass retention system	Reactor type	Optimum HRT (hours)	Optimum Organic Loading Rate (OLR)	Optimum H_2 production index	Reference
Glucose	Granule	CSTR	0.5	10 g glucose/L	H_2 yield 1.81 mol H_2/mol glucose	(Show et al., 2007)
Cheese whey	Granule	UASB reactor	6	20 g COD/L/d	H_2 Production Rate 0.36-0.38 L H_2/L/d	(Carrillo-Reyes et al., 2012)
Food waste	Biofilm	Batch pilot scale up-flow rector (packed with coir pith)	0.50 m/day[a]	50 g COD/L	H_2 Production Rate 9.67 LH_2/L/h	(Pasupuleti et al., 2014)
Food waste (pre-treated with alkali)	Suspended	ASBR (fill: 0.5 h; reaction: 8 h; settle: 3 h & discharge: 0.5 h)	36	30 g COD/L	H_2 yield0.69 mol H_2/mol $hexose_{added}$	(Kim et al., 2010)
Tequila vinasse	Suspended	ASBR (fill: 3 min; reaction: 5.33 h; settle: 30 min & discharge: 7 min) with 50% volumetric exchange rate	12	3 g COD/L	H_2 Production Rate 50.5 mL H_2/L/h	(Buitrón and Carvajal, 2010)
Kitchen waste	Suspended	Inclined plug-flow reactor (inclined at 20°)	168	6.5 kgVS/m^3/d	H_2 yield 72 mL H_2/g VS	(Jayalakshmi et al., 2009)
Municipal food waste & kitchen wastewater	Suspended	Anaerobic baffled reactor (ABR)	38.4	29 g COD_{total}/L/d	H_2 Production Rate 6 L H_2/d	(Tawfik and El-Qelish, 2012)
Glucose	Suspended	Anaerobic membrane bioreactor (MBR)	12 (SRT[b])	5.8 g glucose/L	H_2 Production Rate 640 mL H_2/h	(S.-E. Oh et al., 2004)

[a]Up-flow velocity, [b]SRT: Solid Retention Time

Table 2.8 - Effects of operational temperature and pH on fermentative hydrogen production

Substrate type	Microbial inoculum	Optimum pH	Optimum Temperature (°C)	Reactor type	HRT (days)	Maximum H_2 Yield (mL H_2/g VS)	Major acid type produced	Reference
Food waste	Heat shock treated anaerobic sludge	6.3	35	Leaching Bed Reactor	25	310	Acetate	(Han and Shin, 2004)
Cassava stillage	Heat treated UASB sludge	7	60	Batch CSTR	3.5	53.8	Butyrate	(Luo et al., 2010b)
Cassava stillage	Heat treated UASB sludge	5	60	Batch CSTR	3.5	66.3	Butyrate	(Luo et al., 2010b)
OFMSW	Untreated anaerobic digestate	5.5	37	Semi-continuous CSTR	21	165[a]	Butyrate	(Valdez-vazquez et al., 2005)
OFMSW	Untreated anaerobic digestate	6.4	55	Semi-continuous CSTR	21	360[a]	Acetate	(Valdez-vazquez et al., 2005)
Wheat Straw	Cow dung compost	7.0	36	Batch CSTR	6.25	68.1	Acetate and butyrate	(Fan et al., 2006)
Vegetable kitchen waste	Enriched from kitchen waste compost	7.0	55	Batch CSTR	7.0	12.8[b]	Butyrate and lactate	(Lee et al., 2008)
Cattle wastewater	Mixed wastewater sludge, cow dung compost, chicken manure compost, river sludge	5.5	45	Batch CSTR	1.25	319[c]	Butyrate	(Tang et al., 2008)
Rice straw	Heat treated wastewater sludge	6.5	55	Batch CSTR	6.5	24.8 [d]	Acetate	(Chen et al., 2012)

[a] mL H_2/g $VS_{removed}$, [b] mL H_2/g COD, [c] mL H_2/g $COD_{consumed}$, [d] mL/g TS

2.1.7 Substrate pre-treatment for enhanced H_2 yield

Fermentative biohydrogen production from lignocellulosic substrates is limited by biological hydrolysis (Monlau et al., 2013b). The complex organic substrates cited earlier, such as lignocellulosic biomasses, demand physical, chemical, biological or a combination of these pre-treatments to enhance the degradation process, system performance and biogas production (Hendriks and Zeeman, 2009; Mussoline et al., 2012; Taherzadeh and Karimi, 2008; Zheng et al., 2014). These pre-treatment methods reduce the crystallinity of the cellulose and increase the surface area of the materials to improve the separation of the lignin and hemicellulose fractions (Saratale et al., 2008). There have been some studies on the effect of the pre-treatment on fermentative biohydrogen production (Chairattanamanokorn et al., 2009; Kongjan and Angelidaki, 2010; Pan et al., 2010; Zhu et al., 2005). These pre-treatment methods have in most cases a positive influence on the H_2 yield, as the biohydrogen production depends on the soluble fraction of sugars or carbohydrates.

Physical pre-treatment methods which generally include mechanical comminution (chopping, grinding, milling), irradiation with gamma-rays, electro-beam or microwaves, hydrothermal treatment, high pressure steaming or pyrolysis are effective in breaking the crystallinity, increasing the accessible surface area and decreasing the degree of polymerization (Taherzadeh and Karimi, 2008). Chemical methods such as ozonolysis, acid or alkaline hydrolysis, solvent extraction, explosion with steam ammonia fiber or CO_2 are effective in increasing the surface area, delignification and also decreasing the crystallinity and rendering the partial or complete hydrolysis of hemicelluloses. These physical and chemical treatment methods can be promising for industrial applications as they are rapid. However, these methods demand energy and chemical inputs. Moreover, lignocellulosic substrates can also be biologically treated with fungi and actinomycetes which provide delignification and partial hydrolysis of cellulose, while some enzymes (hemicellulase and cellulase) can aid in the hydrolysis and degradation of the lignocellulosic materials (Mussoline et al., 2012).

The physical pre-treatment, especially the reduction of substrate particle size, has an effect on the biogas yield and process kinetics (Esposito et al., 2008; G Esposito et al., 2011; G. Esposito et al., 2011). Chen et al. (2012) investigated the effects of the rice straw particle size and concentration on cumulative dark fermentative biohydrogen production.

They used a meshed rice straw concentration of 30 g TS/L with five particle sizes (<0.297, 0.297-0.58, 0.58-1.19, 1.19-10 and >10 mm) as the substrate at an initial cultivation pH 6.5 and temperature of 55 °C. The results of the study showed that rice straw of a particle size <0.297 mm gave the highest cumulative H_2 production (191 mL H_2/L) with a H_2 yield of 6.4 mL/g TS. The substrate with a larger particle size had an extended lag phase and lower hydrogen production. This can be explained by the fact that decreasing the particle size increases the substrate availability for microbial hydrolysis and fermentation.

Kongjan and Angelidaki (2010) pretreated wheat straw at 180 °C for 15 min to obtain a hydrolysate which mostly contained hemicellulose leaving the cellulose and lignin in solid form. The hydrolysate was used as the substrate for fermentative hydrogen production. Similarly, Zhang et al. (2007) reported the use of acid pretreated corn stalks for fermentative biohydrogen production. The biohydrogen yield from acid pretreated corn stalks was higher than of that of untreated waste. However, mostly physical pretreatment methods are applied in combination with chemical or biological pre-treatment methods to obtain better and rapid hydrolysis of substrates (Table 2.9).

The effect of pre-treatment methods for different lignocellulosic substrates have a diverse effect on the hydrolysis of soluble sugars and release of inhibitory products (Jönsson et al., 2013; Monlau et al., 2013a; Palmqvist and Hahn-Hägerdal, 2000; Parawira and Tekere, 2011). This needs to be further investigated for the selection of suitable pre-treatment methods. These studies have shown that the pre-treatment methods can enhance the system performance enhancing the biogas production. However, the selection of a pre-treatment process should be based on effectiveness, energy balance, economic feasibility and environmental sustainability (Ariunbaatar et al., 2014).

In addition, some studies have reported that during the pre-treatment of lignocellulosic biomass, various undesirable compounds are released which exert inhibitory effects on microorganisms (Jönsson et al., 2013; Palmqvist and Hahn-Hägerdal, 2000; Parawira and Tekere, 2011; Quéméneur et al., 2012). The most commonly reported inhibiting substances which are released during the pre-treatment processes are furfural, hydroxyl-furfural and phenolic substances. Quéméneur et al. (2012) and Monlau et al. (2013c) investigated the inhibition and control of these inhibitors on the biohydrogen production. Thus, the selection of pre-treatment methods for lignocellulosic substrates should also consider these aspects.

Table 2.9 - Examples of different pre-treatment methods applied to complex substrates used in DF

Substrate	H_2 yield (mL H_2/g VS)	Pretreatment methods	Reactor mode	Temperature (°C)	Reference
Rice straw	24.8[a]	Size reduction, <0.297mm	Bath	55	(Chen et al., 2012)
Wheat stalks	17.6	Size reduction, 1 mm	Batch	35	(Yuan et al., 2011)
Wheat straw	212[b]	Hydrothermal (180 °C for 15 min)	Continuous UASB reactor	70	(Kongjan and Angelidaki, 2010)
Corn Stover	2.84[c]	Steam explosion, 190.220 ◦C for 3-5 min	Batch	35	(Datar et al., 2007)
Corn Stover	3.0[c]	Acidic steam explosion (1.2% H_2SO_4), 180 and 200 ◦C for 1–3 min	Batch	35	(Datar et al., 2007)
Beet-pulp	66.7 ±10.1[d]	Alkaline at pH 12 using 2 M NaOH for 30 min. + Microwaves (170 °C for 30 min)	Batch	35±2	(Ozkan et al., 2011)
Bagasse	300	100 °C for 2 h +4% NaOH (w/v) + cellulase (20 FPU/g)	Batch	55	(Chairattana manokorn et al., 2009)
Grass	72.2[e]	4% HCl (w/v), boiled 30 min.	Batch	35	(Cui and Shen, 2012)
Grass	39.5[e]	4% NaOH (w/v), boiled 30 min.	Batch	35	(Cui and Shen, 2012)
Corn stalks	209.8	1.5% H_2SO_4, 121 °C for 60 min+ 9.4 IU/g of cellulase 52 °C at pH4.8 in 0.1 M sodium citrate buffer at 5% (w/v)	Batch	36±1	(Pan et al., 2011)
Corncobs	107.9	100 °C, 30 min and 1% HCl (w/w))	Batch	36	(Pan et al., 2010)

[a]mL/g TS, [b]mL H_2/g sugars, [c]mol H_2/mol glucose, [d]mL H_2/g COD, [e]mL H_2/g dry grass

Addition of nutrients and trace elements

Microorganisms in fermentation processes require nutrients for bacterial activity and growth. Thus, nutrients such as nitrogen, phosphate, metal ions and other micronutrients

are needed in fermentation processes for enzymatic activities and biomass growth, which affects the H_2 production. Biomass rich in carbohydrates such as wheat wastes and palm oil effluents may be deficient in nutrients (such as nitrogen, phosphorous) or minerals (such as trace metals). Therefore, nutrients or micro nutrients must be provided as supplement for optimum microbial activities for biohydrogen conversion from carbohydrate rich substrates (Argun et al., 2008b; Lin and Lay, 2005, 2004).

Nitrogen and phosphorous

Nitrogen has great significance for hydrogen producers, as it is an important component of proteins, nucleic acids and enzymes. Similarly, besides being an important nutrient, phosphate also serves in buffering the biochemical reactions (Wang and Wan, 2009). In the thermophilic DF of palm oil mill effluents (POME), O-Thong et al. (2008, 2007) showed that supplementing iron (257 mg Fe^{2+}/L), adjusting the C/N ratio from 95 to 74 (using peptone as nitrogen source) and the C/P ratio from 650 to 559 (using $Na_2HPO_4 \cdot 2H_2O$) could enhance H_2 production. In these studies, the hydrogen production rate increased by 60% (O-Thong et al., 2008) and COD removal efficiencies improved from 35.5±9.8 % to 62.2±2.8% (O-Thong et al., 2007) compared to raw POME without nutrient supplementation.

Likewise, Argun et al. (2008b) studied the effects of the C/N and C/P ratio on the hydrogen yield and specific H_2 production rate in DF of wheat powder solution by supplementing nitrogen and phosphorous. The results of the study showed the highest H_2 yield of 281 NmL H_2/g starch were obtained at a C/N ratio of 200 and C/P ratio of 1000. However, there are some disagreements in the carbon to nitrogen and phosphorous ratios. Lin and Lay, (2004) achieved a 500% and 80% increased hydrogen yield and hydrogen production rate at a C/N ratio of 47 compared with the blank. Similarly, O-Thong et al. (2008, 2007) attained an optimum hydrogen production and COD removal at a C/N ratio of 74 and a C/P ratio of 559. Several studies have used the integration of co-substrates as a strategy to maintain an appropriate C/N ratio, examples include the use of swine manure as a source of nitrogen in co-fermentation with vegetable waste (Tenca et al., 2011) and use of cassava starch in co-fermentation with the microalgae *Chlorella pyrenoidosa* (Xia et al., 2014).

Metal ions and micronutrients

Higher concentrations of metal ions exert inhibitory effects on the hydrogen producers (Li and Fang, 2007a, 2007b; Lin and Shei, 2008). However, trace amounts of some metal ions enhance the reactor performance (Karadag and Puhakka, 2010). Karadag and Puhakka (2010) found that iron and nickel improved the reactor performance and H_2 production was enhanced by 71%. O-Thong et al. (O-Thong et al., 2008) obtained the optimal hydrogen production when the substrate contained 257 mg Fe^{2+}/L during the thermophilic DF of POME.

Inhibition due to heavy metals

Toxic heavy metals such as cadmium (Cd), chromium (Cr), zinc (Zn), copper (Cu), nickel (Ni), and lead (Pb) which may be present in industrial and municipal solid waste may lead to upset or ultimately failure of anaerobic reactors (Li and Fang, 2007a). Altaş (2009) studied the inhibitory effect of heavy metals on methane producing anaerobic granular sludge. The order of toxicity for the individual heavy metals in decreasing order was: Zn (most toxic, 7.5 mg/L) >Cr (27 mg/L) >Ni (35 mg/L) ≈ Cd (least toxic, 36 mg/L).

Lin and Shei (2008) showed the relative toxicity of the heavy metals to fermentative hydrogen production was in the order of Zn>Cu>Cr. The maximum concentration of these metals that reduced the hydrogen producing activity by 50% was 4.5 mg Zn/L, 6.5 mg Cu/L and 60 mg Cr/L (Lin and Shei, 2008). However, Li and Fang (2007b) reported the relative toxicity to H_2 production in the following order: Cu (most toxic)>>Ni~ Zn > Cr > Cd > Pb (least toxic). The bioactivity of the sludge was reduced to 50% of the control at 30 mg Cu/L, 1600 mg Ni and Zn/L, 3000 mg Cr/L, 3500 mg Cd/L and >5000 mg Pb/L.

2.1.8 Use of by-products

The low process yield and the incomplete conversion of organic biomass are two major bottlenecks for commercial dark fermentative biohydrogen production (Gómez et al., 2011; Ren et al., 2011). As overviewed in Section 4, dark fermentative biohydrogen can be enhanced by suitable substrate selection, inoculum enrichment strategies, and optimal operation of bioreactor or substrate pre-treatment. However, a single DF system cannot achieve beyond the highest yield of 4 moles H_2 per mole hexose, as DF has a maximum yield of 33% (on sugars) (Gómez et al., 2011). Besides, DF residues mainly contain volatile fatty acids, major by-products of the DF process, which need to be utilized to achieve complete conversion of the organic biomass. Dual systems are integrated by the

conversion of carbohydrates to organic acids in the first stage (DF) and the conversion of by-products in the second stage, either to H_2 (photofermentation, bioelectrochemical cells) or CH_4 (AD). Also, AD can be considered as the final stabilization stage to stabilize the residues of DF, photofermentation and bioelectrochemical cells. Figure 2.5 shows an example of different possibilities of integrating DF to other post treatment processes.

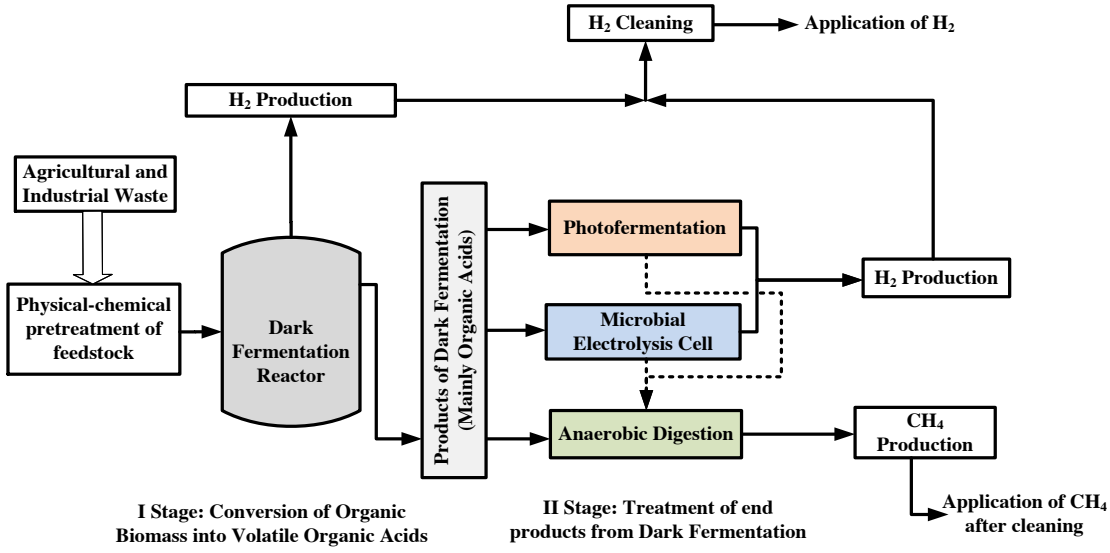


Figure 2.5 - Different strategies for integrating DF with post treatment processes for improved biofuel production

A number of studies have been carried out combining DF either with photofermentation (Argun and Kargi, 2010; Chen et al., 2008; Hema and Agrawal, 2012; Nath et al., 2005; Redwood and Macaskie, 2006; Su et al., 2010, 2009a, 2009b) or/and using bio-electrochemically assisted microbial reactors (Cheng and Logan, 2007; Jeremiasse et al., 2010; Liu et al., 2005, 2012; Wang et al., 2011) for improving the biohydrogen yield or with the AD process for improving the economic viability (Cavinato et al., 2009; Lin et al., 2012; Liu et al., 2006; Liu et al., 2013; Ruggeri et al., 2010; Venetsaneas et al., 2009). Light dependent fermentative processes can be a good option for a second stage H_2 production, because of their higher substrate conversion efficiency, and being less energy intensive and environmental friendly (Chen et al., 2010). On the other hand, bio-electrochemically assisted microbial fuel cells are also an option to treat the effluents from DF and increase the H_2 yield (Logan et al., 2008). Likewise, the economic viability of the DF process can be enhanced by AD as a final step. Table 2.10 gives some examples of integrated processes of DF combined with post treatments.

Photofermentation

Under anaerobic conditions, purple non sulfur photosynthetic bacteria carry out anaerobic photosynthesis using light as energy source for synthesizing hydrogen (Adessi and De Philippis, 2014; Eroglu and Melis, 2011). The purple non sulfur bacteria use the captured light energy to produce ATP and high energy electrons through reverse electron flow which reduces ferredoxin (Figure 2.6). Then, the ATP and reduced ferredoxin drives the proton reduction to hydrogen by nitrogenase (Hallenbeck and Ghosh, 2009). The research attention to these organisms is increasing because of their higher biohydrogen yield potential and better light utilization proficiency, as they are able to absorb and utilize both visible (400 – 700 nm) and near infrared (700 – 900 nm) light. Moreover, they are able to use a wide variety of substrates (Eroglu and Melis, 2011).

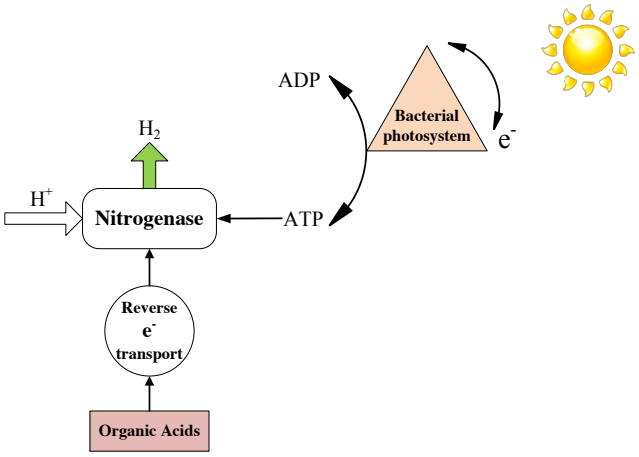


Figure 2.6 - Schematic presentation of photofermentation (adapted and modified from Hallenbeck and Ghosh (2009))

The ability of purple non sulfur bacteria to convert the organic acids to biohydrogen makes photofermentation a good post treatment for biohydrogen production from DF effluents. An example of integrated dark and photofermentative conversion of acetic acid to biohydrogen is:

DF: $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$ (2.8)

Photofermentation: $CH_3COOH + 2H_2O \xrightarrow{\text{Light energy}} 4H_2 + 2CO_2$ (2.9)

Depending on the operating conditions of the bioreactors and other parameters described earlier (See section 4), DF might follow different pathways rather than only the acetic

acid pathway. Therefore, a theoretical biohydrogen potential of DF effluents containing acetate, propionate and butyrate can be written as (Barbosa et al., 2001; Han et al., 2012):

Lactate: $C_3H_6O_3 + 3H_2O \rightarrow 6H_2 + 3CO_2$ (2.10)

Propionate: $C_3H_6O_2 + 4H_2O \rightarrow 7H_2 + 3CO_2$ (2.11)

Butyrate: $C_4H_8O_2 + 6H_2O \rightarrow 10H_2 + 4CO_2$ (2.12)

Purple non sulfur species such as *Rhodospirillum rubrum*, *Rhodopseudomonas palustris*, *Rhodobacter sphaeroides*, *Rhodobacter capsulatus* and *Rhodopseudomonas faecalis* have been widely used in photofermentation studies for H_2 production (Adessi and De Philippis, 2014; Eroglu and Melis, 2011), while some studies have been done with mixed cultures isolated from wastewater sludge (Cheng et al., 2012; Venkata Mohan et al., 2008; Xia et al., 2013). Yangling et al. (Yanling et al., 2008) evaluated the microbial community dynamics in a mixed photofermentative culture enriched from a digestate from the AD of pig dung and found the prevalence of mostly *Rhodopseudomonas palustris*.

Redwood et al. (Redwood et al., 2008) presented different integration strategies for combining two step dark and photofermentation processes. Nath et al. (Nath et al., 2005) studied the combined dark and photofermentation for biohydrogen production using glucose as the substrate. DF was carried out by *Enterobacter cloacae* strain DM11, followed by photofermentation by *Rhodobacter sphaeroides* strain O.U.001 using the spent medium from the DF, which mainly contained acetic acid. The combined hydrogen yield was higher than a single biohydrogen system, i.e. 1.86 mol H_2/mol glucose in DF and 1.5–1.72 mol H_2/mol acetic acid in the photofermentation. Similarly, combining the two fermentation processes, Chen et al. (Chen et al., 2010; Tao et al., 2007) and Tao et al. (Tao et al., 2007) attained a total yield of 10.25 mol H_2/mol sucrose and 6.63 mol H_2/mol sucrose respectively. In a study by Su et al. (Su et al., 2009a), a yield of 4.16 mol H_2 mol/mol glucose was obtained from photofermentation of DF effluents using glucose as the substrate, which increased the total yield to 5.48 mol H_2/mol glucose.

Other researchers have used effluents from DF of diverse substrate types in photofermentative biohydrogen production. Argun et al. (Argun and Kargi, 2010) used the DF effluent of a ground wheat solution with a H_2 yield of 781 ml/g total VFA. In another studies by Su et al. (Su et al., 2010, 2009b), cassava starch and water hyacinth

were used as the substrates in DF and its effluent was utilized successfully for photofermentative biohydrogen production. The studies reported the increase in total H_2 yield from 240.4 mL H_2/g starch to 402.3 mL H_2/g starch (Su et al., 2009b) and 76.7 to 596.1 mL H_2/VS (Su et al., 2010) using *Rhodopseudomonas palustris*. These studies have shown that combined dark and photofermentation is a potential technology for biohydrogen production using diverse substrates.

Some drawbacks of photofermentative systems include the inherent high energy demand associated with the nitrogenase enzyme, lower solar conversion efficiencies and economic issues of anaerobic photobioreactors covering large areas (Hallenbeck and Benemann, 2002). However, these inefficiencies can be overcome by developing an efficient photobioreactor (Dasgupta et al., 2010; Gebicki et al., 2010). Besides the presence of light conditions, the culture medium of photofermentation should be under ammonia limitation and oxygen should be absent, as both inhibit the nitrogenase activity (Argun et al., 2008a; Eroglu et al., 1999; Koku et al., 2003) . Higher ammonia concentrations (in excess of 2-5 mmol) can be detrimental to hydrogen production (Argun et al., 2008a; Lee et al., 2011). Thus, the effective removal of ammonia from DF residues can be a bottleneck in coupling photofermentation with DF processes. Therefore, substrates with a high C/N ratio seem more suitable for H_2 conversion in these systems. Nonetheless, several ammonia removal strategies such as stripping, natural zeolites and selective membranes can be applied which could facilitate the coupling of the two processes (Androga et al., 2012a; Redwood et al., 2012b).

In addition to biohydrogen production, accumulation of poly-hydroxybutyrate (PHB) could raise future interests, as it possesses economic value as a precursor of biodegradable polymer (Koku et al., 2002). Thus, energy recovery and economic sustainability of the commercial development of DF also depends on the development of post-treatment processes like photofermentation.

Microbial Electrolysis Cells

Biohydrogen production from DF residues is also possible through an emerging technology known as electrohydrogenesis or biocatalyzed electrolysis or microbial electrolysis (Chookaew et al., 2014; Das and Veziroglu, 2001; Gómez et al., 2011; Li et al., 2014; Liu et al., 2012; Moreno et al., 2015). Electrochemically assisted Microbial Fuel Cells (MFCs), Microbial Electrolysis Cell (MECs) or Bioelectrochemical Systems

(BES) use microorganisms to catalyze the biochemical reactions at the anode and/or cathode, producing protons and electrons from the oxidation of organic matter (Jeremiasse et al., 2010; Liu et al., 2005; Logan et al., 2008). MECs should not be confused with MFCs, the former is an electrolysis reactor which produces hydrogen, while a MFC is a fuel cell that produces electricity (Logan et al., 2008). In MECs, on oxidizing acetate under standard biological conditions (25 °C, 1 bar pressure and pH 7) H_2 can be produced at the cathode by applying a small circuit voltage, theoretically 0.14 V (Logan et al., 2008) (Figure 2.7). Some exoelectrogenic microorganisms which are capable of electron transfer to an electrode (anode) include the genera *Geobacter*, *Shewanella* and *Pseudomonas sp*. (Liu et al., 2005; Logan et al., 2008; Moreno et al., 2015), while the function and the community composition of the microorganisms at the cathode are not known (Logan et al., 2008). The evolution of hydrogen in BESs can be represented in the following reactions:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (2.13)$$

$$\text{Anode: } CH_3COOH + 2H_2O \rightarrow 2CO_2 + 8e^- + 8H^+ \quad (2.14)$$

$$\text{Cathode: } 8H^+ + 8e^- \rightarrow 4H_2 \quad (2.15)$$

A minimum theoretical voltage required to produce hydrogen at pH 7 is - 410 mV (Normal Hydrogen Electrode). However, the anode potential produced by the oxidation of organic matter is approximately –300 mV. Thus, hydrogen can theoretically be produced at the cathode by applying a circuit voltage higher than –110 mV (i.e. $V_{applied} = V_{anode} - V_{cathode} = -410-(-300)$ mV), though it has been found that a minimum applied voltage of more than 250 mV is needed due to ohmic resistance and electrode overpotential (Das and Veziroglu, 2008; Liu et al., 2005). This applied voltage required is considerably lower than –1210 mV, the theoretical voltage needed for hydrogen production via electrolysis of water at neutral pH conditions (Liu et al., 2005).

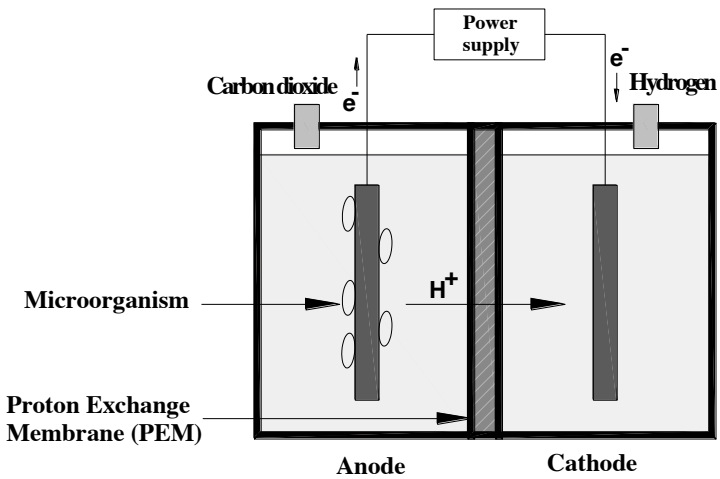


Figure 2.7 - Schematic diagram of two chambered MEC separated by a proton exchange membrane and power supply (adapted and modified from Liu et al. (2005))

Lalaurette et al. (2009) tested a two-stage process, combining DF using cellulose as a substrate and MEC systems for hydrogen production. This improved the total hydrogen yield to 9.95 mol H_2/mol glucose from the fermentative hydrogen yield of 1.64 mol H_2/mol glucose using cellulose. Similarly, Liu et al. (2012) used the volatile acids accumulated in the fermentation of waste activated sludge as a carbon source for biohydrogen production in a MEC with a H_2 yield and production rate of 1.2 mL H_2/mg COD and 120 mL H_2/g VSS/d, respectively. The results from the analysis of the electrohydrogenesis end products showed that more than 90% of the acetate and propionate were converted to hydrogen, but with lower conversion of n-butyrate and n-valerate (<20%). Likewise, Moreno et al. (2015) obtained 94.2 L H_2/kgVS from two stage DF-MEC systems using cheese whey wastewater.

The MECs are still under research and development. One of the challenges of MECs is to suppress the methanogenic activity during the electrohydrogenesis with mixed cultures as it negatively affects the H_2 production rate. Hu et al. (2008) has proposed to inhibit the methanogenic activity by exposing the cathodes to air. They studied a single chambered MEC to investigate the hydrogen production using mixed and pure (*Shewanella oneidensis* MR-1) cultures. The major objective was to reduce the potential losses associated with the membrane and increase the energy recovery of the process. Studies of the long term performance of MEC systems are needed to further develop and achieve the technical and economic edge of this technology.

Anaerobic digestion

Anaerobic Digestion (AD) is a proven biological waste treatment method for volume reduction, waste stabilization and biogas recovery (CH_4) from organic waste (Esposito et al., 2012). The AD process can be combined with DF to achieve further conversion of end products of DF and the residues from photofermentation and MECs systems (Figure5). Photofermentation requires a clear medium for efficient light utilization. Thus, the residue from the filtration of DF effluents, microbial biomass produced in photofermentation and the residues from MECs (if any) can be utilized in AD for the final stabilization. The two stage processes, combining biohydrogen production in the first stage and AD in the second stage, not only increase the sustainability of the process, but also guarantee the complete treatment of the organic waste (Gómez et al., 2011).

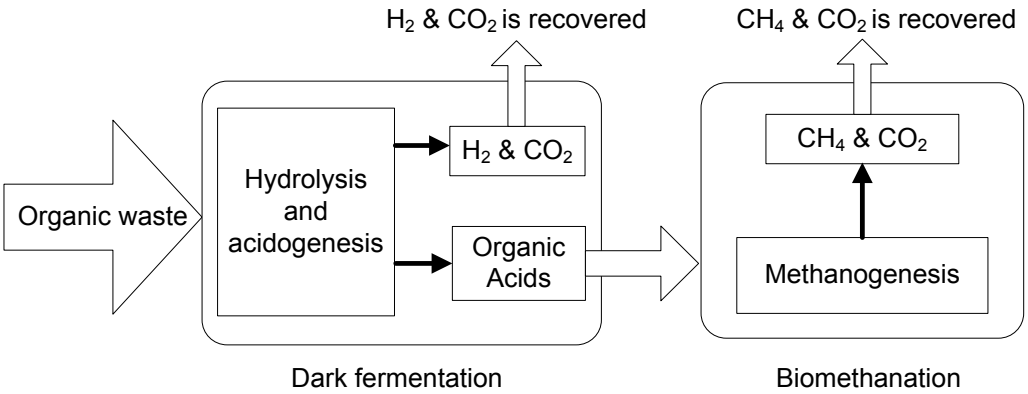


Figure 2.8 - Two-stage process for hydrogen and methane production from organic waste

A number of studies have been done on dual systems (Cavinato et al., 2009; Lin et al., 2012; Liu et al., 2006; Z. Liu et al., 2013; Ruggeri et al., 2010; Venetsaneas et al., 2009; Wieczorek et al., 2014). DF followed by AD (Figure 2.8) has shown technical and economic feasibility of the integrated process up to pilot scale (Cavinato et al., 2012; Lee and Chung, 2010). Wang and Zhao (2009) ran a successful pilot scale unit consisting of hydrolysis-acetogenesis for H_2 generation in a rotating drum of 200 liters, followed by a methanogenesis stage in 800 liters reactor. Likewise, Cavinato et al. (2009) established successful two stage conversion of hydrogen and methane from organic waste. Similarly, Antonopoulou et al. (2008) investigated two stage hydrogen and methane production using sweet sorghum with a H_2 yield of 10.41 L H_2/kg sweet sorghum and a methane yield of 29 L CH_4/kg sweet sorghum utilizing DF residues as a sole substrate in AD, while Kvesitadze et al. (2011) obtained a cumulative H_2 and CH_4 yield of, respectively, 104 L

H_2/g VS and 520 L CH_4/g VS using OFMSW. Similarly, Antonopoulou et al. (2008) showed the feasibility of a two stage hydrogen-methane process using cheese whey.

Jung et al. (2010) showed that two stage H_2-CH_4 conversion from molasses is economically feasible. Ruggeri et al. (2010) used the energy balance as a tool to determine the sustainability of integrated DF and AD, which showed the positive energy gain. Similarly, Schievano et al. (2014) reported 8%-43% increment in energy production in two stage systems in comparison to a single stage AD. Thus, in light of recent popularity of two stage AD processes for treating high strength wastewater or concentrated solids, the former stage can be modified to be used for hydrogen production (Guo et al., 2010). Also, the DF process can be seen as a pre-treatment stage if the organic waste of interest is subjected to complete stabilization (Wang and Zhao, 2009). Thus, in order to improve the economic competence of commercial DF, AD could provide an attractive solution (Ljunggren and Zacchi, 2009).

Other applications

Besides the conversion of volatile fatty acids and other reduced carbon sources to biomethane or biohydrogen in biological processes, VFAs can be used in various applications: biological nutrient removal from wastewater (Elefsiniotis et al., 2004; Lim et al., 2000), sulfur and sulfate reduction (Finke et al., 2007; Sørensen et al., 1981), biopolymer (such as polyhydroxybutyrate) production (Ntaikou et al., 2009) and microbial lipids production (Fei et al., 2011; Fontanille et al., 2012). Lim et al. (2000) studied the use of volatile fatty acids produced from food waste as carbon sources in the removal of nitrogen and phosphorous from municipal wastewater in a sequential batch reactor (SBR) (Lim et al., 2000). Similarly, Elefsiniotis et al. (2004) studied the denitrification process (20 to 200 mg NO_3^- -N/L) using VFA generated from the AD of starch rich industrial and municipal wastewater as a carbon source in batch reactors.

Similarly, a “carboxylate platform” or third biorefinery platform has been introduced to generate a mixture of carboxylates as intermediates for the production of complex fuels utilizing waste biomass (Agler et al., 2011). Ntaikou et al. (2009) investigated the combined production of biohydrogen and biopolymers from the DF of olive mill wastewater and the use of DF effluents which mostly contained VFAs in SBR using polyhydroxyalkanoates (PHAs) producing bacteria culture.

Tuna et al. (2009) used the volatile fatty acids produced in DF processes for hydrogen production by electrohydrolysis. Hydrogen was generated by applying a low voltage in the range of 1-3 V DC current to DF effluents of wheat powder containing different VFAs concentrations. The applied voltage of 2 V and 10.85 g/L of total VFA gave the highest energy efficiency (56%).

Table 2.10 - Examples of operational conditions and system performances of integrated DF systems

First Stage: DF								Second Stage: Photofermentation						Reference
Substrate type	Microbial Inoculum	pH	T (°C)	HRT days	Max. H_2 Prod. Rate	Max. H_2 Yield	Dominant end products	Process & Microbial Inoculum	pH	T (°C)	HRT days	Max. Biogas Prod. Rate	Max. H_2 Yield	
Sucrose	*Clostridium pasteurianum*	7	37	-	-	3.85 mol H_2/mol sucrose	Butyrate and acetate	*Rhodopseudomonas palustris* WP3-5	7.1	32	96 hours	25.2 mL H_2/L/h	4.03 mol H_2/mol sucrose	(Chen et al., 2010)
Sucrose	Heat treated Cattle dung and sludge from biogas plant	6	38	Batch	360 mL H_2/L/h	3.67 mol H_2/mol sucrose	Butyrate and acetate	*Rhodobacter sphaeroid*s SH2C	7	30	Batch	-	4.06 mol H_2/mol sucrose	(Tao et al., 2007)
Acid hydrolyzed sugarcane bagasse	*Enterobacter aerogenes* MTCC 2822	6.8	38	Batch	1000 mL H_2/L	-	Butyrate and acetate	*Rhodopseudomonas* BHU 01	6.8	34	Batch	755 ml/L	-	(Rai et al., 2014)
First Stage: DF								Second Stage: AD						Reference
Substrate type	Microbial Inoculum	pH	T (°C)	SRT days	Max. H_2 Prod. Rate	Max. H_2 Yield	Dominant end products	Process & Microbial Inoculum	pH	T (°C)	SRT days	Max. CH_4 Prod. Rate	Max. CH_4 Yield	
OFMSW	Heat treated sludge from biogas plant	5.2	37	2	640 mL H_2/d	43 mL H_2/g VS_{added}	Acetate and butyrate	Sludge from biogas plant	7.5	37	15	7500 mL CH_4/d	500 mL CH_4/g VS_{added}	(Liu et al., 2006)
Food waste	Indigenous microbial cultures from food waste	5.2–5.8	40	6.66	-	65 mL H_2/g VS	Acetate and butyrate	Anaerobic granular sludge from UASB	6.8	40	26.67	-	546 mL CH_4/g VS	(Wang and Zhao, 2009)

Micro algae (*Chlorella vulgaris*)	*Clostridium thermocellum*	-	55	Batch	-	53.4 mL H_2/g VS	Acetate and butyrate	Anaerobic granular sludge from ASBR	-	55	batch	22.38 mL CH_4/g VS·d	320.6 mLCH_4/g VS	(Lü et al., 2013)
First Stage: DF								Second Stage: Bioelectrochemical systems						
Substrate type	Microbial Inoculum	pH	T (°C)	HRT days	Max. H_2 Prod. Rate	Max. H_2 Yield	Dominant end products	Process & Microbial Inoculum	pH	T (°C)	HRT days	Max. H_2 Prod. Rate	Max. H_2 Yield	Reference
Molasses	-	-	-	Batch	700 mL H_2/L/d	0.27 mol H_2/mol COD	Ethanol, acetic and butyric acid	Domestic wastewater	6.7-7.0	25	Batch	1410 mL H_2/L/d	-	(Lu et al., 2009)
Corn Stover	*Clostridium thermocellum*	6.8	50	Batch	0.25 L H_2/L/d	1.67 mol H_2/mol-glucose	Acetic acid and ethanol	Inoculum from microbial fuel cell Wastewater	7.3	-	Batch	1±0.19 L/L/d	750±180 mL/g COD	(Lalaurette et al., 2009)
Corn stalk	*Microwave irradiation pre-treated cow dung*	7.0	36	Batch	1.73 m^3 H_2/m^3/d	129.8 mL H_2/g corn stalk	Acetate, butyrate, propionate, ethanol	Spent dark fermentation medium (Single chambered cell)	7.0	36	Batch	3.43 ±0.12 m^3 H_2/m^3 d	257.3 mL H_2/g corn stalk	(Li et al., 2014)

2.1.9 Pilot scale applications

Most DF studies have been carried out at laboratory scale batch, semi-continuous or continuous reactors. To the best of our knowledge, no studies have reported the DF process at industrial or full scale. Limited studies have been done on pilot-scale applications of DF processes (Cavinato et al., 2012; Jayalakshmi et al., 2009; C. M. Lee et al., 2010; Lee and Chung, 2010; Lin et al., 2011; Ren et al., 2006). Ren et al. (2006) studied a 1.48 m^3 continuous flow anaerobic reactor for 200 days at an OLR of 3.11-85.57 kg COD/m^3/d fed with molasses. The maximum hydrogen yield was 26.13 mols H_2/kgCOD removed in the OLR range of 35–55 kg COD/m^3/d and a maximum production rate of 5.57m^3 H_2/m^3 reactor/d was reached. Jayalakshmi et al. (2009) worked with a plug-flow inclined DF reactor of volume 0.15 m^3 with kitchen waste as the substrate. The reactor gave a H_2 yield of 72 mL H_2/gVS added.

Another reported long term pilot-scale study was carried out at Fen Chia University (Taiwan), comprising of two feedstock storage tanks (0.75m^3 each), a nutrient storage tank (0.75m^3), a mixing tank (0.6 m^3), an agitated granular sludge bed fermenter (working volume 0.4 m^3), a gas-liquid-solid separator (0.4 m^3) and a control panel. A pilot-scale high-rate reactor was operated for a period of 67 days under mesophilic conditions (35 °C) at an OLR of 40-240 kg COD/m^3/d with sucrose as the substrate. An OLR of 240 kg COD/m^3/d gave a hydrogen production rate of 15.59 m^3/m^3d and a hydrogen yield of 1.04 mol H_2/mol sucrose. In another study, Cavinato et al. (Cavinato et al., 2012) carried out a two-stage pilot-scale thermophilic DF and AD of food waste for the production of, respectively, biohydrogen and methane with recirculation of AD effluents to DF to control the pH (5-6). The organic loading rate of 16.3 kgTVS/m^3d was maintained with a HRT of 3.3 days in the DF stage, yielding 66.7 L H_2/kg TVS.

2.1.10 Challenges and future prospects

Modeling and simulation

Several researches have been proposed to integrate DF processes with AD, photofermentation or bioelectrochemical systems to utilize the VFAs produced to increase its viability. Modeling of kinetic parameters and biohydrogen production becomes important for the design, analysis and operation of the fermentative processes. Also, the predictive capacity of the model for end products helps to design the

downstream processes. Several models have been proposed to describe the biohydrogen production, growth of hydrogen fermenters, substrate consumption and intermediate biochemical processes (Arudchelvam et al., 2010; Gadhamshetty et al., 2010; Wang and Wan, 2009). With increasing research on DF, the modeling of the biohydrogen production process could be of primary interest to achieve a better understanding of the DF pathways and control of the process.

Parameters such as substrate concentration, pH, temperature, and HRT affect the H_2 yield and production rate and the nature of the end products (See section 4). Wang and Wan (Wang and Wan, 2009) reviewed existing mathematical models such as the Modified Gompertz model for product formation (H_2 production), the Logistic model for biomass growth (Mu et al., 2006), substrate utilization based on Monod Kinetics, the Arrhenius model for temperature effects, pH inhibition models based on the IWA Anaerobic Digestion Model no. 1 (ADM1) (Batstone et al., 2002) and the Modified Luedeking-Piret models for the formation of by-products (Mu et al., 2006).

There is, however, a need to upgrade the different kinetic models, including complex biochemical processes, which involve the fermentative biohydrogen production such as hydrolysis, acidogenesis and H_2 production from complex substrates (Figure2). The IWA ADM1 has been used extensively to model AD processes (Batstone et al., 2002; Blumensaat and Keller, 2005; Esposito et al., 2008; G Esposito et al., 2011). ADM1 is a structured mathematical model based on the COD balance of composite substrates and includes a number of biochemical processes involving disintegration of substrates such as hydrolysis, acidogenesis, acetogenesis and methanogenesis, biomass growth and decay processes and the physical interaction of the gas-liquid phases. Because of the similarity of some initial biochemical and physical processes, a modified ADM1 has been proposed to model dark fermentative biohydrogen production processes (Arudchelvam et al., 2010; Gadhamshetty et al., 2010; Peiris et al., 2006). Nonetheless, a model that can simulate the process and predict the formation of all the major intermediates and biohydrogen considering all influencing parameters is a necessity.

Energy balance and COD conversion

The net energy gain in DF processes is an important issue that has been addressed by few researchers (Perera et al., 2010; Ruggeri et al., 2010; Tommasi et al., 2012). The energy balance is an important factor for the process sustainability. Higher culture temperatures have been suggested in the literature (Cavinato et al., 2011; Chen et al., 2012; Lee et al., 2008; Shin et al., 2004; Valdez-vazquez et al., 2005) for maximizing H_2 yield, without considering the net energy gain (Perera et al., 2010). Some studies (Perera et al., 2010; Ruggeri et al., 2010) suggested that DF processes have to be operated at ambient temperature in order to obtain a positive net energy. After evaluation of literature data on DF of different substrates, Perera et al. (2010) reported the net energy gain in dark fermentative processes is positive when the process temperature is below 25 °C. In another study by Ruggeri et al. (2010), the optimum working temperature of 20 °C has been recommended, which offers 20% of the available energy. However, these studies have suggested to couple DF processes with AD, microbial fuel cells, bioelectrochemical systems or photofermentation to obtain a more positive net energy balance from the recovery of energy from the DF end-products and residues.

Perera et al. (2010) reported that DF combined with BES or DF with AD can result in a positive energy yield. Similarly, Ruggeri et al. (2010) found that the AD step after DF can deliver a positive net energy with 40-90% available energy. Su et al. (2009b) obtained a higher conversion efficiency of the heat value in DF from 13.3% to 46.0% when combined with photofermentation. This was due to an increase of the H_2 yield from 1.59 to 5.48 mol H_2/mol glucose.

Lower rates of COD reduction efficiencies are a concern if the DF process aims to treat waste biomass. The conversion of COD to hydrogen is low; theoretically 16 g of COD reduction is achieved per mole of H_2 obtained. However, the COD remains in the by-products as VFAs and alcohols. Mohammadi et al. (2011) obtained 0.41 mmol H_2/g COD from mesophilic DF of POME with a COD removal efficiency of 86%. In another study, O-Thong et al. (2008) obtained a COD removal efficiency of 55% with H_2 yield of 0.142 L H_2/L POME. Nonetheless, it has been suggested from the studies (Table 10), that combining DF processes with AD, BES or PF will not only improve the energy recovery, but give higher COD reduction efficiencies and provide complete treatment of organic waste biomass.

pH control

Unlike AD processes where the production of acidity from VFAs generation is balanced by alkalinity of the systems, DF processes are unstable because of the continuous production of acidity (VFAs production). As discussed earlier (Section 4.2.3), a very low pH can inhibit the hydrogen production, while the acidic range (5-6) favors H_2 production depending on the type of substrate. The use of an excessive amount of buffers, acids or base to maintain the pH acidic can decrease the economics and sustainability of the process as well as increase the salt concentration of the DF effluents.

One of the sustainable solutions could be to explore substrates with a higher pH or alkalinity to equilibrate the system. (Choi and Ahn (2013) suggested the use of substrates with a high pH to replace the use of buffers. Life Cycle Analysis (LCA) of two-step thermophilic DF followed by photofermentation of potato peels, showed that most of the impact was generated by the use of the phosphate buffer during the process (Ochs et al., 2010). To provide natural buffering, Cavinato et al. (2011) recycled the reject water (effluent) from the AD step in the two-step DF and AD. The AD reject water provided alkalinity to maintain the pH in the DF step around 5.5, giving a H_2 yield of 51 L/kgVS of food waste fed with a H_2 content of 37% in the biogas. However, the major concern with the recirculation of the AD reject water is the activity of methanogens present in the reject water, which can affect the purity of the biohydrogen produced in the DF step. In addition, inhibition of H_2 production due to higher levels of ammonia present in the reject water could be another concern as reported in a study by Cavinato et al. (Cavinato et al., 2012). Thus, long term studies to assess the effect of reject water recirculation from the AD step on the H_2 content in biogas produced from DF could open further doors to ensure the sustainability of DF systems.

