Bioenergy Production From Sewage Sludge Via Enhanced Anaerobic Digestion And Dark Fermentation.

by

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The candidate confirms that the work submitted is his own, except where the work has formed part of a jointly-authored publication and has been included. Also, appropriate credit has been given within the thesis where reference was made to the work of others.

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DEDICATION

This thesis is dedicated to

my lovely family

for their unconditional support and love

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What a journey…

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ABSTRACT

Energy demands have dramatically increased over the last decade, and a revolution in technology and lifestyles has led to the use of fossil fuels to cover this demand, negatively impacting our environment and causing an increase in greenhouse gases (GHG) and global warming. Aside from their environmental impact, fossil fuels are an unsustainable source of energy. Hence, researchers have focused for many years on finding new sources of energy. Sewage sludge is a sustainable source of energy production and has long been used as feedstock for anaerobic digestion (AD) systems for biogas production. Ever stricter environmental restrictions on sewage sludge discharge/disposal and the reliance of wastewater treatment plants (WWTPs) on AD (as the preferred technology for sewage sludge handling) has led to an increase of sewage sludge volume in recent years. This situation is challenging for WWTP; moreover, AD increases GHG (30–40% of produced biogas is carbon dioxide). Therefore, current sewage sludge management needs to upgrade the produced biogas by increasing methane yield and reducing carbon dioxide. There is potential to upgrade the biogas through hydrogenotrophic pathway by addition of hydrogen gas into AD to be combined with carbon dioxide and produces extra methane gas and, therefore, achieving higher energy output, sewage sludge utilisation and volume reduction. This study investigates the hydrogen potential of sewage sludge by using the dark fermentation (DF) process, a promising biological hydrogen production method. Results reveal need to apply an upgraded method of optimising biogas production and increasing methane yield; the need for an inoculum pre-treatment prior to DF; and the importance of a substrate pre-treatment, such as enzymatic hydrolysis (EH). Further investigation and assessment of the DF process is required to create other opportunities to use sewage sludge as feedstock for sustainable hydrogen production.

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS/ABBREVIATIONS

AD	Anaerobic digestion
ADBA	Anaerobic digestion biogas association
ADS	Anaerobically digested sludge
AST	Acid shock pre-treatment
ATP	Adenosine triphosphate
BD	Anaerobic biodegradability
BHP	Bio-Hydrogen potential
BMP	Biochemical methane potential
BST	Basic shock pre-treatment
C/N	Carbon to Nitrogen ratio
CSTR	Continuously stirred tank reactor
Defra	Department for Environment, Food and Rural Affairs
DF	Dark fermentation
EH	Enzymatic Hydrolysis
EU	European Union
FID	Flame ionization detector
FW	Food waste
G	Glucose
GC	Gas chromatography
GSW	Gelatin solid waste
HPLC	High-performance liquid chromatography
HRT	Hydraulic retention time
HSS	Hydrolyzed sewage sludge
HST	Heat shock pre-treatment
HTP	Hydrothermal treatment plant
ISR	Inoculum to substrate ratio
NADH	Nicotinamide adenine dinucleotide
NNFCC	National Non-Food Crops Centre
OFMSW	Organic fractions of municipal solid waste
OLR	Organic loading rate
PMS	Paperboard mill sludge
PS	Primary sludge
PSI	Photosystem I
SAS	Thickened secondary sludge
sCOD	Soluble chemical oxygen demand
SRT	Solid retention time
SS	Sewage sludge
TCD	Thermal conductivity detector
TCOD	Total chemical oxygen demand
THP	Thermal hydrolysis process
TMP	Theoretical methane potential
TNb	Total Nitrogen
TS	Total solids
VFA	Volatile fatty acids
VS	Volatile solids
WAS	Waste activated sludge
WRAP	Waste Resources Action Programme
WWTP	Wastewater treatment plant

Chapter 1
Introduction

This chapter presents an overview of the current challenges faced by the wastewater industry, particularly with regard to the long-term sustainability of treatment processes that are energy-intensive and the opportunities arising from in-house production of renewable energy. The up- and downstream processes required for bioenergy production from sewage sludge are described, starting from sewer-based collection and the transportation of sewage to wastewater treatment plants (WWTPs); then, the thesis addresses types of treatments (physical, chemical and biological) and, finally, discusses sewage sludge processing via anaerobic digestion (AD) for bioenergy production. This chapter also describes alternatives to carbon-based bioenergy production and the role of biohydrogen gas production in reducing net-carbon emissions by upgrading biogas in AD units, considering conventional and unconventional hydrogen production methods. Furthermore, it gives an overview of bio-hydrogen production from sewage sludge via dark fermentation (DF), including process limitations, operation parameters and optimising methods. The research problem and gaps are mentioned in this chapter, as well as the project aim, scope and objectives. A brief description of each chapter in this thesis is included in the thesis structure section at the end of this chapter.

1.1 Overview

Energy demand has dramatically increased since the industrial revolution, as advances in technology and changes in people's lifestyles have