Solid State Dark Fermentation (SSDF)

Anaerobic reactors are generally categorized into wet (<10% TS), semi-dry (10–20% TS) and dry (>20% TS) processes (Karthikeyan and Visvanathan, 2012). However, some categorized wet digestion for low (<15% TS) substrates and dry digestion of high solids (>15% TS) processes (Motte et al., 2013). By increasing the TS content, dry fermentation processes can be operated at a high OLR with little water addition, which offers advantages such as smaller reactor volume, easy handling of the digestate residues and technical simplicity (Karthikeyan and Visvanathan, 2012; Motte et al., 2013). This could

be attractive for commercialization of these processes. However, the drawbacks of SSDF are the low H_2 yields due to mass and energy transfer limitations, which affects the product formation (Robledo-Narváez et al., 2013).

Using agro-industrial wastes (70% sugarcane bagasse, 15% of pineapple peelings and 15% of waste activated sludge) under mesophilic conditions, Robledo-Narváez et al. (2013) found a decrease in H_2 yield (3 mmol H_2/g TS) at a TS content higher than 18 %TS in a tested TS content range from 15-35%. Similar results were obtained by Valdez-Vazquez and Poggi-Varaldo (2009), where the highest H_2 productivity and yield (463.7 NmL/kg/d and 54.8 N mL/g VS removed, respectively) was obtained at a TS of 20.9 % using organic solid waste (paper (40%) and food (60%) wastes) for the tested TS range from 20.9 – 35.1 %TS. Likewise, Motte et al. (2014) also reported 19 %TS as the limit to achieve higher H_2 production performance during the DF of wheat straw, as metabolic pathways shifted towards lactic acid formation at higher TS content. Further research is required on SSDF in order to elucidate the mechanisms involved during dark fermentation at high TS contents. This research could provide practical solutions for biohydrogen production from organic solid waste.

2.1.11 Conclusions

DF technology has an excellent future potential for biohydrogen production as renewable biomass can be used as a feedstock and the integration with other systems could foster a higher H_2 yield and economic feasibility. The economic considerations and production at industrial scale recommend a continuous bioprocess. Thus, more research on continuous DF processes needs to be carried out to demonstrate the long-term operational feasibility of continuous processes. Microbial community of hydrogen producers and innovative substrates needs to be explored. The use of spent dark fermentation residues in photofermentation and or electrochemical systems as a secondary step could pave the way towards sustainable biohydrogen production in up-scaled systems. Finally, anaerobic digestion is required to further stabilize the residues generated from the upstream processes. The future design and configuration of industrial scale dark fermentative processes is expected to be similar to anaerobic digestion processes, with some modifications in process parameters. Existing two stage methane-producing plants can be modified for dark fermentation, while SSDF fermentation opens new opportunities for biohydrogen production from renewable biomass.

2.2 Valorization of dark fermentation effluents via photo fermentative production of biohydrogen and biopolymers

This chapter presents the research advances in utilization of dark fermentation effluents, which mainly contain volatile fatty acids (VFAs), to produce biohydrogen (H_2) and biopolymers by photofermentation (PF) processes. The recent and past studies of PF of organic substances, mainly organic acids, using the purple non-sulfur bacteria (PNSB), are presented. The different laboratory and pilot scale PF studies carried out and the conditions necessary for optimal H_2 production and/or synthesis of biopolymer, polyhydroxybutyrate (PHB), using PNSB strains are reported. This review also focuses on the design considerations of the photobioreactors and economics of production. In the context of increasing application of PF process for waste valorization via H_2 and PHB production, this work provides a state of art of the technology in operational parameters such as bacterial strains, substrate types, light intensity, concentration, culture pH and temperature and the design consideration for photobioreactors for the valorization of dark fermentation effluents by the application of PNSB as future reference.

2.2.1 Introduction

Most energy fuels, chemicals and raw materials in our daily lives are derived from petroleum based refineries. However, depleting fossil fuel reserves and increasing greenhouse gas emissions and severe pollution problems as the consequence of by-products from fossil fuel utilization is driving interests towards biorefineries for the production of energy and useful chemicals (Cherubini 2010; Menon and Rao 2012). In the energy and environmental sector, hydrogen (H_2) has gained considerable interests owing to its higher specific energy content (122 MJ/kg) as well as water and energy being the sole by-products (Balat and Kırtay 2010). At present, H_2 production for industrial applications is mainly derived from thermo-catalytic and gasification processes, which are highly dependent on fossil fuels. In comparison to the energy intensive physico-chemical routes for H_2 production, biological processes can be operated at ambient conditions and are advantageous as they can utilize renewable biomass (Ghimire et al. 2015; Das and Veziroglu 2001).

Based on the light dependency as an energy source for the biochemical reactions, biological H_2 production pathways can be broadly categorized into light dependent and

independent processes (Das & Veziroglu 2008; Hallenbeck & Ghosh 2009). The light dependent photo-hydrogen production systems can be further classified into i) direct photolysis, where water is broken down into H_2 and O_2 gas by algae and cyanobacteria, ii) indirect photolysis in which cyanobacteria or cyanophytes synthesize H_2 in the presence of light and inorganic carbon, and iii) photofermentation (PF), carried out by photosynthetic bacteria where photodecomposition of organic compounds occurs. The light independent processes include i) dark fermentation (DF), which involves fermentative hydrogen production from carbohydrate rich organic biomass, and ii) H_2 from bio-electrochemical systems or microbial electrolysis cells.

DF is a well studied biological route for the production of hydrogen from organic biomass, including waste, owing to its higher H_2 production rates than light dependent processes (Ghimire et al. 2015). However, due to the thermodynamic constraints, dark fermentative conversion of carbohydrate rich organic biomass offers lower H_2 yields and gives incomplete conversion of organic biomass, i.e. organic acids and alcohols remain as major fermentation by-products. On the brighter side, the PF processes can convert these dark fermentative by-products to biohydrogen. Moreover, PF processes have higher H_2 yields and generate less residues compared to DF processes (Li and Fang 2009; Lo et al. 2010). A dual system can integrate the conversion of carbohydrates to organic acids in the first stage (DF) and the utilization of its by-products in the second stage (PF) (Redwood et al. 2008).

Using light as a source of energy, purple non-sulfur bacteria (PNSB) synthesize H_2 by carrying out an anaerobic photosynthesis. In PNSB, this takes place in the presence of the nitrogenase enzyme and light, with reduced carbon sources such as organic acids. In addition, under certain operating conditions, PNSB also synthesize cell reserve materials or biopolymers, i.e. polyhydroxybutyrate (PHB) molecules (Khatipov et al. 1998; De Philippis et al. 1992).

An example of PF is the conversion of acetic acid to biohydrogen and/or biopolymers. It can be expressed by the following equations (2.16 and 2.17):

$$2CH_3COOH + 4H_2O \xrightarrow{\text{Light energy}} 8H_2 + 4CO_2 \tag{2.16}$$

$$2CH_3COOH + 2[H] \rightarrow \text{PBH-monomer} + H_2O \tag{2.17}$$

Photofermentative H_2 production systems are attractive because of their higher H_2 yield potential, i.e. 66.67 mmol H_2/g COD (Eqn. 2.16) from PF systems compared to only 22.22 mmol H_2/g COD from the DF process with acetate as sole by-product (Eqn. 2.1). Moreover, the biopolymer production can add an economic value to the PF process. However, photofermentative production of H_2 and PHB are competing processes (Wu et al., 2012; Khatipov et al. 1998). Nonetheless, a concomitant production of H_2 and PHB is also possible, as shown in a study by Montiel-Corona et al. (2015). The photofermentative H_2 and PHB production depends on several operating conditions, such as nutrients availability (carbon to nitrogen ratio (C/N) ratio), PNSB strain (mixed or pure culture), pH, light intensity and presence of physical-chemical stress, for example the presence of inhibitors of H_2 formation such as ammonium in the culture medium (Adessi and De Philippis 2014; Chen et al. 2011; Li and Fang 2009).

The ability of PNSB to convert reduced carbon sources such as organic acids and alcohols to H_2 and PHB makes PNSB based PF a good post treatment process for dark fermentation effluents (DFE) (Cheng et al. 2015; Chookaew et al. 2015; Dipasquale et al. 2015; Nasr et al. 2014; Rai et al. 2014). Moreover, the potential of the PF process to be operated as stand alone system for wastewater treatment has also been reported (Li and Fang 2009; Eroğlu et al. 2008; Hülsen et al., 2014). With the increasing application of DF processes for H_2 production, the integrated DF-PF process can enhance H_2 yields, thus providing sustainability to scaled-up biohydrogen production processes. Likewise, the potential of PF processes for the production of biopolymers can give further economic gain.

This chapter aims to summarize the state of the art of PF processes for H_2 production by overviewing existing understanding of the microbiology of the PF process, different photobioreactor (PBR) design, conversion efficiencies of different PNSB strains, process operational parameters such as pH, temperature, nutrient requirements. This competence can be applied for the valorization of DFE and wastewater through H_2 and PHB production. In addition, this work presents current approaches of the mathematical modeling of PF as well as highlights the economics of the process.

2.2.2 Microbiology and phototrophic metabolism of PNSB

Bacterial photosynthesis

Bacterial photosynthesis can be divided into two types depending on the presence or absence of oxygen for the metabolism of bacteriochlorophyll, a bacterial photosynthetic pigment. Oxygenic photosynthesis is carried out by cyanobacteria and prochlorophytes, whereas anoxygenic photosynthesis can be generally mediated by purple bacteria, green sulfur bacteria, heliobacteria and others (Kim and Gadd 2008). Photosynthetic anoxygenic bacteria are a very diverse groups of bacteria which carry out bacteriochlorophyll dependent photosynthesis as a metabolic process (McEwan 1994). The anoxygenic phototrophic bacteria can be broadly grouped into different classes (Figure 2.9), based on their photosynthetic pigments and electron donors (Kim and Gadd 2008; McEwan 1994). Depending on the electron donors used, purple bacteria can be further divided into purple sulfur bacteria (use sulfur compounds as electron donors) and non-sulfur bacteria (use organic substances as electron donor).

Some drawbacks of this photofermentative system as pointed by Hallenbeck & Benemann (2002) include inherent high energy demand associated with the nitrogenase enzyme, lower photo conversion efficiencies and economic issues of anaerobic photobioreactors covering large areas. These drawbacks can be overcome by effective design and operation of the photobioreactors (PBRs) and selecting proper strains or enrichment of PNSB for an efficient conversion to photo-H_2.

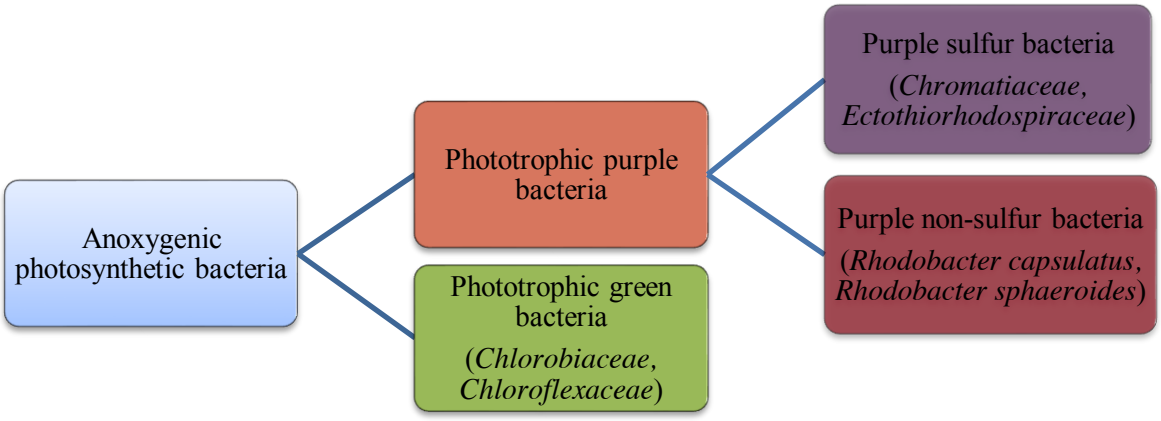


Figure 2.9 - Classification of anoxygenic photosynthetic bacteria

Purple non-sulfur bacteria (PNSB)

Among the anoxygenic bacteria, the PNSB exhibit very diverse morphological, biochemical and metabolic properties (Imhoff et al. 1984). PNSB are gram-negative photo-heterotrophs, which normally carry out photosynthesis under anaerobic conditions. Although PNSB are facultative anaerobes, they can also grow chemotropically under oxygenic conditions using oxygen as electron acceptor (McEwan 1994). Different from purple sulfur bacteria, which use elemental sulfur as the electron donor, PNSB typically use organic electron donors such as organic acids, however, they can also use hydrogen gas as electron donor (Kim and Gadd 2008).

PNSB can utilize various types of carbon sources such as short-chain organic acids and glucose. The theoretical photofermentative conversion of different organic acids, typically present in DFE, to H_2 can be expressed by the reactions presented in Eqns. 2.9 - 2.12.

However, the conversion ability of different PNSB for different substrates varies (Barbosa et al. 2001; Bianchi et al. 2010). Some species prefer a certain sole carbon source, while H_2 yields seem to be higher with mixed sources of carbon (Han et al. 2012). The variation in H_2 production from different carbon sources can be explained by differences in their reduction states and the associated metabolism for the assimilation of different carbon sources (Kars and Gündüz, 2010; Han et al., 2012; Wang et al. 2014). Similarly, when the carbon source is acetate, most of the reducing power of the PNSB is utilized for the synthesis of PHB rather than H_2 (Hustede et al.,1993; Kars and Gündüz 2010).

Photosystem of PNSB

The photosynthetic apparatus of PNSB is simple as it contains only one photosystem (PS), unlike the two PS in algae and cyanobacteria. PNS bacterial cells contain bacteriochlorophyll α or β located on cytoplasmic membrane. The PS of PNSB contains the light harvesting complexes that absorb photons initiating a charge (electron-hole) separation through excitation (Figure 2.10). Electrons that are liberated from organic acids are transported around through a number of electron carriers, i.e. the cytochrome C_2 complex, cytochrome bc_1 complex (Cyt bc_1) and quinone Q (Figure 2.10). The transfer of electrons across the membranes creates a large proton gradient which drives the

synthesis of ATP from ADP by ATP synthase (Figure 2.10) (Akkerman et al., 2002; Hu et al., 2002). The extra energy in the form of ATP will be used to reduce ferredoxin-fd. Then, the ATP and reduced ferredoxin drives the proton reduction to hydrogen by nitrogenase (Hallenbeck and Ghosh 2009). Thus, as a result of anoxygenic photosynthesis, conversion of organic substances into H_2 takes place.

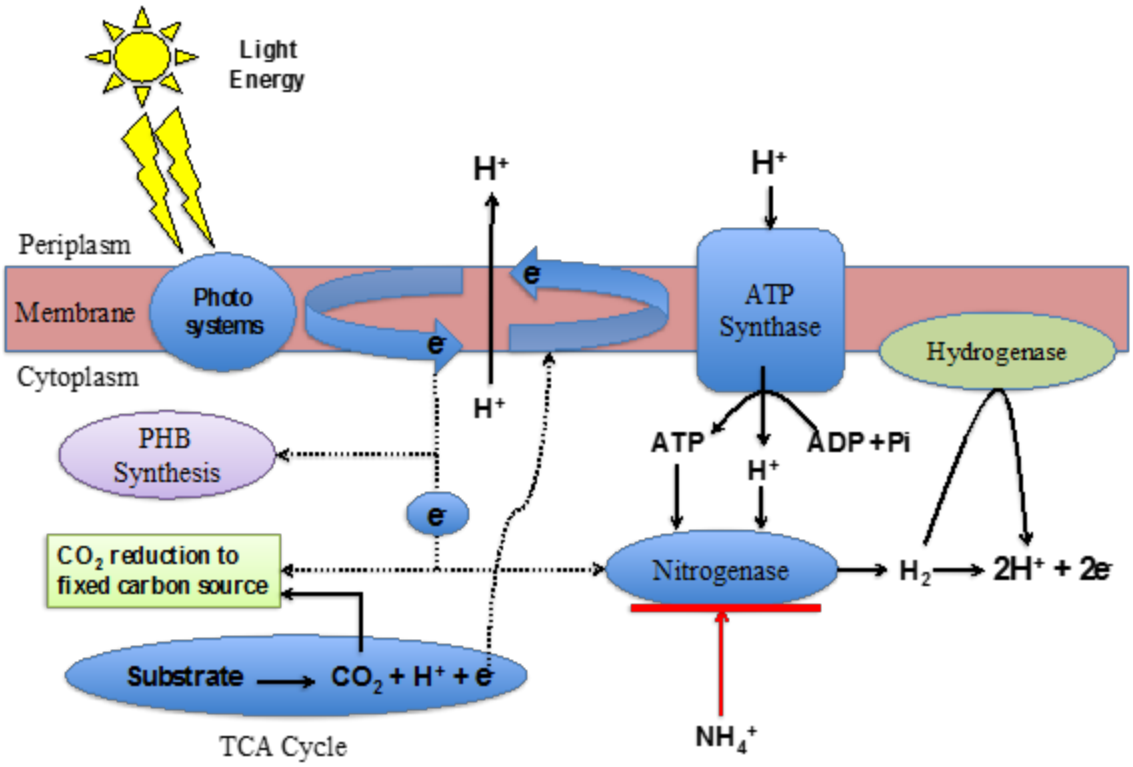


Figure 2.10 - Schematic representation of mechanisms of photofermentative H_2 and PHB production in PNSB (Adapted and modified from Adessi and De Philippis (2014); Akkerman et al. (2002); Kars and Gündüz (2010))

Nitrogenase and hydrogenase are the two enzymes that strongly influence hydrogen production: nitrogenase promotes its production, whereas hydrogenase consumes hydrogen (Figure 2.10). Besides the light conditions, the PF culture medium should be under nitrogen limitation and oxygen should be absent, as their presence inhibits the nitrogenase activity (Koku et al. 2002; Li and Fang 2009; Kars and Gündüz 2010). The activity of the nitrogenase enzyme is of fundamental importance for efficient photo-H_2 production (Hallenbeck and Benemann 2002). Equations 2.18 and 2.19 explain the effect of N_2 on the metabolism of PNSB (Das and Veziroglu 2001):

With dinitrogen: $N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi$ (2.18)

Without dinitrogen: $8H^+ + 8e^- + 16ATP \rightarrow 4H_2 + 16ADP + 16Pi$ (2.19)

The presence of nitrogen, either in gaseous form or in the culture medium, can thus inhibit the activity of the nitrogenase enzyme that synthesizes molecular H_2. Therefore, substrates with a high C/N ratio are more suitable for H_2 conversion in these systems.

PHB accumulation by PNSB

PNSB accumulate poly-β-hydroxybutyrate (PHB), an intracellular storage of carbon and energy formed under physiological stress, particularly, at high carbon to nitrogen (C/N) ratio, higher ammonia concentration or sulphur deprived conditions (Khatipov et al. 1998; Eroglu and Melis 2011; Waligórska et al. 2009). The production of PHB and H_2 functions as the way to dissipating the excess reducing power and the PHB synthesis competes with the H_2 production (Figure 2.10). Thus, depending on the aim of the process, the PF can be directed towards H_2 production by suppressing the PHB synthesis through genetic engineering of the PNSB (Kim et al. 2011). Kars and Gündüz (2010) reviewed the different genetic manipulation strategies to improve photofermentative biohydrogen production. They proposed to modify the acetate assimilation pathways that share the common biosynthetic route of PHB.

After the deletion of the PHB producing gene from *R. sphaeroides* KD131, the H_2 production rate was increased from 36.1 ml H_2/l/h to 43.8 ml H_2/l/h (Kim et al. 2011), in accordance with the study of Hustede et al. (1993) who observed an increase in cell growth and H_2 production when eliminating the gene for PHB synthesis in *Rhodobacter sphaeroides*.

In addition, PNSB produce light harvesting bacterial pigments (bacteriochlorophylls and carotenoids) that can be of commercial interests (Venil et al., 2013). This ability of PNSB has been highlighted in a few older studies and need to be explored again (Schmidt 1971; Cohen-Bazire et al. 1965).

Photo-hydrogen conversion efficiencies

Akkerman et al. (2002) suggested three parameters to evaluate the photo-H_2 production process: H_2 production yield, the yield coefficient of H_2 produced relative to the carbon source consumed and the photochemical efficiency (PE). Table 2.11 compares PF and DF systems in terms of H_2 yields from substrate conversion and production rate. PF systems are superior in terms of substrate to H_2 conversion, while they have slower H_2

production kinetics than DF systems. Considering the theoretical conversion of substrate to H_2 from Eqns. 2.9 – 2.12 and the experimental results reported in past studies (Table 2.11), PNSB have a very versatile metabolism and high substrate to H_2 conversion efficiency (Bianchi et al. 2010; McEwan 1994; Rupprecht et al. 2006). Their PF system lacks oxygen sensitivity issues that are encountered in biophotolysis. Moreover, their light utilization proficiency is high, as PNSB can absorb and utilize both visible (400 – 700 nm) and near infrared (700 – 900 nm) light. Also, PNSB use a wide variety of substrates (Eroglu & Melis 2011). The application of PNSB can be promising for PF systems, as they not only give a higher substrate to product conversion and higher H_2 yield, but also benefit in their capability to reduce pollution loads, e.g. treatment of effluents (organic acids) from DF, with the added economic benefit in the form of PHB production, a valuable biopolymer.

Table 2.11 - Comparison of photo and fermentation dark systems for biohydrogen production

Bio H_2 systems (*Microorganisms*)	Carbon source	H_2 production rate mL H_2/L/h	H_2 Yield mL H_2/g COD[a]	References
Photofermentation				
R. palustris WP3-5	DFE	25.2	235.1	(Chen et al., 2010)
R. sphaeroides RV	Succinate	16.5	158.7	(Han et al., 2012)
Mixed culture	DFE	5.7	568.5	(Montiel-Corona et al., 2015)
Dark fermentation				
Kitchen waste compost	Vegetable waste	1000	38	(Lee et al., 2010)
Clostridium thermocellum 7072	Corn stalks	740	140	(Cheng and Liu, 2011)
Klebsiella sp. TR17	Glycerol	48	128.6[b]	(Chookaew et al., 2015)

[a]mL H_2/g COD is calculated from the data provided in the publications
[b]mL H_2/g COD consumed

The photofermentative H_2 production efficiency can also be measured as photochemical efficiency (PE), which is an efficiency parameter with which the light is utilized to produce energy stored as hydrogen in a PF process. The PE depends on the

photosynthetically active radiation (PAR) range, which determines the light energy absorbed by the photofermentative species. For example, green algae have a PAR range of 400-700 nm, while the range for PNSB is 400-950 nm (Figure 2.11). Akkerman et al. (2002) reported the PE values vary between 3 to 10% in green algae. Redwood et al. (2012) achieved a 71% increase in combined photosynthetic activity by illuminating both *Rhodobacter sphaeroides* and *Arthrospira (Spirulina) platensis* by dividing a single beam of simulated sunlight using a dichroic mirror.

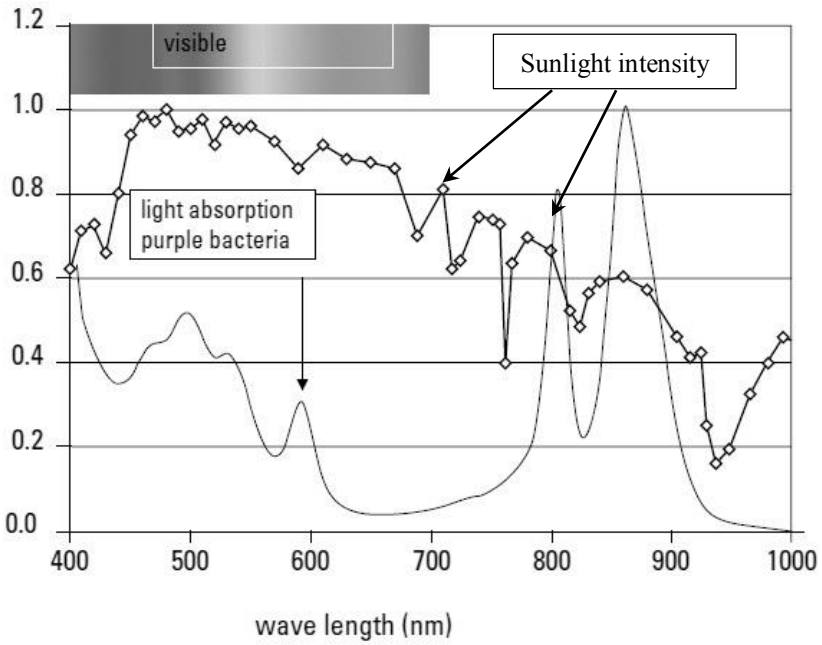


Figure 2.11 - Sunlight and light absorption by purple bacteria (Akkerman et al., 2002)

In addition to H_2 yield and the other parameters mentioned before, the performance of a PF process can be evaluated by the light conversion efficiency. Light or solar energy conversion efficiency can be calculated as the ratio of total energy produced, i.e. stored in the form of H_2, to the total energy input to the bioreactor (energy as photons in case of solar conversion efficiencies). The light conversion efficiency (η) can be calculated with an empirical formula, i.e. the ratio of the total energy (heat of combustion) value of hydrogen to energy input to the PBR by solar radiation (Koku et al. 2002). The η can be evaluated as

$$\eta\,(\%) = \frac{[33.61 \cdot \rho_{H_2} \cdot V_{H_2}]}{[I \cdot A \cdot t]} \cdot 100 \qquad (2.20)$$

Where,

V_{H2} is the volume of produced H_2 in l,

ρ_{H2} is the density of the produced hydrogen gas in g/l,
I is the light intensity in W/m^2,
A is the irradiated area in m^2 and
t is the duration of hydrogen production in hours.

Koku et al. (2002) reported a varying range of light conversion efficiencies between 1–5% on the average for different strains of *Rhodobacter sphaeroides*. According to the best of knowledge of the authors, a PE higher than 10% has not been reported so far. However, a wide range of approaches to increase the PE has been suggested in the literature, including the design of efficient PBR and improving lighting conditions (Adessi and De Philippis 2014; Chen et al. 2011), genetic modifications of PNSB for enhancing nitrogenase activity (Ozturk et al. 2006), reduction of the pigment content for higher light uptake (Kondo et al. 2002), deletion or inactivation of the genes responsible for PHB synthesis (Kim et al. 2011; Franchi et al. 2005) and developing hydrogenase deficient (*hup*$^-$) mutant PNSB strains responsible for H_2 uptake (Franchi et al. 2005; Uyar et al. 2015).

2.2.3 Operating conditions of PF

PNSB inoculum

PNSB are widely distributed in nature and prefer aquatic environments with low oxygen concentrations, significant amounts of soluble organic matter, moderate temperatures and weak as well as stronger light conditions (Imhoff et al. 2005). Besides freshwater, members of the PNSB group can also be found in marine and hypersaline environments and even in sediments that are exposed to light. These organisms can also thrive in thermal springs and alkaline soda lakes (Imhoff et al., 2005). An eutrophic lake is an example of a favorable habitat for members of these genera (Imhoff et al. 2005; Bianchi et al. 2010).

Rhodopseudomonas palustris, *Rhodobacter sphaeroides*, *Rhodobacter capsulatus* and others are the most studied PNSB stains. However, PNSB strains capable of utilizing the substrates and light at higher conversion efficiencies are of research interest. Many studies have successfully isolated H_2 producing PNSB strains from different mixed consortia.

Some examples of isolated H_2 producing PNSB and their H_2 yields and production rates are presented in Table 2.12.

Afsar et al. (2011) carried out PF studies using different PNSB strains, which showed the PF efficiency highly depends on the effluent composition and bacterial strain used. The PF was carried out using the effluents from the thermophilic DF of glucose and potato steam peel hydrolysate as carbon source under indoor batch conditions. The PNS strains, such as *Rhodobacter capsulatus* (DSM1710), *Rhodobacter capsulatus* hup$^-$ (YO3), *Rhodobacter sphaeroides* O.U.001 (DSM5864), *Rhodobacter sphaeroides* O.U.001 hup$^-$ and *Rhodopseudomonas palustris*, were used in the study. The results showed that *Rb. sphaeroides* gave the highest amount of hydrogen from PF of glucose dark fermentation effluents, while *Rb. capsulatus* produced better results on effluents from the dark fermentation of potato steam peels hydrolysate.

However, the use of pure cultures of bacterial strains demands maintenance of sterile conditions in the bioreactors. The varying PF efficiencies of different PNS bacterial strains on different substrates suggests, for substrates such as DFEs which contain mixed organic acids, the use of mixed consortia of PNSB bacteria in order to exploit the substrate utilization capacity of different PNS bacterial strains. In a study by Montiel-Corona et al. (2015), the H_2 yields from enriched mixed PNSB cultures was higher (1478 ± 17 mL H_2/L) than from pure *R. capsulatus* cultures (1252 ± 20 mL H_2/L).

Inoculum Age

The selection of inoculum culture age can be critical to obtain a higher performance of PF systems. It has been found that the PNSB inoculum from the exponential phase of the growth curve is suitable for better performance of PBRs for biohydrogen production (Basak and Das 2007). Koku et al. (2003) found vast differences in total H_2 production, H_2 production rates and the overall substrate conversion rates when *Rhodobacter sphaeroides* O.U. 001 of two different inoculum ages were used in the PF of malic acid. The inoculum harvested from the mid-exponential phase gave a higher total gas production (357 mL H_2), gas production rate (0.009 mL H_2/L/h) and overall substrate conversion rate (35%) than from an inoculum harvested at the stationary phase, which gave a lower total gas production (236 mL H_2), gas production rate (0.003 mL H_2/L/h) and overall substrate conversion rate (24%).

In a study by Sasikala et al. (1991) on the effect of culture age on the photo-production of hydrogen by *R. sphaeroides* O.U. 001, the inoculum with a 20 hour culture period gave the highest H_2 evolution (60 mL H_2/L reactor), while it was lower for a short (4 h) or long (38 h) culture period. A range of optimal inoculum ages has been reported in the literature. Akroum-Amrouche et al. (2011) reported an optimum inoculum age of 36 - 48 hours in PF using *Rhodobacter sphaeroides* CIP 60.6, while Liu et al. (2011) reported an inoculum age of 24 hours for *Rhodoseudomonas faecalis* RLD-53 as optimum.

The aged inoculum can give poor performance in terms of H_2 production and large retention times may shift the metabolic pathways to accumulation of poly-β-hydroxybutyrate (PHB) (Koku et al. 2003). They also reported that a repeated culture of PNSB might lead to loss of H_2 production capacity due to a decline in the activity of the electron carrier ferredoxin.

Cell immobilization

Studies have used different cell immobilization techniques in order to have the advantage of operating the PF process in the exponential growth phase for an infinite period of time and protect the culture strains from the inhibitory effects of chemicals which might be present in influent (Chen & Chang 2006; Liu et al. 2011; Zhu et al. 1999a; Zhu et al. 1999b). However, a major limitation in cell immobilized PF systems is the penetration and transmission of light through the immobilization media. Also, the cell immobilization technology might not be practical when the PNSB cells are required to be harvested for PHB production.

Zhu et al. (1999a) used cationic polyelectrolytes, such as chitosan, poly-L-lysine (PLL), polyethyleneimine (PEI) and trimethylammonium glycol chitosan iodide (TGCI), to entrap *Rhodobacter sphaeroides* in order to prevent the inhibitory effect of NH_4^+ on H_2 production. In another study by Chen & Chang (2006), a small amount of solid carrier, e.g. activated carbon, silica gel, or clay, was used for immobilization of *Rhodopseudomonas palustris* WP3-5 cells. The results of the study showed 67.2–50.9% and 37.2–32.5% increases in H_2 production rate and H_2 yield, respectively, when clay and silica gel were used. Similarly, Zhu et al. (1999b) demonstrated that the immobilization in agar gels could protect the PNS strains from inhibitory effects of the ammonium ion in photofermentative hydrogen production from tofu wastewater using *Rhodobacter sphaeroides*.

Table 2.12 - Comparison of photo-H_2 production by different isolated and mixed PNSB strains from various inoculum sources

Microbial Inoculum sources	Isolated PNSB members	Highest H_2 producing stain	Main Carbon source	Temp. °C	pH	Light intensity	Maximum H_2 yield	Maximum H_2 production Rate (mL H_2/L/h)	References
Pig dung	Not reported	*Rhodopseudomonas palustris*	Acetate	30	7	5,000 lx	660 ml at 13th day	-	Yanling et al., 2008
Wastewater ponds	*Rhodobacter sps.*	*Rhodobacter sphaeroides* ZX-5	Butyrate	30	6–9	4,000 lux (Tungsten lamps)	-	118	Tao et al., 2008
Water and lake bed samples	Not reported	Unidentified PNSB strain TN1	Acetate	30	-	3,000 lux	1.85 mol H_2/mol acetate	43	Suwansaard, et al., 2009
Freshwater pond sludge	Not reported	*Rhodopseudomonas faecalis* strain RLD-53	Malate	35	7	4,000 lux (Incandescent lamp)	3.55 mol H_2/mol acetate	25	Ren et al., 2009
Lake water and sediment samples	*Rb. Capsulatus, Rs. rubrum, Rb. Sphaeroides, R. palustris* stain AV33	*Rhodopseudomonas palustris* stain AV33	Lactate	30	6.8	200 mmol (photons) m^2/s (Incandescent lamp)	-	50.7	Bianchi et al., 2010
Activated sludge	*R. palustris*	Unidentified PNSB mixed culture	DFE of starch wastewater	31	5.5	190 W/m^2 (Tungsten lamps)	0.97 ± 0.1 L/g $COD_{consumed}$	120.8±7	Tawfik et al., 2014
Activated sludge	Not reported	Unidentified enriched IZT PNSB	DFE	30	7.0	3000 lux (LEDs and halogen lamps)	1478 ± 17 mL H_2/L	5.7	Montiel-Corona et al., 2015
Silt sewage, pig manure, and cow dung	Not reported	Unidentified PNSB mixed culture	Enzymatic hydralysate of corncob	30	7.0	4000 lux (LED lamps)	11.5 L H_2 /L	165	Zhang et al., 2015

Carbon sources and nutrients sources

The substrate types and their concentration used in PF can influence the H_2 production rates and yields. Han et al. (2012) studied the effect of different carbon sources and their concentrations on the photo-H_2 production using a batch culture of *Rhodobacter sphaeroides* RV. The substrates used were either individual substrates such as acetate, propionate, butyrate, lactate, malate, succinate, ethanol, glucose, citrate or sodium carbonate or mixed carbon sources such as malate and succinate, or lactate and succinate. The results of the study showed that the H_2 production for the mixed substrates is higher (794 mmol H_2/mol substrate for 2.02 g/L lactate and 2.0 g/L succinate) than using a single substrate (424 mmol H_2/mol substrate for 0.8 g/L sodium propionate). This makes PF prominent for the application in the treatment of DFE that typically contains more than one organic acid (Nasr et al. 2014; Rai et al. 2014).

Effect of OLR and HRT

Similarly, the OLR and HRT could affect the performance of PBRs as they determine the substrate degradation efficiency and the hydrogen production rate. Mohan et al. (2008) studied the effect of different OLRs on photo-H_2 production and substrate degradation efficiency. The synthetic wastewater gave the maximum substrate degradation efficiency (1.4 kg COD/m^3/day) at an OLR of 2.45 kg COD/m^3/day, while higher specific H_2 production (19.29 mol H_2/kg $COD_{removed}$) was achieved at an OLR of 1.4 kg COD/m^3/day with 45% COD removal. In another study, Tawfik et al. (2014) studied the effect of varying OLR (3.2 to 16 kg COD/m^3/day) using mixed PNSB cultures, which resulted in maximum H_2 production at an OLR of 6.4 kg COD/m^3/day. Increasing OLR caused VFAs accumulation, which might inhibit the PNSB. Therefore, inhibition of the nitrogenase activity resulted in decreasing H_2 production when the OLR was higher than 6.4 kg COD/m^3/day (Tawfik et al. 2014). This is supported by another PF study carried out with acid hydrolyzed wheat starch and a pure culture of *Rhodobacter sp*. (Kapdan et al. 2009). The results of the study showed that, upon increasing the initial sugar concentration from 2.2 to 13.0 g/L, the H_2 yield (H_2Y) increased, with a maximum H_2Y achieved at 5 g/L (143.5 mL H_2/g COD).

A range of optimum HRT, varying from 2.5 h (Tawfik et al., 2014) to 3 days (Ozmihci and Kargi 2010) has been reported in the literature for achieving higher photo-H_2

production in a continuous reactor. Tawfik et al. (2014) found an optimum HRT at 2.5 h (0.97 ± 0.12 $LH_2/gCOD_{removed}/d$), when studying a range of HRT from 0.9 to 4.0 h. They also observed the improvement in removal efficiency of butyrate and lactate when the HRT was increased. Similarly, another study carried out with mixed PNSB by Zhang et al. (2015) showed that varying HRTs from 12 to 72 h significantly affected the H_2Y with the highest H_2Y of 482.4 mmol H_2/L obtained at a HRT of 36 h. In contrast, Ozmihci and Kargi (2010) obtained the highest H_2Y and production rate at an HRT of 72 h during PF of DFE using *Rhodobacter sphaeroides*. The differences in optimum HRT may be attributed to differences in PNSB strains, substrate concentration, carbon to nitrogen ratio (C/N) and other operating conditions such as pH, temperature and light intensity.

Effect of C/N ratio

The carbon to nitrogen ratio plays an important role in the growth of PNSB, photo-H_2 and PHB production. However, higher levels of nitrogen inhibit H_2 production while higher C/N ratios enhance the production of PHB (Eroglu et al. 1999; Koku et al. 2003; Argun et al. 2008; Waligórska et al. 2009). A low C/N ratio can result in the accumulation of ammonia, which inhibits the nitrogenase and thus the H_2 production process. Therefore, it is always desirable to have nitrogen-limited conditions in the PBR. Due to the nitrogen requirements for bacterial photosynthetic metabolism and inhibition of nitrogenase at higher ammonium concentrations, there is a tradeoff between the minimum amount of nitrogen for bacterial growth and non-inhibiting levels.

A range of C/N ratios has been reported in the literature, i.e. from as low as 8 to as high as 120. Eroglu et al. (1999) reported the optimum C/N ratio of 15 mM to 2 mM (malic acid to glutamic acid) for the maximum hydrogen production rate. In another study, Boran et al. (2010) reported a C/N ratio of 45 with 40 mM of acetic acid and 2 mM of sodium glutamate in PF by *Rhodobacter capsulatus* in a solar tubular photobioreactor under outdoor conditions. Similarly, Argun et al. (2008) reported the optimum total VFAs and NH^+_4-N concentrations of 2350 mg/L and 47 mg/L, respectively, for increasing the H_2 production by *Rhodobacter sphaeroides* strains. In another study (Eroğlu et al. 2009), the highest H_2 production potential of 19.9 m^3 H_2/m^3 was obtained from olive mill wastewater with the highest C/N molar ratio of 73.8.

Waligórska et al. (2009) found that accumulation of PHB increased by 30 fold when the C/N ratio increased from 6 to 120 in *R. sphaeroides*. However, the amount of PHB

accumulation mainly depends on the PNSB strains and the other process operational conditions (De Philippis et al., 1992; Montiel-Corona et al., 2015). As PHB biosynthesis is a H_2 competing pathway, its concomitant production with H_2 could raise future interests, as PHB possesses economic value as a biodegradable polymer (Koku et al. 2002). Some of the results from previous studies on H_2 and PHB production in PF processes are summarized in Table 2.13.

Micronutrients

Microorganisms need different micronutrients such as iron and nickel for their metabolism and growth. The PF process relies on the photosynthetic electron transport systems from which bacteria obtain their energy (Figure 2.10). The constituents of the electron transport systems such as cytochromes are Fe protein complexes and PNSB strains have 24 Fe atoms in each nitrogenase (Zhu et al., 2007). Another electron carrier, ferrodoxin, also contains Fe. Thus, Fe limitation can influence the metabolism of PNSB and production of H_2.

Uyar et al. (2009) found that the hydrogen yield increases from 0.3 to 1.0 $L/L_{culture}$ when iron was added to micronutrient. They suggested 0.1 mM of ferric citrate as optimum concentration for hydrogen production. Similarly, Zhu et al. (2007) studied the effect of ferrous ion (0 - 3.2 mg/l) on PF using *Rhodobacter sphaeroides* and found that the photo-H_2 production was significantly suppressed when Fe^{2+} was limited. The H_2 production increased when increasing the Fe^{2+} concentration and reached the maximum at the concentration of 2.4 mg/l. In another study, Rai et al. (2014) studied the effects of Ni^{2+}, Fe^{2+} and Mg^{2+} on the PF of cheesewhey for H_2 production, and showed significant effects of Ni^{2+} and Fe^{2+} supplementation on H_2 yields. However, the presence of nickel might also enhance the hydrogenase activity, which takes up the H_2 produced by the nitrogenase activity, thus decreasing the net H_2 production yield (Li and Fang, 2009).

Presence of bicarbonate

Some studies have shown that addition of bicarbonate and carbonate ions enhances the H_2 production in PF (Montiel-Corona et al., 2015; Takabatake et al., 2004). Bicarbonate and carbonate function as electron acceptors and enhance the utilization of butyric and propionic acids, while their absence unbalances the oxidation-reduction potential resulting in decreased H_2 production. Takabatake et al. (2004) reported that the presence

of carbonate improves assimilation of ammonium (NH_4^+) and VFAs. They also observed that the uptake of acetate releases carbonate, however it was not enough to promote butyrate and propionate consumption, which are more oxidative than bacterial cells. For PNSB growth on butyrate, each mole of butyrate requires 0.7 mol of CO_2 (Montiel-Corona et al. 2015).

Effect of light intensities and wavelength

The light conversion efficiency (η) varies for different PNSB strains because of their different light harvesting antenna pigments, thus they have a different photosynthetically active radiation (PAR) range. However, η also depends on the light intensity, illuminated area of the PBR, reactor design and other operational conditions of the PF process. Generally, the intensity of light has a positive influence on the H_2 production. There are some studies dedicated to assess the effect of the light intensity on growth and H_2 production by PNSB (Koku et al. 2002; Uyar et al. 2007; Sevinç et al. 2012; Androga et al. 2014; Akman et al. 2015).

Uyar et al. (2007) studied the effect of intensity of light, light wavelength and illumination protocol on the growth and H_2 production by *Rhodobacter sphaeroides* O.U. 001 in photobioreactors (Figure 2.12). The hydrogen production increased with increasing the light intensity and the highest production was reached at 270 W/m^2. The results also showed the decrease in photoproduction of hydrogen by 39% when there is a lack of infrared light (750-950 nm wavelength). The substrate conversion efficiency was increased and hydrogen production was stimulated when the light was illuminated after inoculation and no hydrogen was produced during the dark periods.

Sevinç et al. (2012) studied the effect of temperature (20, 30 and 38 °C) and light intensity (1500, 2000, 3000, 4000 and 5000 lux) on the kinetic parameters and hydrogen production in PF of acetic and lactic acid using *Rhodobacter capsulatus*. The results of the study reported the maximum hydrogen production at 5000 lux for 20 °C and 3000 lux for 30 and 38 °C. In a more recent study, Androga et al. (2014) established an optimal light intensity and temperature of 287 W/m^2 (4247.6 Lux) and 27.5 °C, respectively, in PF tests carried out using *R. capsulatus* DSM 1710 in a medium containing acetate, lactate and glutamate. In another recent study, Akman et al. (2015) reported an optimum light intensity of 263.6 W/m^2 (3955 lux) in a PF study carried out with acetate as the carbon source and *R. capsulatus,* which is in accordance with the study from Androga et al.

(2014), that established 287 W/m^2 as optimum light intensity in PF carried out using *R. capsulatus*.

Future development of PF systems requires an economical solution to provide the sources of light, so that outdoor systems utilizing natural sunlight become a practical option. Therefore, research interests have been growing to exploit the natural sunlight in PF processes (Androga et al. 2012a; Montiel-Corona et al. 2015; Avcioglu et al. 2011; Androga et al. 2011). Even though sunlight cannot ensure continuous light conditions, there are some studies that have shown that the dark and light cycles might not have significant effects on photo-H_2 production (Li et al. 2011) or have positive effects on H_2 production depending on the exposure duration of the light and dark conditions (Sargsyan et al. 2015). Montiel-Corona et al. (2015) reported a 40.25% reduction in H_2 yields during PF using mixed PNSB in comparison to indoor conditions. However, H_2 yields obtained from outdoor reactors can be comparable to those under indoor conditions (Androga et al. 2011). In addition to the type of light source, photofermentative H_2 production also depends on other operating conditions of the PBRs, such as mixing conditions that affects the distribution of light, culture temperature and pH. Furthermore, harnessing the natural light in upscale applications of PF might reduce the cost of long-term PBRs operation.

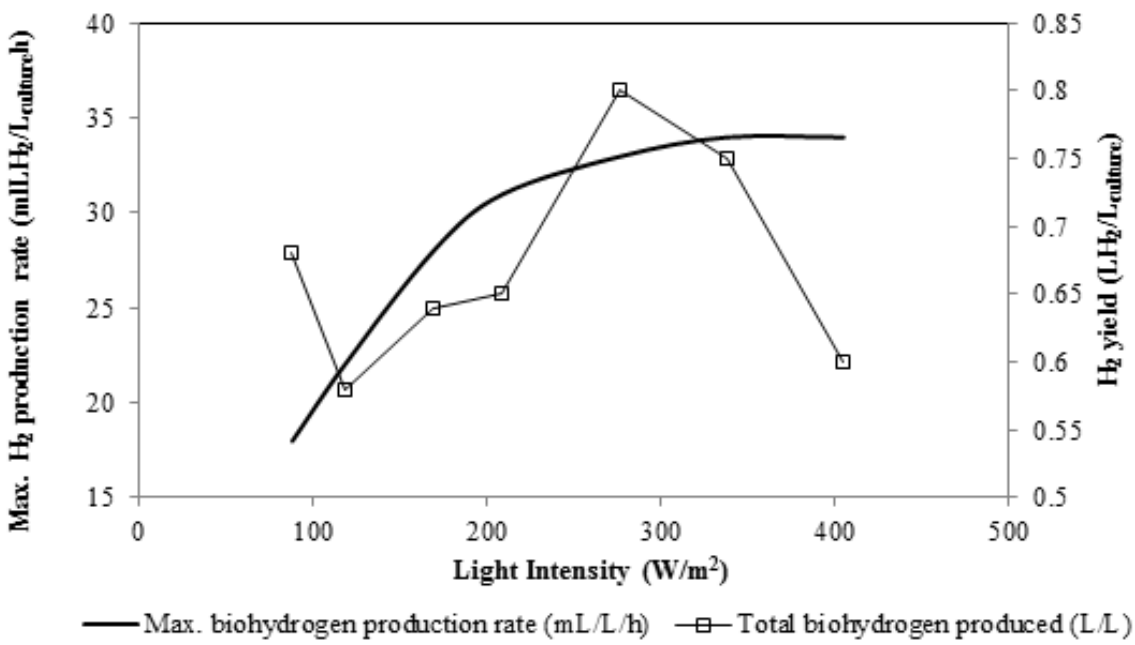


Figure 2.12 - Effect of light intensity on biohydrogen production by *Rhodobacter sphaeroides* O.U. 001 (Uyar et al., 2007)

Table 2.13 - Comparison of hydrogen and PHB production by different isolated strains and enriched mixed cultures of PNS via photofermentation of various carbon sources

Microbial inoculum sources	Main carbon and nitrogen source	C/N ratio	Light intensity	PHB (% Dry cell weight)	Volumetric H_2 yield (mLH_2/L)	References
Rhodobacter sphaeroides 17023 (wild type)	30 mM acetate and 7 mM glutamic acid	8.6	1500 lux of incandescent light	70	0	(Hustede et al., 1993)
Rhodobacter sphaeroides 17023 (wild type)	30 mM acetate and 7 mM glutamic acid	12.86	1500 lux of incandescent light	24	2310	(Hustede et al., 1993)
Enriched photoheterotrophic culture	DFE (11.61 g/L butyric, L 1.76 g/L propionic and 1.01 g/L acetic acid and 0.78 g/L total ammonia		3000 lux of LEDS and halogen lamps	5	1478 ± 17	(Montiel-Corona et al., 2015)
Rhodobacter capsulatus		10.63		29	1252 ± 20	
Rhodobacter sphaeroides O.U. 001 (DSM 5648)	Sugar refinery wastewater (30% v/v in medium)	-	200 W/m^2	70.4	648	(Yiğit et al., 1999)
Rhodobacter sphaeroides strain RV	40 mM Acetate only	-	5000 lux incandescent light	38	0	(Khatipov et al., 1998)

Culture temperature and pH

The operating temperature of a culture is one of the important parameters that affects the bacterial metabolism or metabolic pathways as well as substrate conversion efficiency and thus H_2 production. Basak and Das (2007) reported 31 to 36 °C as optimum temperature for *Rhodobacter sp*., while Androga et al. (2014) reported 26.8 °C (and 285 W/m^2) as optimum culture temperature for a higher H_2 yield. Moreover, culture pH affects the biochemical reactions as it determines the ionic form of the active sites for enzymatic activity (Chen et al., 2011). PF studies have been carried out in the pH range varying between 5.5 to 7.5 (Table 2.12 and 2.14). Akroum-Amrouche et al. (2011) reported an optimum pH of 7.5 (± 0.1) for the H_2 production by *Rhodobacter sphaeroides,* while Nath and Das (2009) have reported an optimum H_2 production at pH 6.5 for the same PNSB species. This difference of change in optimum pH can be attributed to the difference in substrate type used in PF experiments as lactate was used as a sole carbon source in the former, while DF spent medium was used in the latter study. In another study, Koku et al. (2002) reported an optimum pH of 7.1 - 7.3 for the activity of the nitrogenase enzyme, while the range of 6.5 to 7.5 is optimum for the activity of the hydrogenase enzyme.

During most of the PF tests, pH has shown an increasing trend which could be due to PHB production (Khatipov et al. 1998; Nath and Das 2009). Eroglu et al. (1999) reported a slight decrease in pH during the bacterial growth phase and pH increase during H_2 production. The effluents from DF are generally in the acidic pH range (Ghimire et al. 2015), and are required to be adjusted to a pH range 6.5 – 7.5 to ensure the optimum operating conditions in the PF process. However, the range of optimum pH seems to be dependent on the PNSB species. Some studies by Tawfik et al. (2014) and Tao et al. (2008) have shown the feasibility of H_2 production by mixed PNSB at pH 5.5 - 6.0, which is generally an ideal pH range of DFE obtained from DF processes.

Effect of mixing

Mixing is required in PBRs to keep the PNSB biomass suspended and uniformly distribute the substrates and nutrients in the culture medium. Moreover, mixing ensures the uniform distribution of light throughout the PBRs, avoiding light gradients. It also helps to maintain sufficient mass transfer, which generally includes the exchange of gases, i.e. H_2 and CO_2. Akroum-Amrouche et al. (2011) found unstable H_2 production

with a 13.0% and 60.8% reduction of the average and maximum H_2 production rate when mixing was stopped during the exponential phase of PF. In another study, Li et al. (2011) reported that mixing during the H_2 production phase of the PNSB stationary growth phase as vital for higher H_2 yields than during the exponential cell growth phase. Moreover, the type of mixing system may also affect the photo-H_2 production performance. Zhang et al. (2015) showed that baffled PBRs can outperform magnetic-stirred PBRs as supported by higher H_2 yields as well as faster cell growth and substrate conversion. This higher H_2 production can be attributed to enhanced gas transfer and distribution of light in the PBRs due to well mixing conditions.

Inhibition of photo-H_2 production

Nitrogenase plays an important role in the hydrogen generation. Thus, the presence of chemical substances that disrupt the nitrogenase activity decreases the photo-H_2 production. Koku et al. (2002) reported that the presence of N_2 and NH_4^+ inhibit the H_2 production. Also CO, EDTA and O_2 are likely to inhibit the nitrogenase activities. Similarly, an elevated level of CO_2 inside the reactor inhibits the photo-H_2 production, while lower levels (4 - 18% w/v) favor the growth phase of PNSB and thus H_2 production (See Carbon sources and nutrients requirements). Furthermore, a lower C/N ratio does not favor photo-H_2 production as it could result in the accumulation of ammonium and inhibition of nitrogenase in a PF process for H_2 production.

Table 2.14 - Variation of different operational parameters in PF studies

PNS strains	Carbon (& nitrogen) source	Culture type (Reactor type)	Culture Temp. °C	pH	Light intensity	Maximum H_2 yield	Maximum H_2 production rate	References
Rhodobacter sphaeroides O.U.001 (DSM586)	Dark fermentation effluents of glucose	Batch	30°C	buffer 6.4	150 - 200 W/m^2 (Tungsten lamp)	484 mmol H_2/L DFE	1.18 mmol H_2/L/h	(Afsar et al., 2011)
Rhodobacter capsulatus (DSM1710)	Dark fermentation effluents of potato steam peels hydrolysate	Batch	30°C	buffer 6.4	151 - 200 W/m^2 (Tungsten lamp)	117 mmol H_2/L DFE	0.5 mL H_2/L/h	(Afsar et al., 2011)
Rhodobacter capsulatus (Hup$^-$)	Acetic acid (glutamate)	Continuous Tubular PBR	<40°C	below 8	Natural sunlight (Outdoor conditions)	0.35 mol H_2/mol acetic acid	0.40 mol H_2/(m^3·h)	(Boran et al., 2012)
Rhodobacter sphaeroides CIP 60.6	Lactate (glutamate)	Batch	30°C	7	4,500–8,500 lux (Tungsten lamp)	-	39.88 L/m^3/h	(Akroum-Amrouche et al., 2011)
Rhodobacter capsulatus YO3(hup$^-$)	Acetate (glutamate)	Fed-batch panel PBR	35°C	7	Natural sunlight (Outdoor conditions)	-	11.42 LH_2/m^3/h	(Androga et al., 2011)
*Rhodopseudomonas palustris*WP 3-5	Formic, acetic, butyric, lactic acid (glutamate)	Continuous Column PBR	28–35°C	6.8	4,000 -7,000 lux	-	13.26 LH_2/m^3/h	(Lee et al., 2011)

Rhodobacter sphaeroides O.U.001	Malate (glutamate)	Batch Annular PBR	32°C	6.8	15 W/m^2	4.5 mol H_2/mol malic acid	6.5 L H_2/m^3/h	(Basak & Das, 2009)
Mixed culture	Acetate (glutamate)	Batch	34°C	6-7	4,000 lux (Fluorescent light)	-	3.51 mol H_2/Kg COD/d	(Venkata Mohan et al., 2009)
	Butyrate (glutamate)					-	3.33 mol H_2/Kg COD/d	
Rhodobacter capsulatus (DSM 155)	Dark fermented effluents of miscanthus hydrolysate (with iron addition)	Batch	30–33°C	6.6-6.8	4000 lux	1.0 L H_2/L culture	-	(Uyar et al., 2009)
Rhodobacter sphaeroides O.U.001 (DSM 5864)	Malate	Flat panel PBR	32°C	6.8	200 W/m^2 Tungsten lamp	4.6 mol H_2/mol malate	10 mL H_2/L/h	(Eroglu et al., 2008)
Rhodopseudomonas palustris WP3-5	Butyrate (glutamic acid)	Batch	32°C	7.1	10,000 lux (Tungsten lamp)	5.74 mol H2/mol butyric acid	24.9 mL H_2/L/h	(Chen et al., 2007)

2.2.4 PBR systems

PBR reactor configurations

The design considerations of PBRs for photo-H_2 production are similar to those of PBRs for algal biomass production. However, anaerobic conditions are required for the PF process using PNSB. Most of the published reviews on the design of PBRs for biohydrogen production are based on bioreactors for algal biomass production (Akkerman et al. 2002; Dasgupta et al. 2010; Carvalho et al. 2006). In some more recent works, Adessi and De Philippis (2014) and Chen et al. (2011) have summarized the knowledge on the design, illumination and culture strategies of PBR systems aimed at enhancing photo-H_2 production with PNSB.

The most common reactor types reported in the literature are presented in Figure 2.13. More insight has been provided in the performance of different reactors with more elaboration on tubular and flat panel reactors, as these reactors configurations have been the subject of major interest because of their practicability in scaled-up PF processes.

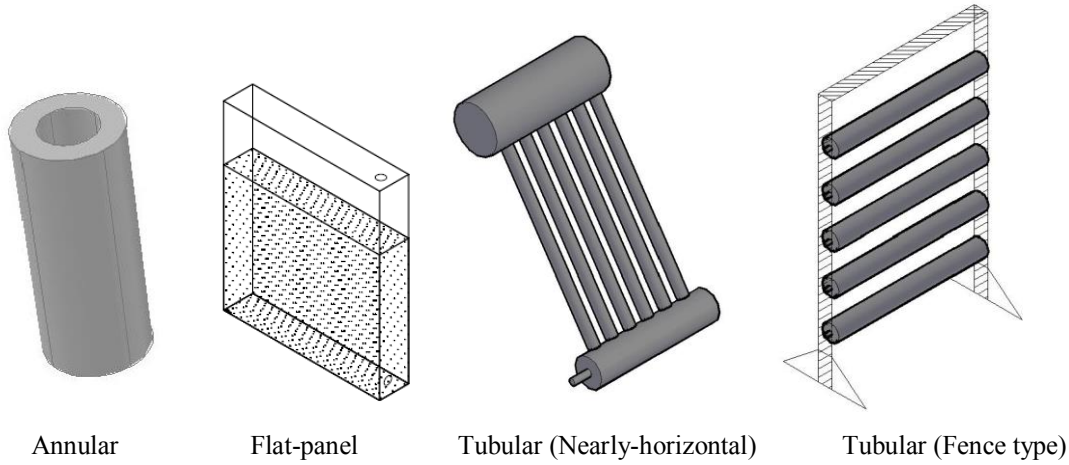


Figure 2.13 - Schematic representation of the potential PBRs for PF

Plate reactors

Plate reactors are flat panels which consist of a rectangular transparent box with a depth vary between 1-5 cm (Akkerman et al. 2002). These reactors have received research attention for photo-H_2 production because of their large illumination area and possibilities of scaling up and suitability in outdoor conditions. Flat plate PBRs are constructed with

cheap materials, which are generally transparent to achieve the maximum utilization and conversion of solar energy.

Eroglu et al. (2008) investigated the performance of an 8 L flat plate PBR under outdoor operating conditions using a culture of *Rhodobacter sphaeroides* O.U.001. Among the different carbon sources such as malate, lactate, acetate and olive mill wastewater used in the PF, the highest hydrogen production rate (10 $mLH_2/L/h$) was reached with malate as carbon source and formate was found to be the dominant end product. Ugwu et al. (2008) reported the following potential problems that flat plate systems can face during scale up:

- requirement of many compartments and support materials
- difficulty in operational temperature control
- wall growth resulting in reduced light penetration

Tubular reactors

Tubular PBRs contain a long transparent tube with a length ranging from 10 to 100 meters and diameters ranging from 3 to 6 cm (Akkerman, et al. 2002). These PBRs are one of the most suitable reactors for outdoor conditions. Generally, tubular PBRs are constructed with transparent glass or plastic tubes. The culture is recirculated with a mixing system (such as a pump) to provide efficient mass transfer and equal light distribution.

Boran et al. (2010) successfully developed and demonstrated a pilot scale (80 L) tubular PBR for photofermentation of acetate using *Rhodobacter capsulatus* in outdoor operating conditions (during winter seasons) in Ankara (Turkey). The PBR gave an average molar productivity of 0.31 mol $H_2/m^3/h$ during daylight hours and the gas contained 99% hydrogen and 1% carbon dioxide by volume. The system provided an overall hydrogen yield of 0.6 mol H_2/mol acetate and the H_2 production with respect to the total illuminated surface area amounted to 0.112 mol $H_2/m^2/day$.

Ugwu et al. (2008) reported some limitations that tubular PBRs face during scale up:

- difficulty in operational temperature control
- fouling and growth on the walls of the tubes
- large space requirements

One of the major problems during the scaling up of tubular PBRs is the decrease in illumination surface to volume ratio because of the increase in diameter of the tube. This

causes a decrease in light intensity (light shading effect) for the cells at the lower part of the tube, which negatively affects the cell growth. However, a good mixing system provides also an efficient light distribution (Ugwu et al. 2003).

Vertical-column reactors

Vertical-column reactors have been subject of research for algal biomass production as they are compact, have low cost and are easy to operate (Ugwu et al., 2008). Bubble columns, airlift reactors and annular column reactors are common vertical-column PBR configurations (Posten 2009). Bubble column reactors have a larger diameter than tubular reactors and are frequently used indoor (at a larger lab scale) or outdoor. Because of the larger diameters in these reactors, darker zones are created at the center of the column, which might be disadvantageous for photosynthetic bacterial growth. Besides these three major reactors types, laboratory scale PF research has been carried out in internally illuminated reactors (Chen et al. 2010). Dasgupta et al. (2010) have briefed the possibility of using different configurations such as torus shaped and helical reactors.

The concept of an annular column reactor aims to overcome the problem associated with the central darker zones in bubble column reactors (Posten 2009). The major advantages of this reactor configuration are high mass transfer rate, good mixing conditions with less shear on bacterial cells, low energy consumption and potential for industrial application. However, the small illumination surface makes this configuration less competitive than other counterparts.

Comparison between panel and tubular PBRs

Table 2.15 compares studies done in various configurations of PBRs. Flat panel and tubular reactors have the highest theoretical efficiencies and have been used at pilot scale under outdoor conditions (Boran et al. 2010; Eroglu et al. 2008; Gebicki et al. 2010). These studies opened perspectives for scaling up of these two promising PBRs for photo-H_2 production using PNSB cultures. In some lab scale studies, higher H_2 productivities were obtained with flat panel PBRs, while some studies with tubular PBRs have shown good performance under outdoor light conditions. Moreover, tubular PBRs are easier to manage and scale-up.

Photo-H_2 production through PF can be a promising technology for clean energy recovery. In addition, recovery of PHB can be of further interest. To establish PF as post

treatment of DFE, more research needs to be performed for improving the system efficiency through optimization of different operating parameters. The system efficiency can be improved by providing optimum culture conditions and bioreactor design. The PF systems have been presented as the bottlenecks in the integrated DF-PF process because of their higher production cost. Thus, innovative low-cost mixing, heating and cooling systems need to be explored and PBR designs for improving the surface area to volume (A/V) ratio require future research. Moreover, PHB can add economic value to the PF process. Using mixed PNSB to utilize the conversion efficiencies of different microbial consortia can give an economic advantage by the reducing cost of H_2 production.

Table 2.15 - Comparison of Tubular and Plate PBRs under outdoor conditions

PNS strains	Carbon source	PBR type	Volume in liters (Dimension)	Operations conditions	Maximum H_2 yield	Maximu H_2 production rate $LH_2/m^3/h$	Productivity per illuminated surface area $LH_2/(m^2 \cdot d)$	Productivity per ground area $LH_2/(m^2 \cdot d)$	Light conversion efficiency	References
Rhodobacter capsulatus DSM155	Acetate, sodium lactate and glutamate	Flat-panel	4×25 L	Summer (Aachen, Germany)	-	12.3	3.69	29.52	0.20%	(Gebicki et al., 2010)
Rhodobacter capsulatus DSM156	Acetate, sodium lactate and glutamate	Tubular	60 L (0.12 m dia. & 0.65 m length)	Summer (Aachen, Germany)	-	6.3	3.35	3.35	0.19%	(Gebicki et al., 2010)
Rhodobacter capsulatus DSM 1710	Acetate, lactate and glutamate	Tubular	80 L	Winter (Ankara, Turkey)	15%	6.9	2.46	1.74	1%	(Boran et al., 2010)
Rhodobacter capsulatus YO3 (Hup–)	Acetate and glutamate	Flat-panel	4 L	Summer (Ankara, Turkey)	53%	11.4	1.5	4.93	-	(Androga et al., 2011)
Rhodobacter capsulatus YO3 (Hup–)	Acetate and glutamate	Tubular	90 L	Outdoor Conditions (Ankara, Turkey)	35%	0.4 mol $H_2/(m^3 \cdot h)$	0.432 mol $H_2/(m^2 \cdot d)$	0.3 mol $H_2/(m^2 \cdot d)$	0.20%	(Boran et al., 2012)
Arthrospira platensis M2 (cyanobacteria)	CO_2	Tubular	34 L	Summer (Florence, Italy)	-	1.26 mol $H_2/(L \cdot d)$	32.95 mol $H_2/(m^2 \cdot d)$	-	5.6% [a]	(Tredici & Zittelli, 1998)
Arthrospira platensis M2 (cyanobacteria)	CO_2	Flat-panel	5.4 L	Summer (Florence, Italy)	-	1.09 mol $H_2/(L \cdot d)$	30.65 mol $H_2/(m^2 \cdot d)$	-	4.8% [a]	(Tredici & Zittelli, 1998)

[a] Photosynthetic efficiency of the cultures was calculated by multiplying the reactor productivity by the mean enthalpy value of the biomass of *A. platensis* M2 cultivated outdoors (21.56 kJ g^{-1}) and divided by the mean visible solar energy input on the culture surface (14.08 MJ/d). Other photosynthetic efficiency was calculated using equation 2.20.

2.2.5 Design considerations for PBRs

In addition to the physical parameters such as quantity of light penetrating into the bioreactor, a good PBR design should consider various physiochemical parameters such as pH, temperature, dissolved oxygen and CO_2, shear due to agitation, C/N ratio, carbon sources and availability of nutrients. As mentioned earlier, these parameters influence various biochemical pathways and ultimately the H_2 production in PBRs.

A general consideration to achieve a good design of PBRs as reported by Dasgupta et al. (2010) includes the following physicochemical parameters which affect the performance of PBRs:

- high light penetration into PBRs
- high surface area to volume ratio (higher illumination area)
- temperature and pH control
- good mixing system
- better gas exchange or mass transfer
- transparency and durability of the materials

Surface area to volume (A/V) ratio

The amount of light absorbed by a reactor system is a limiting factor in PBR systems. Surface area to volume ratio is one of the important parameters to be considered during the design of PBRs as it determines the amount of light entering into the system. The higher the A/V ratio, the larger will be the surface area for receiving light for growth and metabolism. Therefore, the A/V ratio can be directly co-related with cell concentration and the volumetric productivity of the system (Dasgupta et al. 2010).

Gebicki et al. (2009) compared hydrogen productivities of a flat panel (A/V ratio of 20 m^{-1}) and an inclined horizontal tubular (A/V ratio of 15.38 m^{-1}) PBR with respect to illuminated surface area and ground area occupied by the reactor. The mean hydrogen productivity of the flat panel reactor was 1250 $mlH_2/(m^2_{illuminated\ surface}/day)$, while that of the tubular reactor was 1100 $mlH_2/(m^2_{illuminated\ surface}/day)$. The illuminated area per unit ground area occupied by the panel reactor was 8.9 times higher than that of the tubular reactor, which gives the economic edge of the comparison. However, a fenced type

tubular PBR (Figure 2.13) could be a research interest in the future as this reactor configuration occupies less space compared to inclined horizontal tubular PBR.

Mixing systems

Mixing systems in PBRs could include pumping, mechanical stirring and airlift mixers. Ugwu et al. (2003) proposed a static mixer for tubular bioreactors. The selection of the type of mixing system is important as the pumps used for mixing or recirculation exert shear forces that might be harmful to PNSB. Another disadvantage of the mixing system is the additional cost due to the required energy for its operation.

Construction materials

Selection of materials during the construction of PBRs not only determines the economy, but also the performance of the system. Several factors should be considered while selecting the construction materials. PBRs can be constructed from glass, polyvinyl chloride (PVC) material, low-density polyethylene (LDPE), poly-methyl methacrylate (PMMA) and fiberglass. Dasgupta et al. (2010) reported the following considerations for the selection of the construction material for PBRs:

- high transparency
- durable and low cost
- non-toxic to PNS strains and resistant to chemicals and metabolites produced by the PNS strains
- high weathering resistant and easiness in cleaning

The results of the Net Energy Analysis (NER) of three different materials, viz. glass, LDPE and PMMA, done by Gebicki et al. (2010) suggests the use of LDPE for the construction of tubular and panel PBRs.

2.2.6 Mathematical modeling of growth and product kinetics of PNSB

Knowledge on the kinetics of the biological process becomes vital to have a better design and control of the process. The strong influence of operational parameters such light intensity and substrate concentrations on photofermentative H_2 and PHB synthesis has been demonstrated (Uyar et al. 2007; Androga et al. 2014; Hustede et al. 1993; Han et al. 2012; Wu et al. 2012). However, very limited work has been done on the kinetic analysis

of the photofermentation process (Gadhamshetty et al. 2008; Zhang et al. 2015; Koku et al. 2002).

Biomass growth

Few mathematical models have been proposed to study growth kinetics of PNSB cultures. A theoretical cell growth rate can be expressed as:

$$\frac{dX}{dt} = \mu X - mX \qquad (2.21)$$

Where: X is the cell dry weight concentration (g/L), m is maintenance coefficient for biomass (decay rate) and μ is the specific growth rate (h^{-1}). Gadhamshetty et al. (2008) proposed the Monod equation to provide the expression for μ to model the growth curve in a batch PBRs with the assumptions that sufficient light and optimal C/N ratio is available under stressful nitrogen concentrations. The proposed model simulates the biomass growth under substrate-limited conditions as:

$$\frac{dX}{dt} = \mu X = \left(\frac{\mu_m S}{K_s + S}\right) X \qquad (2.22)$$

where: the specific growth rate μ (hr^{-1}) depends on both maximum specific growth rate μ_m (hr^{-1}) and the half saturation constant K_S (mg/l).

However, the growth curve obtained for *R. sphaeroides* O.U. 001 deviated from the Monod model (Koku et al., 2003). The Equation 2.22 needs to include the substrate inhibition and inhibition due to higher biomass concentration. Moreover, the inhibition from higher substrate levels could be due to osmotic stress and/or the presence of one or more unknown inhibitors such as pigments (Gadhamshetty et al. 2008). Besides higher biomass concentration reduces the light intensity inside the PBR, causes self-shading effects and limits the substrate diffusion, which in turn affects the hydrogen production. Thus, the specific growth rate (μ) in Equation 2.22 is modified in Equation 2.23 to include the two inhibitory effects:

$$\mu = \frac{\mu_m S}{K_s + S + \frac{S^2}{K_{Xi}}}\left(1 - \frac{X}{X_m}\right) \qquad (2.23)$$

The inhibitory effect due to biomass concentration is provided by a Logistic model. The term "X_m" is the maximum cell dry mass concentration at which growth will cease. The

specific growth rate in the Equation 2.23 is further modified to include the effect of the light exposure on PNSB. The modification included the declining effect of excess light on biomass growth as the surplus absorbed light energy may results in damage and degradation of the reaction center involved in the photosynthetic process. The final equation is expressed as:

$$\mu = \frac{\mu_m S}{K_s + S + \frac{S^2}{K_{Xi}}}\left(1 - \frac{X}{X_m}\right)\left(\frac{I}{K_{XI} + I + K_I I^2}\right) \qquad (2.24)$$

The smaller the value of K_I, the larger is the inhibition effect of light on PNSB growth.

Consumption of substrate

The Contois model can be used to describe the consumption of the substrate:

$$\frac{dS}{dt} = -\frac{\mu_m S}{Y(S + K_S X)} X \qquad (2.25)$$

or

$$\frac{dS}{dt} = \frac{1}{Y_{S/X}} \mu X \qquad (2.26)$$

Relation between biomass growth and product formation

Mu et al. (2006) used the Modified Luedeking-Piret model to establish the relationship product (P_i) formation, substrate (S) degradation and biomass (X) growth for the DF hydrogen production by mixed anaerobic cultures. The following Luedeking-Piret model could be used to describe the relationship between three parameters. The Luedeking–Piret model and its modified form can describe the relationship between formation of H_2 and PHB as products and biomass:

$$\frac{dP_i}{dt} = -Y_{\frac{Pi}{X}} \frac{dX}{dt} + \beta X \qquad (2.27)$$

$$\frac{dP_i}{dt} = -Y_{\frac{P_i}{X}} \frac{dX}{dt} \qquad (2.28)$$

where: 'P_i' is the concentration of the product 'i' and '$Y_{Pi/X}$' is the yield of product 'i' with respect to biomass 'X'.

Similarly, the formation of products with respect to consumption of substrate can be written as:

$$\frac{dP_i}{dt} = -Y_{\frac{P_i}{S}} \frac{dS}{dt} \qquad (2.29)$$

where: 'P_i' is the concentration of the product 'i' and 'Yp_i/s' is the yield of product 'i' with respect to substrate 'S'.

The growth of biomass can be expressed in relation to the substrate consumption as:

$$\frac{dX}{dt} = -Y_{\frac{X}{S}} \frac{dS}{dt} \qquad (2.30)$$

where: 'X' is the concentration of the biomass and 'Y_X/s' is the yield of biomass with respect to the substrate 'S'.

On integrating Equation 2.30 from initial concentration (S_0) to final substrate concentration (S) and product (from initial concentration of 0 to final product concentration Pi), it is possible to write the following equations:

$$dP_i = -Y_{P_i} dS$$

$$\int_0^{P_i} dP_i = -Y_{P_i} \int_{S_o}^{S} dS \qquad (2.31)$$

$$P_i = -Y_{P_i}(S_0 - S) \qquad (2.32)$$

with i = H_2 and PHB.

These relationships can be applied to model the kinetics of substrate consumption, PNSB growth and products formation (H_2 and PHB) in the PF process.

2.2.7 Future perspectives

Economics

There are very few studies aimed at determining the economics of photo-H_2 production (Benemann 1997; HYVOLUTION 2011). Benemann (1997) presented an economic analysis of a conceptual two-stage process where microalgae are used to produce a carbohydrate rich biomass cultivated in large open ponds and hydrogen will be produced in tubular photobioreactors. The paper reported the estimated overall total hydrogen production costs of 9.5 $/GJ.

An integrated process of biohydrogen combining thermophilic dark fermentation followed by photofermentation had a biohydrogen production cost of 10 €/GJ (~13.42 $/GJ), i.e. 1.21 €/kg$H_2$ (~1.62 $/kg$H_2$) based on the lower heating value of H_2 (HYVOLUTION, 2011). In the integrated concept, the total cost per kilogram of biohydrogen is 56 € (~75.15 $), which is much higher than that of hydrogen from coal (0.36 - 1.83 $/kg$H_2$) and natural gas (2.48 - 3.17 $/kg$H_2$) (Bartels et al., 2010; HYVOLUTION, 2011). The standalone thermophilic dark fermentation process costs 21 €/kgH_2 which is lower than the combined system, thus the PF needs improvement to lower the per unit production cost. In their study, HYVOLUTION (2011) estimated the capital cost of commercial scale tubular and flat panel photofermenters at 91 and 332 million € (122.11 and 445.51 $), respectively. However, the capital cost depends on several factors such as reactor size, material and labor cost.

The light conversion efficiencies of the PF play an important role in determining the economics of photo-hydrogen production. In addition, the substrates and the PNSB strains are also crucial factors. The selection of PBRs also influences the capital and operational cost and in the end, the unit cost of the photo-hydrogen production (HYVOLUTION 2011).

Integration with dark fermentation

PF can be applied as a post treatment stage on DFE, which mostly contains organic acids and alcohols (Figure 2.14). The integrated DF-PF process has been demonstrated by several studies (Rai et al. 2014; Tawfik et al. 2014; Yang et al. 2015). DF has the unique capability to utilize a wide range of complex waste biomass that can ensure the future supply of feestock, and combining the two processes (DF + PF) can provide the complete conversion of organic substrate in addition to enhanced H_2 yields. Typical chemical reactions of conversion of organic acids produced in mixed type fermentation to photo-H_2 are presented in Equations 2.9 – 2.12.

Redwood et al. (2008) reviewed different possible integration strategies for coupling DF-PF processes. In general, DF-PF systems can be integrated in three possible ways; i) utilizing DFE produced in PF systems, ii) cultivating dark and photofermentative microorganisms in one reactor system (Chandra et al. 2015; Liu et al. 2010) or iii) separating the two systems by a physical barrier such as a membrane (Redwood et al.

2011; Liu et al. 2015). DF followed by photo-H_2 production is well studied by many researchers (Ghimire et al. 2015).

Depending on the process operating parameters such as pH, substrate loading and substrate type, DFE generally has an acidic pH (< 6.0) and inhibiting levels of ammonia and organic acids. Therefore, the DFE requires pre-treatment such pH adjustment, dilution and removal of ammonia before feeding into a PF process. Ammonia concentrations exceeding 2 - 5 mM inhibit the photo-H_2 production (Lee et al. 2011; Argun et al. 2008). Therefore, substrates with a higher C/N ratio are usually preferred for PF. Depending on the DFE requirements, several ammonia removal strategies such as stripping, treatment with natural zeolites and membrane processes can be applied (Androga et al. 2012b; Redwood et al. 2012). However, most continuous dark fermentative processes lack high ammonia levels due to incomplete conversion of proteins or amino acids present in the substrates, making them ideal substrates for the PF processes.

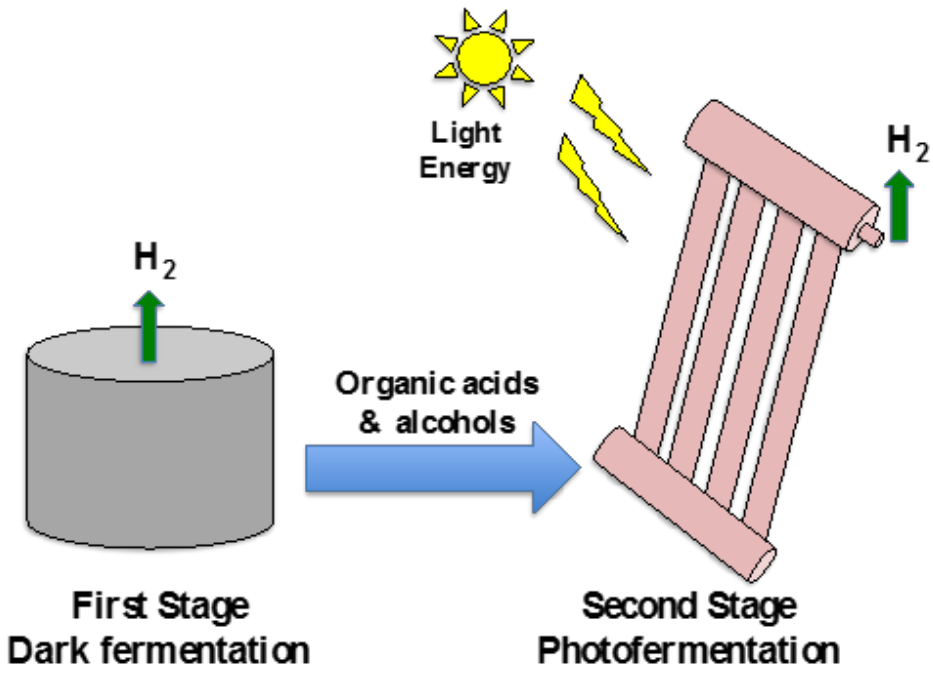


Figure 2.14 - Sequential DF-PF process

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CHAPTER 3

OPERATIONAL STRATEGIES TO IMPROVE DARK FERMENTATIVE H_2 PRODUCTION USING COMPLEX WASTE BIOMASS

The section 3.1 has been published as Ghimire, A., Frunzo, L., Salzano, E., Panico, A., Lens, P.N.L., Pirozzi, F., Esposito, G. (2015). Biomass enrichment and scale-up implications for dark fermentation hydrogen production with mixed cultures. Chem. Eng. Trans. 43, 2015, 391–396.

The section 3.2 has been published as Ghimire, A., Sposito, F., Frunzo, L., Pirozzi, F., Escudie, R., Trably, E., Lens, P.N.L., Esposito, G. (2015). Effects of operational parameters on dark fermentative hydrogen production from biodegradable complex waste biomass. Waste management (In press)

3.1 Evaluation of methods for obtaining H_2 producing seed inoculum for dark fermentation

This section summarises the results of the study performed on the enrichment of microbial communities for enhancing hydrogen production in dark fermentation (DF) using mixed culture, which considerably affect the overall performance. This work evaluates the following pre-treatment methods: acid treatment, heat shock (at 95 °C and 105 °C) and load shock pre-treatment, keeping into account scaling-up of DF systems. Further insights are also provided on the safety aspects concerning the production and storage of H_2, and on the importance of operational costs and feasibility of the pre-treatment methods.

3.1.2 Introduction

The progressive running down of fossil fuel reserves coupled with the need of reducing the greenhouse gas (GHG) emissions in the atmosphere has made the development of new, renewable and environmental friendly energy sources very crucial. Hydrogen (H_2) biologically produced from organic wastes seems to be really promising, due to its efficient hydrogen to power conversion coefficient (3.0 kWh/Nm^3), high energy density (142 MJ/kg) and harmless combustion by-products (Cardoso et al., 2014). To this aim, either photo fermentation (PF) or dark fermentation (DF) processes have been successfully used to biologically produce H_2 from organic sources. However, DF is usually preferred to PF due to lower operational costs and process conditions at ambient temperature and pressure (Das and Veziroglu, 2008).

The biological conversion of organic sources into H_2 is obtained by using biomasses either consisting of pure cultures or composed of mixed cultures. Mixed systems are generally less performing in terms of H_2 yields, but are easier and less expensive to handle as they do not require any asepsis procedure and can be fed with several different substrates, as reported in previous studies (Valdez-vazquez et al., 2004). Here it is worth noting that mixed bacteria communities with the ability of producing H_2 are intrinsically present in soils, sediments, sludge from wastewater treatment plants, compost, cow dungs, municipal organic solid wastes (Wong et al., 2014). Hence, these communities can be enriched by appropriate pre-treatment methods, although higher H_2 production rate can be only obtained if H_2 consuming organisms such as methanogens and homoacetogens are inhibited (Wang and Wan, 2009).

The most commonly and successfully used biomass pre-treatment methods include heat (Wang and Wan, 2008), acid (Wang and Wan, 2008), base (Zhu and Beland, 2006), and load shock (Luo et al,. 2010) as well as aeration (Giordano et al., 2014). These methods are based on the observation that when the biomass experiences hostile environmental conditions, H_2 producers survive due to their ability in forming spores (e.g. *Clostridium*) that protect them from the adverse conditions, hence returning to be effective again when the environmental conditions turn to be favourable as the spores germinate (Li and Fang, 2007). Besides, the H_2 consumers may not survive unless with same capacity.

The effectiveness of these pre-treatments on H_2 production depends on nature of biomass, which in turn can cause the occurrence of inconsistency in results from lab scale experiments (Wang and Wan, 2009). Therefore a deeper knowledge of the effects that pre-treatment methods have on H_2 production from DF is necessary before operating the scaling up of these methods as well as, being H_2 highly flammable and explosive, safety aspects in large-scale reactors are also a primary concern.

The aim of this paper is to evaluate the effectiveness of the following pre-treatment methods (i) acid shock treatment, (ii) heat shock treatment and (iii) load shock pre-treatment on H_2 production through bio-H_2 potential DF batch (BHP) tests. The evaluation has been done by analysing the following parameters from the BHP tests: (i) cumulative H_2 production; (ii) H_2 production rate; (iii) length of the lag phase; and (iv) production of process intermediates. Furthermore, this study also deals with the safety aspects concerning the production and storage of H_2 (USEPA, 2011) and highlights the relevance of operational cost, feasibility and complexity of the pre-treatment methods in scaled up systems.

3.1.3 Materials and methods

Biomass used to perform the BHP tests was collected from the anaerobic digester treating dairy waste produced by the factory "La Perla del Mediterraneo" located in Capaccio (Salerno, Italy). The total solids (TS) and volatile solids (VS) content of biomass were 2.79 ± 0.05 % (w/w on wet mass) and 67.2 ± 0.4 % (w/w on dry mass). The sludge was stored at 4 °C before being used. The BHP tests were fed with glucose.

All BHP tests were carried out in 1,000 mL transparent borosilicate glass bottles GL 45 (Schott Duran, Germany) used as DF batch reactors and placed in a water bath maintained

at 34 ± 1 °C by a thermostat (ALEAS AL 2201, 150 W). In the batch reactors, airtight conditions were provided with caps sealed with silicon. Each bottle was equipped to sample the internal mixture and spill out the gas. BHP tests were carried out in duplicates at the initial pH of 7.

Heat shock treatments were carried out by heating the biomass at 105 °C for 4 h (HST-105°C) and at 95 °C for 45 min (HST-95°C); acid shock treatment (AST) was performed by adjusting the pH of the biomass at pH 3 using 1 M HCl for 24 h and then turning pH back at 7 using 1 M NaOH; load shock (LST) treatment was carried out by feeding the batch reactors with 85 g COD/L of glucose followed by acidification process for 4 days and finally extracting the supernatant after a settlement process and replacing the extracted liquid volume with distilled water. A substrate to biomass ratio of 0.85 g COD glucose/g VS biomass was maintained in all BHP tests. Once the cumulative H_2 production in the reactors reached a stable value (Load I), the reactors were furthermore fed with 4.5 g of glucose (Load II).

The volume of gas produced from each BHP tests was measured on daily basis by acid solution (1.5 % HCl) displacement method. The biogas volumes were corrected for moisture at 0°C and 1 atm (NmL) and reported as the daily average. H_2, CO_2 and CH_4 content in gas were measured with Varian Star 3400 gas chromatograph equipped with ShinCarbon ST 80/100 column provided with a thermal conductivity detector and argon as carrier gas. Samples of the digesting mixture collected from each reactor to measure the volatile fatty acids (VFAs) content and their composition were preliminarily extracted at 80 °C according to the head space-solid phase micro-extraction technique (HS-SPME) (Abalos et al., 2000) and subsequently analysed with gas chromatograph equipped with mass spectrometry provided with helium as carrier gas. The pH was measured with a pH meter (WTW, inolab, pH level 2). The TS and VS content of biomass and organic wastes were determined according to Standard Methods (APHA, 2005).

The modified Gompertz relationship (equation 3.1) was used to model the H_2 production from BHP tests (Wang and Wan, 2008). The equation contains 3 parameters: i) cumulative H_2 production potential H_o (mL), ii) H_2 production rate R (mL/h), iii) lag time λ (h). H_o, R and λ were estimated from BHP test by using the Curve Fitting Toolbox in MATLAB®.

$$H(t) = H_o \cdot \exp\left\{-\exp\left[\frac{R.e}{H_o}\right](\lambda - t) + 1\right\} \quad (3.1)$$

Where t is the time.

3.1.4 Results and discussions

The results from BHP tests are shown in Figures 3.1 and 3.2 and Tables 3.1 and 3.2. In Figure 3.1, the effects of different biomass pre-treatment methods are represented by plotting the average cumulative H_2 production, whereas in Tables 3.1 and 3.2, the same effects are evaluated comparing the specific H_2 production and the parameters calibrated by using equation 3.1.

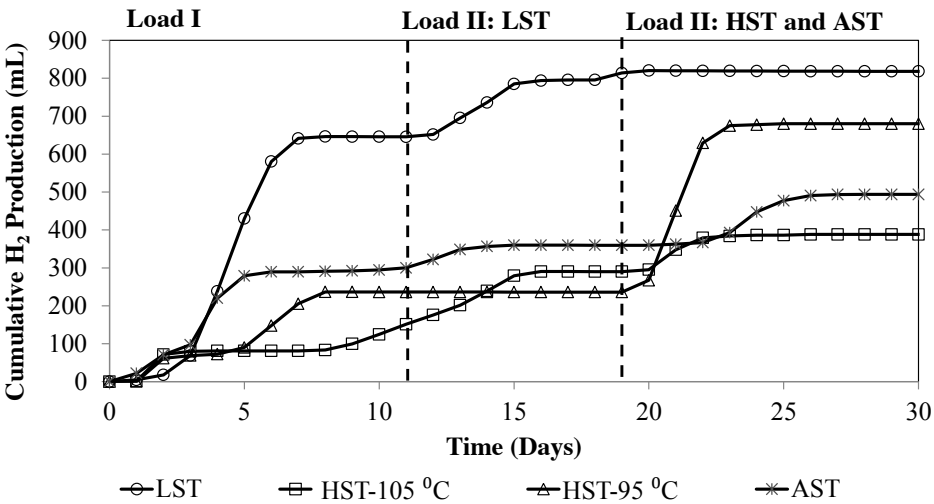

Figure 3.1 - Average Cumulative H_2 Production in BHP tests

From Figure 3.1 and data in Tables 3.1 and 3.2, it can be noted that LST gave better H_2 production performance with highest specific H_2 production (143.5 ± 13.2 NmL/g glucose), H_2 production rate (9.4 NmL/h) and a lag phase slightly longer (0.53 h) than HST-95°C, while AST showed the lowest lag time (13.57 h). AST also gave good cumulative H_2 production (373.1 NmL) whereas BHP in the tests with HSTs was low. From the analysis of methane content in biogas, it can be concluded that there were negligible methanogenic activities in the tests with LST, HST-105°C and AST whereas the BHP tests with HST-95°C was unable to completely inhibit the methanogenic microorganisms, which could explains the lower H_2 production.

Table 3.1 - Effects of biomass pre-treatment methods on biohydrogen production performance during Load I

Pre-treatment method	Modified Gompertz model[a]			
	Ho (Nml)	*R* (Nml/h)	λ (h)	R^2
LST	657.8	9.40	69.94	0.9980
HST-105 °C	341.6	1.28	138.98	0.9880
HST-95 °C	238.9	2.44	69.41	0.9910
AST	373.1	1.52	13.57	0.9953

[a]The parameters were determined based on average cumulative daily H_2 production during Load I

After the batch reactors were fed with a second load of glucose (Load II), the H_2 yield decreased in the BHP tests with LST, HST-105 °C and AST whereas it increased in tests with HST-95C (Figure 3.1). In Table 3.2 the specific H_2 production obtained from the first (Load I) and the second (Load II) feeding operation as well as the respective pH values at the beginning and at the end of the BHP tests are compared. Figure 3.2 shows the major fermentative products accumulated at the end of the BHP tests. The production of intermediates (VFAs) and pH values were monitored in order to evaluate the performance of DF process. A possible reason for the lower H_2 yield than expected when a LST was performed could actually be explained with the occurrence of the inhibiting effect due to the high butyric acid accumulation in the reactor, as indicated in the study published by Van Ginkel and Logan (2005), whereas a low pH (3.7±0.44) could be the cause of the lower H_2 production in AST during Load II.

Table 3.2 - Comparison between Load I and Load II feeding operations

Pre-treatment Method	NmL H_2/g glucose (Load I)	mL H_2/g glucose (Load II)	Initial pH	Final pH Load I	Final pH Load II
LST	143.5±13.2	38.4±17.4	7±0.01	5.3±0.01	4.9±0.02
HST-105 °C	64.5±12.7	21.8±5.1	7±0.01	5.2±0.00	4.5±0.02
HST-95 °C	52.5 ±3.4	98.7±23.9	7±0.01	5.4±0.01	4.6±0.02
AST	79.9±22.3	29.8±5.0	7±0.01	4.5±0.16	3.7±0.44

± indicates data range based on duplicate samples

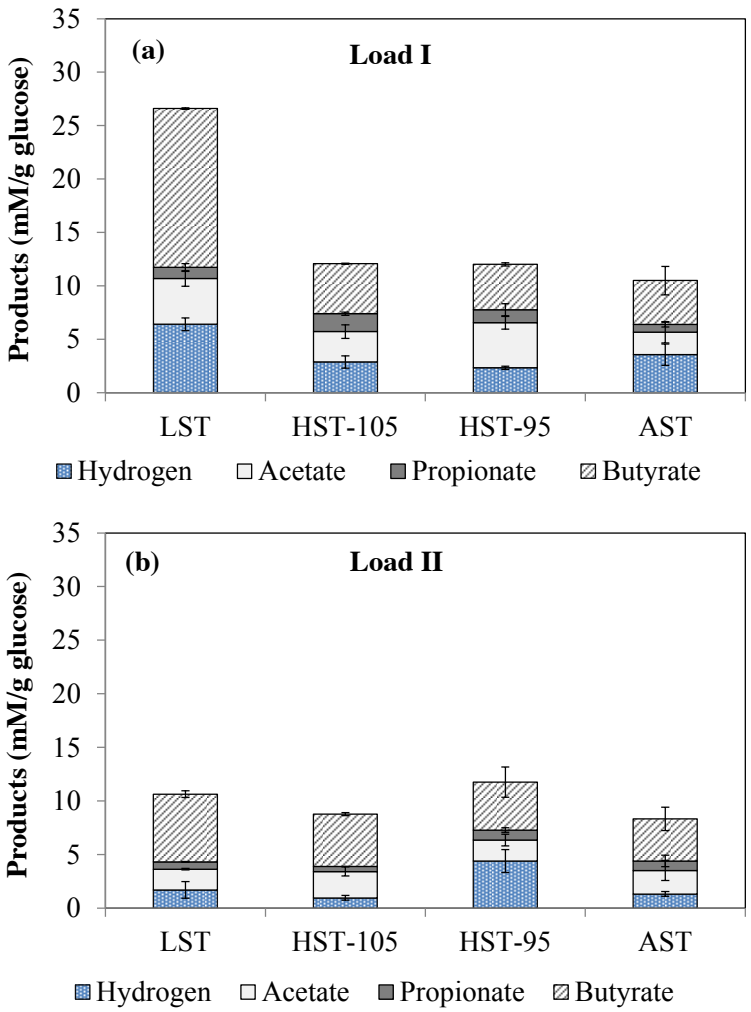

Figure 3.2 - Major fermentative end products in the BHP tests with different biomass pre-treatment methods during (a) Load I and (b) Load II

In order to select and set up a method to pre-treat the biomass in full scale reactor, several parameters need to be considered: the operational costs, the feasibility and complexity of the method as well as the time required to enrich the biomass with H_2 producing bacteria (safety will be considered in the next section) Table 3.3 shows a simple evaluation of the parameters based on this study and literature data for the four pre-treatment methods investigated in this paper. HSTs show a high energy demand, which makes them less attractive in a full scale application. AST requires large amounts of acid and base solutions. LST is more feasible to be used in a full-scale reactor due to lower operational costs compared with the other methods.

Table 3.3 - Evaluation of biomass pre-treatment methods for DF process

Pre-treatment method	Energy Requirement	Chemical Requirements	Operational Costs	Scale-up Application
LST	+ +	+	+	+ + +
HST-105 ^{0}C	+ + +	+	+ + +	+ +
HST-95 ^{0}C	+ +	+	+ + +	+ +
AST	+	+ + +	+ +	+ + +

+ Less intensive; ++ Moderately intensive; +++ Very Intensive (Adapted and modified from Ghimire et al., 2015)

The H_2 production and process performance are strongly influenced by many factors such as physico-chemical properties of substrate and co-substrates, type of biomass sources, reactor configuration, and operational conditions. Luo et al. (2010) actually evaluated the effects of different pre-treatment methods on mixed culture for H_2 production using cassava stillage as substrate and found differences in H_2 yields only when DF was performed in batch reactors, whereas no difference was noticed in continuous DF processes.

Safety considerations on scale-up

Several accidents can be found in the literature due to severe reactivity of biogas. Hence, specific analyses are due for this mixture for the correct design of prevention and mitigation systems (e.g. venting, suppression), and for the structural design of the reactors, including auxiliary and transportation systems (USEPA, 2011).

When batch reactors are adopted, the isochoric-isotherm option should be considered for the hazard of hydrogen mixture. By using the ideal gas equation, the calculated maximum pressure in the lab reactors varied from 2.11 to 2.15 bar, considering a reactor head space 540 mL, reactor temperature of 35 °C and ambient conditions 25 °C and 1 bar for the measurement of the biogas. Quite clearly, due to anaerobic conditions, the reactors are flushed with nitrogen and no hazards are predicable unless oxygen (air) leakage due to rapid depressurisation and oxygen (air entrance). On the other hand, the continuous operations adopted in large-scale reactors are normally operated under ambient conditions and air. Hence, a deflagration or even a detonation of the mixture of hydrogen possibly mixed with several other oxidation components that are typical in large-scale biomass operation as CO, CO_2, methane and other low-weight gases, including toxic H_2S, may occur.

The literature on the safety characterisation of complex biogas mixtures is very scarce and mainly based on experimental observations (Cammarota et al., 2009), as no additive methodologies are applicable for the definition of flammability limits, burning velocity, and for the definition of occurrence of dramatic scenarios as deflagration to detonation transition or combustion-induced Rapid Phase Transitions (Salzano et al., 2012).

In large-scale reactors, H_2 might ranges between 40 % to 50 % v/v however with inerts as CO_2 (50 – 60 % v/v) and water vapour (1 – 5 %) and operation are conducted under thermophilic temperature ranges (55 - 60 °C) in comparison or mesophilic reactors (35-40 °C). For ambient conditions, may be adapted the analysis reported in (Di Benedetto et al., 2009), that clarified the effect of CO_2 on H_2 burning, which is essentially thermal, and the ranges of adiabatic flame temperature (i.e. adiabatic pressure) and laminar burning velocity for the given mixtures obtained by means of both experimental and numerical analysis. Stable flames (for the use in combustion equipment) or, conversely, flame extinguishing (for fire and explosion safety) are obtained, at ambient temperature, for CO_2 larger than 40 % v/v in air, hence in the presence of N_2. The effect at higher temperature has to be defined in future works.

3.1.5 Conclusions

The evaluation of results from the BHP tests and the analysis of different pre-treatment methods suggest that LST of biomass can favour the development and growth of an efficient H_2 producing bacteria community to start-up and handle up-scaled DF systems. Moreover, monitoring of metabolites production and pH can give useful information on process performance and its reliability, thus helping to prevent VFAs accumulation and the subsequently occurrence of inhibition phenomena affecting the H_2 producing biomass activity. Also, safety aspects need to be taken into consideration in the up-scaled DF systems during H_2 production, storage and application.

3.2 Effects of operational parameters on dark fermentative H_2 production

This section presents the findings on the effect of initial pH, combination of food to microorganism ratio (F/M) and initial pH, substrate pre-treatment and different inoculum sources on the dark fermentative H_2 yields obtained using three model complex waste biomass: food waste, olive mill wastewater (OMWW) and rice straw. The cumulative H_2 production, H_2 production rate, lag time for H_2 production and accumulation of metabolites were used as comparison parameters to determine the optimal conditions for H_2 production carried out in a series of batch tests.

3.2.1 Introduction

Dark fermentation (DF) of organic waste is one of the promising technologies for biohydrogen (H_2) production. The DF processes are usually preferred over other light dependent, photofermentation or biophotolysis processes because of the high bioreactor productivities and the potential to utilize a wide range of organic wastes as feedstock (Hallenbeck et al., 2009; Urbaniec and Bakker, 2015). In addition, the associated production of organic acids and alcohols, among others, can be either used in sidestream processes like anaerobic digestion for methane or photofermentative H_2 production for energy recovery, or can be used for the production of platform molecules (Bastidas-Oyanedel et al., 2015; Sarma et al., 2015).

Waste biomass is abundant and can sustain DF processes in scaled-up applications. Easily degradable food waste (the organic fraction of municipal solid waste (OFMSW)), more slowly degradable agricultural residues (i.e. rice straw) as well as agro-industrial waste such as olive mill wastewaters (OMWW) can serve as sustainable feedstock sources for dark fermentative H_2 production (Guo et al., 2010; Kapdan and Kargi, 2006; Ntaikou et al., 2010; Show et al., 2012). A major bottleneck in the utilization of these low cost waste biomasses is the rather low H_2 yields observed in the DF processes (Ghimire et al., 2015a; Urbaniec and Bakker, 2015). Nevertheless, H_2 yields and process kinetics can be enhanced by optimizing operating parameters, such as pre-treatment of inocula, food to microorganisms (F/M) ratio (also substrate to inoculum ratio), pre-treatment of substrates, culture temperature and pH (De Gioannis et al., 2013; Guo et al., 2010; Ntaikou et al., 2010; Wang and Wan, 2009). During recent years, extensive experimental research has been devoted to establish the optimal operational conditions for maximizing H_2

production, with a special focus on operational pH, temperature and substrate utilization (De Gioannis et al., 2013; Ghimire et al., 2015a; Wong et al., 2014).

A wide range of optimal pH values have been reported for different substrates to enhance H_2 yields: an initial pH of 6.5 for food waste (Cappai et al., 2014), initial pH of 8.0 for food waste (Kim et al., 2011), a controlled pH of 7.0 for vegetable kitchen waste (Lee et al., 2008), an initial pH of 6.5 for rice straw (Chen et al., 2012), an initial pH of 6.0 for cheese whey (De Gioannis et al., 2014) and an initial pH of 4.5 for sucrose and starch (Khanal et al., 2004). This considerable variability in culture pH is mainly due to differences in temperature, substrate type and concentration (F/M ratio), inoculum types and their pre-treatment methods.

H_2 yields in DF of organic waste are strongly affected by the operational temperature as it can influence the rate of hydrolysis and the production of volatile fatty acids (VFAs) and thus the final pH of the fermentation (De Gioannis et al., 2013; Ghimire et al., 2015a). A thermophilic temperature has been reported to favor the dark fermentative H_2 production (Shin et al., 2004; Valdez-vazquez et al., 2005). Likewise, the physico-chemical characteristics of the substrates, and most importantly the biodegradability or bioavailability (can also be defined as the fraction of easily accessible carbohydrates for fermentative conversion) crucially affects the H_2 production (Monlau et al., 2013a). Therefore, several studies have established a strong correlation between H_2 yields and the initial carbohydrate fraction (soluble sugars in some cases) present in the substrates (Alibardi and Cossu, 2015; Guo et al., 2013; Monlau et al., 2012).

In this context, alkaline pre-treatment methods have been popularly adopted for the saccharification of lignocellulosic biomass (plant stalks, rice and wheat straw), which could enhance the production of H_2 in DF and CH_4 in DF coupled to anaerobic digestion, respectively and could thus give economic credentials (Monlau et al., 2015, 2013c; Sambusiti et al., 2013). Alkaline pre-treatment of lignocellulosic biomass has been reported to be carried out at different concentrations of alkaline agents (2 - 12% NaOH, weight basis), temperature (40 - 190 °C) and treatment period (30 minutes - 24 hours), with varying level of effectiveness in terms of increase in biogas yields (H_2 and CH_4) with consequent higher net energy recovery and economic return (Monlau et al., 2015, 2013b; Sambusiti et al., 2013). However, alkaline agents (i.e. Na^+ from NaOH) might exert inhibitory effects on dark fermentative microbial communities (Kim et al., 2009).

Consequently, an investigation of selected alkaline pre-treatment conditions for a particular substrate type becomes vital to study the conditions that enhance the H_2 production.

H_2 production from organic waste is influenced by the presence of an effective hydrolyzing, H_2 producing microbial community, which depends on the inoculum source and inoculum pre-treatment method (Abreu et al., 2009; Bellucci et al., 2015; Chen et al., 2012; Pakarinen et al., 2008). Abreu et al. (2009) and Chen et al. (2012) showed that the H_2 yields mainly depend on the inoculum sources. However, the response of fermentative microorganisms towards the presence of inhibiting substances present in a substrate can influence the DF process. In a recent study, Bellucci et al. (2015) reported a varying response of fermentative microbial communities for H_2 production, when the inhibitor 5-hydroxymethylfurfural (HMF) was added. This was linked to the difference in inoculum pre-treatment methods applied. Likewise, the presence of polyphenolic compounds in substrates such as OMWW can exhibit inhibitory effects on fermentative microbial communities and H_2 yields (Hamdi, 1992; Ntaikou et al., 2009). Subsequently, investigating the effect of the inoculum source on H_2 production performance from substrates like OMWW is fundamental to reach an optimum in H_2 production.

Despite some studies attempted to establish the optimal operational conditions of initial pH, F/M ratio, alkaline pre-treatment of substrate and inoculum selection, dissimilarities in H_2 production exist due to the differences between substrate types and experimental conditions. Therefore, it becomes essential to investigate the optimum initial pH for food waste under thermophilic DF conditions. So far, only few studies have considered the combined effects of F/M ratio and initial pH on thermophilic DF of food waste (Ginkel et al., 2001; Pan et al., 2008). Ginkel et al., (2001) revealed a profound impact of the concentration of substrate and pH on the H_2 yields in sucrose DF of, with an optimum pH and substrate concentration at pH of 5.5 and 7.5 g COD/L, respectively. In other study, Pan et al. (2008) established a F/M ratio of 6.0 as optimum for thermophilic DF of food waste, without the consideration of initial pH. Similarly, past studies on pre-treatment of substrates seemed more focused on maximizing the methane yields in anaerobic digestion by adopting higher concentrations of alkaline agents and treatment temperature (Monlau et al., 2013a). Therefore, optimum conditions of alkaline pre-treatment for dark fermentative H_2 production need to be investigated for lignocellulosic agricultural residues such as rice straw. Finally, different inoculum sources can be explored to study

the effect on H_2 production from a typical poorly biodegradable feedstock such as OMWW, which contains polyphenolic compounds (Ntaikou et al., 2009).

The present study aims to investigate the effects of i) the initial pH and combined pH and F/M ratio on food waste, ii) alkaline substrate pre-treatment on dark fermentative H_2 production from rice straw and iii) the effect of inoculum source and pre-treatment on H_2 production from OMWW. Cumulative H_2 production, H_2 yields, H_2 production rates, lag phase and accumulation of DF metabolites (mainly organic acids and ethanol) were used to evaluate the efficiency of these various strategies to improve the H_2 production performance from these complex organic wastes.

3.2.2 Materials and methods

Inoculum

Two types of inoculum, i.e. anaerobic digested sludge (ADS) and waste activated sludge (WAS) were used in the experiments. ADS was collected from the effluent of an anaerobic digestion plant of a dairy farm located in Capaccio (Salerno, Italy). The plant features include a 100 m^3 CSTR operating at a hydraulic retention time of 24 days and operating within a pH and temperature range of 7.4 - 7.5 and 52 - 56 °C, respectively. The plant is continuously fed with buffalo manure, cheese whey of buffalo milk and sludge from an industrial wastewater treatment plant. WAS was collected from a secondary clarifier unit at the Nola Municipal Wastewater Treatment Plant located in Naples (Campania, Italy). The characteristics of the ADS and WAS before pre-treatment are presented in Table 3.4. The inocula were stored at 4 °C until used. The WAS and ADS underwent a heat shock treatment (HST) at 105 °C for 1.5 and 4 hours, respectively, in order to enrich spore forming *Clostridium* sp. and inhibit methanogens (Ghimire et al., 2015b). WAS had a shorter time for HST than ADS because it was obtained from an aerobic activated sludge process.

Preparation of feedstock

Three Three types of waste as reference models of complex waste biomass with different characteristic biodegradability, were used in this study: i) food waste, representative of moderately biodegradable organic waste was selected to study the effect of initial pH and substrate concentration on H_2 yields, ii) rice straw as a representative of slowly degrading lignocellulosic agricultural residues was used to study the technical feasibility of substrate

pre-treatment on biohydrogen production and iii) OMWW was used to study the effect of the inoculum type and its adaptation to toxicants, as OMWW contains phenolic compounds and long chain fatty acid that can affect microbial growth (Hamdi, 1992; Ntaikou et al., 2009). Food waste was a mixed waste with a composition similar to the one reported by VALORGAS (2010) for European countries as (% by weight): fruit and vegetables: 72%, cooked pasta and rice: 10%, bread and bakery: 5%, dairy products (cheese): 2%, meat and fish: 8% and snacks (biscuits): 3%. To prepare the food waste, food was bought fresh from municipal markets in Naples (Italy), shredded with a blender (120 W Black and Decker, Kitchen Blender) for 5 minutes without adding water and immediately stored at frozen conditions (-20 °C) to avoid acidification. The rice straw was harvested from rice fields in Pavia (Italy) in 2012 and stored inside an airtight plastic bag at room temperature. Rice straw was reduced with the help of general paper scissors to a particle size of less than 2 mm (sieved with sieve size of 2mm by 2mm). OMWW was collected from a pressure olive mill of the Frascati area (Lazio, Italy) in autumn 2013 and was stored at < 4 °C until use. The characteristics of the feedstocks are presented in Table 3.4.

Table 3.4 - Characteristics of the substrates and inocula used in this study

Characteristics	Food waste	OMWW	Rice Straw	ADS	WAS
pH	4.4 ± 0.1	4.6 ± 0.1	NA	8.3 ± 0.1	7.0 ± 0.1
Chemical Oxygen Demand (COD)	347.6 ± 47.0 $g/kg_{food\ waste}$	141.5 ± 13.0 g/L_{OMWW}	NA	NA	NA
Total solids	21.0 ± 0.1 %	4.7 ± 0.1 %	92.3 ± 0.2 %	2.33 ± 0.4 %	2.9 ± 0.2%
Volatile solids	20.2 ± 0.1 %	3.1 ± 0.3 %	80.9 ± 0.6 %	1.93 ± 0.1 %	1.8 ± 0.1%
Carbohydrate content	105.8 ± 0.7 $g/kg_{food\ waste}$	12.9 ± 0.2 g/L_{OMWW}	NA	NA	NA
Lipids	17.5 ± 1.0 $g/kg_{food\ waste}$	45.3 ± 4.0 g/L_{OMWW}	NA	NA	NA
TKN	6.4 ± 0.2 $g/kg_{food\ waste}$	0.5 g/L_{OMWW}	NA	NA	NA
NH_4-N	NA	NA	NA	283.5 ± 11.0 mg NH_4-N/L	203.1 ± 3.0 mg NH_4-N/L
Alkalinity	NA	NA	NA	1437.2 ± 14 mg $CaCO_3$/L	2605.7 ± 70.0 mg $CaCO_3$/L
Total phenols	NA	1.16 ± 0.03 g/L_{OMWW}	NA	NA	NA

NA-Not Analyzed

Experimental set-up

Batch tests were carried out in one-liter borosilicate glass bottles (Simax, Czech Republic) maintained in thermophilic conditions (55 ± 2°C) with a thermostat in a water bath. The operating reactor volume in all experiments was 600 mL. The batch reactors were sealed with airtight caps having ports for sampling soluble metabolites and gas. The tests were carried out in duplicates with 30 reactors in total. The different sets of experiments were carried out to study the effect of the different operational parameters using the three selected model substrates (Table 3.5).

Table 3.5 - Experimental conditions applied in the DF batch tests of the tested substrates

Investigation	Substrate	Inoculum	Initial pH	F/M
Effect of initial pH	Food waste	ADS	4.5, 5.0, 5.5, 6.0, 6.5 and 7.0	0.5
Combined effect of food waste and initial pH	Food waste	ADS	5.0 and 6.5	0.5, 1.0 and 1.5
Effect of pre-treatment of substrate	Rice straw	WAS	6.5	7.0
Effect of inoculum source and pre-treatment	OMWW	WAS and ADS	6.0	1.0

Effect of Initial pH and F/M ratios on H_2 yield

The effect of initial pH and F/M ratio on biohydrogen production was studied with food waste and pretreated heat treated ADS as seed inoculum. The effect of the initial pH (4.5, 5, 5.5, 6.0, 6.5 and 7.0) was studied at a F/M ratio 0.5 and under thermophilic conditions (55 ± 2 °C). Another set of experiments was performed at F/M ratios 0.5, 1.0 and 1.5 with the two initial pH values of 5.0 and 6.5. The F/M ratios and two initial pH values were selected due to the fact that they are less affected by acidification at higher F/M ratios and the culture pH in the tests was not buffered with external alkalinity source. In addition, pH 6.5 was previously reported as optimal for food waste by Cappai et al. (2014), and thus considered for investigation in this study. The F/M ratios 0.5, 1.0 and 1.5 were obtained by adding 10 g, 18 g and 27 g food waste respectively, with a 190 g inoculum required to obtain the aimed F/M ratio. The final volume of the mixture was made up to 600 mL by adding distilled water. The initial pH was adjusted once, initially with 1 M HCl and 1 M NaOH prior to the start of the tests.

***Effect of alkaline substrate pre-treatment* on H_2 yield**

Direct conversion of lignocellulosic biomass to biohydrogen is often limited due to their low biodegradability (Monlau et al., 2012; Pan et al., 2010). Biological hydrolysis is one of the limiting factors in DF. The evaluation of the effect of alkaline pre-treatment on H_2 yields was performed on rice straw. This study investigated an alkaline pre-treatment with 4 % NaOH (4 g/100g TS) and 8 % NaOH (8 g/100g TS) at a solid liquid ratio of 1:5 (w/v). This mixture was kept at 55 (± 2) °C for 24 hours in a one-liter borosilicate glass bottle (Simax, Czech Republic). The results were compared with untreated rice straw at thermophilic DF using 200 g of heat-treated WAS as inoculum. The concentration of rice straw was 45 gTS/L and the initial pH was adjusted to 6.5 during the batch tests that gave the optimal dark fermentative H_2 performance for rice straw as reported by Chen et al. (2012).

***Effect of inoculum sources and adaptation using OMWW* on H_2 yield**

Heat shocked WAS and ADS was used as inoculum in a DF of OMWW carried out in batch tests and operated under thermophilic conditions (55 ± 2°C). The F/M ratio was fixed at approximately 1 gVS substrate/gVS inoculum in all sets of batch tests using 200 g of OMWW and a respective volume of ADS and WAS. The initial pH was adjusted to pH 6.0 in all experiments.

Analytical methods

Hydrogen was quantified with a gas chromatograph (VARIAN STAR 3400, USA) equipped with a ShinCarbon ST 80/100 column and a thermal conductivity detector. Argon was used as carrier gas with a front and rear end pressure of 20 psi. The duration of analysis was 14 minutes. The gas volume was measured with a volumetric displacement method. The biogas was passed through acidic water (1.5 % HCl) and the volume was quantified by water displacement (Ghimire et al., 2015c). The volume of hydrogen was calculated from the gas composition. Fermentation end products (lactic, acetic, propionic and butyric acids) were quantified by High Pressure Liquid Chromatography (HPLC) (Chromatography Oven LC 25 Model, Dionex, USA) equipped with a Synergi 4u Hydro RP 80A (size 250×4.60mm) column and an UV detector (AD25 Model, Dionex, USA). Gradient elution consisted of 20% methanol, 10% acetonitrile in 5 mM H_2SO_4 pumped at a rate of 0.9 mL/min by using a gradient pump (GP 50 Model,

Dionex, USA). The elution time was 18.5 minutes. Ethanol and caproic acid were determined with an Aminex HPX-87H column (300 mm on 7,8 mm, Bio-rad), using 5 mM H_2SO_4 as an eluent at a flow rate of 0.4 mL/min. pH was measured with a pH meter (WTW, inolab, pH level 2). The COD of the food waste was measured as reported by Noguerol-Arias et al. (2012). The total lipid content was measured by the Bligh and Dyer chloroform/methanol total lipid extraction method (Bligh and Dyer, 1959). TS and VS concentrations were determined by the Method 2540 (Part 2000), alkalinity by titration (Method 2320, Part 2000) and TKN by macro-Kjeldahl (Method 4500-N_{org}, Part 4000) as described in the Standard Methods (APHA, 2005).

Measurements and data analysis

The biogas accumulated in the reactors was measured daily, except at the starting period of the experiments, i.e. 1 - 3 days, where it was measured twice a day, until the H_2 production completely ceased. The biogas volumes were normalized at 0 °C and 1 atm (NmL) and reported as a daily average. The average values were considered for the evaluations, while the data range based on the duplicate samples is provided and indicated by "±". H_2 yields were calculated by dividing the final cumulative recovery of H_2 by the amount of VS added at the start of the experiment.

De Gioannis et al. (2013) defined a parameter "t_{95}" as the time required to achieve 95% of the maximum H_2 yield. This parameter was used to compare the kinetics associated to different BHP tests, and to evaluate the effect of the experimental conditions.

$$t_{95} = \frac{H_o}{R.e}(1 - \ln(-\ln 0.95)) + \lambda \qquad (3.2)$$

Equation 3.2 corresponds to a rearranged form of the modified Gompertz equation 3.1, that has been widely used to model biohydrogen production kinetics (Gadhamshetty et al., 2010; Wang and Wan, 2009). This empirical formula gives biohydrogen production trends and includes five major parameters: i) cumulative biohydrogen production (or potential) (H_o, mL/g VS), ii) biohydrogen production rate (R, mL/h), iii) e is 2.71828, iv) lag time (λ, hours) and v) total cultivation time (t, hours). The cumulative biohydrogen production is a non-linear curve and in the present study, the parameters H_o, R and λ were estimated using the Curve Fitting Toolbox in MATLAB® (Version MATLAB R2012b, Curve Fitting Toolbox 3.3) with an associated 95% confidence limit. The total cumulative

production, hydrogen production rates and lag phase time were used as parameters to compare the characteristics of the biohydrogen production systems. R software (OSX version 3.1.3) with the package Rcmdr (OSX version 2.1.7) was used for the statistical analysis of data obtained from the experiments. The p value was set at 0.05 and the significance of the results tested with p values: * < 0.05; ** < 0.01; *** < 0.001; while not significant results were with $p > 0.05$.

3.2.3 Results

Effect of the initial pH and combined effect of F/M ratio and pH on H_2 yields

The H_2 yields and the time required to achieve 95% of the maximum H_2 yield were plotted against the initial pH values (Figure 3.3). The H_2 yields showed a decreasing trend to the increasing pH. Figure 3.3 confirmed that H_2 production was favoured at the acidic pH range, i.e. at initial pH 4.5 and 5.0 with H_2 yields of 60.6 (± 9.0) and 50.7 (± 1.0) N mL H_2/g VS, respectively. This result is in agreement with the study reported by Khanal et al. (2004). The fermentative H_2 production patterns at the various pH values investigated are described by a modified Gompertz equation, as presented in Table 3.6 (Modeled plot is provided in Supplementary information S1). The different initial pH values in the tests were characterized by the differences shown in cumulative H_2 production, H_2 production rates and lag phase (Table 3.6). H_2 production rates (R, mL/h) were high at initial pH 7.0, however, higher rates were not co-related with higher H_2 yields (Figure 3.3 and Table 3.6).

Unsurprisingly, the lag phase decreased when increasing the initial pH, which represents the time required for spore forming H_2 producers present in heat-treated ADS to germinate or adapt a sudden change of their environment (Ferchichi et al., 2005; Kim et al., 2011). Figure 3.3 shows the time required to achieve 95% of the maximum H_2 yield decreased by increasing the initial pH, while the rate of H_2 production was higher at initial pH 7.0 (Table 3.6). H_2 production started faster at higher pH and lasted for a short time while it continued for longer time during the tests at lower pH. Thus, a decreasing lag phase did not correspond to an increase in H_2 yields. This can be explained by the methanogenic activities which started at higher initial pH, that was confirmed by the presence of methane in the biogas produced when H_2 production ceased completely. The final pH at the end of the tests was mainly lower than the initial pH (Table 3.6), which is

mainly due to the production of VFAs (Table 3.6). As exception, the final pH in the batch tests with initial pH 4.5 was higher than the initial pH (Table 3.6), which could be due to the higher alkalinity of the inoculum (ADS) and the lower substrate concentration (F/M 0.5) used to avoid the use of chemical buffer. The final pH in all the tests was lower than 5.5, except for tests with initial pH 7.0 where the final pH was 6.6. This can be due to the higher alkalinity (buffering capacity) of the ADS inoculum (Table 3.4).

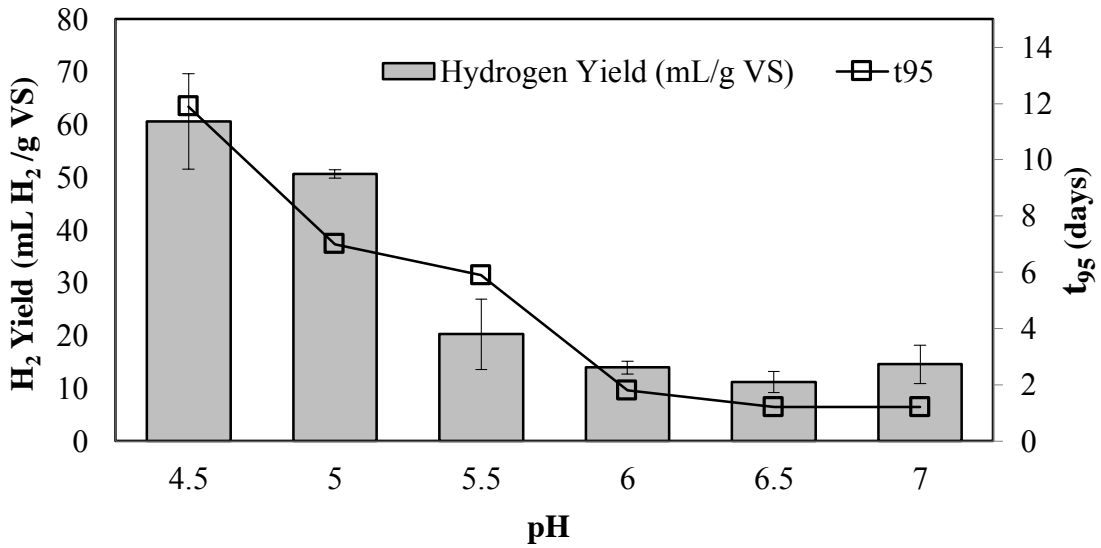

Figure 3.3 - Effect of initial pH on H_2 yield and time required for H_2 production to achieve 95% of the maximum yield during the DF of food waste at F/M ratio 0.5 and thermophilic temperature (55 ± 2 °C) using ADS

The concentrations of the main accumulated metabolites at the end of the tests are summarised in Table 3.6. Results confirm that different fermentation pathways occurred. The presence of propionate and ethanol generally does not indicate H_2 favorable pathways (Kim et al., 2011). The concentration of ethanol was comparatively higher in the tests with initial pH range 6.0 – 7.0, that could be linked to the low H_2 yields. In particular, the butyric to acetic acid ratio (B/A, mM:mM) co-related with the H_2 yields (Figure 3.4). This observation is consistent with a study by Kim et al. (2006), which reported a higher corelation between B/A ratios (1.6 – 9.3) and H_2 yields. However, this ratio might not always give a good indication of high H_2 production. Guo et al. (2013) reported that the homoacetogenic activities can influence the concentration of end-metabolites due to acetate production from H_2 and CO_2. The presence of acetate in higher concentrations

between pH 5.5 – 7.0 might indicate the prevailance of an homoacetogenic activity responsible of lower H_2 yields.

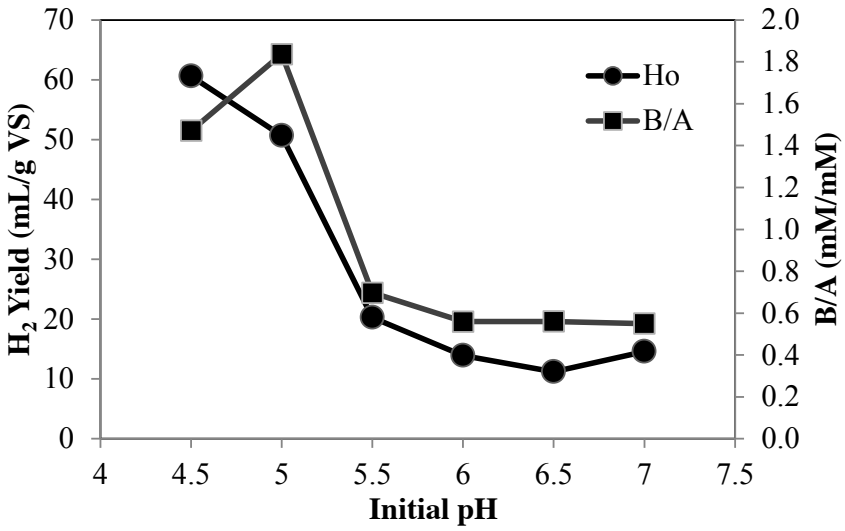

Figure 3.4 - H_2 yields and B/A ratio as a function of pH in the thermophilic DF of food waste at F/M ratio 0.5

The results of the batch tests carried out at F/M ratios 0.5, 1.0 and 1.5 at two initial pH values (5.0 and 6.5) are presented in Table 3.7. Table 3.7 shows the major metabolites accumulated at the end of the tests. At the initial pH 5.0 and F/M ratios of 0.5, 1.0 and 1.5, H_2 yields were 50.7 (± 0.8), 60.3 (± 5.0) and 49.3 (± 12.2) mL H_2/g VS, respectively. Likewise, in tests carried out with an initial pH 6.5, respective H_2 yields of 28.2 (± 4.2), 43.2 (± 2.0) and 54.1 (± 4.4) mL H_2/g VS were obtained. An ANOVA analysis confirmed the significance of difference in H_2 yields at pH 5.0 and 6.5 for an F/M ratio of 0.5 (p value <0.05). However, it was not significant for F/M ratios 1.0 and 1.5 at both initial pH values tested. Likewise, at initial pH 5.0, the differences in H_2 yields were not significant for all the three tested F/M ratios. Interestingly, the differences in H_2 yields were significant (p value <0.05) at an initial pH of 6.5 for F/M ratios 0.5 and 1.5. This implies a combined influence of the F/M ratios and initial pH on dark fermentative H_2 production. The result also suggests that the comparable H_2 yields can be achieved through a combination of pH and F/M ratios by maximizing the utilization of substrates.

The different metabolites yields measured at the end of the batch tests explain the differences in H_2 yields (Table 3.7). The presence of different metabolites suggests a typical mixed type fermentation that can occur in complex substrates like food waste. Acetate yields were higher at initial pH 6.5 compared to pH 5.0, which was also

confirmed in the tests carried out earlier at different initial pH (Table 3.6). Similarly, higher ethanol yields were obtained at increasing F/M ratios and initial pH. High levels of butyrate yield at pH 6.5 and F/M ratios 1.0 and 1.5 can be associated to higher H_2 yields obtained in respective tests, as the production of butyrate is generally co-related to H_2 production (Kim et al., 2011).

Table 3.6 - Effects of initial pH on H_2 production performance and characteristics of accumulated end products

Initial pH	Parameters derived from modified Gompertz model				Characteristics of digestate at the end of DF					
	H_o (mL/gVS)	L (h)	R (mL/h)	R^2	Average final pH	H_2 (mM/kg VS)	Acetate (mM/kg VS)	Propionate (mM/kg VS)	Butyrate (mM/kg VS)	Ethanol (mM/kg VS)
4.5	57.3	113.6	0.7	0.993	4.7 ± 0.1	1341.2 ± 201.3	1854.6 ± 114.0	964.5 ± 99.1	2728.7 ± 359.6	263.7 ± 16.1
5.0	50.9	68.1	1.0	0.999	4.9 ± 0.1	1121.3 ± 17.2	1611.8 ± 412	1686.7 ± 253.3	3018.7 ± 109.7	753.4 ± 290.6
5.5	20.3	41.2	0.4	0.995	5.2 ± 0.6	448.4 ± 148.2	2830.2 ± 381.0	1358.1 ± 392.1	1973.7 ± 374.9	623.7 ± 53.8
6.0	15.4	2.0	0.7	0.997	5.3 ± 0.1	308.0 ± 26.8	3558.9 ± 368.7	959.7 ± 6.4	1992.0 ± 238.1	2340.9 ± 263.7
6.5	11.2	3.3	0.8	0.995	5.5 ± 0.1	247.7 ± 45.3	3900.2 ± 838.5	260.0 ± 34.8	2185.5 ± 580.1	3056.7 ± 32.3
7.0	14.6	25.3	6.7	1.000	6.6 ± 0.1	322.6 ± 80.7	5922.4 ± 43.9	877.2 ± 41.4	3255.6 ± 308.1	1673.6 ± 48.4

R^2 represents the regression coefficient

Table 3.7 - Effects of initial pH and F/M ratio on H_2 production performance and characteristics of accumulated end products in DF of food waste

pH	F/M	Parameters derived from modified Gompertz model					Characteristics of digestate at the end of DF							
		H_o (mL/g VS)	L (h)	R (mL/h)	t_{95} (day)	R^2	Average final pH	H_2 (mM/kg VS)	Lactate (mM/kg VS)	Acetate (mM/kg VS)	Propionate (mM/kg VS)	Butyrate (mM/kg VS)	Ethanol (mM/kg VS)	Caproate (mM/kg VS)
	0.5	50.9	68.1	1.0	7.0	0.949	4.9 ± 0.1	2264.9 ± 34.8	17.5 ± 8.1	1610.7 ± 411.8	1687.0 ± 253.3	3018.7 ± 109.7	753.4 ± 290.6	0.0 ± 0.0
5.0	1.0	58.5	81.9	1.4	9.7	0.997	4.7 ± 0.1	2690.9 ± 206.5	18.1 ± 2.2	1264.0 ± 27.1	3135.4 ± 245.7	2959.9 ± 35.2	1876.5 ± 5.9	0.0 ± 0.0
	1.5	54.2	87.9	0.3	46.5	0.991	4.5 ± 0.1	2202.1 ± 545.2	98 ± 10.3	420.3 ± 119.7	842.8 ± 59.2	2638.1 ± 202.9	1402.9 ± 325.6	0.0 ± 0.0
	0.5	11.2	3.4	0.8	1.2	0.995	5.5 ± 0.1	1259.7 ± 188.4	0.0 ± 0.0	6043.0 ± 357.2	830.3 ± 38.9	2344.0 ± 73.3	3056.7 ± 32.3	0.0 ± 0.0
6.5	1.0	42.6	17.0	1.6	4.6	0.938	5.7 ± 0.1	1928.7 ± 89.3	126.3 ± 124.2	1700.0 ± 305.8	775.8 ± 91.1	2062.9 ± 169.1	3602.1 ± 20.7	70.3 ± 9.4
	1.5	56.9	2.3	1.8	7.0	0.944	5.3 ± 0.1	2413.4 ± 197.0	0.0 ± 0.0	2364.5 ± 216.1	655.5 ± 166.3	2410.5 ± 47.5	2206.0 ± 63.1	263.3 ± 23.1

Effect of substrate alkaline substrate pre-treatment on H_2 yields

Figure 3.5 shows the effects of alkaline substrate pre-treatment on biohydrogen production. The results illustrate that biohydrogen production can be significantly improved with alkaline pre-treatment of rice straw. As expected, the alkaline pre-treatment enhanced the saccharification of sugars from rice straw, which increased along with the concentration of NaOH. The COD values of hydrolysate after pre-treatment with 4% and 8% NaOH were 7.3 (± 0.8) and 8.3 (± 0.7) g/L, respectively, in comparison to the untreated rice straw with 3.8 (± 0.1) g/L soluble COD (determined with solid liquid ratio of 1:5). The results of end-product accumulation (Table 3.8) show that higher H_2 yields corresponded to higher B/A ratios (mM:mM), irrespective of the concentration of acids accumulated at the end of the tests.

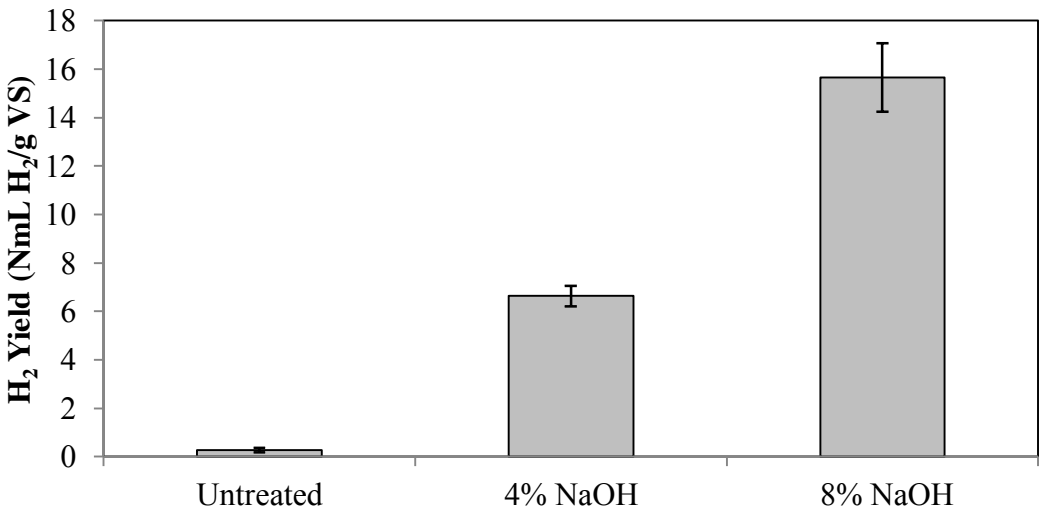

Figure 3.5 - Effect of alkaline pre-treatment of rice straw on H_2 yields

Effect of inoculum sources on H_2 yields

The cumulative H_2 yields and accumulation of end metabolites during the application of two heat treated inoculum sources on biohydrogen production from OMWW is depicted in Figure 3.6 and Table 3.9, respectively. The differences observed when using two inoculum types, i.e. ADS and WAS, at thermophilic temperature gave an indication of the level of inhibition of the polyphenols present in the OMWW on the microorganisms (Hamdi, 1992; Paraskeva and Diamadopoulos, 2006). The initial lag phase observed in Figure 3.6 can give evidence for the adaptation of H_2 producing fermentative microbial communities to phenolic compounds present in OMWW. The maximum H_2 yield from OMWW with WAS was almost 2 fold higher than

with ADS. In addition, WAS sludge required less heat-shock pre-treatment time to inhibit hydrogen consuming methanogens and showed a shorter lag phase (Figure 3.6, Table 3.9). This shows that heat-shocked WAS is an appropriate inoculum for DF of OMWW for higher H_2 recovery.

The lower H_2 yield obtained from OMWW in tests inoculated with ADS is further supported by the analysis of the metabolic pathways (Table 3.9), which showed an accumulation of lactic acid. Metabolic pathways leading to lactic acid are not favorable to H_2 production (Hawkes et al., 2007), which explains the lower H_2 yields observed in the batch tests inoculated with ADS. Likewise, the higher levels of acetate in the tests carried out with WAS than ADS can explain the higher H_2 yields from OMWW, as acetate pathways generally yields to more H_2 per mole of glucose than the butyrate pathways (Hawkes et al., 2007).

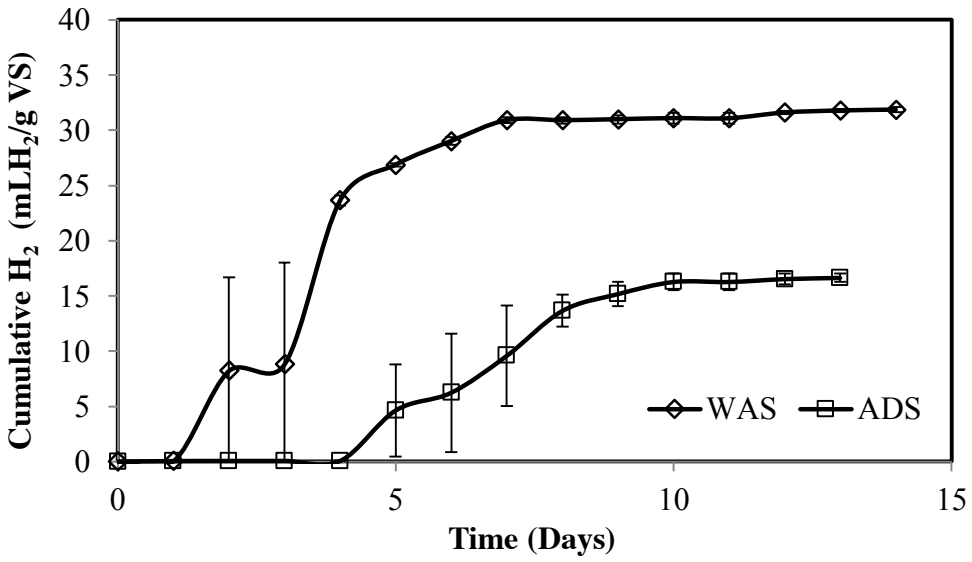

Figure 3.6 - Effect of inoculum sources on cumulative H_2 production from the DF of OMWW using ADS (anaerobic digested sludge) and WAS (waste activated sludge)

Table 3.8 - Effect of substrate pre-treatment on biohydrogen production performance measured by the modified Gompertz model

Pre-treatment method	Parameters derived from modified Gompertz model				Characteristics of digestate at the end of DF						
	H_o (mL/g VS)	L (h)	R (mL/h)	R^2	Average final pH	H_2 (mM/kg VS)	Acetate (mM/kg VS)	Propionate (mM/kg VS)	Butyrate (mM/kg VS)	Ethanol (mM/kg VS)	B/A (mM:mM)
Without treatment	0.3	37.3	0.1	0.958	4.7 ± 0.1	12.8 ± 4.1	462.6 ± 42.7	50.8 ±15.8	46.4 ±13.7	41.0 ± 7.2	0.10
4% NaOH	6.7	23.9	2.9	0.999	4.9 ± 00	296.3 ± 19.2	775.0 ± 13.5	189.4 ±18.5	227.7 ± 38.5	129.4 ± 44.8	0.29
8% NaOH	15.4	11.3	3.6	0.965	5.2 ± 0.6	699.4 ± 62.8	468.6 ± 84.4	55.6 ± 15.4	614.1 ±105.8	148.9 ± 11.8	1.31

Table 3.9 - Effects of inoculum source on H_2 production performance measured by the modified Gompertz model and characteristics of accumulated end products in DF of OMWW

Inoculum type	**Parameters derived from modified Gompertz model**				**Characteristics of digestate at the end of DF**							
	H_o (mL/g VS)	L (h)	R (mL/h)	R^2	Average final pH	H_2 (mM/kg VS)	Lactate (mM/kg VS)	Acetate (mM/kg VS)	Propionate (mM/kg VS)	Butyrate (mM/kg VS)	Ethanol (mM/kg VS)	B/A (mM:mM)
ADS	106.1	101.0	1.0	0.996	5.6 ± 0.1	751.2 ± 15.2	1651.8 ± 573.4	1752.2 ± 510.9	269.5 ± 183.3	4293.5 ± 93.1	3423.2 ± 1104.2	1.95
WAS	204.1	34.4	2.2	0.984	5.5 ± 0.2	1479.7 ± 46.3	0.0 ± 0.0	6823.0 ± 904.1	282.0 ± 217.1	5062.5 ± 131.0	3022.6 ± 0.8	0.44

3.2.4 Discussion

Effect of the pH and F/M ratio on H_2 yield

This study showed that higher H_2 yields can be achieved from easily biodegradable organic waste like food waste, when compared to other complex substrates such as rice straw (Table 3.10). This is mainly a result of the high fraction of easily degradable carbohydrates contained in food waste, as already suggested by Guo et al. (2013). The combination of initial pH and substrate concentration is important to avoid inhibition of H_2 producers through elevated VFA accumulation and consequent pH depletion, and high hydrogen partial pressure (Ginkel et al., 2001). This is likely the case of substrates like food waste which generally show faster hydrolysis kinetics compared to lignocellulosic biomass such as rice straw (Table 3.10), that requires higher optimal substrate concentrations or F/M ratios compared to food waste.

Table 3.10 compares the results of the H_2 yields observed in this study with literature data reported under similar conditions. The highest H_2 yields observed at initial pH 4.5 and 5.0 (60.6 ± 9 and 50.7 ± 1 mL H_2/ g VS food waste, respectively) in this study were in contrast with Cappai et al. (2014), who obtained the highest H_2 yield (56.2 mL H_2/ g VS food waste) at pH 6.5. This difference in optimum initial pH might be due to the higher substrate concentrations used by Cappai et al. (2014) (Table 3.10). Furthermore, two possible explanations can be given for the relationship between initial pH (4.5 and 5.0) and the higher H_2 production: (i) a selection of hydrogen producers at pH range (4.5 – 5.0) and (ii) an inhibition of H_2 consuming methanogens. In addition, the differences in metabolic products accumulating at different initial pH ranges might support the growth of different microbial communinities which can influence the H_2 production as reported in the studies from Fang and Liu (2002) and Lee et al. (2008). Khanal et al. (2004) reported that a microbial shift to solventogenesis did not occur at a pH range 4.5 – 6.5, which provides further evidence of the importance of the initial microbial community and pH to reach higher H_2 yields. In addition, native microorganisms present in the food waste might also influence the DF process in real conditions (waste type and storing conditions). In this study, the storage of food waste at freezing conditions might have impacted native microorganisms. Nevertheless, the comparison of the results between the tests operated at different initial pH remains unaffected as uniform substrates were used.

At lower F/M ratios (0.5 and 1.0), an initial of pH 5.0 favored the H_2 production whereas it was the inverse at a F/M ratio 1.5 and initial pH 6.5. At the initial of pH 5.0 and F/M 1.5, a lower H_2 yield was observed, which might be due to the shock load on the microbial systems. This was also confirmed in the study of Ginkel et al. (2001), who reported an inhibition of H_2 production at higher substrate loading rates due to shock loads. The conversion of substrates to metabolic products at pH 5.0 and F/M 1.5 is lower than at F/M ratios 0.5 and 1.0, which can be due to an inhibition of the substrate conversion. In addition, a low final pH (4.5 ± 0.1) at the end of the test at pH 5.0 and F/M 1.5 (Table 3.7) suggests that H_2 production might be inhibited due to a 'load shock'. This can be supported by the time required to achieve 95% of the maximum H_2 yield (t_{95} = 47 days) (Table 4). Pan et al. (2008) reported that a F/M ratio of 6.0 as appropriate for thermophilic (50 ± 2 °C) fermentation of food waste (Table 3.10). However, the initial pH in their study varied from 6.2 to 6.7. Therefore, in the DF systems where initial pH is not buffered, H_2 production is a combined function of suitable F/M ratio and initial pH. Likewise, an optimal operational pH range could be maitained through subsequent substrate feeding strategies which can garantee higher H_2 production and avoid the H_2 consuming activities i.e. methanogens and homoacetogens.

Table 3.10 - Summary of various strategies to improve the H_2 yields from the substrate with different biodegradability

Substrates	Optimization parameters	Optimal conditions	Substrate concentration (g VS/L)	Culture system	H_2 Yield (NmL/g VS_{added})	Reference
Food waste	Initial pH (4.5-8.5)	pH 6.5	53.1 ± 0.9	Activated sludge, 39 °C, batch	56.2	(Cappai et al., 2014)
Food waste	Initial pH (4.5-7)	pH 4.5 – 5.0	3.4	Anaerobic sludge, 55 ± 2 °C, batch	61.0 ± 9.0 at pH 4.5 51.0 ± 1.0 at pH 5.0	This study
Food waste	F/M ratio (1-10)	F/M ratio of 6.0	18.5	Anaerobic sludge, thermophilic (50 °C), batch	39.0	(Pan et al., 2008)
Food waste	F/M ratio (0.5, 1, 1.5) at pH 5 & 6.5	F/M ratio of 1 at pH 5.0	6.1	Anaerobic sludge, 55 ± 2 °C, batch	60.3 ± 5.0	This study
Sun flower stalks	Substrate pre-treatment (thermo-alkaline)	4% NaOH at 55 °C, 24 hour	5.0	Anaerobic sludge, 35 °C, pH 5.5	4.4 ± 2.6	(Monlau et al., 2013b)
Rice straw	Thermal alkaline pre-treatment	8% NaOH at 55 °C, 24 hour	43.0	Activated sludge, thermophilic (55 °C), initial pH 6.0, batch	15.7 ± 1.0	This study
Rice straw	Inoculum source (MWWS[b], PMS[c] & CDC[d])	MWWS	30.0 g TS/L	55 °C, initial pH 6.5, batch	7.1[e]	(Chen et al., 2012)
OMWW	Inoculum source (activated sludge & anaerobic digestate)	Activated sludge	10.5	55 °C, initial pH 6.0, batch	33.1 ± 1.0	This study

[a]N L H_2/kg total organic carbon; [b]MWWS: Municipal wastewater plant sludge; [c]PMS: Paper Mill Sludge; [d]CDS: Cow Dung Compost; [e]mL H_2/g TS

Effect of alkaline substrate pre-treatment on H_2 yield

The alkaline pre-treatment method applied in this study aimed at improving hydrolysis and solubilization of the organic matter that limit the dark fermentative substrate conversion (Monlau et al., 2015, 2013b). However, the level of effectiveness of the different pre-treatment methods depends on the nature of the substrate (Ariunbaatar et al., 2014; Carlsson et al., 2012). In the study of Monlau et al. (2013c), H_2 yields from sunflower stalks increased from 2.3 (± 0.9) to 4.4 (± 2.6) mL H_2/g VS, while in our study an increase from 0.3 (± 0.1) to 6.6 (± 0.1) from mL H_2/g VS from rice straw as the substrate was achieved under similar conditions of thermo-alkaline pre-treatment (Figure 3.5 and Table 3.8). Meanwhile, H_2 yields further increased to 15.7 (± 1.0) mL H_2/g VS when 8 % w/w NaOH was applied (Figure 3.5). This H_2 yield is lower than the value reported by Chen et al. (2012) with untreated rice straw, i.e. 24.8 mL/g TS at a substrate concentration of 90 g TS/L, whereas, it is 2.2 fold higher when the substrate concentration was 30 g TS/L (i.e. 7.1 mL H_2/g TS). This disagreement might be due to physico-chemical properties of the lignocellulosic substrates, such as particle sizes, soluble carbohydrates content and/or substrate concentration (Monlau et al., 2013a). Chen et al. (2012) reported an increasing trend of H_2 yields, when the particle size of rice straw decreased from 10 mm to < 0.297 mm. In their study, a maximum H_2 yield was obtained with a particle size of < 0.297 mm (6.4 mL H_2/g TS) at a substrate concentration of 30 g TS/L.

The effects of the chemical agents applied (NaOH) and or by-products formed (furfural, phenols) during the pre-treatment process and the response on the dark fermentative microbial community should be taken into consideration while selecting appropriate pre-treatment method. Kim et al. (2009) reported a decrease in H_2 yields when the Na^+ concentration in a continuous DF reactor gradually increased from 0.27 to 21.00 g Na^+/L while the acclimatized fermentative community maintained their activity up to 6.00 g Na^+/L. Nonetheless, in this study, the H_2 yields increased when 8 % w/w NaOH was applied compared to 4 % w/w NaOH (Figure 3.5). Moreover, under similar pre-treatment conditions, 12 % w/w NaOH (i.e. 5.40 g Na^+/L) might either enhance the H_2 yields or exert effect on fermentative microbial community, depending on the inocula type and adaptation to Na^+ concentration. However, the application of pre-treatment methods should be based on the substrate type (biodegradability or bioavailability of easily fermentable carbohydrates), their practicability and economy viability.

Effect of inocula on H_2 yield

The application of two different inoculum types for the DF of OMWW showed differences in response of ADS and WAS in terms of dark fermentative conversion to H_2 and other metabolites (Figure 3.6 and Table 3.9). Comparatively, WAS exhibited better performances in terms of H_2 production as shown by the H_2 production yields and kinetics in Table 6. The difference in H_2 yields might be a result of the effect of polyphenolic substances present in OMWW (total phenols in Table 3.4) on the fermentative communities present in ADS and WAS (Hamdi, 1992; Ntaikou et al., 2009). Ntaikou et al. (2009) used diluted OMWW to avoid growth inhibition, whereas, Hamdi (1992) observed an inhibition mainly on methanogens. Nonetheless, the difference in response of the two inocula could be also due to the difference in heat shock treatment time applied during the HST. ADS required a longer HST time to inhibit the activity of methanogens (Ghimire et al., 2015b) compared to WAS which has an aerobic origin. Therefore, the treatment time could have impacted the microbial communities that could contribute to fermentative H_2 production.

The use of WAS as better inoculum is supported by the studies of Chen et al. (2012) and Kim et al. (2011). Chen et al. (2012) achieved higher H_2 yields with a sludge originated from a municipal wastewater treatment plant when compared with other inoculum sources like cow dung, compost and paper mill sludge. The group attributed higher H_2 yields to the presence of a potential hydrolytic and fermentative bacterial microbial community. Kim et al. (2011) hypothesized that such increase in H_2 yields from sewage sludge addition was due to the presence of iron (Fe), calcium (Ca) and phosphorous (P) at much higher concentrations (no information on speciation was given). Further research on the nutrient and trace metal content in inocula and how these affect the DF rates is thus required.

The selection and application of various optimum operational parameters depends highly on the type of substrate, i.e. mainly its biodegradability. However, the improvement of dark fermentative H_2 production should bear the cost of application of different optimal operational parameters in terms of net energy and economy gain. It should be taken into consideration that DF of waste biomass is not a complete conversion of organic waste, i.e. organic acids and alcohols accumulate in the effluent, for which a subsequent treatment needs to be provided. Valorization of these by-products can support the costs

associated with the optimization of the DF process. Several studies have suggested the integration of DF with processes such as photofermentation (H_2), bioelectrochemical systems (H_2) and anaerobic digestion (CH_4) for further energy recovery and production of platform molecules of economic interest, such as biopolymers (Bastidas-Oyanedel et al., 2015; ElMekawy et al., 2014; Ghimire et al., 2015c; Xia et al., 2013)

3.2.5 Conclusion

This study aimed to investigate the optimal operational parameters in the thermophilic DF of three types of complex waste biomass with varying biodegradability, i.e. food waste, rice straw and OMWW. The DF applied to food waste was favored in the acidic pH range (4.5 - 5.0), though an appropriate substrate concentration must be considered while selecting an acidic pH range. F/M ratios of 0.5 and 1.0 at an initial pH of 5.0 gave, respectively, 1.8 and 1.4 folds higher H_2 yields than at initial pH 6.5. Likewise, F/M ratios and pH can be optimized to achieve higher substrate utilization and H_2 yields. During the tests, higher B/A ratios (mM:mM) were associated with higher H_2 yields, a B/A ratio equivalent to 1.5 was related to the optimal H_2 yield. Similarly, pre-treatment of rice straw with 4% NaOH and 8% NaOH at 55 °C for 24 hours increased the H_2 yield by 26 and 57 fold, respectively. Furthermore, WAS showed adaptability to OMWW containing phenols and gave a nearly 2 fold higher H_2 yield when compared to ADS. In conclusion, the selection and application of the optimal operational parameters for the optimization of H_2 production rely mainly on the substrate biodegradability. Therefore, these parameters should be optimized for each particular type of substrate prior to application in scaled-up DF systems.

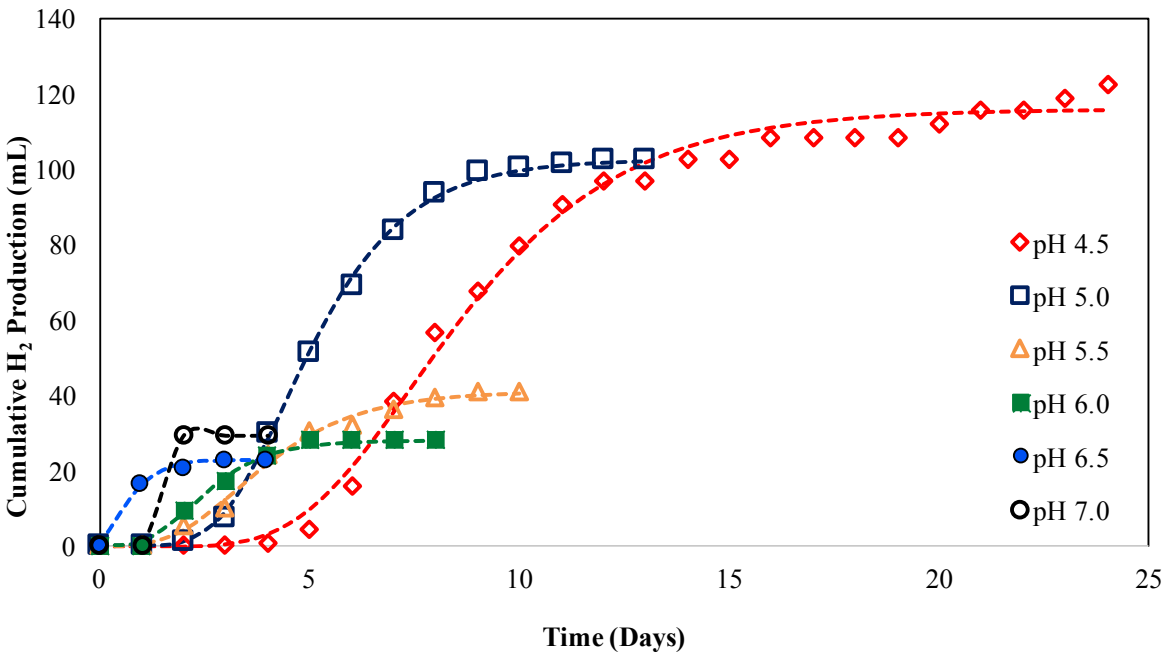

Figure S1. Cumulative H_2 production at different initial pH values using food waste at a F/M ratio 0.5 and ADS as inoculum (dotted lines represents the results from a modified Gompertz model)

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CHAPTER 4

LONG-TERM OPERATION OF HYDROGEN-PRODUCING CONTINUOUS REACTORS

A part of section 4.1 of this chapter has been published as Ghimire, A., Valentino, S., Frunzo, L., Trably, E., Escudié, R., Pirozzi, F., Lens, P.N.L., Esposito, G. (2015). Biohydrogen production from food waste by coupling semi-continuous dark-photofermentation and residue post-treatment to anaerobic digestion: A synergy for energy recovery. International Journal of Hydrogen Energy, 40 (46): 16045–16055.

The section 4.2 of this chapter will be submitted as Ghimire, A., Frunzo, L., Pirozzi, F., Lens, P.N.L., Esposito, G., Continuous biohydrogen production from thermophilic dark fermentation of cheese whey using: use of buffalo manure for pH control and biohydrogen production stability.

4.1 Continuous H_2 production from food waste at low organic loading rates

This section presents the results of the study conducted to demonstrate the long-term continuous H_2 production from dark fermentation food waste conducted in a semi-continuous reactor operated at low organic loading rates (OLRs). The dark fermentation (DF) process was carried out at thermophilic temperature (55±1 ^{0}C) in a reactor of 2 L with a working volume of 1.5 L, for a period of 250 days. The effect of different OLRs and hydraulic retention time (HRT) ranging from 1 to 2.5 VS/L/d and 12 to 4 days, respectively, were assessed on the quantity and the quality of biohydrogen–rich biogas production. A maximum H_2 yield of 139.70±54 NmL H_2/g VS was observed with an OLR of 2.5 gVS/L·d and at 4 days HRT. Soluble metabolic end-products were monitored during this period and it was shown that the butyric acid pathway was mainly responsible of the H_2 production. A transitory accumulation of lactic and/or propionic acids was observed when the OLR (increment) or the HRT (decrement) was changed, causing a decrease in H_2 production. Monitoring of soluble metabolites provided a reasonable indication of DF process performances.

4.1.1 Introduction

Over the past few decades, anaerobic digestion has gained a lot of interest since it constitutes a promising technology for producing an energy-rich biogas from renewable waste and biomass resources such as wastewaters, municipal organic waste, agricultural residues, etc. The inherent characteristics of biohydrogen (H_2) such as higher energy content (143 GJ per ton), energy and water as the only by-products generated from its combustion and the ability to be produced biologically, makes H_2 as a very interesting alternative of future sustainable biofuels (Kotay and Das, 2008). In particular, dark fermentation (DF) systems have the potential to be one of the prominent technologies for H_2 production from renewable waste biomasses (Ghimire et al., 2015a; Urbaniec and Bakker, 2015).

Low cost renewable waste biomasses such as agricultural residues, organic fraction of municipal waste (OFMSW), agro-industrial wastes, etc. might give a competitive economic advantage for the future supply of sustainable feedstock, aiming at the industrial development of DF systems with biological treatment of waste as added benefit (Chong et al., 2009; De Gioannis et al., 2013; Kapdan and Kargi, 2006; Ntaikou et al.,

2010; Wong et al., 2014). In contrast, the use of simpler feedstock sources such as pure carbohydrates (e.g. sucrose and glucose), although it presents higher H_2 conversion rates, could make DF processes less economically competitive (Ren et al., 2011).

OFMSW which is mainly composed of food waste is receiving lot of attention because of its high biodegradability and its potential to be utilized for the production of biofuels and other platform chemicals (Uçkun Kiran et al., 2015). Every year, about 1.3 billion tons per year of food get wasted, which is approximately one-third of the food produced for human consumption (Gustavsson Jenny, Cederbery Christel, Sonesson Ulf, Van van Otterdijk Robert, 2011). Food wastes are generated from the agricultural production, to industrial manufacturing processes and final consumption in households. In the European Union, the total annual production of food waste is estimated at 89.3 Mt, comprising 37.7 Mt generated from household consumption alone (European Commission, 2010). The food waste content in volatile solids ranges from 21 to 27% which shows the high content of organic carbon which can be valorized, and in particular for H_2 production by DF (VALORGAS, 2010). During the past few years, several researches have shown the high potential of food waste to be used as a feedstock in DF processes for H_2 production (Cavinato et al., 2012; Elbeshbishy et al., 2012; Faloye et al., 2013; Han and Shin, 2004; Han, SK. and Shin, 2004; Shin and Youn, 2005; Sreela-or et al., 2011; Valdez-vazquez et al., 2005; Xiao et al., 2013).

With the advantage of a steady operation, continuous DF processes are preferred and scaling-up is more viable in comparison to batch processes which involves regular downtime periods of maintenance (Hawkes et al., 2007). However, stable operation of continuous DF of food waste is mostly influenced by the bioreactor operating parameters such as the pH, temperature, organic loading rate (OLR) and hydraulic retention time (HRT) (Davila-Vazquez et al., 2007; De Gioannis et al., 2013; Guo et al., 2010; Ntaikou et al., 2010; Wang and Wan, 2009). These factors also influence the microbial communities and thus the biochemical pathways that can affect the total H_2 yields in mixed cultures (Li and Fang, 2007).

Hydrogen production rates and total H_2 yield are mainly a function of substrate types and OLRs applied (Ghimire et al., 2015a). A varying range of optimal OLR values has been reported for FW for H_2 conversion carried out in thermophilic DF processes (Ghimire et al., 2015a). Shin et al. (Shin and Youn, 2005) found an optimal hydrogen yield of 126.25

L H_2/kg VS at an OLR of 8 kg VS/m^3/d while the H_2 production decreased when the OLR was increased to 10 kg VS/m^3.d. The authors reported 8 kg VS/m^3.d, 5 days and pH of 5.5, respectively, as optimal OLR, HRT and culture pH. In a study coupling DF and AD, Cavinato et al. (Cavinato et al., 2012) reported 66.7 L H_2/kg VS added at an optimum OLR of 16.3 VS/m^3.d, a HRT of 3.3 days and for a pH maintained in the range of 5-6 through the recirculation of AD effluent. Generally, HRTs in a range of 2-6 days have been reported as optimum for DF of organic FW in a CSTR process (Ghimire et al., 2015a). This range of HRTs is similar to the first stage of two-stage AD process (Aslanzadeh et al., 2014).

Unlike OLR, the HRT is also a function of the substrate types and bioreactor operation parameters. It is well understood that DF processes generate acidic microbial metabolites. Therefore, high OLRs are often responsible for a decrease in pH due to the accumulation of volatile fatty acids (VFAs) present in the DF effluent (DFE). Thus, most of the DF systems require the addition of external alkalinity sources such as alkaline chemicals (NaOH or KOH) or buffering agents (bicarbonate or phosphate buffers) (Shin and Youn, 2005). Meanwhile, few studies have reported the use of recycle water from AD as a solution to reduce the use of external alkaline chemicals (Gottardo et al., 2013; Jung et al., 2013). Moreover, there are additional concerns regarding the decrease in H_2 yields due to hydrogen consuming activities of methanogens or propionic producing bacteria (Jung et al., 2013).

Moreover, thermophilic temperature is mainly favored in DF of food waste since higher H_2 yields are usually observed (Shin et al., 2004; Valdez-vazquez et al., 2005). Moreover, a thermophilic process seems to be more economically interesting owing to its higher yield and less requirement of feedstock in comparison to mesophilic dark fermentation processes (Foglia et al., 2006). Foglia et al. (2006) reported better economic performances of thermophilic DF in comparison to mesophilic operation when the process was operated to convert sugars into hydrogen, CO_2 and organic acids that were further used in a second photofermentation process.

Most of the past studies on continuous and/ or semi-continuous dark fermentative H_2 production were carried out at controlled culture pH with chemical buffering agents such as K_2HPO_4, $NaHCO_3$, Na_2HPO_4 (Carrillo-Reyes et al., 2012; Valdez-vazquez et al., 2005). Likewise, even in a recent pilot scale application of DF has shown the dependency

on buffering agents for stable H_2 production (Elsamadony and Tawfik, 2015). The high amount of chemical buffering agents needed to maintain the operable acidogenic pH (higher than 4.5 – 5.5), which might effect the operational cost of DF bioreactors. Moreover, the effect of use of high concentrations of buffering agents on downstream processes like anaerobic digestion, photofermentation, bioelectrochemical systems is uncertain.

The current study aims at investigating thermophilic DF of food waste for continuous H_2 production at varying low OLR and HRT. The study also considers the recycling of DF effluents to investigate its effect on the performance of bioreactors, which has never been reported in past studies, to knowledge of authors. Moreover, a major aim is to demonstrate a long-term feasibility of continuous H_2 production at varying operational conditions of the bioreactor. The performances of the DF reactor were evaluated by daily monitoring of H_2 and metabolites production rates.

4.1.2 Materials and methods

Preparation of feedstock

An average mix waste composition as found in European countries was prepared at the laboratory paccording to (VALORGAS, 2010). The waste mixture composed of (in % by weight); fruit and vegetables: 72%, cooked pasta and rice: 10%, bread and bakery: 5%, dairy products (cheese): 2%, meat and fish: 8% and snacks (biscuits): 3%. The food waste ingredients were freshly brought from municipal markets in Naples (Italy), shredded with a blender and immediately stored at -20 ^{o}C to avoid acidification. The food waste had a pH of 4.37 ± 0.01 and the characteristics are presented in Table 4.1.

Table 4.1 - Characteristics of food waste

Characteristics	g/kg Food waste
Chemical Oxygen Demand (COD)	347.6±47.4
Carbohydrate content	105.80±0.7
TKN	6.4±0.18
Lipids	17.50±1.19
Total solids (%)	23.79±0.44%
Volatile solids (%)	22.8±0.42%

Experimental setup and operational conditions

An anaerobic digested sludge was collected from an anaerobic digestion plant of the farm "La Perla del Mediterraneo" (Campania, Italy). The sludge was used as start-up seed inoculum, after a thermal pretreatment at 105 ^{o}C for 4 hours to enrich the spore forming clostridium and inhibit the methanogens. The total solids (TS) and volatile solids (VS), ammonia content of the inoculum were 29.54±0.22 gTS/L, 18.36±0.14 gVS/L and 283.47±10.8 NH^{+}_{4}/L respectively. The pH of the inoculum was 8.3±0.1 and the total alkalinity was 1437.20±14.27 mg $CaCO_3$/L.

A semi-continuous stirred 2 L serum bottle (Simax, Czech Republic) with 1500 ml working volume and remaining headspace was setup in the laboratory (Figure 4.1). The reactor was fed with food waste and the effluent was manually extracted on a daily basis. The varying operational conditions investigated during the experimental period are presented in the Table 4.2. Effluent and biogas samples from the reactor were daily analyzed for determining the metabolic intermediates, i.e. VFAs, and the gas composition (H_2 and CO_2). The total volume of gas was measured by volumetric water displacement. The biogas was passed through acidic water (1.5 % HCl) and the volume of water displaced measured the volume of total biogas. Considering this volume and the gas composition analyzed, the volume of H_2 produced was calculated.

Table 4.2 - Experimental design used for the operation of semi-continuous reactor

Experimental periods	I	II	III	IV	V	VI	VII	VIII
OLR (g VS/L·d)	1.0	1.0	1.5	2.0	2.0	2.5	2.5	2.5
HRT (d)	12.0	6.0	6.0	6.0	4.0	4.0	8	4
Concentration (g VS/L)	12	6	9	12	8	10	20	10

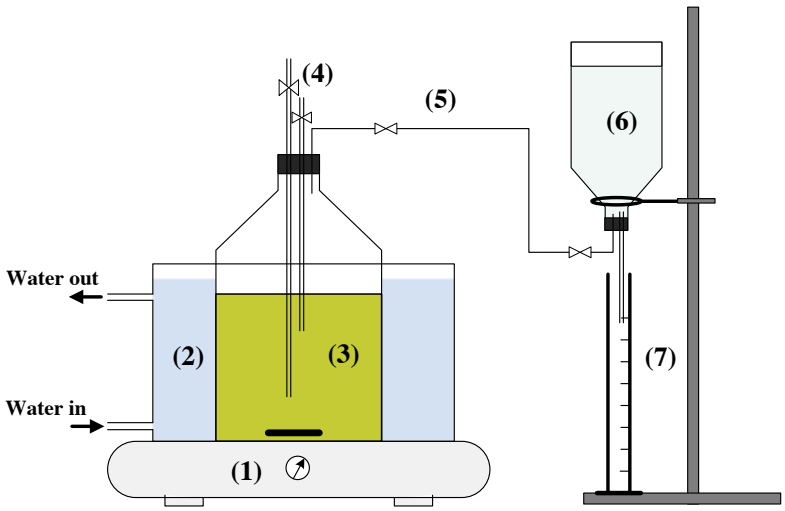

Figure 4.1 - Schematic description of semi-continuous reactors setup for H_2 production 1-Magnetic Stirrer; 2-Thermophilic water circulation bath maintained at 55 ± 2 C; 3-2 L Serum bottle as CSTR reactor; 4-VFA sampling and substrates feeding ports; 5-Gas delivery pipe; 6-1 L serum bottle with 1.5% HCl; 7-Graduated cylinder or bottle for collecting displace acidic water from (6)

Analytical methods

Hydrogen was quantified with a Varian Star 3400 gas chromatograph equipped with ShinCarbon ST 80/100 column and a thermal conductivity detector. Argon was used as carrier gas with a front and rear end pressure of 20 psi. The duration of analysis was 14 minutes. The fermentation products (lactic, acetic, propionic and butyric acids) were quantified by High Pressure Liquid Chromatography (HPLC) (Dionex LC 25 Chromatography Oven) equipped with Synergi 4u Hydro RP 80A (size 250×4.60mm) column and UV detector (Dionex AD25 Absorbance Detector). Gradient elution consisted of 20% methanol, 10% acetonitrile in 5 mM H_2SO_4 pumped at a rate of 0.9 ml/min, using Dionex GP 50 Gradient pump. The elution time was 18.5 minutes. COD of food waste was measured according to a method described elsewhere (Noguerol-Arias et al., 2012). The carbohydrate content was determined according to the Dubois method (DuBois, M., Gilles, K., Hamilton, J., Rebers, P., & Smith, 1956). Total lipids was measured following a Bligh and Dyer chloroform/methanol total lipid extraction method (Bligh and Dyer, 1959). The TS, VS of seed sludge and TKN were determined according to the Standard Methods (APHA, 2005).

Data analysis

Hydrogen production rates (HPR) were expressed in L $H_2/m^3/d$ while the H_2 yields (HY) were determined considering the total daily organic load fed to the reactor and expressed as L H_2 /kg VS added. Average and deviations for daily production were determined during the steady state reached after 3 - 4 days operation. The H_2 Production Stability Index (HPSI) was evaluated by considering the ratio of standard deviation and average HPR as reported by Tenca et al. (2011):

$$HPSI = 1 \quad \frac{S.D.(HPR)}{Avg.HPR} \tag{4.1}$$

A HPSI index closer to 1 represents a stable hydrogen production.

FactoMineR, an extension on R software, was used for multivariate analysis of metabolites distribution from the different experimental periods in relation to the hydrogen yields and co-relation circles of the major metabolites are generated.

4.1.3 Results and discussion

Effect of operational parameters on quality and quantity of H_2

The HPR, HY, H_2 content in gas produced and HPSI during the eight experimental conditions (Table 4.2) are summarized in Table 4.3. Figure 4.1 shows HPR and pH trends during the experimental period. The reactor was operated for 253 days to demonstrate the long-term operation feasibility operation continuous H_2 production in a semi-continuous thermophilic DF reactor. The effect of varying operational conditions of OLR and HRT was investigated. The culture pH was experiment aimed at reducing the amount of chemical buffering agents that are used to maintain an acid pH. Initial pH of the influent was 7 and the pH was not regulated and adjusted itself according to the fermentative activity with the aim at reducing the dependency on chemical buffering agents.

HPR trends showed the increases in H_2 yield with the increase in OLR, the change in HY was not significant (Table 4.3 and Figure 4.2 a). The range of HRT (12 - 4 d) studied does not show a significant effect on HY, as seen from the comparison of HYs during the experimental period IV and V (Table 4.3). PCA analysis, presented in Figure 4.3 (a-b) showed that range of OLR studied (1 - 2.5 gVS/L/d) has more effects on HY than the HRT (12 - 4 d).

The effect of maintaining the culture at pH 5.5 exhibited only insignificant increase in HY, nevertheless, H_2 production was stable during the experimental period (period IV, shown by the shaded region in Figure 4.2 b), also shown by HPSI of 0.86. During the period, the percentage of H_2 in the gas averaged between 59.4 ± 6% while CO_2 averaged 39.1 ± 6%. The H_2 production performances during experimental period V were nearly comparable to period IV. Furthermore, when the OLR was changed to 2.5 g VS/L/d in period VI, the HPSI decreased to 0.63, evident by the unstable HPR (Figure 4.2 (a) and Table 4.3). During period VII, when HRT was increased from 4 days to 8 days, the H_2 production decreased and ceased (Figure 4.2 (a)), this might be attributed to the change in H_2 producing microbial community. However, the when HRT was changed back to 4 d in period VIII, the H_2 production started again.

Moreover, at the end of experimental period VIII (shaded region in Figure 4.1 a), the DF residues after settling for half an hour and removing the supernatant was recycled back into the reactor along with the feed. The recycling the DF residues has insignificant affect on HPR and HY. This gives supports the fact that H_2 production is mainly function of soluble fraction of carbohydrates present in the substrates Guo et al. (2013) and Monlau et al. (2012). Thus, this fraction of DF residues demands further treatment through anaerobic digestion. The H_2 productions during the period V was compared with the previous studies conducted with similar feedstock (Table 4.4), showed that the results obtained from this study is comparable. Therefore, even at the low OLR 2 - 2.5, the HY is comparable to that of the past studies. Additionally, the optimal operating conditions of OLR (2 - 2.5 g VS/L/d) and HRT (4 – 6 d), gives this DF system a potential flexibility to integrate with anaerobic digestion with two stage conversion to H_2 and CH_4 respectively (Aslanzadeh et al., 2014).

Table 4.3 - H_2 production rate, yields and production stability from FW by mixed anaerobic cultures

Exp. Period	HPR (N L/L/d)	HY (N L/kg VS_{added})	H_2 in biogas (%)	HPSI
I	116.9±40.1	116.9±40.1	52.8%±1%	0.66
II	54.1±41.3	54.1±41.3	31.2%±1%	0.24
III	109.5±32.8	73.0±21.9	43.8%±20%	0.70
IV	210.2±29.8	105.1±14.9	59.4%±6%	0.86
V	208.0±34.8	104.0±17.4	57.2%±6%	0.83
VI	303.6±111.4	121.4±44.5	55.8%±10%	0.63
VII	133.2±112.1	53.3±44.8	46.1%±28%	0.16
VIII	408.8±97.6	163.5±39.0	59.4%±40%	0.76

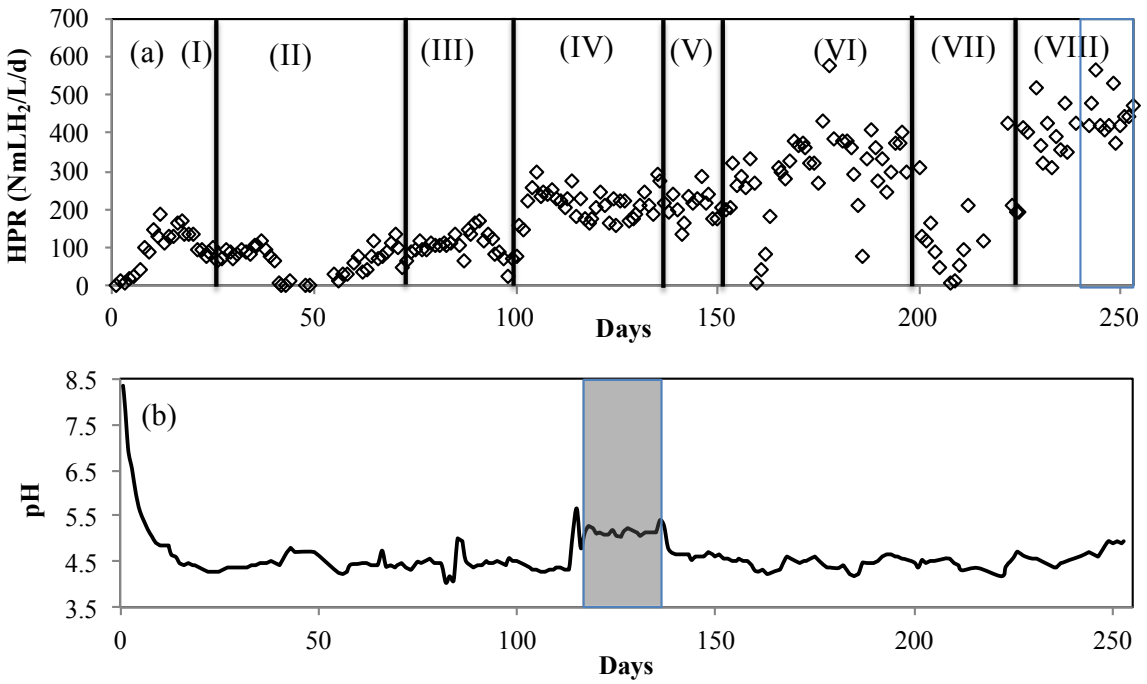

Figure 4.2 - (a) HPR (mL H_2/L/d) (b) pH trends in semi-continuous thermophilic DF bioreactor (the shaded region in Figure 4.2 (a) represents the experimental period when the DF residues were recycled back to the reactor and 1 (b) represents the period when pH was adjusted at pH 5.5)

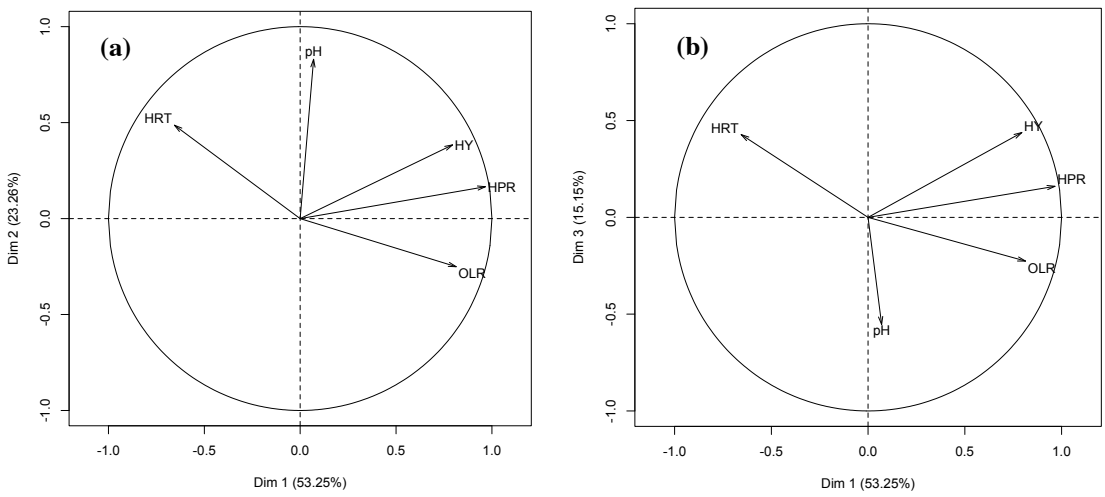

Figure 4.3 - Correlation circle of pH, HRT, OLR, HPR and HY formed by the first three principle components dim1, Dim 2 and Dim 3 representing 53.25, 23.26 and 15.15 % of the total variance, respectively (a) Projections according to the first two factors (Dim 1 and Dim 2). (b) Projects according to the first and third factors (Dim 1 and Dim 3)

Table 4.4 - Comparison of H_2 production from food waste by mixed cultures

Substrate type	Reactor type	Temp. (°C)	pH	OLR (g VS/L·d)	Maximum assessed H_2 yield (Nml H_2/g VS_{added})	H_2 in biogas (%)	Reference
FW	Batch	55	4.5 (initial)	6	46.3	23	(Shin et al., 2004)
Vegetable kitchen waste	Intermittent-CSTR	55	6.0	28[a]	38.1[b]	40	(Lee et al., 2010)
FW and sewage sludge	Batch	35	5.0-6.0	-	122.9[c]	-	(Kim et al., 2004)
OFMSW (FW+paper)	Semi-continuous CSTR	55	6.4	11[d]	360	58	(Valdez-vazquez et al., 2005)
OFMSW	Packed bed reactor	38±2	5.6±0.2	16[e]	99	47	(Alzate-Gaviria et al., 2007)
FW	Semi-continuous CSTR	55	4.5-5	2.5	104.0±17.4	57±6 %	This study

[a]gCOD/L·d, [b] mL H_2/g COD, [c] mL H_2/g carbohydrate COD, [d]g VS/kg wet mass reactor·d, [e]g VS/kg·d, FW=Food waste, OFMSW= Organic fraction of municipal solid waste

Effects on production of overall metabolic products

The concentration of major by-products, i.e. lactate, acetate, propionate, butyrate and ethanol monitored during the fermentation period of 253 days are summarized in Table 5. These metabolites are generally present in the DF of complex substrate using mixed cultures (Guo et al., 2013). PCA was done to understand the relationship between the OLR, by-products and H_2 production (Figure 4.3). It can be seen that H_2 production is more correlated with the butyrate as explained by variable Dim 2. However, the presence of other metabolites does not show a clear relationship to H_2 evolution, while their proximity can suggest that these metabolites can be expected under the DF of complex substrate by mixed consortia. The accumulation of lactate or propionate does not represent the H_2 favorable pathways, which can clarify the lower production of H_2 during the period VII, whereas, the production of butyric in a mixed fermentation pathway indicates higher H_2 yields. The presences of these metabolites for H_2 favorable and unfavorable pathways are further supported by the biochemical reactions shown in the equations 2-9 (Table 4.6).

Although the presence of ethanol and acetate might indicate H_2 production pathways (eqn. 2 and 4). However, from the PCA, they do not show clear relation with the evolution of H_2. Moreover, there might be a biochemical pathways that could favors the production of propionate and acetate which are catalyzed by propionic acid bacteria, shown in equation 7 (Tyree et al., 1991). This fact can be supported by an increase in production of acetic and propionic acid (8.3 ± 3.4 and 23.7 ± 12.6 mM) during the experimental period VII, when HRT increased from 4 days to 8 days. Likewise, no clear co-relation was found between HY and butyric to acetic acid (B/A) ratio as suggested by Hawkes et al. (2007) (data not presented here) (equation 5). Therefore, the presence of acetate might not always give an indication of H_2 production. It can be seen from the results presented in Table 4.5 that the sudden change in OLR or/and HRT could change the metabolic pathways to lactate and propionate production. However, the pathways can be reversible, when the HRT was changed back to 4 days from 8 days the reactor stated to H_2 production. The monitoring of soluble metabolites can aids in an operational management of the DF bioreactors.

Table 4.5 - Characteristics of influent and effluents DF of FW during different experimental periods

Exp. Period	pH_IN	pH_OUT	Lactate (mM)	Ethanol (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)
I	7.00	4.7±0.3	0.1±0.2	4.8±0.2	13.1±3.6	3.8±2.2	10.4±2.8
II	7.00	4.5±0.1	0.6±1.4	5.4±3.5	3.2±2.0	3.4±2.3	6.2±4.2
III	7.00	4.5±0.2	4.0±9.1	8.7±2.7	4.9±0.6	6.0±2.2	11.0±1.6
IV	7.00	4.9±0.4	0.0±0.0	17.2±8.6	8.5±1.8	9.6±2.9	12.0±2.9
V	7.00	4.7±0.2	0.0±0.0	17.1±6.6	6.7±1.9	5.7±2.1	9.9±3.2
VI	7.00	4.4±0.1	0.5±0.9	9.4±5.3	5.7±2.8	5.9±2.7	11.1±7.5
VII	7.00	4.50±0.1	3.9±1.8	10.6±1.8	8.3±3.4	23.7±12.6	14.9±5.8
VIII	7.00	4.47±0.9	0.2±0.2	6.1±2.2	9.0±6.5	8.8±7.7	16.4±10.5

Table 4.6 - Reaction stoichiometry in dark fermentation of glucose

Possible H_2 producing pathways	Metabolic pathways	$\Delta G'_0$[a] (kJ/mol)	Eqn
$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$	Acetate	-206.3	(2)
$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$	Butyrate	-254.8	(3)
$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2OH + CH_3COOH + 2CO_2 + 2H_2$	Ethanol & acetate	-215.7	(4)
$4C_6H_{12}O_6+2H_2O \rightarrow 3CH_3CH_2CH_2COOH + 2CH_3COOH + 8CO_2 + 10H_2$	Butyrate & acetate	-254.0	(5)
Unfavorable and H_2 consuming pathways			
$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O$	Propionate	-359.6	(6)
$1.5\ C_6H_{12}0_6 \rightarrow 2C_2H_5COOH + CH_3COOH + CO_2 + H_2O$	Propionate & Acetate	-310	(7)
$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$	Ethanol	-235.0	(8)
$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH$	Lactate	-198.1	(9)

[a] $\Delta G'_0$ values are adapted from (Kim et al., 2006; Thauer et al., 1977)

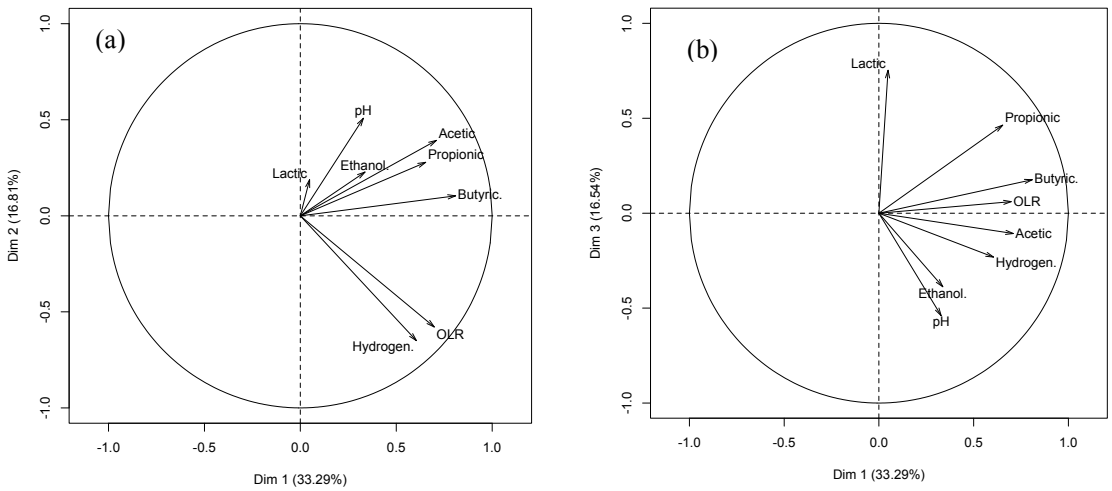

Figure 4.4 - Correlation circle of six metabolites, pH and OLR formed by the first three principle components dim1, Dim 2 and Dim 3 representing 33.29, 16.81 and 16.54 % of the total variance, respectively (a) Projections according to the first two factors (Dim 1 and Dim 2). (b) Projects according to the first and third factors (Dim 1 and Dim 3)

4.1.4 Conclusion

The paper shows the long-term feasibility of continuous H_2 production in semi-continuous reactor using food waste as substrate. The low OLRs have been taken to maintain the culture pH at operable conditions that showed the comparable HPR and HY in relation to past studies. The optimum HPR, HY and HPSI of 208.0 ± 34.8 NmL H_2/L/d, 104.0 ± 17.4 NmL H_2/g VS and 0.83, repectively, was obtained at the OLR of 2.5 g VS/L/d at the HRT of 4 days. The change in operating conditions can affected the metabolic pathways and thus the H_2 production as seen from the inhibition of H_2 production from the accumulation of lactate and propionate in the bioreactor. The recirculation of solid residues present in DFE does not significantly affect the H_2 yield so it can be used feedstock in the anaerobic digestion process for further energy conversion in the form of methane.

4.2 Co-fermentation of cheese whey and buffalo manure for pH control

This section presents investigation on the effect of buffalo manure (BM) addition on pH control and H_2 production stability during the dark fermentation of cheese whey (CHW). Dark fermentation (DF) processes are often favored at acidic pH ranging 5.0 - 6.0 depending on type of substrates and bioreactor operating conditions. H_2 production in DF process is inhibited at lower culture pH (<4.0) and becomes unstable due to lack of production of buffering capacity like in anaerobic digestion for methane. The co-fermentation with substrates contributing to alkalinity such as animal manure can highly reduce dependency on chemical buffering agents for maintaining the optimal pH conditions. CHW and BM, the abundant waste by-products from agro-industrial activities (mozzarella cheese industries) in the Campania Region, Italy, were used as substrates for continuous H_2 production in a semi-continuous thermophilic DF reactor operated at various organic loading rates (OLR). At CHW to BM ratio of 4 g VS/gVS, the maximum H_2 yield, production rate and H_2 content in the biogas of 152.2 ± 43.9 mL H_2/g VS, 215.4 ± 62.1 mL H_2/L·d and 58.01 ± 4.8%, respectively, were achieved at an OLR of 2.1 g VS/L/d of CHW at a hydraulic retention time (HRT) of 12 days. BM addition aided to maintain culture pH around 4.8 – 5 in the dark fermentation reactor. The use of BM as co-substrate improved the H_2 production stability and can give economic sustainability to DF systems in scaled-up applications.

4.2.1 Introduction

Activities associated with dairy industries, either related to livestock farming for milk production or processing of milk products such as cheese, generates large amount of waste (De Gioannis et al., 2014). In 2013, out of the 144 million tonnes of whole milk collected in European Union (EU- 28 countries), 36.2 % was used for production of cheese, butter (28.1%), drinking milk (12.1%), cream (12%), milk powder (3.2%) and other uses (8.4%) (Eurostat, 2013). Cheese manufacturing industries generate liquid waste by-products, mainly cheese whey (CHW) (Carvalho et al., 2013; Venetsaneas et al., 2009). Simultaneously, livestock activities also produce large quantities of solid animal manure waste, fodder waste (which generally contains a lignocellulosic fraction) and wastewater which includes urine and feces which can pose threats to the atmospheric and aquatic environment due to pathogens and high nitrogen (ammoniacal nitrogen) contents (Cantrell et al., 2008). DF of waste biomass can be one of the very promising

technologies, which can provide environmental credentials from recovery of renewable energy in the form of biohydrogen (H_2), as well as organic waste treatment.

Generally, the waste biomass rich in carbohydrates is considered to be most suited for DF processes (Azwar et al., 2014; Ghimire et al., 2015a; Guo et al., 2010; Monlau et al., 2013; Yasin et al., 2013). CHW can be very suitable feedstock for DF processes as it is characterized by high organic loads, comprising mainly soluble form of carbohydrates (lactose), protein and lipids (Marone et al., 2014; Moreno et al., 2015; Teli et al., 2014).

Although animal manure is not considered as suitable substrate for DF processes, it can be used as co-substrate. It has been suggested in previous studies that animal manure can provide macro and micronutrients such as NH_3, P, K and trace metals required for bacterial growth as well as it can act as buffering agent to maintain the alkalinity (Lateef et al., 2012; Marone et al., 2014; Perera and Nirmalakhandan, 2011). In anaerobic digestion processes for methane production, the production of acidity from VFAs generation is balanced by production of alkalinity from ammonia and bicarbonate (Michael H, 2003; Redzwan and Banks, 2010). However, DF processes are not stable due to continuous production of VFAs, lowering the process pH.

Culture pH plays an important role in the biochemical pathways and H_2 yields. Depending on the type of substrates, often an acidic range (5.0-6.0) favors H_2 production while a very low pH can inhibit the hydrogen production (Ghimire et al., 2015a; Khanal et al., 2003). DF processes require nutrient supplements and adequate pH buffering agents to maintain optimal DF conditions, which can inevitably impede the economic sustainability of the DF process in scaled-up applications (Gottardo et al., 2013; Tenca et al., 2011). Choi and Ahn (2013) have suggested the use of substrates with a high pH to replace chemical buffers. There have been very few studies carried out using animal manure as a buffering agent and nutrient amendment in order to get higher H_2 yields (Marone et al., 2014; Tenca et al., 2011; Wu et al., 2009). In particular, reports on the effect of animal manure addition on long-term continuous H_2 production is very scarce (Tenca et al., 2011).

The aim of this work is to study the use of buffalo manure (BM) as a co-substrate for continuous H_2 production using CHW as a main substrate in thermophilic DF process. The study also aims at maximizing the H_2 yields along with optimal process stability. In addition to quantity and quality of daily H_2 productions, major soluble metabolites,

culture pH, total alkalinity, and the ammonium (NH_4^+-N) concentrations were monitored to assess the process performances.

4.2.2 Materials and methods

Start-up, inoculum and feedstock

The heat shocked anaerobic digested sludge collected from an anaerobic digestion plant described elsewhere (Ghimire et al., 2015b) was used as start-up inoculum. The total solids (TS), volatile solids (VS) and ammonia (NH^+_4-N) content of the inoculum are 29.54±0.22 gTS/L, 18.36±0.14 gVS/L and 283.47±10.8 gNH^+_4/L, respectively. The pH of the inoculum was 8.3±0.1 and the total alkalinity was 1437.20±14.27 mg $CaCO_3$/L. The CHW and BM collected from the cheese factory and buffalo farm in Salerno, Italy, were stored at <4 °C for further use in the experiments. The waste composition used in the study is presented in Table 4.7.

Table 4.7 - Characteristics of cheese whey and buffalo manure

Characteristics	Cheese whey	Buffalo manure
pH	4.88±0.01	8.05±0.01
Total solids (%)	6.06±0.03%	5.67±0.04%
Volatile solids (g/L)	50.54±0.22	42.17±1.35
Total COD (g/L)	67.02±6	ND
Soluble sugars (g/L)	12.88±0.34	ND
TKN (g/L)	0.86±0	1.99±0.1
Lactic acid (g/L)	2.52±0.172	ND
Alkalinity (g/L)	0.5±0	4.37±1

ND- Not Determined

Semi-continuous reactor and operating conditions

A continuously stirred tank reactor of 1500 ml working volume and 700 ml headspace was setup with continuous biogas measurement (Figure 4.1). The reactor was fed with CHW and the effluent extracted manually on daily basis. The produced total volume of gas was measured with volumetric displacement method passing through acidic water (1.5 % HCl) and the volume of H_2 was confirmed by the analysis of gas composition. Based on the different feeding strategy of BM and operational conditions of the reactor,

the operation periods were divided into seven experimental periods as shown in Table 4.8.

Table 4.8 - Operational conditions and buffalo manure feeding strategies during different experiments runs

Experimental Periods	CHW:BM (gVS/gVS)	Total OLR (gVS/L/d)	OLR of CHW (gVS/L/d)	HRT (d)
I	0	0.7	0.7	12.0
II	0	2.1	2.1	12.0
III	1	4.2	2.1	12.0
IV	4	2.6	2.1	12.0
V	4	2.6	2.1	8.0
VI	2	3.2	2.1	8.0
VII	4	2.6	2.1	8.0

Analytical methods

The biogas composition was quantified by Varian Star 3400 gas chromatograph equipped with ShinCarbon ST 80/100 column and a thermal conductivity detector. The duration of analysis was 14 minutes. Argon was used as carrier gas with front and rear end pressure of 20 psi. The major fermentation products (lactic, acetic, propionic and butyric acids) were quantified by High Pressure Liquid Chromatography (HPLC) (Dionex LC 25 Chromatography Oven) equipped with Synergi 4u Hydro RP 80A (size 250×4.60mm) column and UV detector (Dionex AD25 Absorbance Detector), as described elsewhere (Ghimire et al., 2015b). Ethanol was also quantified by HPLC (Aminex HPX-87H column (300 mm on 7,8 mm, Bio-rad), as described elsewhere (Ghimire et al., 2015b). COD was determined according to a method described elsewhere (Noguerol-Arias et al., 2012). The carbohydrates were determined by Dubois method (DuBois et al., 1956) and total lipids were measured by Bligh and Dyer chloroform/methanol total lipid extraction method (Bligh and Dyer, 1959). TS, VS of seed sludge and TKN were determined according to Standard Methods (APHA, 2005).

Data analysis

Biohydrogen production rates (HPR) were expressed in mL H_2/L/d while the H_2 yields (HY) were determined considering the total daily CHW and BM fed to reactor and expressed as mL H_2 /g VS added. Average values and corresponding standard deviations

were calculated after allowing the 3 - 4 days of time to achieve the steady state. The H_2 Production Stability Index (HPSI) was evaluated by considering the ratio of standard deviation and average HPR, reported previously by Tenca et al. (2011) given in equation 4.1. Principal Component Analysis (PCA) analysis was carried in statistical analysis was carried in FactomineR extension R Commander (Version 2.1-7 OS X) included in R software (Version 3.1.1 OS X).

4.2.3 Results and discussion

Effect of CHW:BM ratio on HY, HPR and HPSI

The HY, HPR and HPSI during seven experimental periods of 110 days are summarized in Table 4.9 and Figure 4.5. The addition of buffalo manure improved the H_2 production stability to 0.66 and 0.71 during the experimental periods III and IV, respectively. The HY during the start-up of the reactor, i.e. periods I and II increased for some days, however the production of H_2 was not sustained due to pH depletion as a result of VFA accumulation. The HY decreased at the end of the period IV, even though the pH was stable around 4.8 ± 0.1. This might be attributed due to the increase in total metabolites concentration in the reactor. One of the best strategies to avoid VFA accumulation is facilitating its removal from the reactor by decreasing the HRT without washing out of the microbial biomass.

During the experimental period V, the H_2 production decreased further when the HRT of the reactor was decreased from 12 to 8 days. This might be due to washing out of the biomass which was evident by the decreased in fermentative acitivities seen from lower yields in other metabolic by-products (Table 4.10). Moreover, another reason for low HY can be the sudden decrease in the culture pH. Consequently, BM fraction in the feed was increased (Table 4.8) as a strategy to increase alkalinity in the reactor. The H_2 production increased for a while, however it did not lasted longer. This might be due to proliferation of H_2 consumers such as methanogens present in the BM (Cheong and Hansen, 2006; Wu et al., 2009). As a control strategy CHW:BM ratio was increased again, decreasing the BM in the influent feed. This eventually increased the H_2 production (Figure 4.5).

Table 4.9 - H_2 production performance during the dark fermentation at different CHW:BM ratio

Exp. Periods	CHW:BM (gVS/gVS)	HY	HPR	HPSI	H_2 %	CO_2 %
I	0	123.8±85.1	73.8±45.7	0.38	37.04±7.0%	40.13±10.4%
II	0	95.3±64.1	134.9±90.7	0.33	46.69±7.1%	40.37±9.6%
III	1	139.8±47.8	197.8±67.7	0.66	51.85±9.0%	44.43±6.7%
IV	4	152.2±43.9	215.4±62.1	0.71	58.01±4.8%	39.13±4.6%
V	4	51.8±29.3	73.4±41.4	0.44	38.47±12.6%	27.80±4.7%
VI	2	76.2±76.1	183.0±107.7	0.41	38.38±21.2%	37.32±12.0%
VII	4	131.7±44.6	186.3±63.7	0.66	51.10±6.3%	46.34±6.4%

Table 4.10 - Characteristics of effluents from the DF of CHW with BM as co-substrate during different experimental periods

Exp. Periods	**Ammonia (mg NH_4^+-N/L)**	**Total Alkalinity (mg $CaCO_3$/L)**	**pH_OUT**	**Lactate (mM/gVS)**	**Ethanol (mM/g VS)**	**Acetate (mM/gVS)**	**Propionate (mM/gVS)**	**Butyrate (mM/gVS)**	**Hydrogen (mM/gVS)**
I	157.9±0.0	1019.3±145.0	5.6±0.3	0.00±0.0	17.27±4.2	21.19±11.6	3.43±2.9	14.80±7.3	7.44±4.5
II	71.8±0.0	337.4±82.9	4.6±0.1	1.42±0.7	2.60±0.1	9.60±11.1	4.09±6.5	8.99±2.7	4.60±3.1
III	389.3±59.2	1327.6±388.2	5.0±0.2	0.69±0.8	5.74±1.9	2.61±1.3	0.31±0.2	7.51±2.4	3.38±1.1
IV	179.4±34.0	1184.4±373.6	4.8±0.1	0.29±0.5	10.45±3.3	4.18±2.1	0.51±0.4	14.12±6	5.88±1.7
V	111.2±3.6	394.5±127.0	4.5±0.1	2.99±1.5	13.70±0.0	2.20±0.9	0.17±0.2	9.64±3.9	2.00±1.1
VI	86.1±35.9	878.6±265.8	5.2±0.1	1.18±1.2	8.48±0.8	2.10±1.7	0.10±0.1	6.69±3.7	2.46±2.4
VII	81.2±8.2	619.7±152.8	4.8±0.1	1.41±1.4	12.27±1.7	3.28±1.4	0.22±0.2	10.72±3.7	5.09±1.7

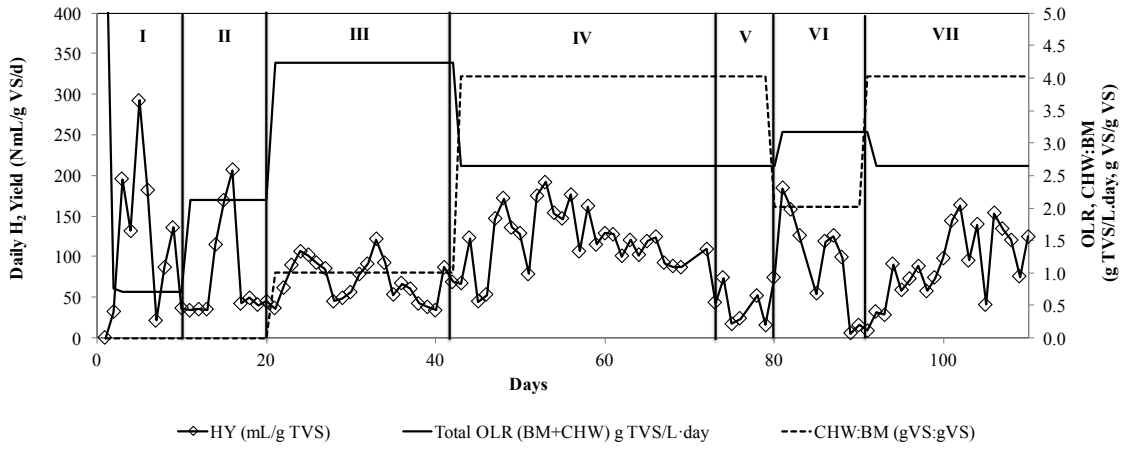

Figure 4.5 - Daily H_2 yields during the different buffalo manure feeding strategies in semi-continuous DF reactor using CHW as main substrate and BM as co-substrate

Effect of BM addition on pH, alkalinity and ammonia concentration

The trends of alkalinity, ammonia and pH during the different BM feeding strategies are presented in Figure 4.6. The production of organic acids that followed the DF are responsible for decreases in the culture pH. The culture pH has profound impact on the selection and growth of fermentative microbial communities and thus their metabolic pathways (H_2 production). Therefore, it is necessary to maintain the culture pH in the DF process below the inhibitory levels of 4.5, which favours solventogenesis. Figure 4.6 (a) and (b) show alkalinity, ammonia and pH trends, repectively, during different BM feeding strategies (CHW:BM ratios). Addition of BM to the reactor resulted in an increased in alkalinity, which stabilized the culture pH during the process around 4.8 to 5. Similarly, Figure 4.7 (b) tried to establish the relationship between the Total alkalinity (Tak) to Total acids (Tac) ratio and HPSI. The higher HPSI was obtained when the Tak/Tac ratio is between 3 – 4. This suggests requirement of constant alkalinity source to maintain a stable culture pH and H_2 production during the DF process.

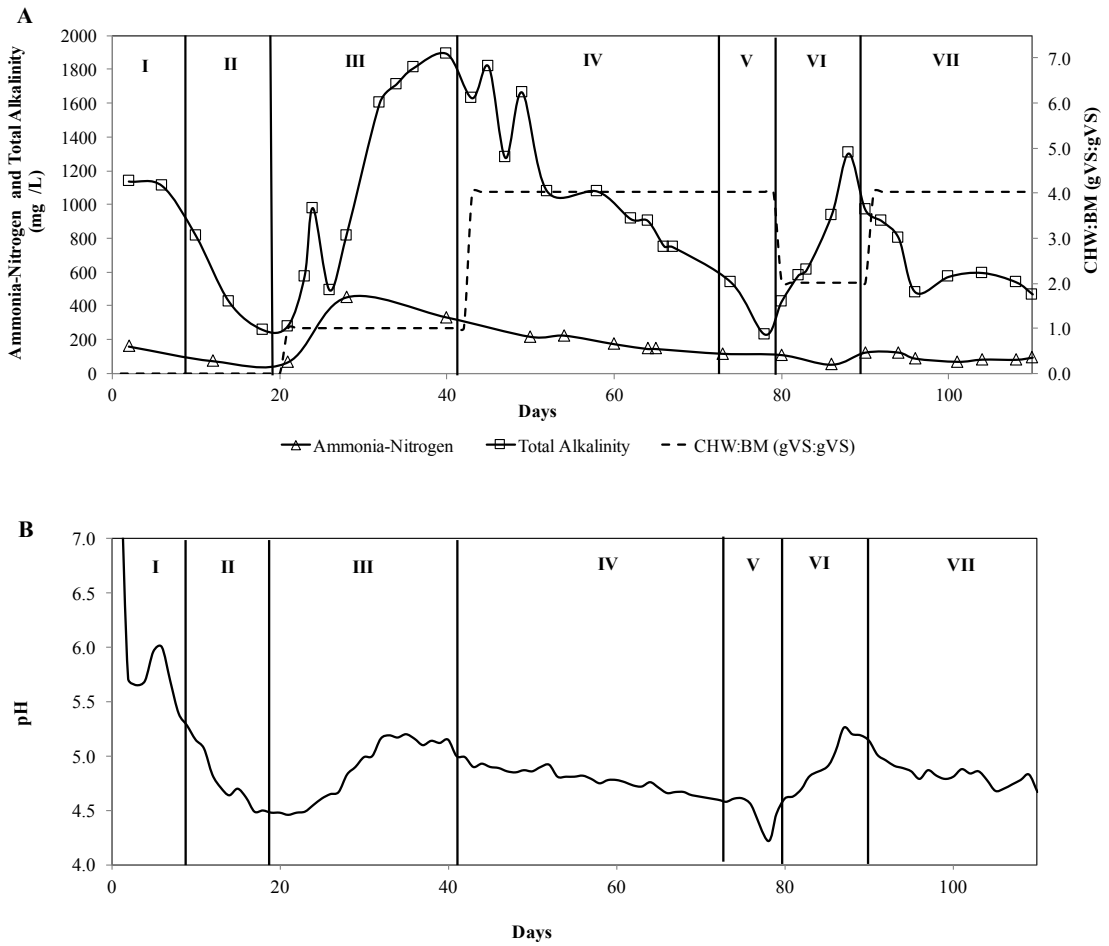

Figure 4.6 - CHW:BM ratio, total alkalinity (as mg $CaCO_3$/L), and ammonium concentration (as NH_4^+N/L) (A) and pH trends (B), during the different buffalo manure feeding strategies in a semi-continuous DF reactor

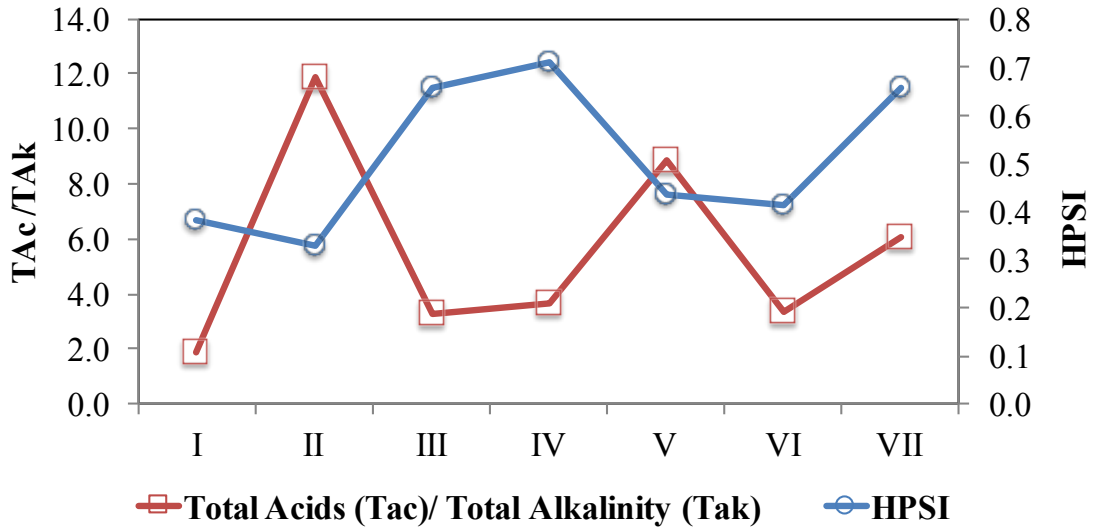

Figure 4.7 - Total/Alkalinity ratio and HPSI during the different operational strategies

The limitation in the use of animal manure might be a need for physical or chemical treatment for inhibiting methanogenic activities which consume H_2 (Cheong and Hansen, 2006; Wu et al., 2009). However, this study did not consider any pre-treatment for BM before feeding. Moreover, another, limitation in the use of BM could be an inhibition of the H_2 production due to higher ammonia content in BM. The animal manure such as swine, poultry and dairy manure have a low C/N ratio (C/N ratio of swine manure: 12.8) (Yin et al., 2014) and higher levels of ammoniacal nitrogen (cattle slurry: 1040 -1925 mg/l and chicken manure 7000 - 12,800 mg/L) (Callaghan et al., 2002) that might cause inhibition of microbial community. Cavinato et al. (2012) reported the decrease in H_2 production at total ammoniacal nitrogen concentration higher than 2 g N/L. However, ammonia levels in our study were lower than the inhibitory levels reported in literature.

Metabolites production

Major metabolites such as lactate, acetate, propionate, butyrate and ethanol, produced during the different experimental periods are summarized in Table 4.10. With the different concentrations of organic acids and alcohols present in the dark fermentation effluent, H_2 production can be related to more than one biochemical pathways presented in Table 4.6. The principal component analysis of co-relation circles presented in Figure 4.8 suggests that the H_2 production was mainly due to the butyrate and acetate pathways which are well co-related with the HY. Unsurprisingly, other metabolites such as propionate, lactate or ethanol, which normally do not represent H_2 favorable pathways, were not well correlated.

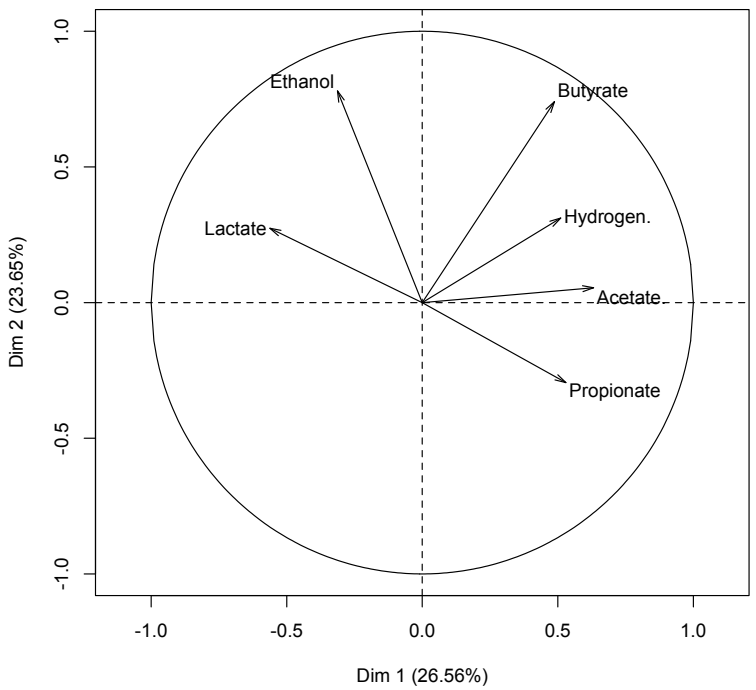

Figure 4.8 - Correlation circle of six metabolites formed by the first two principle components dim1 and Dim 2 representing 26.56 and 23.65 % of the total variance, respectively.

4.2.4 Conclusion

DF of acidic wastewater could be challenging due to decrease in pH by the consequent production of organic acids during the DF process without source of alkalinity. A long-term continuous production of H_2 has been demonstrated in this work using CHW and BM. HY of 131.8±38.0 mL H_2/g VS was obtained with HPSI of 0.71 when the CHW to BM ratio was 3.4 g VS/gVS. However, use of BM characterized by higher alkalinity could be applied as co-substrate for maintenance of operable pH during the DF process around 4.8 – 5. Therefore, addition of BM can aid in the stability of the continuous dark fermentative H_2 production and remove the dependency on chemical-buffering agents. Furthermore, BM can provide the source of nutrients (nitrogen) during the DF of carbohydrate rich substrates like CHW. Hence, a co-fermentation of CHW with BM could give economic sustainability in scaled-up applications of DF processes that use locally available feedstock sources.

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CHAPTER 5

INTEGRATION OF DARK FERMENTATION IN A BIOREFINERY CONCEPT

A part of section 5.1 of this chapter has been has been published as Ghimire, A., Valentino, S., Frunzo, L., Trably, E., Escudié, R., Pirozzi, F., Lens, P.N.L., Esposito, G. (2015). Biohydrogen production from food waste by coupling semi-continuous dark-photofermentation and residue post-treatment to anaerobic digestion: A synergy for energy recovery. International Journal of Hydrogen Energy, 40(46) 16045–1605.

A section 5.2 of this chapter has been published as Ghimire, A., Valentino, S., Frunzo, L., Pirozzi, F., Lens, P.N.L., Esposito, G. (2016). Valorization of by-products from thermophilic dark fermentation of food waste via concomitant production of biohydrogen and polyhydroxybutyrate using adapted Rhodobacter sphaeroides and mixed photofermentative culture. Bioresource Technology (In press).

A section 5.3 of this chapter will be submitted as as Ghimire, A., Frunzo, L., Pirozzi, F., Trably, E., Lens, P.N.L., Esposito, G., Cazier, E.A., Escudie, R., Solid State Dark Fermentation for waste biomass valorization by production of biohydrogen and platform molecules: Effect of total solids contents and hydrogen partial pressure on substrate conversion.

5.1 Integration of dark-photo fermentation and anaerobic digestion for enhanced energy yields

This sections presents the results of the study aimed at maximizing the energy yields from food waste in a three-step conversion scheme coupling dark fermentation (DF), photofermentation (PF) and anaerobic digestion (AD). The DF effluents mainly contained volatile fatty acids (VFAs) and alcohols as metabolites and un-hydrolyzed solid residues. The supernatant, after separation, was used to recover H_2 in a PF using *Rhodobacter sphaeroides*. The solid residual fraction along with PF effluent was converted into methane by anaerobic digestion.

5.1.1 Introduction

The inherent characteristics of hydrogen (H_2), such as higher energy content (142 MJ per kg), energy and water as the only by-products generated from its combustion, application in fuel cells for electricity generation and the ability to be produced biologically, makes H_2 a very interesting alternative future sustainable energy carrier (Kotay and Das, 2008). Among several biological technologies proposed for H_2 production, dark fermentation (DF) is emerging as one of the prominent options, shown by the increasing research interests in this technology (Ghimire et al., 2015a). The advantages such as the flexibility to operate under different conditions of temperature and pressure, higher production rates, possibility to use renewable waste biomass as feedstock and the treatment capability make the DF process attractive. Waste biomass such as agricultural residues, the organic fraction of municipal solid waste (OFMSW) and agro-industrial wastes are economically competitive when considering a supply of sustainable feedstock, aiming at the industrial development of DF systems for biological treatment of waste (Chong et al., 2009; De Gioannis et al., 2013; Ntaikou et al., 2010).

It has been well documented that dark fermentative H_2 production is generally due to the conversion of the initial soluble fraction of carbohydrates present in the complex organic biomass, that will lead to accumulation of volatile fatty acids (VFAs) and alcohols in DFEs (Guo et al., 2013; Monlau et al., 2012). Some recent studies have shown the potential of these DFEs to be utilized in PF processes for H_2 production (Chookaew et al., 2015; Rai et al., 2014). Combining DF with PF, Su et al. (Su et al., 2010) achieved an increase in H_2 yield from 76.7 to 596.1 L H_2/kg VS from water hyacinth. Meanwhile, Rai et al. (Rai et al., 2014) achieved 43% higher volumetric H_2 yields from acid hydrolyzed

sugarcane bagasse in two step DF-PF systems. However, during the conversion of complex organic biomass like FW, a part of the unhydrolyzed solid residues will remain that can be further valorized in AD systems producing methane (CH_4) in a three steps conversion scheme (Figure 5.1). Xia et al. (Xia et al., 2013a, 2013b) reported that a three-step conversion of algal biomass combining DF-PF-AD can achieve 1.7 and 1.3 times higher energy yields in comparison to a two-stage DF-AD and an one stage AD process, respectively.

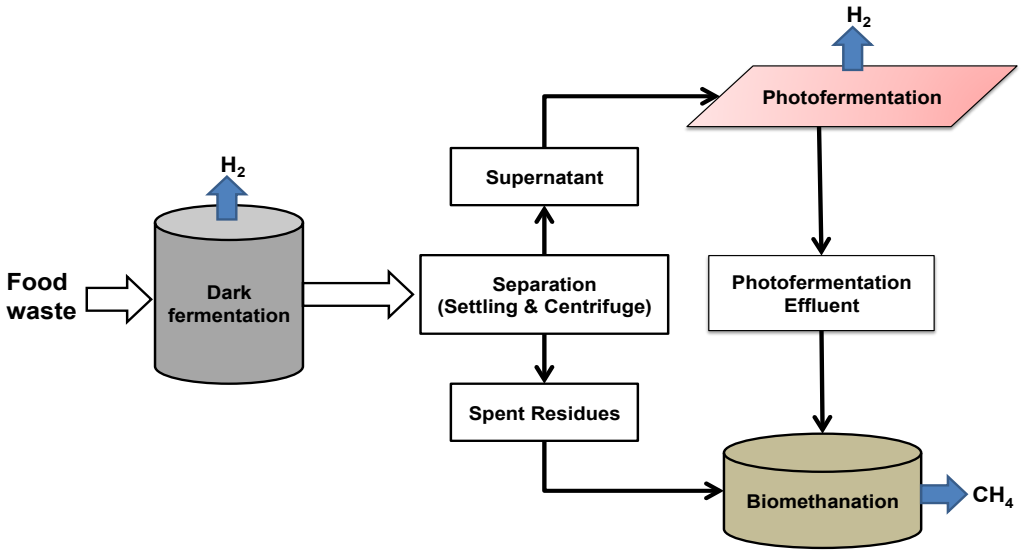


Figure 5.1 - Schematic of the three-stage conversion of FW to hydrogen and methane.

High OLRs are often responsible for a decrease in culture pH due to the accumulation of VFAs present in DFE. Thus, most of the continuous DF systems utilizing acidic substrates such as food waste require constant addition of external alkalinity sources such as alkaline chemicals (NaOH or KOH) or buffering agents (bicarbonate or phosphate buffers) (Elsamadony and Tawfik, 2015; Shin and Youn, 2005; Valdez-vazquez et al., 2005). A long-term study of continuous H_2 production at varying operating conditions of OLR and HRT to establish a long-term operability for continuous H_2 production in relation with the production of metabolites could provide further insights for the development of scaled-up DF systems. Similarly, a three-step conversion process (DF, PF and AD) might contribute to an increase in overall energy yield and could provide the biological treatment to the by-products generated from DF systems.

This study aims to demonstrate the long-term operational feasibility of continuous H_2 production from FW using a semi-continuous thermophilic DF reactor at various low OLRs and HRTs without pH control. The experiment also aimed at reducing the

dependency on chemical buffering agents that are used to maintain the culture pH at working conditions. H_2 production through different possible biochemical pathways was discussed in relation to major metabolites present in DFEs, obtained during the varying experimental conditions. The potential of coupling DF with photofermentative H_2 production was investigated in batch PF experiments by using the liquid fraction of the DFE after physical separation. Further, the waste streams generated from the coupling of DF-PF were utilized in AD to maximize the energy yields and provide integrated waste treatment solutions.

5.1.2 Materials and methods

Preparation of feedstock

An average composition of waste, as found in European countries, was prepared as cited elsewhere (VALORGAS, 2010). The waste mixture was prepared at the laboratory and was composed of (in % by weight): fruit and vegetables 72%, cooked pasta and rice 10%, bread and bakery 5%, dairy products (cheese) 2%, meat and fish 8% and snacks (biscuits) 3%. The FW ingredients were freshly bought at municipal markets in Naples (Italy), shredded with a blender and immediately stored at -20 ^{0}C to avoid acidification. The FW characteristics were (in g/kg FW): chemical oxygen demand (COD), 347.6 ± 47.4; carbohydrate content, 105.80 ± 0.7; total Kjeldahl nitrogen (TKN), 6.4 ± 0.18; lipids, 17.50 ± 1.19; total solids (TS), 23.79 ± 0.44%; volatile solids (VS), 22.8 ± 0.42% and the pH was 4.4 ± 0.1.

DFE were collected from the outlet of the fermenter and had a pH of 4.5 ± 0.1. After undergoing settling for 30 minutes and centrifugation at 4500 rpm for 20 minutes, the supernatant was collected. The DFE characteristics are presented in Table 5.1. The DFE was supplemented with KH_2PO_4, 3 g/L; $NaHCO_3$, 0.7 g/L; ferric citrate 24.5 mg/L and 10 mL of a trace metals solution (for composition, see below). pH was adjusted to 6.5 and then the DFE medium was autoclaved at 121 ^{o}C for 20 minutes.

Table 5.1 - Characteristics of the DFE used in PF experiments.

Parameters	Values (mg/L)
Chemical Oxygen Demand (COD)	3561.8±131.1
TKN	208.0±7
NH_4^+-N	1.14±0.3
Phosphate	130.5±1
Total iron (Total-Fe)	≤ 0.7
Lactic Acid	33.0
Acetic Acid	466.0
Propionic Acid	449.6
Butyric Acid	1075.4
Ethanol	323.0

The solid residues left after settling and centrifugation of DFE along with the PF effluents mainly containing photofermentative biomass were used as feed for AD. The characteristics of the solid residues generated from solid-liquid separation was comprised of undigested FW which had a pH of 4.5 ± 0.1 and solid DF residue with a content of: COD 2.64 ± 0.4 g/kg residue; TS 2.42 ± 0.02% and VS 2.31 ± 0.02%. The PF effluent had a pH of 7.26 ± 0.01; and contained a soluble COD of 1407.7 ± 109 mg/L; with 0.71 ± 0.01 % TS and 0.28 ± 0.01 % VS contents.

Experimental setup and operational conditions

Dark fermentation bioreactor

The experimental set-up and start-up of DF reactor is explained in Section 4.1.2. The reactor was operated in semi-continuous mode with three different HRTs and four OLRs in six different operational conditions (Table 5.2).

Table 5.2 - Experimental design used for the operation of semi-continuous reactor

Experimental periods	I	II	III	IV	V	VI
OLR (kg VS/m^3/d)	1	1	1.5	2	2	2.5
HRT (d)	12	6	6	6	4	4
Concentration (kg VS/m^3)	12	6	9	12	8	10

Photofermentation bioreactor

Rhodobacter sphaeroides AV1b (kindly provided by professor Roberto De Philippis, University of Florence, Italy) was previously isolated from the Averno lake in Naples (Italy) as described elsewhere in Bianchi et al. (Bianchi et al., 2010) and was used as

inoculum for PF. *R. sphaeroides* AV1b was first grown in a medium as previously described by Bianchi et al. (Bianchi et al., 2010), which was composed of (in g/L): DL-malic acid, 2; sodium glutamate, 1.7; K_2HPO_4 , 0.5; KH_2PO_4, 0.3; $MgSO_4.7H_2O$, 0.4; NaCl, 0.4; $CaCl_2.2H_2O$, 0.075; ferric citrate, 0.005; yeast extract, 0.4 and 10 mL of trace metals solution containing (in mg/L): $ZnSO_4.7H_2O$, 10; $MnCl_2.4H_2O$, 3; H_3BO_3, 30; $CoCl_2.6H_2O$, 20; $CuCl_2.2H_2O$, 1; $NiCl_2.6H_2O$, 2 and $Na_2MoO_4.2H_2O$, 30.

The *R. sphaeroides* AV1b pre-culture was grown again in a DFE supplemented with appropriate chemicals and autoclaved, as explained earlier. It was mainly composed of (in mg/L): acetic acid, 848; propionic acid, 457; butyric acid, 1184; NH_4^+, 6; phosphate (as PO_4^{3-}), 35.8 and total Fe 0.045. Ten mL of the culture (1.52 g TSS/L) that represents 2.5 % V/V of the reactor working volume was used as inoculum in the PF experiments with DFE (Table 5.1).

Transparent 500 mL borosilicate serum glass bottles (Simax, Czech Republic) with 400 mL working volume were used as photofermentative batch reactor. The batch reactors were maintained at room temperature (24 ± 2 °C, April-May) under a luminance of about 4000 Lux and positioned on the top of the stirrers. Caps of the reactors presented two separate ports for biogas and culture medium sampling. The bottles were sealed with silica and flushed with argon to ensure anaerobic conditions and eliminate the nitrogen gas (N_2) from the headspace since N_2 can inhibit the activity of the nitrogenase enzyme responsible for photofermentative H_2 production (Koku et al., 2002). The H_2 production was quantified as in DF process.

AD of residues from DF-PF process

A batch test was carried out in 1 liter transparent borosilicate serum glass bottles (Simax, Czech Republic) and was maintained at 34 ± 1°C in a water bath. The working volume of the reactor was 600 mL with an initial S/X ratio of 0.5 with a substrate concentration of 4.5 g VS/L. A low S/X ratio 0.5 was selected to assess the biomethane potential of the feed used. Based on the substrate type, a range of S/X ratio 0.5 - 2.3 gVS substrate/gVS inoculum is suggested to prevent the acidification of the AD reactor (Esposito et al., 2012). The source of inoculum used in the tests was the same as the start up inoculum used in the semi-continuous DF reactor. The characteristics of the inoculum were (in g/L): TS, 23.71 ± 0.17; VS, 14.55 ± 0.11; ammonium (NH_4^+-N), 0.46 ± 0.02; and had a pH 8.2 ± 0.1. The tests were carried out in duplicates.

Analytical methods

Hydrogen was quantified with a Varian Star 3400 gas chromatograph equipped with a ShinCarbon ST 80/100 column and a thermal conductivity detector. Argon was used as the carrier gas with a front and rear end pressure of 20 psi. The duration of analysis was 14 minutes. The fermentation products (lactic, acetic, propionic and butyric acids) were quantified by High Pressure Liquid Chromatography (HPLC) (Dionex LC 25 Chromatography Oven) equipped with a Synergi 4u Hydro RP 80A (size 250×4.60mm) column and UV detector (Dionex AD25 Absorbance Detector). The gradient elution consisted of 20% methanol and 10% acetonitrile in 5 mM H_2SO_4 pumped at a rate of 0.9 mL/min, using a Dionex GP 50 Gradient pump. The elution time was 18.5 minutes. Ethanol was quantified by HPLC (Aminex HPX-87H column (300 mm on 7,8 mm, Bio-rad) using 5 mM H_2SO_4 as an eluent. The COD of the FW was measured as described elsewhere (Noguerol-Arias et al., 2012). The carbohydrate content was determined according to the Dubois method (DuBois, M., Gilles, K., Hamilton, J., Rebers, P., & Smith, 1956). Total lipids were measured following a Bligh and Dyer chloroform/methanol total lipid extraction method (Bligh and Dyer, 1959). The light intensity was measured with a light meter (Lutron-LX-107). The TS and VS of the seed sludge and TKN were determined according to the Standard Methods (APHA, 2005).

Data analysis

Hydrogen production rates (HPR) were expressed in L $H_2/m^3/d$ while the H_2 yields (HY) were determined considering the total daily organic load fed to the reactor and expressed as L H_2 /kg VS added. Average and deviations for daily production were determined during the steady state reached after 3-4 days of operation. The H_2 Production Stability Index (HPSI) was evaluated by considering the ratio of standard deviation and average HPR as reported by Tenca et al. (Tenca et al., 2011):

$$HPSI = 1 \quad \frac{S.D.(HPR)}{Avg.HPR} \tag{5.1}$$

A HPSI index closer to 1 represents a stable hydrogen production.

FactoMineR, an extension on R software, was used for multivariate analysis of the metabolite distribution from the different experimental periods in relation to the hydrogen yields and co-relation circles of the major metabolites were generated.

5.1.3 Results and discussion

Effect of operational parameters on H_2 production rate and yield

The results in terms of H_2 yields (HY), hydrogen production rates (HPR) and H_2 Production Stability Index (HPSI) during the different OLRs and HRTs investigated in the six operation periods (Table 5.2) are summarized in Table 5.3. Figure 5.2 shows the HPR (a) and pH trends (b), over the operation period of 193 days. The results show an increase in HPR when OLRs were increased. During the operating periods II, III and IV at a constant HRT of 6 days, the HPR increased from 54.1 ± 41, to 109.5 ± 33 and 210.2 ± 30 N L/m^3/d, when the OLR was increased from 1 to 1.5 and 2 g VS/m^3/d, respectively (Tables 2 and 3). Meanwhile, the overall HY increased from 54.1 ± 41.3 N L/kg VS_{added} to 105.1 ± 14.9 N L H_2/kg VS_{added}. During the experimental period IV, the H_2 production had a comparatively better stability as shown by a HPSI of 0.86. However, no significant effect was observed on the total HY and HPR when the HRT changed to 4 days during operational period V (Table 5.3). When the OLR was changed from 2 to 2.5 kg VS/m^3/d during period VI, both HY and HPR increased. However, the H_2 production was not stable, supported by a lower value of HPSI of 0.63. This instability could be explained by the accumulation of acids and a subsequent decrease in pH to 4.4 ± 0.1, which might have affected the microbial community.

During a short operation period (at the end of period IV), the culture pH inside the reactor was regulated manually to an initial culture pH 5.5 with 1 M NaOH, during feeding, with the objective to assess the influence of pH on the H_2 production performance (Figure 5.2 b). However, pH regulation did not show any effect on the HPR (Figure 5.2 a). Nevertheless, the increased HPSI (Table 5.3) showed that H_2 production was stable during that period in comparison to the experimental period when the culture pH was uncontrolled. The percentage of H_2 and CO_2 in the gas averaged 59 ± 6% and 39 ± 6%, respectively, when the H_2 production stabilized. However, the H_2 production performances in experimental period IV (HPR: 210.2 ± 29.8 N L/ m^3/d and HY: 105.1 ± 14.9 N L/kg VS_{added} at a HRT of 6 days and OLR 2 kg VS/m^3/d) were comparable to experimental period V (HPR: 208.0 ± 34.8 N L/m^3/d and HY: 104.0 ± 17.4 N L/kg VS_{added} at a HRT of 4 days and OLR of 2 g VS/L/m^3/d). Thus, the operational conditions of period V were considered as ideal for the DF of FW in thermophilic semi-continuous reactors,

as a lower HRT are generally more economically efficient in terms of bioreactor design and operation.

Table 5.3 - H_2 production rate, yields and production stability from FW by mixed anaerobic cultures

Exp. Period	HPR (N L/m^3/d)	HY (N L/kg VS_{added})	H_2 in biogas (%)	HPSI
I	116.9±40.1	116.9±40.1	52.8%±1%	0.66
II	54.1±41.3	54.1±41.3	31.2%±1%	0.24
III	109.5±32.8	73.0±21.9	43.8%±20%	0.70
IV	210.2±29.8	105.1±14.9	59.4%±6%	0.86
V	208.0±34.8	104.0±17.4	57.2%±6%	0.83
VI	303.6±111.4	121.4±44.5	55.8%±10%	0.63

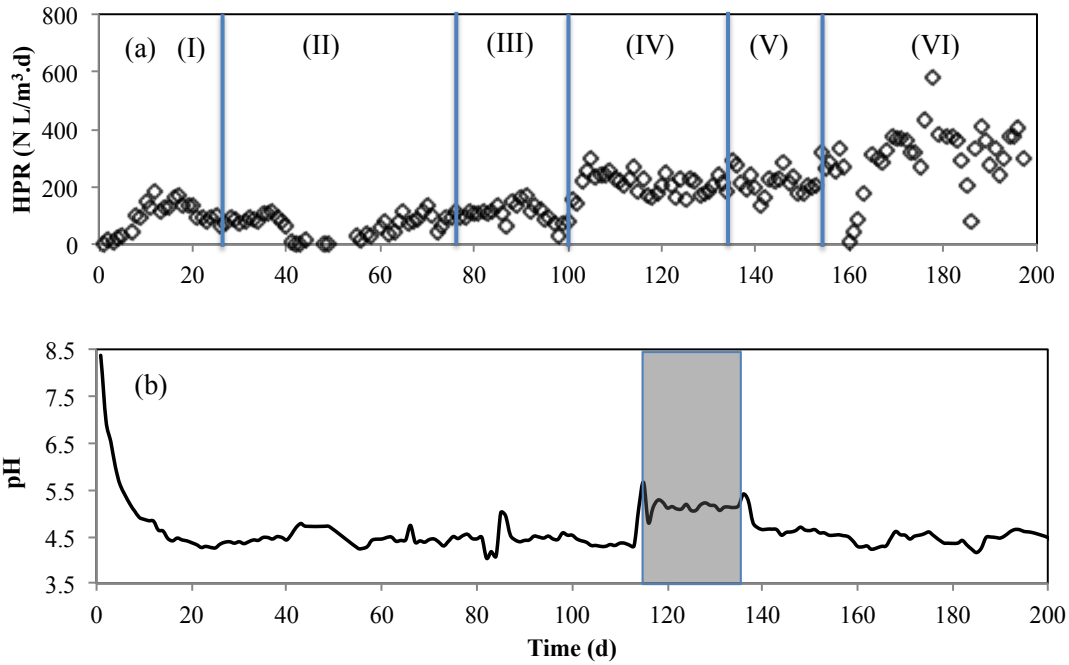


Figure 5.2 - HPR (L H_2/m^3/d) (a) and pH trends in semi-continuous thermophilic reactor (b); shaded region represents the experimental period when the culture pH inside the reactor was adjusted daily to pH 5.5 during the feeding operation.

A comparison of previous studies on dark fermentative H_2 production from FW with the results from this study (Table 4.4) suggests that comparable results in terms of H_2 production can be achieved even at low OLRs and without pH control. Nonetheless, the characteristics of FW can also affect the overall HY as H_2 production is mainly function of the soluble fraction of carbohydrates present in the substrate (Guo et al., 2013). The OLRs reported in the past studies were higher than in this study, and thus a source of

alkalinity to balance the pH conditions at optimum was required. Valdez-Vazquez et al. (Valdez-vazquez et al., 2005) used $NaHCO_3$ and K_2HPO_4 to maintain the optimum pH at 6.4, while Lee et al. (Lee et al., 2010) used NaOH and H_3PO_4 to maintain the culture pH at 6. Thus, this pH decrease resulting from the production of acids can be minimized by the use of lower OLRs. Higher OLRs can exert detrimental effects on the microbial community, and thus H_2 production, by decreasing the pH due to the accumulation of metabolites (Van Ginkel and Logan, 2005).

Metabolic intermediates

Lactate, acetate, propionate, butyrate and ethanol were the main metabolic intermediates observed during the different experimental periods. Such a mixture of intermediates is characteristic of mixed fermentation pathways occurring with complex substrates (Guo et al., 2013). Average concentrations of the main metabolites during the six different experimental periods are summarized in Table 5.4. There can be a number of possible H_2 production pathways during mixed type fermentation, as represented by equations 2 – 5 (Table 4.6), whereas H_2 consuming or unfavorable pathways presented in equations 6 – 9 might exist at the same time (Hawkes et al., 2007; Li and Fang, 2007). The presence of ethanol, acetate and butyrate are evidences for the presence of an ethanol-acetate or butyrate-acetate pathway for H_2 production in the DF of the FW investigated. On the other hand, the presence of lactate or propionate can be attributed to fluctuations in H_2 production resulting in low H_2 yields.

Table 5.4 - Characteristics of influent and effluents from DF of FW during different experimental periods

Exp. Period	pH_IN	pH_OUT	Lactate (mM)	Ethanol (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)
I	7.00	4.7±0.3	0.1±0.2	4.8±0.2	13.1±3.6	3.85±2.21	10.4±2.8
II	7.00	4.5±0.1	0.6±1.4	5.4±3.5	3.2±2.0	3.44±2.33	6.2±4.2
III	7.00	4.5±0.2	4.0±9.1	8.7±2.7	4.9±0.6	5.97±2.16	11.0±1.6
IV	7.00	4.9±0.4	0.0±0.0	17.2±8.6	8.5±1.8	9.65±2.91	12.0±2.9
V	7.00	4.7±0.2	0.0±0.0	17.1±6.6	6.7±1.9	5.70±2.15	9.9±3.2
VI	7.00	4.4±0.1	0.5±0.9	9.4±5.3	5.7±2.8	5.89±2.70	11.1±7.5

Figure 5.3 shows the plot of correlation circles of the five major metabolites and the HY. Figure 5.3 (a) shows that the butyrate and acetate concentration is well correlated with the HY values. Not surprisingly, propionate, lactate and ethanol are in the Dim 2 and are not correlated with the HY, which is supported by equations 6 - 9 (Table 4.6) in a DF

with glucose as model substrate. However, the pathways leading to ethanol-acetate also yield H_2, as shown in Equation 4 (Hwang et al., 2004; Lin and Hung, 2008). Nonetheless, Figure 5.3 shows that the ethanol is not correlated with acetate. Therefore, most of the H_2 yields can be attributed to the butyrate-acetate pathway, which showed a good correlation and is explained in Dim 1. The variable Dim 3 is mostly explained by lactate concentrations (Figure 5.3 b), which correlated oppositely with HY and is an orthogonal and independent variable. The proximity of butyrate, ethanol and propionate suggests that these metabolites can be expected from DF by mixed microbial consortia. This is also supported in a study by Hwang et al. (Hwang et al., 2004) who obtained butyrate, ethanol and propionate as the major metabolites during the DF at a pH range of 4-4.5, 4.5-5.0, 5.0-6, respectively.

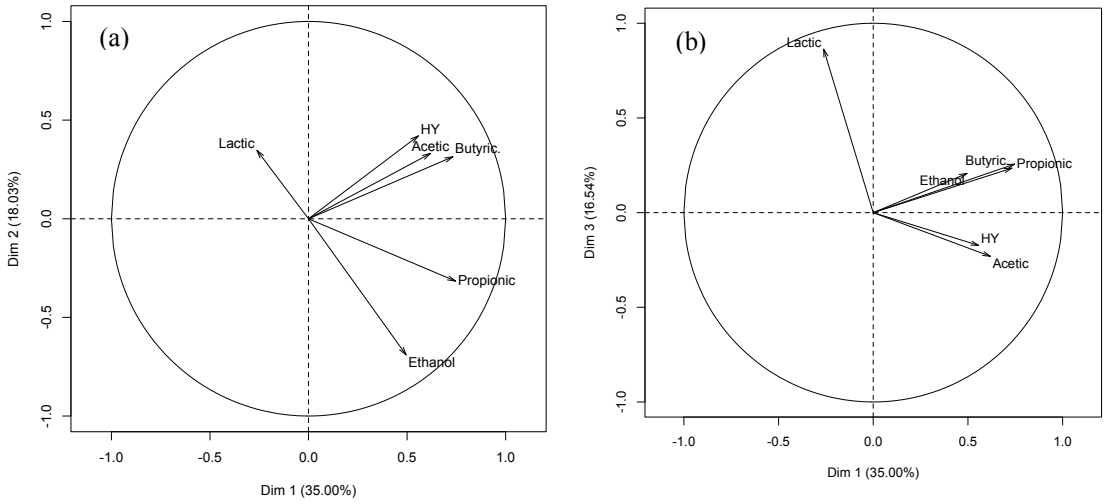


Figure 5.3 - Correlation circle of five metabolites and HY formed by the first three principle components Dim1, Dim 2 and Dim 3 representing 35.00, 18.03 and 16.54 % of the total variance, respectively. Projections according to the first two (Dim 1 and Dim 2) (a) and first and third factors (Dim 1 and Dim 3) (b)

Photofermentative H_2 production from the liquid fraction of DF

The DFE from the semi-continuous DF reactor obtained during experimental period VI was further converted to H_2 by *R. sphaeroides* AV1b in a PF process. Cumulative H_2 production and VFA consumption trends during the PF experiments are shown in Figure 5.4 (a) and (b), respectively. VFA and ammonium concentrations in the DFE medium (shown in Table 5.1) were both non-inhibiting levels for photofermentative H_2 production. Han et al. (Han et al., 2012) reported that concentrations equal to 9.8 mM, 10.9 mM and 4.2 mM, respectively, for acetate, butyrate and propionate gave the

optimum H_2 yield using *R. sphaeroides*. However, concentrations up to 30 mM of acetate have been reported by Hustede et al. (Hustede et al., 1993). Similarly, the ammonium concentration was at non-inhibitory levels, as only a concentration higher than 2 - 5 mM of NH_4^+-N has been reported to inhibit the photofermentative H_2 production (Argun et al., 2008; Lee et al., 2011).

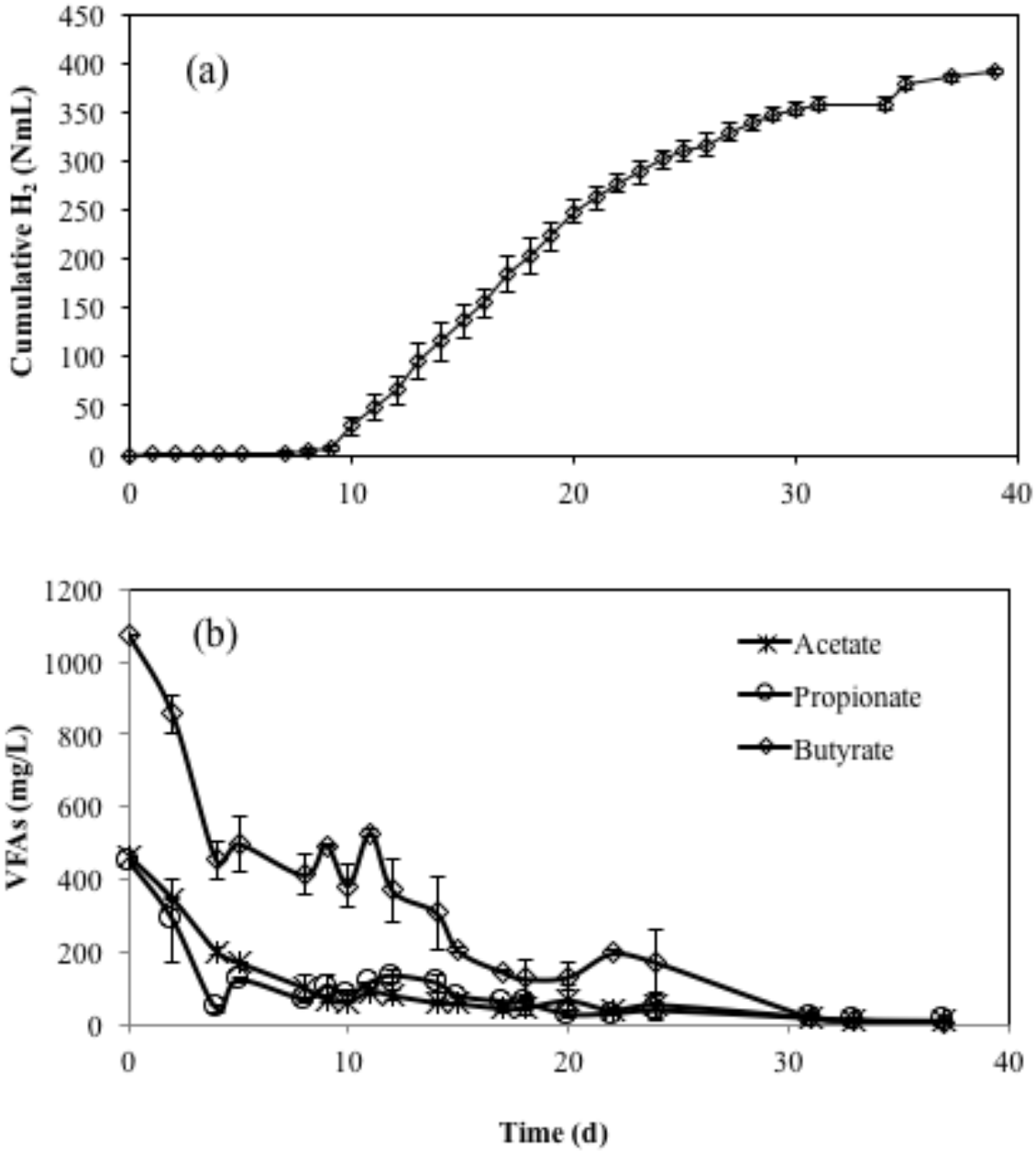


Figure 5.4 - Cumulative hydrogen production (a) and depletion of major VFAs (acetate, propionate and butyrate) (b) in a PF tests using DFE and *R. sphaeroides* AV1b.

The PF of spent DFE yielded a cumulative production of 365.6 ± 3.2 NmL H_2, corresponding to a volumetric yield of 914 ± 8 N L H_2/m^3 and a substrate yield of 427 ± 6 N L H_2/kg COD consumed. The batch experiments were carried out for 40 days until the H_2 production completely ceased (Figure 5.4 a). This is longer than any H_2 production time reported elsewhere (Rai et al., 2014; Xia et al., 2013b). The long lag phase (9 days) can partly explain this result. The final effluents were analyzed for COD, VFAs and biomass concentration which showed a COD reduction of 60.1%, while more than 98 ± 1% of VFAs were removed to reach a final biomass concentration of 1.6 g TSS/L. Theoretical COD removal calculated from the VFA concentration in final effluents showed a COD removal efficiency of 99.2%. However, the production of biomass and other bacterial carotenoids increased the final total COD of the PF effluent and thus reduced the total COD removal efficiency. This was evident by the reddish brown color of the effluent. The maximum percentage of H_2 in the biogas was 89% with 8.9% of CO_2.

The volumetric H_2 production obtained in this study (914 ± 8 N L H_2/m^3) is higher than the study of Rai et al. (2014) using *Rhodopseudomonas* BHU 01 with a volumetric H_2 yield of 755 L H_2/ m^3. In another study by Uyar et al. (2009) using *Rhodobacter capsulatus* (DSM 155) as biomass and DFE of *Miscanthus* hydrolysate as substrates, a volumetric yield of 1000 L H_2/ m^3 was obtained, which is slightly higher than in this study. The present study showed the potential of an integrated DF-PF system to achieve higher H_2 yields. Thus, the combined DF-PF processes can help in the industrial development of DF processes using FW. The residues generated from the downstream of these processes can, nevertheless, still be treated with anaerobic digestion in order to provide additional conversion of organic matter to further recover energy.

AD of DF-PF waste stream

The solid residues generated by the coupled DF-PF process can be ideal for AD as the undigested FW residues from the DF process and the PF effluent containing biomass generated from the PF can be converted to methane in a biorefinery model (Figure 5.1). The result of the average cumulative methane production trends during the biomethane potential test using the waste stream generated from the DF-PF process is presented in Figure 5.5. The cumulative CH_4 production stabilized after 50 days and the average cumulative CH_4 production was 871 ± 16 mL, corresponding to a total average yield of 324 ± 6 N L CH_4/kg VS added (feed) and 0.9 kg COD/kg VS removed (calculated from

CH_4 produced), evaluated after subtracting the endogenous methane produced in the controls. The initial and final average pH in the BMP tests was 7.0 and 7.7, respectively, while the pH of the DF and PF residues were respectively, 4.33 and 7.26. The pH was not adjusted with a buffering agent because the alkalinity of the inoculum was sufficient to maintain the pH, this further adds the to practicability of the AD as a post-treatment option.

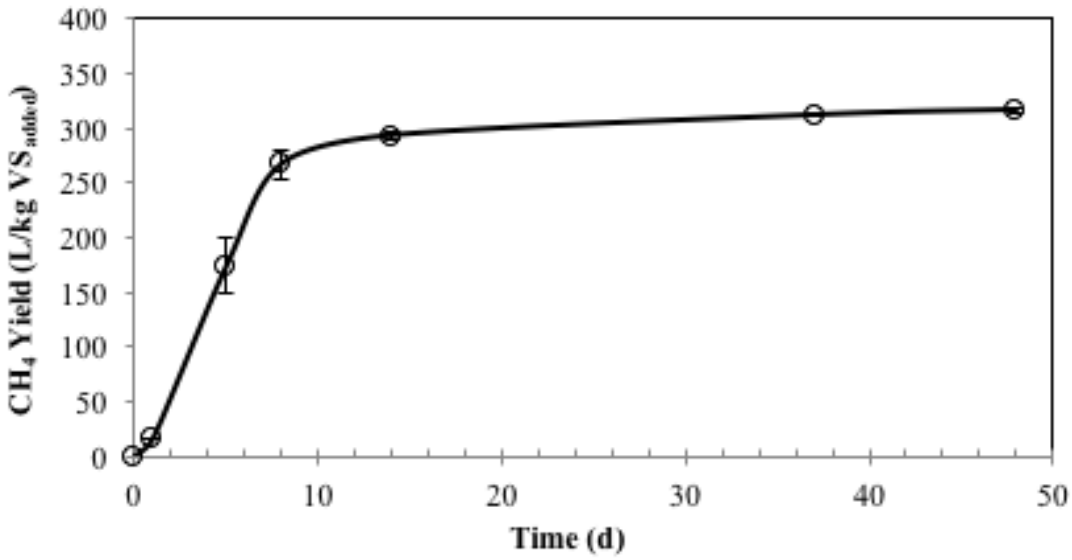


Figure 5.5 - Methane yields from mesophilic AD of waste stream generated in the coupled DF-PF processes

Energy yields from gas biofuels produced from food waste

When considering the conversion of the initial VS added at the beginning of the DF process, the overall average H_2 yield from coupling of the DF-PF process was increased from 105.1 N L H_2/kg $VS_{initial}$ to 184.3 N L H_2/kg $VS_{initial}$, with an additional 79.2 N L H_2/kg $VS_{initial}$ from PF and 99.3 N L CH_4/kg $VS_{initial}$ from AD. The increase in energy yields obtained in his study was compared with energy yields from the coupled process previously reported in the literature (Table 5.5). The energy yields of hydrogen and methane from the stand alone DF as well as the two stage DF-PF and DF-AD was calculated based on the heating values of H_2 (242 kJ/mol) and methane (801 kJ/mol). These calculated energy yields represent the energy gain from the conversion of substrates by biological processes. However, the net energy gain can be estimated by considering the energy input in the processes, which is not representative in lab scale reactors and thus not calculated in this study.

Table 5.5 - Comparison of energy yields from gaseous biofuels produced out of FW as feedstock using stand alone or coupling of different technologies

Feedstock	Process/ type	H_2 yield from DF / DF+PF (N L H_2/kg VS)	[a] Energy yield from H_2 (MJ/kg VS)	CH_4 yield from AD (L CH_4/kg VS)	[a]Total energy yield (MJ/kg VS)	Reference
FW+paper	Semi-continuous DF	360	3.89	-	3.89	(Shin et al., 2004)
FW	DF+PF (batch)	671[b]	7.25	-	7.25	(Zong et al., 2009)
Vinegar residue treated by HCl	DF+AD (batch)	53.2	0.57	192	7.4	(Z. Wang et al., 2015)
FW	DF+AD (batch)	55	0.60	94	3.96	(Nathao et al., 2013)
N. oceanica [c]	DF+PF+AD (batch)	183.9	1.98	161.3	7.74	(Xia et al., 2013b)
C. pyrenoidosa	DF+PF+AD (batch)	198.3	2.14	186.2	6.66	(Xia et al., 2013a)
FW	Semi-continuous DF + PF (batch) +AD (batch)	184	1.99	99.3	5.55	This study

[a] The energy yield was calculated from the yield of biogas based on the heating values of hydrogen (242 kJ/mol and methane (801kJ/mol); [b] L H_2/kg food waste; [c] Algal biomass pre-treatment by microwave heating with dilute H_2SO_4; [d]Algal biomass pre-treatment by steam heating with dilute H_2SO_4

By coupling DF with PF and AD processes, an additional 4.4 MJ/kg VS of energy yield can be achieved from food waste, which is higher than the coupled DF - AD process or stand alone DF processes (Table 5.5). Out of the overall energy recovered from the three-stage conversion (DF-PF-AD) of food waste, H_2 contributes only 35.8% out of 5.55 MJ/kg VS. However, this may be a positive add-on to the overall economic return compared to CH_4 productivity only. Therefore, the three-step process can definitely increase the recovered energy yield. Moreover, it is a very good solution for waste treatment as a higher FW conversion was accomplished. Table 5.5 shows that the energy yield of DF and PF from the study of Zong et al. (2009) is higher than the energy yield reported in this study. This is likely because of the difference in H_2 yield achieved in these studies. In other studies by Xia et al. (2013a, 2013b) and Wang et al. (2015), although the overall energy yields obtained from the respective three and two step conversion were high, the pre-treatment of the substrate required an energy input. Therefore, the overall

energy yields obtained from the coupling of various processes depends on the H_2 and CH_4 yields and production rates in individual processes, which are mainly a function of process operational conditions such as pH, temperature, HRT and OLR as well as carbohydrate content and nature of the feedstock. Moreover, the coupling of the PF and AD processes in the downstream process is not only advantageous from the energy point of view, but it also provides biological treatment of the waste stream generated by the DF processes (COD and pathogen removal) (Ward et al., 2008).

5.1.4 Conclusion

This study has shown the long-term feasibility of continuous H_2 production as well as the possibility to further recover energy through integration of PF and AD using FW as the substrate. In addition, the viability of H_2 production at low OLRs without the culture pH control can minimize the excessive use of chemical buffering agents for pH control. The integration of DF with PF can increase the overall H_2 yield 1.75 fold. On the other hand, applying AD for the post treatment of waste streams generated by the coupling of the DF-PF processes can further increase the overall energy yield by 5.55 MJ/kg VS of food waste, adding a synergistic effect to the overall energy recovery during the conversion of food waste.

5.2 H_2 and biopolymer production by phototofermentation

This section presents results of the study of concomitant production of biohydrogen and polyhydroxybutyrate (PHB) from photofermentation (PF) using spent medium produced from thermophilic dark fermentation (DF) of food waste mainly containing volatile fatty acids (VFAs) and alcohols as soluble metabolites. This study showed that DF-PF coupling not only yields energy and economic benefits in terms of H_2 and PHB productions but it also provides post treatment of residues by removal of COD.

5.2.1 Introduction

Biological hydrogen (bio-H_2) processes have gained much interest as they could lead to low cost and renewable hydrogen production technologies which are environmentally benign (Das and Veziroglu, 2008). Biological hydrogen production processes can be categorized into light dependent processes such as biophotolysis and light independent processes such as dark fermentation (DF) and bioelectrochemical systems or microbial electrolysis cells (Ghimire et al., 2015a). In light dependent processes, water is broken down into H_2 and O_2 gas by algae and cyanobacteria. Alternatively, cyanobacteria or cyanophytes can also synthesize H_2 from water and inorganic carbon. Moreover, photofermentation (PF) is carried out by photosynthetic bacteria, where photodecomposition of organic compounds into H_2 occurs (Das and Veziroglu, 2008; Hallenbeck and Ghosh, 2009).

DF systems are a promising biological route for H_2 production due to its mild operational requirements (ambient temperature and pressure), higher conversion rates to H_2 and wide range of complex low cost waste biomass that can be used as feedstock (Ghimire et al., 2015a; Guo et al., 2010). However, dark fermentative conversion of complex organic biomass to H_2 produces by-products, mainly volatile fatty acids (VFAs), lactic acids and alcohols as soluble metabolites and un-hydrolyzed solid residues, leaving incomplete conversion of the organic biomass (Xia et al., 2013). Dark fermentative biohydrogen production is strongly correlated with the initial soluble carbohydrate fraction present in the substrates (Alibardi and Cossu, 2016; Guo et al., 2013; Monlau et al., 2012). Nonetheless, the soluble metabolites (organic acids and alcohols) present in DF residues can be further converted to biohydrogen through PF (Chookaew et al., 2015; Ghimire et al., 2015b; Rai et al., 2014).

Under anaerobic conditions, purple non-sulfur bacteria (PNSB) carry out an anaerobic photosynthesis using light as the energy source synthesizing bio-H_2. In PNSB, this takes place with reduced carbon sources such as organic acids by the nitrogenase enzyme in the presence of light (Barbosa et al., 2001). Photofermentative bio-H_2 production systems are attractive owing to their higher substrate to H_2 conversion potential compared to dark fermentative systems (Han et al., 2012).

Moreover, a theoretical H_2 potential of 12 moles of H_2 per mole of hexose could be realized by integrating a PF process with DF systems (Han et al., 2012). Thus, the integration of DF-PF can provide a practical solution to H_2 production along with the enhanced conversion of organic biomass. The integrated DF-PF process has been demonstrated by several studies (Rai et al. 2014; Tawfik et al. 2014; Yang et al. 2015). DF has the unique capability to utilize a wide range of complex waste biomass that can ensure the future supply of feestock, and combining the two processes (DF + PF) can provide the further conversion of organic substrate in addition to enhanced H_2 yields.

The majority of the past studies carried out on combined DF-PF processes for H_2 production have used synthetic pure substrates containing major VFAs and pure microbial cultures (Chen et al., 2010; Tao et al., 2007). However, low cost complex waste biomass such as agricultural residues, organic fraction of municipal solid waste (OFMSW) and industrial wastes are attractive substrates for economically sustainable scaled up applications of dark fermentative. A number of studies have recently shown the possibility of combined DF-PF processes using waste biomass as the substrate (Chookaew et al., 2015; Rai et al., 2014; Zong et al., 2009). In this scenario, the use of dark fermentation effluents (DFE) generated from DF of complex organic waste and the application of PNSB for its capability to produce H_2 from DFE is attractive.

In addition to H_2 production, PNSB can synthesize poly-β-hydroxybutyrate (PHB) under certain conditions of physiological stress, such as high Carbon/Nitrogen (C/N) ratio or sulfur deprivation (Eroglu and Melis, 2011; Waligórska et al., 2009). Similar to H_2 production, PNSB synthesizes PHB as a way to dissipate the excess reducing power (Waligórska et al., 2009). PHB is a polyhydroxyalkanoate, an interesting biodegradable polymer having applications in bioplastics production and medicine (Kemavongse et al., 2008). The amount of PHB accumulation depends on the PNSB strains and the process

operational conditions (De Philippis et al., 1992; Montiel-Corona et al., 2015). In *R. sphaeroides*, Waligórska et al. (2009) found that accumulation of PHB increased 30 fold when the C/N ratio rose from 6 to 120. Although PHB biosynthesis is a H_2 competing pathway, its concomitant production with hydrogen raises future interests, as PHB possesses economic value as a precursor for biodegradable polymers (Koku et al., 2002).

Use of a mixed culture of PNSB is important for practical applications, as it reduces the asepsis costs involved when waste residues from DF systems are utilized. PF by pure cultures using spent DF residues generated from complex waste biomass has been reported in a few studies, i.e. sugarcane bagasse (Rai et al., 2014), glycerol (Chookaew et al., 2015) and cassava (Zong et al., 2009). However, there are limited studies that have been conducted using mixed PNS cultures for DFE conversion to H_2 (Montiel-Corona et al., 2015; Tawfik et al., 2014). In a recent study, Ghimire et al. (2015b) reported the 1.75 fold increase in H_2 yield from the integration of DF and PF processes using adapted *R. sphaeroides* cultures as inoculum. However, a long lag phase for H_2 production was observed, which was attributed to the initial PHB accumulation (Ghimire et al., 2015b).

The aim of this study was to investigate the concomitant production of H_2 and PHB from DFE (with and without dilution) obtained from the thermophilic DF of food waste, using adapted pure and mixed PNSB cultures under sterile and non-sterile conditions, respectively. H_2 production, PHB quantification and COD removal efficiency were the major parameters taken into consideration during this study of DFE valorization. Other hydrogen production performance parameters such as lag phase and time required to achieve 95% of the maximum H_2 production were considered for the evaluation of the photofermentative H_2 production performance.

5.2.2 Materials and methods

Dark fermentative H_2 production

A thermophilic DF process, described elsewhere by Ghimire et al. (2015b), was set-up for continuous hydrogen production from food waste. A semi-continuous stirred 2.0 L serum bottle with a 1.5 L working volume and 500 mL headspace was used as DF reactor. The culture pH was 4.5 (± 0.2). The H_2 yields and production rates were 104 (± 17 NmL) H_2/g VS and 208 (±35) NmL H_2/L/d at organic loading rates (OLRs) of 2 gVS/L/d and hydraulic retention time (HRT) of 4 days (described in section 4.1).

Photo fermentative H_2 production

PF inoculum

R. sphaeroides AV1b (kindly provided by professor Roberto De Philipis, University of Florence, Italy), isolated from the Averno Lake (Naples, Italy by Bianchi et al. 2010), was used as inoculum for PF tests RS-I and RS-D. *R. sphaeroides* AV1b was first grown in RPN medium as described by Bianchi et al. (2010) containing (g/L): DL-malic acid, 2; sodium glutamate, 1.7; K_2HPO_4, 0.5; KH_2PO_4, 0.3; $MgSO_4.7H_2O$, 0.4; NaCl, 0.4; $CaCl_2.2H_2O$, 0.075; ferric citrate, 0.005; yeast extract, 0.4 and 10 ml of trace metal solution containing (mg/L): $ZnSO_4.7H_2O$, 10; $MnCl_2.4H_2O$, 3; H_3BO_3, 30; $CoCl_2.6H_2O$, 20; $CuCl_2.2H_2O$, 1; $NiCl_2.6H_2O$, 2 and $Na_2MoO_4.2H_2O$, 30. Similarly, a reddish brown hydrogen producing mixed PNSB culture was obtained after 7-10 days incubation in the RPN medium.

R. sphaeroides AV1b was adapted in autoclaved (121 °C for 20 min) DFE, centrifuged and supplemented with buffer and other essential nutrients as described in the preparation of PF medium. The DFE contained (in mg/L): acetic acid, 848; propionic acid, 457; butyric acid, 1,184; NH_4^+-N, 6.0; Phosphate (PO_4^{3-}), 35.8 and total Fe^{2+}, 0.045. The DFE medium for the *R. sphaeroides* AV1b culture was first autoclaved at 121 °C for 20 minutes to avoid the growth of opportune microorganisms. The inoculum was added after cooling while culture mediums for the mixed PNSB was not sterilized. Each photofermentative test was inoculated with 10 mL (1.5 g TSS/L, 2.5 % of working reactor volume) culture.

Preparation of PF medium

For each experimental test, the DFE was collected from the DF reactor during the 160-180 days operation period of the DF reactor described above, after settling for 30 minutes. The supernatant was collected after centrifuging at 4500 rpm for 20 minutes and had a pH of 4.5 (± 0.2). The DFE was supplemented with KH_2PO_4, 3g/L; $NaHCO_3$, 0.7 g/L; ferric citrate 24.5 mg/L and 10 mL of the trace metals solution. The DFE was supplemented with the above mentioned trace metals to provide all the necessary trace elements for the PF process (Bianchi et al., 2010; Montiel-Corona et al., 2015). Phosphate buffer (KH_2PO_4) was added to maintain the optimum pH around 6.5 – 6.8. Moreover, sodium bicarbonate ($NaHCO_3$) as it can act as an electron acceptor and can aid during the

uptake of propionic and butyric acid due to unbalances in the oxidative and reductive potential (Montiel-Corona et al., 2015). The pH of the DFE medium was adjusted to 6.5 with 1 M NaOH.

The characteristics of the three different DFE media, namely RS-I, RS-D and PM-D used for PF tests are presented in Table 5.6. The characteristics of undiluted DFE presented in the first column were used for PF tests using a pure culture of *R. sphaeroides* AV1b (labelled as "RS-I", reported in Ghimire et al. (2015b)). The second and the third columns of Table 5.6 refer to diluted DFE (1:2 ratio with milli Q water) used for PF tests with the pure *R. sphaeroides* AV1b culture and mixed PSNB culture (labelled as "RS-D" and "PM-D", respectively)

Table 5.6 - Characteristics of substrates used in photofermentative experiments

Characteristics of substrates used in photofermentative experiments

Characteristics	RS-I (mg/L)	RS-D (mg/L)[a]	PM-D (mg/L)
Chemical Oxygen Demand (COD)	3561.8 ± 131.1	2182.2 ± 303.0	2400.9 ± 149.0
Total Kjeldahl Nitrogen (TKN)	208.0 ± 7.0	189.1 ± 24.0[b]	189.1 ± 24.0
NH_4^+-N	1.1 ± 0.3	1.6 ± 0.3	0.9 ± 0.3
Lactic Acid	33.0	36.1	23.4
Acetic Acid	466.0	277.0	288.1
Propionic Acid	450.0	197.4	224.6
Butyric Acid	1075.4	636.1	547.0

[a] Analyzed after autoclaving the DFE
[b] Analyzed before autoclaving the DFE

PF experiments

Three sets of experiments were conducted to assess effect of dilution and use of the pure and mixed PNSB culture for H_2 and PHB production by PF of DFE medium. Transparent borosilicate glass bottles (Simax, Czech Republic) with a 500 mL capacity and a 400 mL working volume were used as photofermentative batch reactors. The batch reactors were maintained at a room temperature 24 (± 2) °C (April-June) under the luminance of approximately 4000 Lux (20 W compact florescent light) and positioned on a continuous stirrer (250 rpm). A long lag phase was observed during the H_2 production in the study of Ghimire et al. (2015b). The PHB concentration was analyzed in the samples collected every 3-5 days during the tests. The reactors were provided with arrangements for sampling of gas and culture medium. The bottles were flushed with argon to provide the anaerobic conditions and eliminate the nitrogen from the headspace.

Analytical methods

Hydrogen was quantified by a Varian Star 3400 gas chromatograph equipped with ShinCarbon ST 80/100 column and a thermal conductivity detector. Argon was used as carrier gas with of 20 psi front and rear end pressure. The duration of analysis was 14 minutes. The fermentation products were quantified by High Pressure Liquid Chromatography (HPLC) (Dionex LC 25 Chromatography Oven) equipped with a Synergi 4u Hydro RP 80A (size 250×4.60 mm) column and UV detector (Dionex AD25 Absorbance Detector) as described by Ghimire et al. (2015b). Gradient elution consisted of 20% methanol and 10% acetonitrile in 5 mM H_2SO_4, pumped at a rate of 0.9 ml/min by using a Dionex GP 50 Gradient pump. The elution time was 18.5 minutes.

For PHB analysis, samples were vacuum dried and the polymer was extracted according to Oehmen et al. (2005). PHB was quantified by gas chromatography (GC) equipped with a mass spectrometer (MS) and HP 5MS (Agilent) column and helium as the carrier gas. The light intensity was measured with a light meter (Lutron-LX-107).

The COD was determined by the Closed Reflux method and Total Kjeldahl Nitrogen (TKN) by macro-Kjeldahl as described in the Standard Methods (APHA, 2005). Biomass growth was quantified by spectrophotometric measurements of the Optical Density at 660 nm (OD660) (Photolab Spektral, WTW, Germany). Dry Cell Weight (DCW) was determined after filtering 20 mL of PNSB culture samples on GF/F Whatman filters dried at 105 ºC for 24 hours. DCW was correlated to the OD660 measurements using the calibration curves OD660 = 3.6876*DCW (R = 0.99823) and OD660 = 3.1839*DCW (R = 0.99865), respectively, for *R. sphaeroides* AV1b and mixed PNSB cultures.

Data analysis

The H_2 production was quantified with water (acidified with 1.5% HCl) displacement, and was normalized at standard conditions described else where in Ghimire et al. (2015b). The modified Gompertz equation (5.2) allowed to compare the kinetics associated to different PF tests, and to evaluate the effect of the experimental conditions.

$$H(t) = H_o \cdot \exp\left\{-\exp\left[\frac{R.e}{H_o}\right](\lambda - t) + 1\right\} \quad (5.2)$$

$$t_{95} = \frac{H_o}{R.e}(1 - \ln(-\ln 0.95)) + \lambda \quad (5.3)$$

The empirical equation (5.2) gives five major parameters: i) cumulative biohydrogen production (or potential) (H_o, mL), ii) bio-H_2 production rates (R, mL/h), iii) e = 2.71828, iv) lag time (λ, hours) and v) total cultivation time (t, hours). The equation 5.2 can be rearranged to equation 5.3 in order to calculate the time required to produce 95% of the maximum H_2 production (t_{95}). The parameters H_o, R and λ were estimated using the Curve Fitting Toolbox in MATLAB® with an associated 95% confidence limit.

5.2.3 Results and discussions

Concomitant production of H_2 and PHB

Undiluted versus diluted DFE

Figure 5.6 A presents bio-H_2 production (Fig 5.6 A) and concomitant depletion of VFAs and PHB production (Figure 5.6 B) in photofermentation of DFE medium (RS-I) using *R. sphaeroides* AV1b. A cumulative volumetric yield of 914 (± 8) N mL H_2/L was obtained at the end of the 40 days of incubation (Figure 5.6 A). The maximum composition of H_2 and CO_2 in the biogas reached 89.0% and 8.9%, respectively. The VFAs concentration decreased gradually, in particular, the acetate and propionate concentration decreased sharply until 10 days, while butyrate concentration decreased steadily until 30 days. The decrease in VFA concentration was followed by the increase in PHB concentration (Figure 5.6 B). The maximum PHB concentration of 1864.5 (± 76.4) mg/L, corresponding to 39.2 ± 9 % DCW, was obtained after 33 days from the reactor.

When the VFAs were completely degraded (Figure 5.6 B), a decrease in PHB accumulation was observed with a final PHB accumulation of 32.5 (± 3%) of DCW. This trend of PHB accumulation is in accordance with the literature, as PNSB can accumulate PHB as cell reserve material, which they use during a famine stage when substrate is depleted, as explained in the "feast-famine" theory (Johnson et al., 2009). James et al. (1999) reported that microorganisms use PHB as an energy source for survival during the low nutrient environments. Therefore, *R. sphaeroides* AV1b might have used PHB for their growth and metabolism when VFA depleted (Figure 5.6 A and B). This phenomenon can be supported by a decrease in PHB concentration and small increment in H_2 production after day 35, when the VFAs were completely depleted (Figure 5.6 B). However, this small increment in H_2 production can be also due to conversion of more

complex or recalcitrant organic matter, i.e. carbohydrates, that might be present in the DFE medium as reported also by Montiel-Corona et al. (2015). The cumulative H_2 production from RS-I tests (914 ± 8 mL H_2/L) is comparable to that of Uyar et al. (2009), who obtained a maximum H_2 production of 1000 mL H_2/L from the DFE obtained from DF of *Miscanthus* hydrolysate using *Rhodobacter capsulatus*. The H_2 yield is a function of reactor operational parameters, PNSB species used and substrate type (Eroglu and Melis, 2011). The average biomass concentration was 1.6 (± 0.1) g TSS/L at the end of the test.

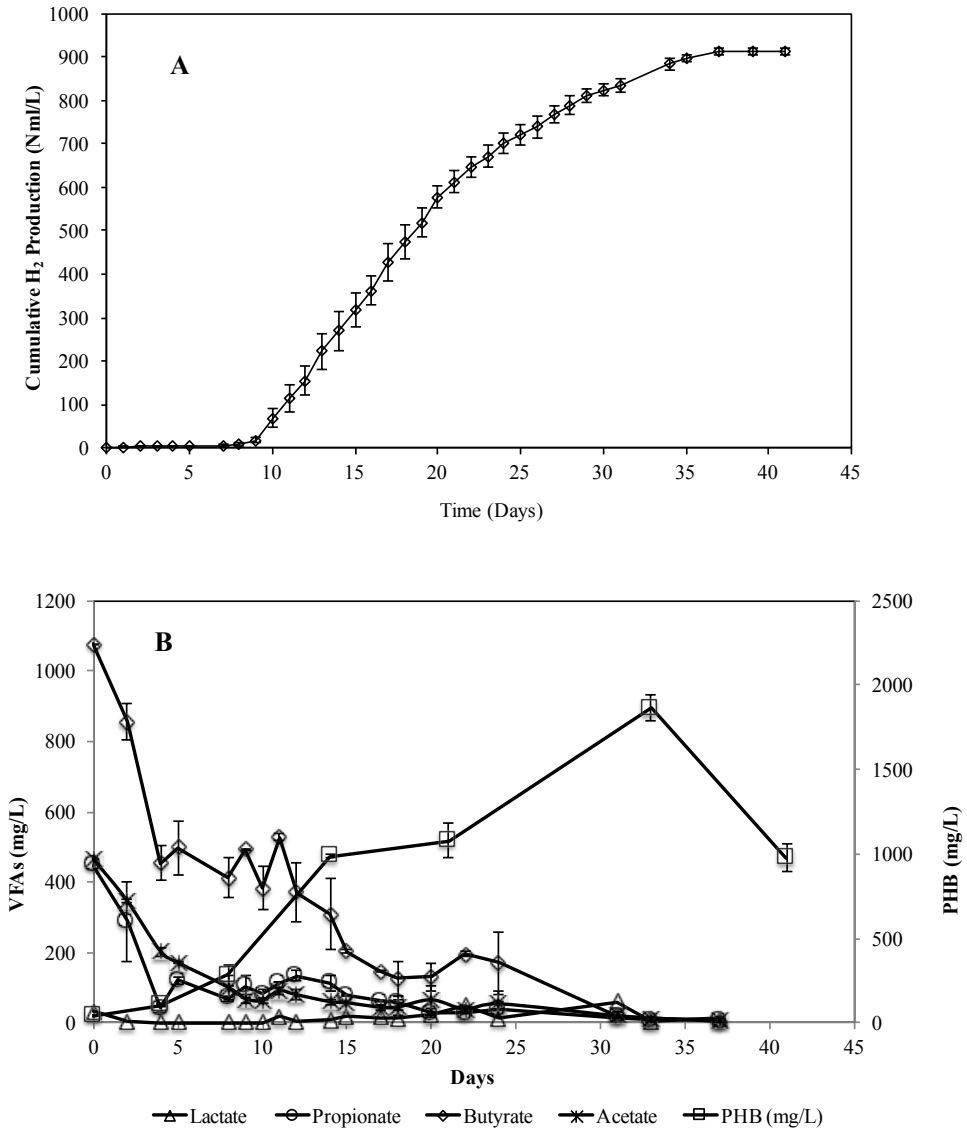


Figure 5.6 - Cumulative H_2 production (A) and VFAs depletion and PHB concentration (B) in the reactor during the test using RS-I medium and *Rhodobacter sphaeroides* AV1b

Table 5.7 - Summary of photo-H_2 performance estimated by modified Gompertz model

Tests	Volumetric Yield	NmL H_2/g COD_{added}	Kinetic model parameters of photo-H_2 production				
	NmL H_2/L		Ho (mL)	L (d)	R (mL/L·h)	t_{95} (d)	R^2
RS-I	914.1±8	256±2	368.8	9.3	2.2	26.0	0.9985
RS-D	358.0±25	164.0±12	144.2	4.3	0.9	20.0	0.9984
PM-D	168.7±14	71.3±6	69.8	2.1	0.4	22.3	0.9754

The analysis of kinetics parameters of bio-H_2 production in RS-I incubation obtained from the modified Gompertz model is presented in Table 5.7. A long initial lag phase of 9 days was observed and the 95% of the maximum production was reached after 26 days (Table 5.7). The longer lag phase can be attributed to the time required for biomass growth or to the competitive nature of PHB and H_2 production. The concentration of individual VFAs present in DFE for RS-I tests (Table 5.6) was not in the inhibiting range as reported by Han et al. (2012). As a long lag phase was observed with the *R. sphaeroides* AV1b using undiluted DFE (RS-I), PF tests were carried out with diluted DFE (1:2 ratios with ultrapure water) as well. A pure *R. sphaeroides* AV1b and a mixed PNSB culture was incubated in the diluted DFE, RS-D and PM-D, respectively (Table 5.6).

The results of the tests RS-I, RS-D and PM-D are presented in Table 5.7. Figure 5.7 and Table 5.7 show that the lag phase decreased to half when the DFE was diluted. Moreover, the time required for achieving 95% of the maximum production also decreased by 4 - 6 days. This might be due to the lower biomass concentration in the culture during the RS-D tests (Figure 5.7 B) compared to RS-I tests (1.6 g TSS/L). The biomass concentration strongly influences the availability of light for H_2 producing activity by PNSB (Koku et al., 2003). This can be supported by the 33.6 % decrease in the H_2 yield in RS-D tests. This shows that the H_2 production performance was compromised by dilution. However, this decrease in the H_2 yield can be due to the decrease in carbon source (COD) concentration in the culture medium i.e. DFE (Table 5.6). Besides, the H_2 production activity in *R. sphaeroides* AV1b might decrease with time during batch cultures due to decline in the activity of the electron carrier ferredoxin (Koku et al., 2003). Therefore, a continuous PF reactor can be adopted to eliminate the issues with a longer lag phase and

reduction in the H_2 production activity of the PNSB cultures to maintain optimal H_2 production performance.

Pure culture versus mixed PNSB culture)

Figure 5.7 compares the results of volumetric H_2 production, biomass growth, PHB accumulation and major VFAs depletion in the RS-D (A, B and C) and PM-I (D, E and F) tests, respectively. The mixed PNSB culture gave lower H_2 yields in comparison to the pure *R. sphaeroides* AV1b cultures. The lower H_2 yields of the mixed PNSB might be due to the absence of a H_2 producing PNSB population in the mixed PNSB culture. Likewise, the opportune microorganisms present in unsterilized DFE medium (PM-D) during application of mixed PNSB culture might consume available COD which is supposed to be utilized by PNSB for H_2 and / or PHB production. Montiel-Corona et al. (2015) reported a H_2 yield of 591.2 N mL H_2/g COD by an mixed PNSB culture. Thus, the H_2 yields of DFE via a mixed PNSB culture depends on the enrichment of H_2 producing PNSB in a mixed culture. The continuous or semi-continuous operation of PF processes in different reactor types, such as CSTR, tubular and flat panel reactors could efficiently enrich the mixed community of H_2 producing PSNB. In addition, bioaugmentation with pure PNSB cultures can be considered as an option to increase the H_2 yields from PF of DFE. Nasr et al. (2015) reported a H_2 yield of 166.83 (± 27.8) mL H_2/g $COD_{removed}$ from a continuous photofermentative reactor, in comparison to this study which gave a lower H_2 yield of 96.8 (± 6) NmL H_2/g $COD_{removed}$ in a batch process inferring an effect of the enrichment of an active mixed PNSB culture and reactor operational conditions.

The trends of PHB production showed that the maximum PHB concentration is reached close to the fermentation time period when 95% of the maximum H_2 production was achieved (Figure 5.6 and Table 5.7). In all experiments (Figure 5.7 and Table 5.7), it can be seen that the PHB concentration decreases when all VFAs are depleted in the medium. Thus, the bacterial biomass should be harvested during this period to recover the maximum amount of PHB. The H_2 and PHB yields for pure cultures are higher than those of mixed PNSB, this might be attributed to the absence of effective PNSB species that are responsible for H_2 production or gives lower PHB yields, i.e. *R. palustris* have a comparatively lower capacity to accumulate PHB compared to *R. sphaeroides* (Montiel-Corona et al. 2015).

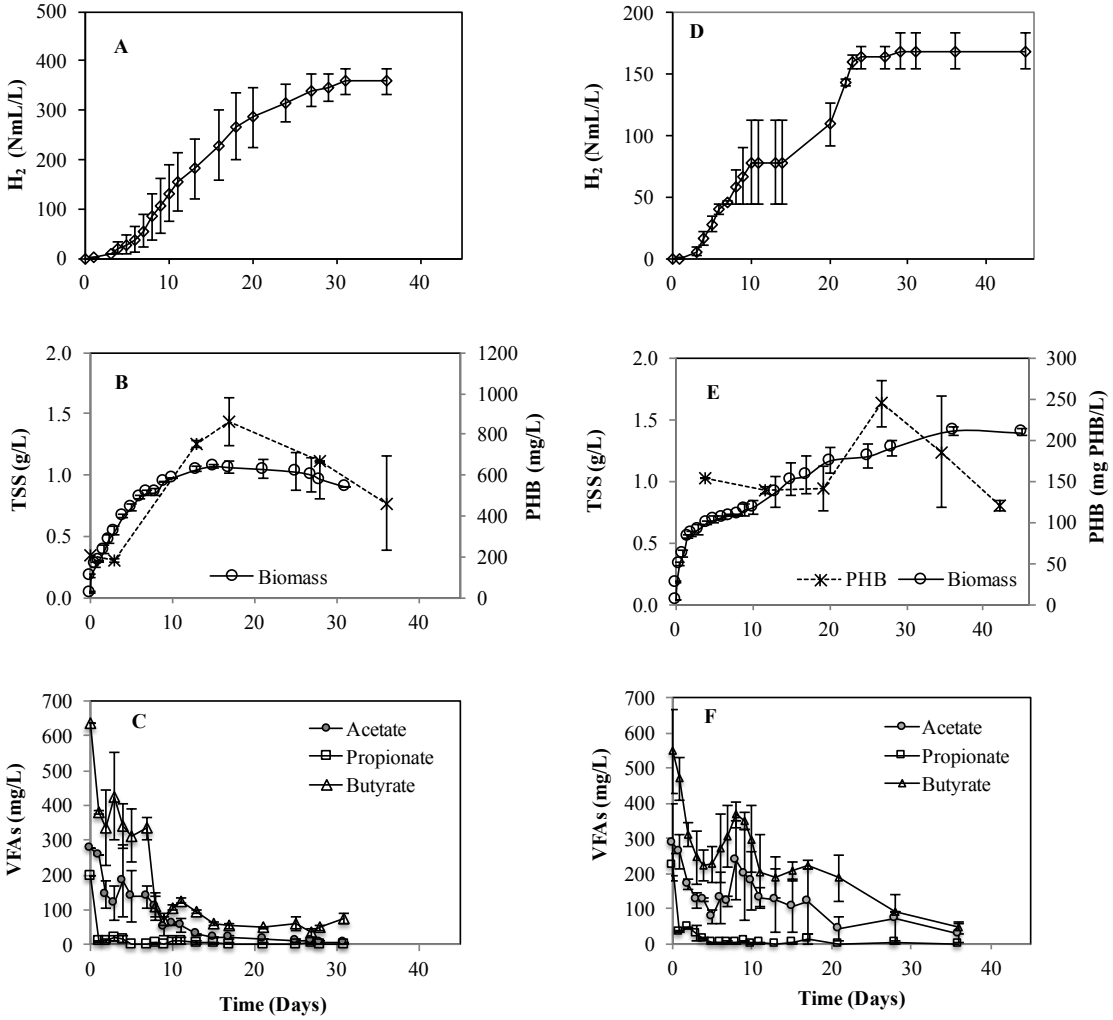


Figure 5.7 - Cumulative hydrogen production (A, D) and biomass and PHB concentrations (B, E) and depletion of major VFAs (C, F) in RS-D (left) and PM-D (right) tests

Substrate conversion efficiency and COD removal

The theoretical photofermentative conversion of organic acids, typically present in DF residues, to H_2 and PHB can be expressed by equations 3 - 7 in Table 5.8 (Barbosa et al., 2001; De Philippis et al., 1992; Han et al., 2012). The conversion ability of different PNSB varies on difference in substrate types (Barbosa et al., 2001; Bianchi et al., 2010). Identical to H_2 production (Table 5.8), PHB yields also depend on the type of VFAs present in the DFE. During the cultivation of *R. sphaeroides* in aerobic dark conditions, Kemavongse et al. (2007) reported that the addition of propionate (40 mM) and valerate (40 mM) to acetate (40 mM) in the substrate can induce the production of poly-β-

hydroxybutyrate-co-β-hydroxyvalerate (PHBV), a copolymer. Moreover, the presence of valerate gave 4 times more PHBV than propionate. Nonetheless, in our tests the propionate concentrations were low and valerate was not present (Table 5.6). Therefore, the production of PHBV in significant amounts was not expected.

Depending on the operational parameters such as C/N ratio, pH and substrate concentration, the hydrogen production in PNSB competes with PHB production (Hustede et al., 1993). Montiel-Corona et al. (2015) reported a negative correlation between the H_2 and PHB production in photofermentation using DFE obtained from DF of fruit and vegetable wastes. Nevertheless, they also reported that the conditions such as substrate type, concentration, argon flushing and alkaline culture pH can induce PHB accumulation along with H_2 production. Likewise, this study shows that the concomitant production of hydrogen and PHB is possible through PF of DFE.

Table 5.8 - Possible photofermentative pathways

Eqns.	Source of carbon	Possible photofermentative pathways	Major product
(5.4)	Lactate	$C_3H_6O_3 + 3H_2O \rightarrow 6H_2 + 3CO_2$	H_2
(5.5)	Acetate	$C_2H_4O_2 + 2H_2O \rightarrow 4H_2 + 2CO_2$	H_2
(5.6)	Propionate	$C_3H_6O_2 + 4H_2O \rightarrow 7H_2 + 3CO_2$	H_2
(5.7)	Butyrate	$C_4H_8O_2 + 6H_2O \rightarrow 10H_2 + 4CO_2$	H_2
(5.8)	Acetate	$2CH_3COOH + 2[H] \rightarrow$ PHB-monomer $+ H_2O$	PHB

Figure 5.8 summarizes the major products yields from the conversion of DFE into H_2, PHB and biomass per unit g COD added. Figure 5.8 also shows that the dilution slightly affects the soluble COD removal from DFE. The COD removal increased from 60.1 (± 1) % to 80.2 (± 1) % and 73.6 (± 0) % for, respectively, *R sphaeroides* AV1b in RS-I DFE, RS-D DFE and mixed PNSB for PM-D. This COD removal efficiency is comparable to that reported by Montiel-Corona et al. (2015). COD removal from DFE depends on several parameters such as the type of PNSB culture, initial influent COD concentration, dilution factor or reactor operating conditions (Montiel-Corona et al., 2015). Nonetheless, initial COD concentrations and type of PNSB species applied seems to strongly influence the COD removal. With diluted DFE, the mixed PNSB cultures gave higher COD removal efficiencies compared to pure cultures (Table 5.9). The higher COD removal efficiency can be due to the functioning of several microbial consortia present in the culture, supported by the results from Montiel-Corona et al. (2015) and this study

(Table 5.9). Moreover, the different COD removal efficiency in RS-I (60.1 ± 1 %) and RS-D (80.2 ± 1%) tests can be due to varying initial COD concentrations, 3.6 ± 0.1 and 2.2 ± 0.3 g/L, respectively. This was supported in a study by Tawfik et al. (2014), who reported a decrease in COD removal by mixed PNSB culture when the organic loading rate (OLR) increased from 3.2 to 16.0 g COD/L/day. However, H_2 production increased to an OLR of 6.4 g COD/L/day and decreased gradually on further increasing the OLR. This is due to inhibition due to VFAs accumulation present in the DFE medium. Likewise, Montiel-Corona et al. (2015) correlated the decrease in H_2 yields at higher COD concentrations (at 9.0 and 13.6 g/L) and the associated higher nitrogen content of the DFE to the interference in light penetration because of higher biomass concentration.

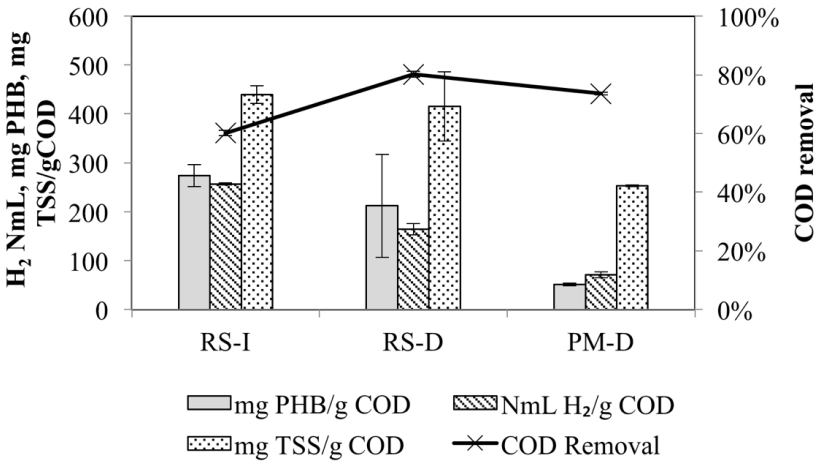


Figure 5.8 - PHB, H_2 and biomass yield per gram of COD and soluble COD removal (%) in different PF experimental runs

Table 5.9 shows comparison of the H_2 and PHB yields and COD removal obtained in this study with studies reported in the literature. The H_2 yields obtained in RS-I tests (914 ± 8 mL H_2/L) is higher compared to Yiğit et al. (1999) (648 mL H_2/L), while the H_2 production with mixed PNSB (168.7 ± 14 mL H_2/L) is lower compared to Montiel-Corona et al. (2015) (1478 ± 17 mL H_2/L) (Table 5.9). The lower H_2 production can be attributed to the lower initial COD concentration of 2.2 ± 0.3 g/L used in this study compared to 4.6 g/L in Montiel-Corona et al. (2015). In addition, the enrichment of PNSB cultures that determine a healthy population of H_2 producers can strongly influence H_2 yields. Therefore, the differences in H_2 and PHB yields might be attributed mainly to differences in substrates types and concentration and PNSB cultures. In *R. sphaeroides*, PHB yields are higher when acetate is a sole substrate in PF medium (Hustede et al.,

1993) (Table 5.9). Similarly, the competitive nature of H_2 and PHB can be clearly seen from the fact that the higher H_2 yields are obtained when PHB yields are lower (Table 5.9). On the contrary, studies conducted with complex substrates such as DFE (mixed organic acids) and wastewater have shown the concomitant H_2 and PHB production (Montiel-Corona et al., 2015; Yiğit et al., 1999), which is in agreement to this study.

In addition to production of valued added products, PNSB can efficiently remove COD from the DFE, which makes PF process attractive for environmental engineering applications. Considering the removal of major VFAs (acetate, butyrate and propionate) and lactate from the DFE, the total VFAs removal efficiencies exceeding 99, 95 and 85%, respectively, were achieved in the tests with RS-I, RS-D and PM-D, respectively. However, the biomass in the final PF medium, evidenced by the reddish brown color of the effluent due to the presence of colloidal bacterial pigments, contributes to a fraction of the final total COD of the effluent. Nevertheless, concentrated PNSB biomass can be used as feedstock for the anaerobic digestion processes for the recovery of methane, as shown by Ghimire et al. (2015b).

Table 5.9 - Comparison of hydrogen and PHB production by different isolated strains and enriched mixed cultures of PNS via photofermentation of various carbon sources

Microbial Inoculum sources	Carbon and nitrogen source	PHB (% DCW)	Volumetric H_2 Yield (mL H_2/L)	COD Removal (%)	References
Rhodobacter sphaeroides 17023 (wild type)	30 mM acetate and 7 mM glutamic acid	70	0	-	Hustede et al., 1993
	30 mM lactate and 7 mM glutamic acid	24	2310	-	
Enriched photoheterotrophic culture IZT	DFE (11.61 g/L butyric, L 1.76 g/L propionic and 1.01 g/L acetic acid and 0.78 g/L total ammonia	5	1478 ± 17	89	Montiel-Corona et al., 2015
Rhodobacter capsulatus		29	1252 ± 20	65	
Rhodobacter sphaeroides O.U. 001 (DSM 5648)	Sugar refinery wastewater (30% v/v in medium)	70.4	648	-	Yiğit et al., 1999
Rhodobacter sphaeroides RV	40 mM Acetate only	38	0	-	Khatipov et al., 1998
Rhodobacter sphaeroides AV1a	DFE (RS-I, Table 5.6)	32.5 ± 3	914.1 ± 8	60.1 ± 1	This study
Enriched photoheterotrophic culture	DFE (PM-D, Table 5.6)	6.3	168.7 ± 14	73.6 ± 0	This study

5.2.4 Conclusions and future perspective

Concomitant H_2 and PHB production was demonstrated using undiluted and diluted DFE by pure and mixed PNSB cultures. Higher H_2 and PHB yields were obtained from *R. sphaeroides* AV1b with undiluted DFE. H_2 and PHB yields from mixed PNSB cultures were lower than *R. sphaeroides* AV1b cultures. Moreover, the use of mixed cultures could be more appropriate for the treatment of DFE in scaled-up applications, as it can give high COD removal efficiency, save the associated asepsis costs and a wide range of waste biomass can be used. Nonetheless, pure *R. sphaeroides* cultures can be applied for PHB production.

5.3 Solid State Dark Fermentation for production of H_2 and organic acids

This section presents the results of the investigations carried to evaluate the potential of Solid State Dark Fermentation for the production of biohydrogen and organic acids. The main aim is the assessment of the respective effects of total solids content and H_2 partial pressure on substrate conversion, using food waste and wheat straw as model substrates.

5.3.1 Introduction

Dark fermentation (DF) is emerging as a potential biological pathway for production of hydrogen and useful by-products utilizing organic biomass (Liu et al., 2013; Azwar et al., 2014; Ghimire et al., 2015; Wang et al., 2015). Low cost renewable waste biomasses such as agricultural residues, organic fraction of municipal waste (OFMSW) and agro-industrial wastes might give competitive economic advantage for the future supply of sustainable feedstock which may be used industrially for DF systems with biological treatment of waste as an added benefit (De Gioannis et al., 2010; Ntaikou et al., 2010; Urbaniec & Bakker, 2015). OFMSW and lignocellulosic residues such a wheat and rice straws could be potential substrate sources for this purpose as their future supply is abundant and they do not compete with the food crops like the substrates used for first generation biofuels. Food waste has high volatile solids (VS) (21 to 27% VS) content and can be valorized by the concomitant production of biohydrogen and platform molecules as organic acids and alcohols (VALORGAS, 2010; Uçkun Kıran et al., 2015; Wang et al., 2015). DF of food waste has several benefits, along with the production of H_2 as clean energy carrier, volatile acids and alcohols as by-products, which can have wider applications. The soluble by-products of DF can be applied in i) wastewater treatment (Elefsiniotis et al., 2004), (ii) production of platform molecules such as biopolymers (Ntaikou et al., 2009), (iii) microalgal lipids production (Turon et al., 2015), (iv) H_2 production by photo fermentation, (v) feed for microbial electrolysis cells for production of H_2 and other value added chemicals (ElMekawy et al., 2014) and (vi) anaerobic digestion for energy recovery in the form of H_2 and CH_4 (Ghimire et al., 2015).

Recently, Motte et al. (2015) have propose to combine dry DF and mechanical pre-treatment process as a measure to reduce the energy demands and effluents generation. This configuration also enhances the overall substrate conversion, which makes it more plausible for lignocellulosic biomass to be applied in a biorefinery concept. Therefore,

Solid State Dark Fermentation (SSDF) can serve as biological pre-treatment for the utilization of feedstock in a biorefinery concept. A SSDF process can offer several advantages over conventional wet processes, which are usually operated under the low total solids (TS) contents (often less than 10%). A commercial dry AD process is usually operated at TS content higher than 20%. The operational advantages include high substrate loading rates and low water addition. Therefore, SSDF can offer i) economic benefits by reducing the reactor volume and specific energy requirements, (ii) an efficient handling of digestate and (iii) a higher technical simplicity.

The past studies have shown that an increase in TS content impacts the substrate degradation and biogas production (Abbassi-Guendouz et al., 2012; Motte et al., 2013). Fernández et al. (2008) reported decrease in degradation of OFMSW by 17% in SS-AD when the TS content increased from 20 to 30%. In fact, high-solids processes can be restricted by mass transfer limitations that impact the biogas yields as well as the microbial metabolic pathways (Abbassi-Guendouz et al., 2012; Abbassi-Guendouz et al., 2013; Bollon et al., 2013; Liotta, et al., 2014). The mass and energy transfer limitations are driven by the low water content of the system (Motte et al., 2014; Valdez-Vazquez & Poggi-Varaldo, 2009). Thus, microbial activity can be impacted by the transport of soluble components (i.e. substrates, intermediate and end-metabolites). Some studies have shown the dependency of H_2 production on TS content (Motte et al., 2013; Motte et al., 2014; Robledo-Narváez et al., 2013; Valdez-Vazquez & Poggi-Varaldo, 2009). During the study of the effect of TS content on H_2 production from DF of wheat straw (WS), Motte et al. (2013) reported significant decrease in H_2 production at 19 % TS along with the decrease in substrate conversion. In another study, Motte et al., (2014) showed the reduction in H_2 yields, in addition to the favouring the growth of lactic acid producing microbial community in WS. However, very few studies (Valdez-Vazquez & Poggi-Varaldo, 2009) have addressed the issues of effect of increasing TS content in the DF of food waste (FW).

Moreover, in a recent study, Cazier et al. (2015) showed an inhibition of biomass hydrolysis in (SS-AD) anaerobic digestion due to a high hydrogen partial pressure (p_{H2}) in WS. However, the effect of high TS content on acidogenesis and H_2 yields is rather unknown, but inhibition of substrate hydrolysis by high local H_2 partial pressure in SSDF process is probable. The impact of these parameters under SSDF of FW is not well

studied. It is important to understand the limitation of SSDF of organic waste to increase its potential and to open new paths for its industrial application for the production of biofuels and biochemicals.

The present study aims to investigate the effect of the TS content on organic waste conversion in SSDF using FW and WS as representatives of substrates with high and low biodegradability, respectively. In addition, the particular effect of p_{H2} was also studied to investigate the effect of p_{H2} in biomass hydrolysis and metabolic pathways.

5.3.2 Materials and methods

Inoculum source and feedstock

Experiments were designed to study the effect of TS content and p_{H2} on substrate degradation and biochemical pathways in batch SSDF tests. FW was prepared in the laboratory with the composition similar to the one described in Ghimire et al. (2015b). Heat shocked (90 °C, 15 min) waste activated sludge obtained from a municipal wastewater treatment plant in Limoges (France) was used as inoculum. This inoculum was centrifugated (at 6500 rpm for 20 min, 4 °C) to obtain 11% total solids (TS) and 9% volatile solids (VS) content. Similarly, WS with TS and VS content of 95% and 97%, respectively, was used as a representative of lignocellulosic biomass.

Experimental set-up

Effect of TS content on H_2 production and substrate conversion

Batch tests in triplicates were designed at 10%, 15%, 20%, 25% and 30% TS content to investigate the effect of TS on substrate conversion of FW. In each 600 ml flask, 53.4 g of digestate (38.87 % final TS content), composed of FW (20.3 g) and inoculum (4.1 g) in a ratio of 10 g VS substrate/g VS inoculum, i.e. S/X, 16.0 g 2-(N-morpholino) ethanesulfonic acid (MES) buffer, 12 ml 3.2 % NaOH and 1 ml of trace metal solution (containing $FeCl_2$ 2g/L, $CoCl_2$ 0.5 g/L, $MnCl_2$ 0.1 g/L, $NiCl_2$ 0.1 g/L, $ZnCl_2$ 0.05 g/L, H_3BO_3 0.05g/L, Na_2SeO_3 0.05g/L, $CuCl_2$ 0.04 g/L, Na_2MoO_4 0.01g/L) were added. This mixture had an initial pH of 5.5. The amount of distillated water to be added was calculated with a mass balance on TS contents including substrate, inoculum, buffer and other solution addition to obtain the final TS content of 10.0 ± 0.01, 14.98 ± 0.03, 19.89

± 0.04, 24.92 ± 0.02, 30.0 ± 0.07 %TS, in each set of experiments. The batch tests were then incubated at 37 ± 1 °C for 14 days.

Effect of partial pressure of H_2 on substrate conversion

To study the effect of p_{H2}, batch tests were carried out in four replicates with FW and WS at a S/X ratio 10 and final TS content of 25 ± 1 %. The tests were carried out with a thin layer of digestate (<1 cm), approximately 22 ± 2 g, in order to minimize the effect of gas diffusion (Cazier et al., 2015). H_2 was initially added in the headspace of the 600 ml serum bottles in two sets of tests; in one set p_{H2} was equivalent to 542 ± 32 mbar (33 ± 2 % H_2 in the headspace, named as "A") and in the other set it was 1087 ± 29 mbars (66 ± 1 % H_2 in the headspace, named as "B"). A control with only N_2 in headspace was carried out (named as "C") and the final total pressure at the start of the tests for all the conditions was 1500 mbars. The initial culture pH was maintained at 5.5 with MES buffer and the culture was incubated at mesophilic temperature (37 ± 1 °C) for two fermentation periods of 14 and 21 days.

Analytical methods

Gas composition was measured by gas chromatograph (Perkin Clarus 580) equipped with a thermal conductivity detector at 150°C and an injector heated at 250°C and two capillary columns heated at 60°C. The first column was an RtUbond for the CO_2 while the second column was an RtMolsieve used for the detection of the O_2, H_2, N_2 and CH_4. Argon at pressure of 350 kPa and flow rate 31.8 mL/min was used as carrier gas. The gas production was monitored with increase in gas pressure, which was measured with a digital manometer (2000, Leo2 Keller).

5.0 g of digestate were diluted in 5 g of deionized water, mixed during 30 minutes, centrifuged at 18,000 rpm during 20 min at 4°C and then filtrated at 0.2 µm with a nylon membrane. The liquid was then used to measure VFAs, others metabolites and soluble sugars. Dark fermentation metabolites in the digestate were measured at the beginning and end of the experiments. VFAs were quantified with gas chromatograph (Perkin Clarus 580) and Elite FFAP crossbond® carbowax® 15 m column connected to a flame ionization detector at 280°C and N_2 as carrier gas at the flow rate of 6 mL/min, described elsewhere (Cazier et al., 2015). High performance liquid chromatography (HPLC) was used to quantify other metabolites and soluble sugars, that comprised of Aminex HPX-87H

column (300 mm on 7.8 mm, Bio-rad), a pre-column to filter residues (Micro guard cation H refill cartbridges, Bio-rad) and an automatic sampler (Water 717). Sulfuric acid 0.005 M was used as eluent at the flow rate of 0.4 ml/min.

Data analysis

Substrate degradation was estimated computing a theoretical chemical oxygen demand (COD) mass balance by calculating the difference in metabolic end-products (accumulated in both gaseous and liquid phase) at the initial and final state. The COD measurements of the complex organic residues such as lignocellulosic biomass and FW may vary more than 10 % while the overall ubstrate degradation during the process might be lower than 10% COD, thus direct measurement of COD was not considered in this study (Cazier et al., 2015). Therefore, total substrate degradation is calculated as the amount of COD produce from the DF of substrate estimated per kg of TS added initially and calculated as:

$$\textbf{Total Substrate Degradation} = \text{COD of Final State} - \text{COD of Initial State}$$

$$\textbf{Total Substrate Degradation} = \frac{\mathbf{A_{H_2,f}} + \mathbf{A_{met,f}} + \mathbf{A_{GC}}}{\textbf{kg TS}} - \frac{\mathbf{A_{H_2,i}}}{\textbf{kg TS}} \qquad \text{(Equation 5.9)}$$

Where, $A_{H_2,f}$ is the amount of H_2 remaining at the end in the headspace, $A_{met,f}$ the final amount of metabolites accumulated, A_{GC} the total amount of gas (H_2) sampled for analyses, $A_{H_2,i}$ the initial amount of H_2 added and $A_{met,i}$ the initial amount of metabolites in the medium.

R software (OSX version 3.1.3) with the package Rcmdr (OSX version 2.1.7) was used for the statistical analysis of data obtained from the experiments. The P value was set at 0.05 and the significance of the results tested with P values: * < 0.05; ** < 0.01; *** < 0.001; while not significant results were with P >0.05.

5.3.3 Results

Influence of TS content

Figure 5.9 shows the effect of TS content on substrate degradation after 14 days of fermentation period. The substrate conversion decreased and biohydrogen production was significantly inhibited when the initial TS content increased and concomitant shift in the metabolic pathways was observed (Figure 5.9 b). The maximum and minimum substrate

degradation of 134.44 ± 22 and 51.45 ± 3 g COD/kg TS was achieved at 10 and 30 % TS, respectively.

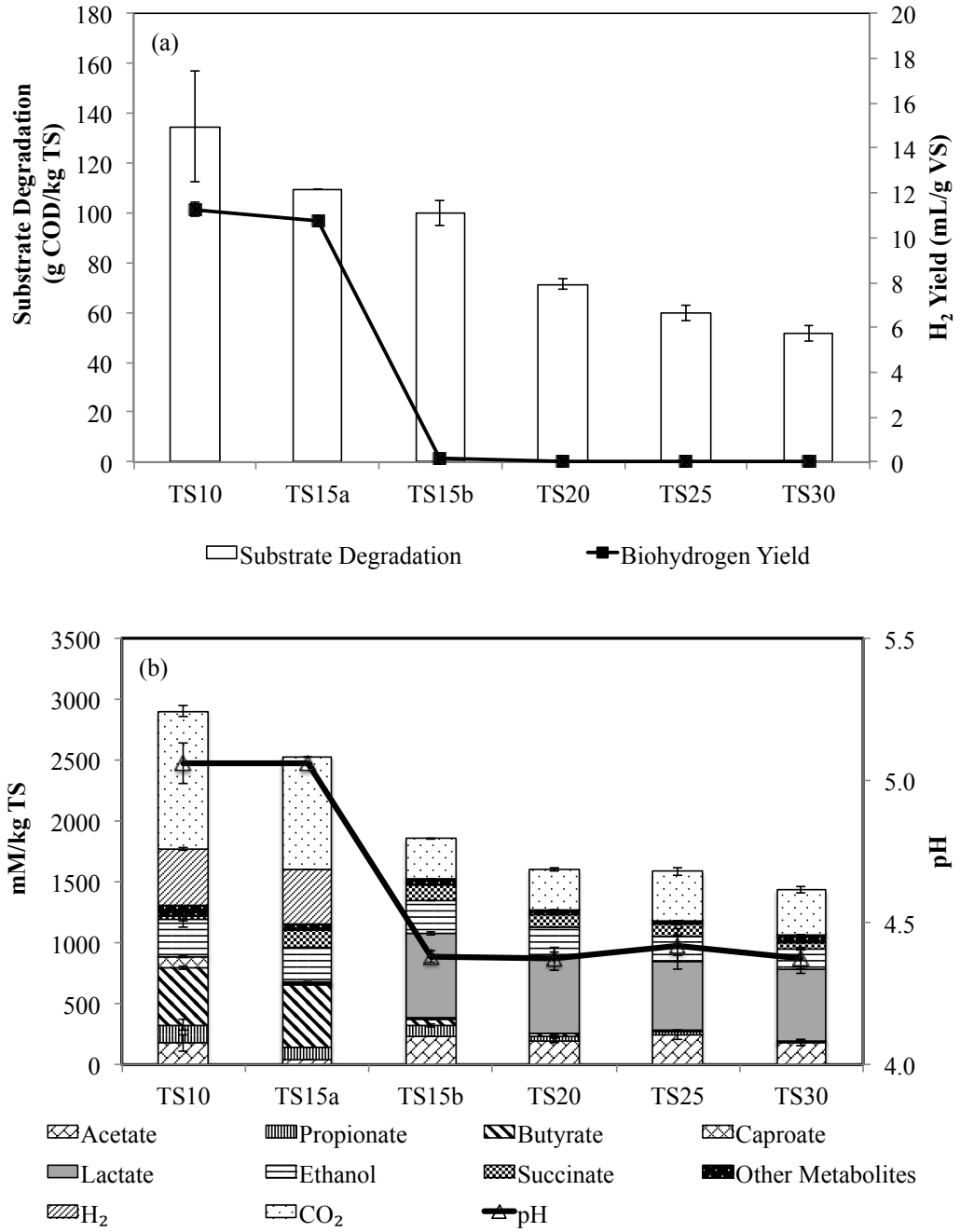


Figure 5.9 - Substrate degradation (in g COD per kg of initial TS) (a); End metabolites accumulation (mM per kg of initial TS) (b); at different TS content

The H_2 production decreased drastically when the TS content increased more than 15% TS and the metabolic pathways favored the lactic acid production, which can be attributed to insignificant amount of H_2. H_2 was produced only in the TS content 10% and 15%. On the basis of biohydrogen production and nature of end-metabolites, two distinct behaviors

were observed at TS 15%. Only one of the three replicates (named TS15a) showed similar nature behavior of DF as TS 10% while the other two replicates showed comparable nature of fermentation as in higher TS content (Figure 5.9 a and b). At TS content higher than 15%, the metabolic pathways mainly shifted towards lactic acid conversion that might explain the decrease in H_2 production and substrate conversion (Figure 5.9 b). Figure 5.9 (b) presents the molar yield (mmol/kg TS) of all the major metabolic end products after 14 days of fermentation at different TS content. The highest substrate conversion of 2901.13 ± 143 mM/KgTS was obtained at wet TS conditions (10%) while the lowest value of 1435.2 ± 13 mmol/kg TS was obtained at TS 30% (Figure 5.9 b). PCA correlation plot of metabolites and hydrogen production is presented in Figure 5.10.

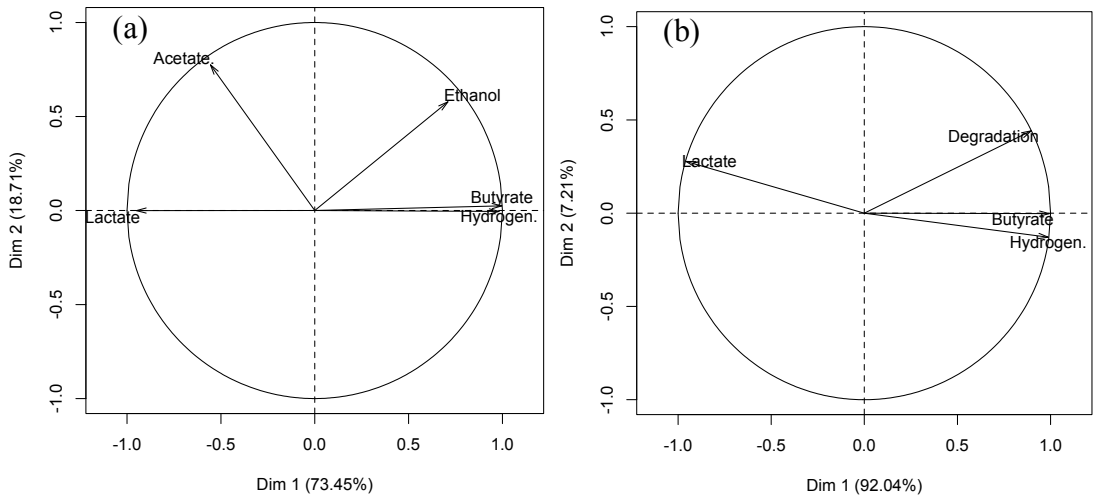


Figure 5.10 - Principal component analysis correlation circle plot (a) Hydrogen and major metabolic by-products production. (b) Substrate degradation and metabolic products.

Effect of p_{H2}

The different initial p_{H2} during the SSDF process was tested to investigate its effect on substrate conversion of FW and WS at higher TS content (25 %TS). Figure 5.11 (a) shows the total substrate degradation values (expressed as g COD/kg TS) after 14 and 21 days of DF at different p_{H2} using FW. The level of inhibition of p_{H2} on substrate hydrolysis was determined based on difference in level of substrate degradation (Figure 5.11 a). No significant effect of initial p_{H2} on hydrolysis of biomass (ANOVA test, P-values > 0.05) was observed at 25% TS as in SS-AD (Cazier et al., 2015). This was further evident in metabolic products accumulated at the end of the experimental periods, which show no noteworthy shift (Fig 5.11 b). Substrate degradation slightly increased with fermentation

time, and lactic acid and ethanol as the major metabolites were observed in all the tested p_{H2} with FW.

These results are in contrast with the study of Cazier et al. (2015), which reported an inhibition of H_2 on the hydrolysis of WS during SS-AD. In this work, such an inhibition started at p_{H2} > 742 mbars and substrate degradation decreased from 90 ± 10 to 20 ± 10 g COD/kg TS in the controls and at p_{H2} 1555 mbars, respectively, followed by the decrease in production of methane and acidogenic metabolic products.

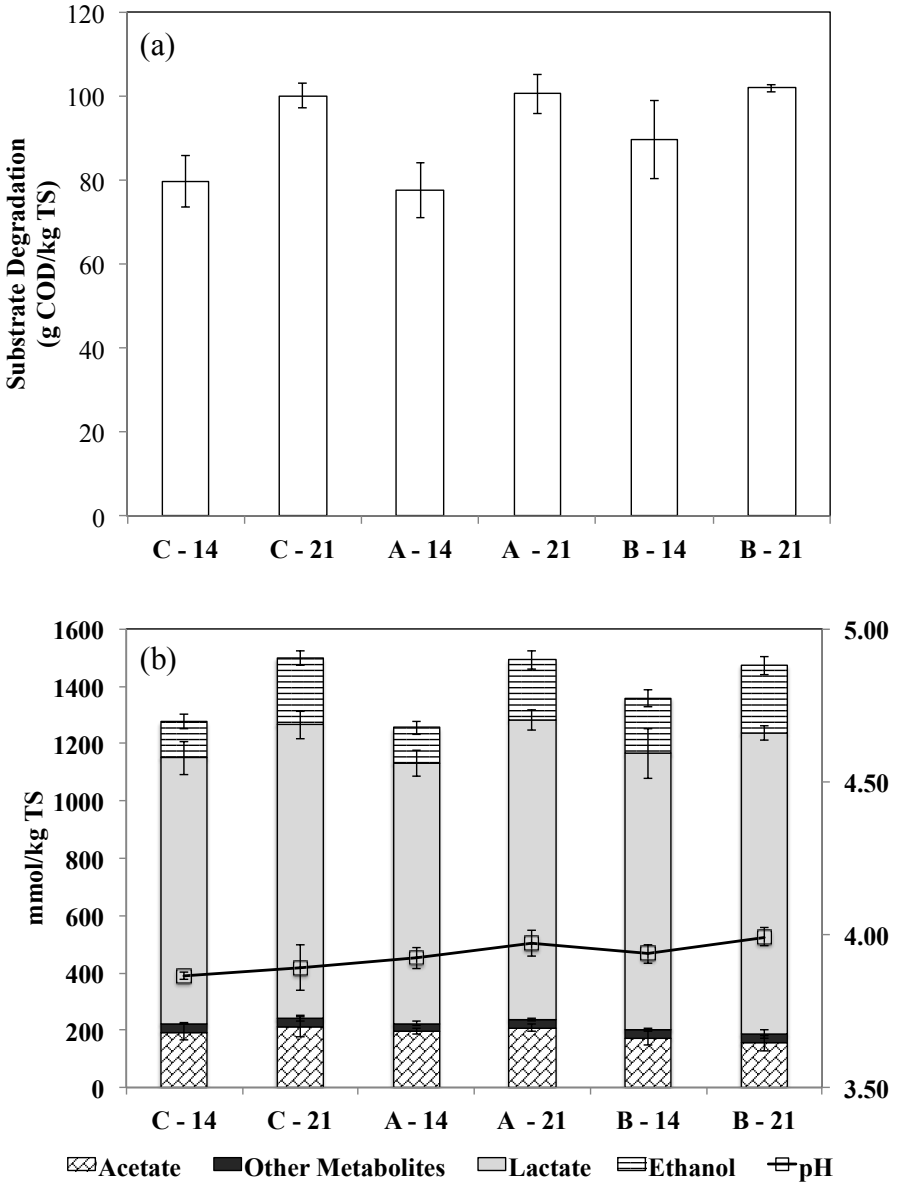
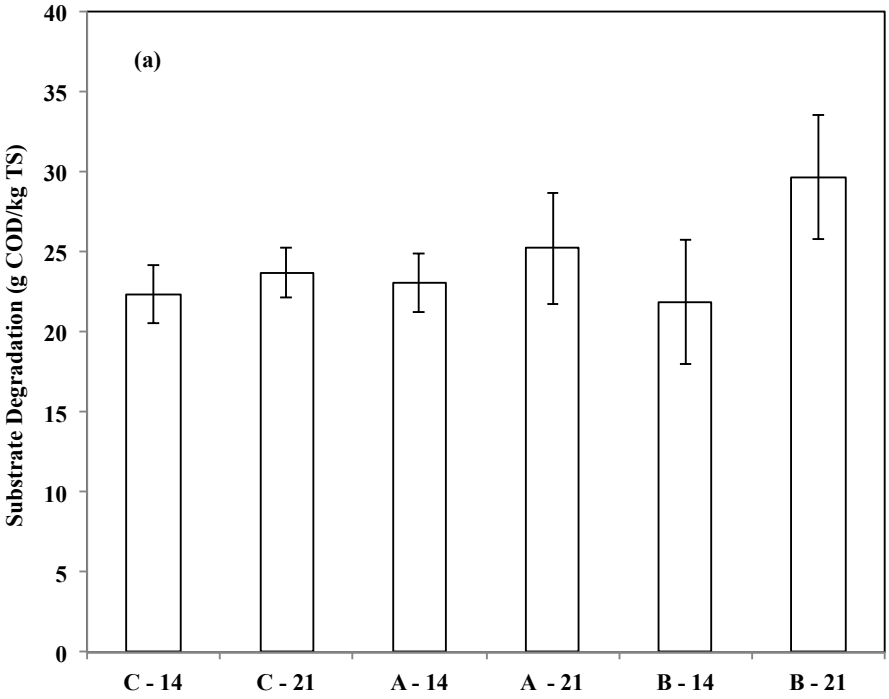


Figure 5.11 - Substrate degradation using FW (in g COD per kg of initial TS) (a); Substrate degradation using FW (in g COD per kg of initial TS) (b); at the end of two fermentation times (14 ≥14 d, 21 ≥ 21 d) and different p_{H2} of of A = 532 ± 33 mbar, B = 1,086 ± 29 mbar and C = 0 mbar at 25 % TS content

The effect of p_{H2} on substrate conversion was not clearly evident during SSDF of readily degradable substrate like FW. The culture pH decreased sharply regardless of adjusting the initial pH with a buffering agent (i.e. MES). Therefore, further tests were carried out with WS, a representative of substrate with low biodegradability, under the similar experimental conditions with an objective to confirm the results obtained with FW. Nonetheless, the pH did not significantly decrease at the end of the experimental period. However, the effect of p_{H2} was not evident, as seen from Figure 5.12 a and b, which was further verified with ANOVA (P-values >0.05). Obviously, the H_2 production was inhibited in the tests with higher p_{H2} (tests A and B). Similarly, lactic acid or ethanol was not present in the metabolic products as in the tests with FW. Interestingly, the substrate degradation of WS during the control tests, i.e. 22.4 ± 2 g COD/kg TS (at pH 5.4), is similar in the study of Cazier et al. (2015), i.e. 20 ± 10 g COD/kg TS, when the maximum inhibition of hydrolysis occurred at p_{H2} 1555 mbars at pH 8-9.



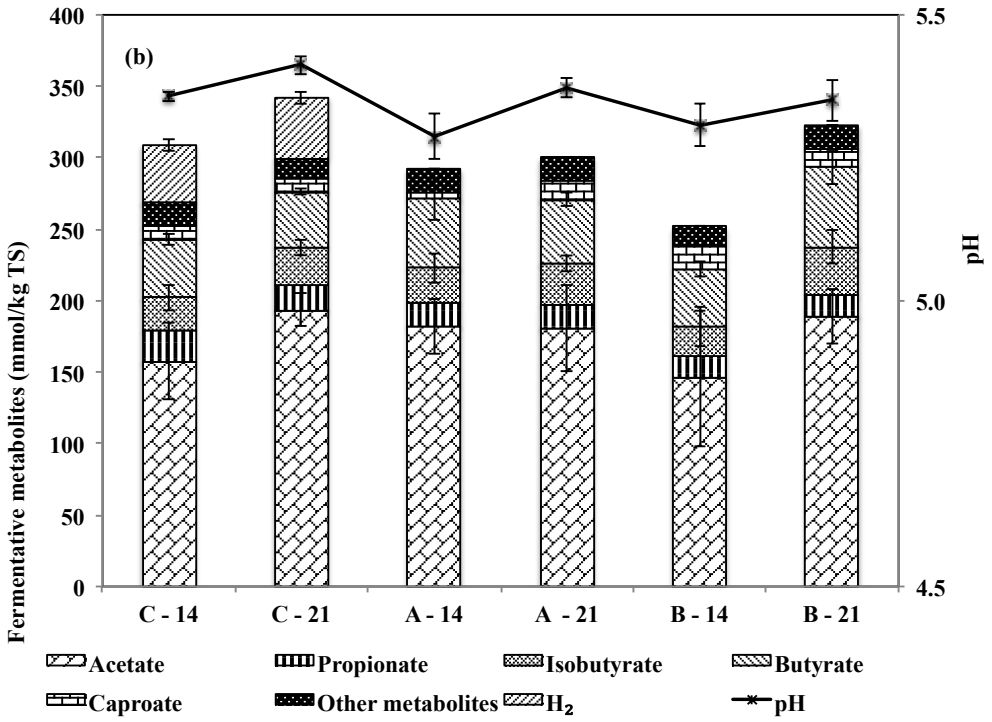


Figure 5.12 - Substrate degradation in WS (in g COD per kg of initial TS) (a); Substrate degradation using WS (in g COD per kg of initial TS) (b); at the end of two fermentation times (14 ≥14 d, 21 ≥ 21 d) and different p_{H2} of A = 552 ± 31 mbar, B= 1,087 ± 30 mbar and C = 0 mbar at 25% TS

5.3.4 Discussion

The results of the SSDF tests carried with FW at different TS content have shown that the H_2 production is impacted by the increase in TS contents, which was in accordance with the earlier studies (Motte et al., 2014; Valdez-Vazquez & Poggi-Varaldo, 2009). In particular, the results from the study of the effect of TS content (Figure 5.9 a and b) further suggest that the limiting effect of TS content starts between 15 and 20% as in agreement with Motte et al. (2014), that reported a metabolic shift at 19% TS with WS as substrate. The metabolites in all the tested % TS were analysed for the possible biochemical pathways.

Theoretically, presence of acetate and butyrate in metabolic by-products are generally correlated with hydrogen production pathways (Ghimire et al., 2015; Guo et al., 2013); however, in this study, the H_2 production was only correlated with butyrate production as shown by the PCA correlation plot in Figure 5.10 (a). Similarly, Figure 5.10 (b) showed that the H_2 production followed substrate degradation, while lactate production is not well

correlated with conversion of substrate. Two possible explanations for lactate production at higher TS content are the following: i) lactic acid bacteria (LAB) are more adaptable to harsh environmental conditions which enable them to inhabit in moisture limited conditions at higher TS content (Sikora et al., 2013); ii) a decrease in pH related to the higher substrate concentration and production of VFA can affect the microbial community structure. However, pka of lactic acid is 3.86 in comparison to 4.75 and 4.78 for acetate and butyrate. Therefore, the decrease in pH is most likely due to the production of lactic acid. This shift to LAB at higher TS contents has been also shown in a study by Motte et al. (2014), regardless of the pH which was maintained constant at 5.50. This further strengthens the fact that the moisture lacking conditions create harsh environmental conditions, which trigger the growth of LAB. In addition, from the decrease in pH in this study, it can be concluded that the alkalinity requirements at high solids systems are higher than in the wet conditions.

Furthermore, Abbassi-Guendouz et al. (2012) showed significant inhibition of methane yields at 30% TS due to accumulation of intermediates such as organic acids and dissolved hydrogen. Thus, p_{H2} might impact the substrate conversion in SSDF, with the accumulation of H_2 in the medium, as reported in a recent study by Cazier et al. (2015) in SS-AD. However, it has been confirmed from this study that the accumulation of H_2 does not impact on the hydrolysis of substrate under SSDF (Figure 5.11 and 5.12), in contrast to the results obtained by Cazier et al. (2015) under SS-AD. This could be due to the fact that the substrate degradation is already under limitation under DF conditions, which is supported by the relationship established between H_2 production (or substrate conversion) and soluble carbohydrates present in the substrates, as reported by Guo et al. (2013) and Monlau et al. (2012). In addition, the hydrolysis of substrate seems to be also a function of culture pH as shown by Veeken et al. (2000).

Moreover, this difference in results can be also attributed to lower operational pH in the present study (pH 3.7 - 5.5) compared to SS-AD (pH 8 - 9) as reported by Cazier et al. (2015). The pH might also affect the conversion of substrates and metabolic products. Veeken et al. (2000) reported the decrease in hydrolysis of complex substrates with the decrease in culture pH. The hydrolase enzyme of hydrolyzing bacteria functions at an optimal neutral pH (Parawira et al., 2005). Lin et al. (2006) reported that xylose removal decreased from 85% to 37% when the culture pH decreased from 8 to 5. Similarly, Fang

and Liu (2002) also reported the decrease in glucose degradation by 10% when pH decreased from 5.5 to 4.

Table 5.10 - Production of VFA from different types of fermentation

Feedstock	Inoculum	Operating conditions	VFA Production	Reference
Food waste	Anaerobic digested sludge	Controlled pH 6.0, 35 °C	799 g COD/kg VS_{added}	(Wang et al., 2015)
Food waste	Anaerobic activated sludge	Controlled pH 6.0, 30 °C	918 g COD/kg $VS_{removal}$	(Wang et al., 2014)
Kitchen waste	Waste activated sludge	Controlled pH 8.0, 37 °C	692.4 g COD/kg VS_{added}	(Chen et al., 2013)
Wheat straw	Anaerobic digestate	Initial pH >8.0, Final pH 5.2, 37 °C, 23 TS content, 64 days SRT (batch)	140 ± 6 g COD/kg TS_{added}	(Motte et al., 2015)
Waste activated sludge	No inoculum addition	Controlled pH 8, 55 °C, 9 days SRT (batch)	368 g COD/kg VS_{added}	(Zhang et al., 2009)
Food waste	Waste activated sludge	Initial pH 5.5, Final pH 5.1, 37 °C, 10 % TS, 14 days SRT (batch)	134.4 ± 22g COD/kg TS_{added}	This study
Wheat straw	Waste activated sludge	Initial pH 5.5, 37 °C, 25 % TS, 14 days SRT (batch)	22.3 ± 2 g COD/kg TS_{added}	This study

VFAs yields obtained in this work are compared with the anaerobic fermentation studies reported in the literature (Table 5.10). All the SSDF tests carried out in this study shown lower VFAs yields (Table 5.10), which can be explained by the difference in operating conditions during the fermentative studies. The production of VFAs under fermentative conversion process is significantly affected by operating parameters such as culture pH, temperature and substrate concentration (Cho et al., 2015; Wang et al., 2014, 2015). Most of the studies reported in Table 5.10 are carried out at pH 6.0 and higher under controlled pH conditions. This could explain the higher conversion of the waste biomass into fermentative products reported in these studies, compared to the results of this study

where the hydrolysis was inhibited due to low pH conditions (3.7-5.1). Likewise, under dry dark fermentative process, the conversion of substrate was dependent on culture pH and TS content. However, the effect of p_{H2} was not clearly evident on substrate hydrolysis. This might be due to the fact that substrate conversion in the tests with applied p_{H2} was already under inhibited conditions due to high TS content (25% TS). In addition the culture pH was in the range of 5 – 5.5 with WS and (3.7 – 5.3) with FW. In addition, the accumulation of lactic acids during these limiting conditions suggests that LAB are dominant in harsh and nutrient rich environment such as FW at higher % TS and not in WS (Sikora et al., 2013). Likewise, the lactic acid fermentation pathways is not favourable for conversion of substrates.

5.3.5 Conclusion and future perspectives

This study highlights the effect of limiting parameters on substrate conversion during SSDF. Initial TS content has shown significant effect on the substrate degradation and metabolic by-products. The biohydrogen production ceased at TS content higher than 15%, therefore the TS content in the SSDF has to be maintained lower than 15 %, if the process is aimed at biohydrogen production. The investigation of the p_{H2} effect on hydrolysis of FW and WS showed that accumulation of H_2 as gaseous product does not have inhibitory effect on hydrolysis of organic substrates in SSDF. However, in general, the hydrolysis of substrate seemed to be limited under DF conditions due to low pH conditions.

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CHAPTER 6

DISCUSSION AND FUTURE PERSPECTIVE

6.1 Introduction and objectives

Several factors such as greenhouse gas emission and pollution problems from the by-products of combustion of the fossil fuels are driving biobased economy for the production of bioenergy and useful chemicals (Cherubini, 2010; Menon and Rao, 2012). In this regards, current research technologies need to be directed towards biorefinery, based on renewable sources such as waste biomass. Specifically, creation of hydrogen (H_2) based economy could hold the potential for future supply of energy. Moreover, production of H_2 from the biological pathways that utilize the renewable resources such as organic waste biomass can be promising and could ensure sustainable production of H_2. Among the different biological technologies, dark fermentation (DF) is one of the potential technologies for H_2 production and valuable by-products such as organic acids and alcohols. These by-products can be either recovered or further converted to other valuable biofuels and platform chemicals in physical, chemical and or biological systems (Bastidas-Oyanedel et al., 2015, Bonk et al., 2015).

DF processes utilizing waste biomass in scaled-up application are limited by low H_2 yields and use of process by-products (Ghimire et al., 2015). The H_2 production from complex waste biomass by dark fermentative mixed culture is strongly influenced by physico-chemical properties of the substrate and co-substrates, types of inocula, food to microorganism (F/M) or substrate/inoculum (S/X) ratio, substrate concentration, organic loading rates (OLR) in continuous bioreactors, pre-treatment of substrates, culture temperature, pH reactor configuration and hydraulic retention times (HRT) (De Gioannis et al., 2013; Ghimire et al., 2015a; Guo et al., 2010; Ntaikou et al., 2010; Urbaniec and Bakker, 2015; Wang and Wan, 2009). The H_2 yield and production rates from DF process can be enhanced by the optimization of these parameters (Figure 2.4). Furthermore, by-products from DF processes, which mostly include organic acids, alcohols and un-hydrolyzed residues can be utilized in other biological systems for their valorization by energy recovery (Figure 2.5). The DF effluents (DFEs) could be utilized in photo fermentation (PF) processes, which could increase the total H_2 yields from the substrate (Figure 2.14). In addition, biopolymer (polyhydroxybutyrate or PHB) can be produced concomitantly via PF processes, while the waste stream generated from coupling of DF-PF processes can be utilized in anaerobic digestion (AD) for further energy recovery as methane (Figure 5.1). Likewise, Solid State Dark Fermentation (SSDF) processes which

benefits from higher process yields and low energy and water requirements are attractive for biorefinery applications.

This study undertakes the aims to investigate the potential of DF of various complex waste biomasses for enhanced H_2 production. The results presented in Chapter 3 elucidate the effects of various operating parameters in dark fermentative H_2 production from a range of different waste biomass. The investigation on long-term operational feasibility of DF process for continuous H_2 production and application of co-substrates to support stability in H_2 production are demonstrated in Chapter 4. Moreover, a biorefinery concept is introduced in Chapters 5 by utilizing dark fermentation effluents in PF and AD for production of energy and biopolymers (PHB) and to investigate the limitations in the application of SSDF. The present chapter 6 summarizes and discusses the future implications of the major research findings in the application of DF processes for production of H_2 and other valued by-products by using mixed culture and complex waste biomass as feedstock. Moreover, the significance of integration with other biological systems for valorization of DF by-products is discussed in a biorefinery framework.

6.2 Major findings and highlights

6.2.1 Effect of operational parameters on dark fermentative H_2 yields

A number of studies have investigated the optimal operational conditions (e.g. culture pH, temperature, substrate utilization and inoculum enrichment) for maximizing H_2 production in DF(Cappai et al., 2014; De Gioannis et al., 2013; Ghimire et al., 2015a; Luo et al., 2010; Wang and Wan, 2011; Wong et al., 2014). However, selection of optimal operating parameters depends higly on substrate type. Therefore, investigations becomes vital in order to establish optimal operating conditions in the dark fermentative H_2 production from a particular feedstock type. Moreover, this study recommends that the biodegrability of the feedstock strongly influences the selection and application of various operating conditions (Chapter 3).

In a DF by mixed culture, the presence of H_2 producing microbial communities is important to achieve higher H_2 yields (Wong et al., 2014). In a scaled-up DF system utilizing waste biomass, mixed cultures are comparatively easier and less expensive to handle compared to pure cultures, as they do not require any asepsis procedure (Hawkes et al., 2007). This study evaluated the different H_2 producing inoculum preparation

methods for starting-up a DF process. Three types of inoculum pre-treatment methods that are commonly reported in literature studies, namely acid treatment, heat shock treatment (conducted at 95 °C and 105 °C) and load-shock treatment were applied to anaerobic digestate obtained from an anaerobic digester treating buffalo manure and cheese whey (Chapter 3, Section 3.1). The effectiveness of the inoculum pre-treatment methods was evaluated for H_2 production performance parameters such as cumulative H_2 production, H_2 production rate, length of the lag phase and process intermediates production in biohydrogen potential (BHP) tests fed with glucose (Figures 3.1 and 3.2, Table 3.1). Moreover, further evaluations were done based on operational costs and feasibility of the inoculum pre-treatment methods for scaled-up application of DF (Table 3.3). The results shown that load shock on anaerobic digestion can favor higher H_2 yields. This can be due to development and growth of an efficient H_2 producing bacteria community as reported by O-Thong et al. (2009). Therefore, load shock pre-treatment can be effective to prepare start-up inoculum for up-scaled DF systems. However, it should be taken into account that in a continuous DF reactor the selection of H_2 producing communities is a function of reactor operating conditions rather than only inoculum preparation or pre-treatment methods (Li and Fang, 2007). Nonetheless, this load shock method can be applied to adapt the biomethanation process for dark fermentative H_2 production. This could have application for the two-stage anaerobic digestion plants for biohythane (biohydrogen and methane) production (Figure 2.8).

Another set of BHP tests were carried to investigate the effects of initial culture pH (Figures 3.3, 3.4 and Table 3.6), combination of food to microorganism ratio (F/M) and initial culture pH (Table 3.7), substrate pre-treatment (Figure 3.5 and Table 3.8) and type of inoculum source (Figure 3.6 and Table 3.9) on the dark fermentative H_2 yields. Three model organic wastes, i.e. food waste, olive mill wastewater (OMWW) and rice straw were used as representative of readily, moderarately and slowly biodegradable substrates, respectively. BHP tests with food waste and heat treated anaerobic digestate have shown that a decrease of initial culture pH from 7.0 to 4.5 and 5.0 can increase the H_2 yields by 4.2 fold (60.6 ± 9 mL H_2/gVS) and 3.5 fold (50.7 ± 1 mL H_2/gVS), respectively. Furthermore, BHP tests carried out at pH 5.0 and 6.0 with F/M ratios 0.5, 1 and 1.5 have shown that the lower F/M ratios (0.5-1) at the initial pH 5 favored H_2 production in comparison to pH 6.5. Moreover, raw rice straw with alkaline treatment with 4% and 8% NaOH at 55 °C for 24 hours increased the H_2 yields by 26 and 57 fold, respectively.

Similarly, in the DF of OMWW, the H_2 yield was doubled when heat-shock pre-treated activated sludge was used as inoculum in comparison to anaerobic digestate. The anaerobic digestate took longer time to adapt to OMWW, which could be due to phenolic compounds present in it (Figure 3.6). This study recommends that the selection and application of different operating parameters to maximize the H_2 yields depends strongly on the biodegradability of the substrates (Table 3.10). These results have implications in the design of high rate DF reactors using complex waste biomass as substrate.

6.2.2 Continuous biohydrogen production

Continuous or semi-continuous processes are generally preferred for continuous H_2 production as they are more viable for scaling-up. Therefore, the future development of DF process at industrial scale relies on the successful operation of continuous processes, that can offer the advantages of steady operation compared to batch processes which involves regular downtime periods of maintenance (Hawkes et al., 2007). This study established a semi-continuous thermophilic DF process for H_2 production at low organic loading rates without controlling the culture pH. The continuous DF processes are not stable due to the decrease in culture pH as a result of the production of organic acids. Therefore, they require sources of alkalinity to maintain the culture pH at non-inhibiting acidogenic pH range (4.5 - 6). Most of the studies conducted on continuous and/ or semi-continuous dark fermentative H_2 production relied on addition of chemical buffering agents such as K_2HPO_4, $NaHCO_3$, Na_2HPO_4 (Carrillo-Reyes et al., 2012; Elsamadony and Tawfik, 2015; Valdez-vazquez et al., 2005). The high amount of chemical buffering agents might increase the operational cost of DF bioreactors at scaled-up production. It further adds uncertainty in the downstream processes applied for the treatment of DF effluent (DFE). This study has demonstrated a long-term feasibility of continuous H_2 production at varying operational conditions of the DF reactor (Section 5.1, Table 5.3). The optimal operational OLR equivalent to 2.5 g VS/L/d and HRT of 4 days have been established in a DF of food waste. These ranges of OLR and HRT values can be applied to the first stage of a two-stage AD process for the production of H_2 and CH_4, repectively (Aslanzadeh et al., 2014).

In another study presented in section 4.2, H_2 production stability was investigated in the DF of cheesewhey with buffalo manure as co-substrates. The results showed that buffalo manure charaterised by higher alkalinity could be used to maintain a culture pH at a range

4.8 - 5, during the DF process. The use of co-substrate aided in the stability of the continuous dark fermentative H_2 production (Table 4.9). This can give economic sustainability for a DF process inscaled-up applications, as it helps to achieve stability of H_2 production in an economical way, removing the dependency on chemical-buffering agents. Moreover, the co-fermentation can provide the biological treatment of waste that otherwise can pose environmental threats in places like Campania Region of Italy where cheese whey and buffalo manure are abundant by-products of agro-industrial activities (mozzarella cheese industries) (Ghimire et al., 2015b).

6.2.3 Integration of dark fermentation in a biorefinery concept

In addition to low process H_2 yields, an incomplete conversion of organic biomass adds another bottleneck in the commercialization of dark fermentative H_2 production (Gómez et al., 2011; Ren et al., 2011). Chapters 3 and 4 have shown that the higher H_2 yields and process stability can be achieved by optimizing the different operational parameters. However, higher substrate conversion of complex waste biomass cannot be achieved with a sole DF system and thus demands downstream process/es (Figure 2.5) (Gómez et al., 2011). Similarly, there have been increasing interests in incorporating DF into biorefinery concept utilizing it as a biological pre-treatment step (Bastidas-Oyanedel et al., 2015; Motte et al., 2015; Sambusiti et al., 2015; Venkata Subhash and Venkata Mohan, 2014). Recently, Motte et al. (2015) have reported higher substrate conversion of lignocellulosic biomass through integration of SSDF in a biorefinery approach. SSDF is advantageous in terms of higher process yields, due to its operation at high substrate loading rates and low water addition. Benefits, such as reduced reactor volume and specific energy requirements, simplicity in operation and handling of digestate, result in economic advantages of the SSDF process. In this context, Chapter 5 covers these aspects, which have been addressed by few studies.

Section 5.1 (Chapter 5) investigated the influence of integrating DF, PF and AD on total energy yields from three-step conversion of food waste (Figure 5.1 and Table 5.5). The supernatant, after separation of DFE, was used to recover H_2 from a PF process using *Rhodobacter sphaeroides* that increased H_2 yield from the food waste by 1.75 fold. The solid residual fraction of DFE along with PF effluent was converted into methane by AD, increasing the total energy yield from 1.13 to 5.55 MJ/kg $VS_{foodwaste}$ added. The three-stage conversion can achieve the higher energy yields compared to stand-alone DF or DF-

PF systems. In addition, the integration provided the biological treatment of residues. This was supported in studies from Xia et al. (2013a, 2013b), who reported that a three-step conversion of algal biomass combining DF-PF-AD can achieve 1.7 and 1.3 times higher energy yields in comparison to a two-stage DF-AD and a one stage AD process, respectively.

Moreover, purple non-sulfur bacteria (PNSB) can concomitantly synthesize H_2 and polyhydroxybutyrate (PHB) in PF process under certain conditions of physiological stress such as high Carbon/Nitrogen (C/N) ratio and sulfur deprivation (Waligórska et al., 2009; Eroglu and Melis, 2011). The capability of PNSB to utilize DFE generated from DF of complex organic waste for conversion to H_2 and PHB could be of economical interest (Figure 5.8). The PF of DFE by enriched mixed culture of PNSB and adapted culture of *Rhodobacter sphaeroides* provided several benefits, e.g. treatment of effluent by COD reduction and recovery of H_2 and PHB as added value products. Thus, integration of DF into a biorefinery concept can provide the economic sustainability to the scaled-up DF processes.

Furthermore, the last section of Chapter 5 dealt with the limitations of dry fermentation processes. SSDF processes are generally operated at total solids (TS) content higher than 15% and are constrained due to mass transfers limitations (Abbassi-Guendouz et al., 2012; Motte et al., 2013). Therefore, the TS content in SSDF could impact in conversion of feed to the desired fermentative products. In addition, accumulation of H_2 could also limit the conversion of substrates, as revealed in a study by Cazier et al. (2015) during Solid State Anaerobic Digestion (SS-AD). The affect of TS content and accumulation of H_2 on substrate conversion during SSDF was investigated with food waste and wheat straw as representative model substrates for readily and slowly degradable substrates.

During the SSDF of food waste, H_2 production was inhibited at a TS content higher than 15%, resulting in a lactic acid accumulation (Figure 5.9). This suggests that the TS content plays a vital role and for the case of SSDF of food waste, TS content has to be less than 15% if the process is aimed at H_2 production. Moreover, the accumulation of H_2 as gaseous products does not exhibit inhibitory effects on hydrolysis of organic biomass during SSDF in contrast to SS-AD (Figures 5.11 and 5.12). This could be due to the operational culture pH of these two different processes. The lower hydrolysis during DF process can be due to the inhibition of hydrolase enzyme at acidic pH (Parawira et al.,

2005). However, in general, the hydrolysis of substrate seemed to be limited under DF conditions as found in this study.

6.3 Future research prospective

The current study addressed several issues and potential in the application of DF of waste biomass interlinked in a biorefinery concept. However, more research needs to be directed at the pilot to full scale implementation of DF process based on real feedstock such as organic waste biomass (Bonk et al., 2015). Moreover, integration of DF with other physical, chemical and or biological systems can improve energy yields which could lead to reduction of the operational costs associated with DF and create possibility for revenues from the recovery of added value chemicals in side stream process.

Based on the local availability, novel feedstock sources, which do not compete with food and agriculture supply chain, should be utilized in DF process. The agricultural residues (straw, corn stover, manure, waste timber cuttings), municipal, agro-industrial waste and biomass sources such as micro and macro algae could serve as the future supply of feedstock for DF based biorefinery. A major difficulty in the utilization of this feedstock sources is poor biological hydrolysis that limits the complete conversion of biomass into intended products, as the efficient dark fermentative conversion depends on the presence of readily available depolymerized carbohydrates (monomeric sugars) (Guo et al., 2013; Monlau et al., 2012). However, different physical, chemical, biological and the combination of these pre-treatment methods can be applied to enhance biological hydrolysis depending on the physico-chemical characteristics of biomass (Carrere et al., 2015; Monlau et al., 2013). More studies regarding technical and economical feasibilities of pre-treatment methods could elucidate their application to DF process.

The selection of optimum operational parameters, such as culture pH, temperature, substrate concentration, substrate, loading rate, food to microorganism ratio and reactor configuration during DF of waste biomass is strongly dependent on substrate type and source of inoculum (Tables 2.1, 2.5, 2.7 - 2.9 and 3.9). Therefore, the selection of these parameters for a particular substrate type and experimental conditions needs investigations before full-scale implementation of the process. Moreover, additional research is required in process control with an aim to enhance the yield and recovery of other targeted metabolic by-products such as acetate, butyrate, propionate, ethanol, etc. In recent years, DF was extensively reviewed and aimed for H_2 production. However,

dark fermentative H_2 production accounts only 4% of the total products conversion with the maximum theoretical conversion, while 67% remains as by-products in liquid phase, i.e. acetic acid in a DF of glucose (Bastidas-Oyanedel et al., 2015).

Similarly, microbial community analysis during the varying operating conditions could further elucidate the existence of different fermentative communities responsible for yields of varying metabolites. The identification and enrichment of fermentative communities could be a precursor for future application of DF process in the production of targeted biomolecules in a biorefinery concept.

The residues generated from the DF process needs down stream process/es for the complete utilization of waste biomass. Biopolymer production in PF utilizing DFE can be economically interesting and requires investigations in process optimization for PHB production (Section 5.2). Moreover, investigations in the technologies for the economic recovery of H_2 (gas cleaning and purification) and associated biochemicals (VFAs and alcohols) from DF fermentation will determine the future application and development of the DF process (Bonk et al., 2015).

Compared to wet fermentation processes which require large reactor volume and have consequently higher energy requirements to treat same quantity of biomass, SSDF can offer benefits in terms of higher volumetric production rates due to higher substrate concentration (Elsamadony and Tawfik, 2015; Romero Aguilar et al., 2013). This research work investigated the technical limitations of SSDF (Section 5.3, Chapter 5). However, additional research is essential in the operational control of the SSDF process towards intended metabolites production in SSDF, as this technology could hold greater promise in the creation of future biorefinery for the production of biohydrogen and biomolecules.

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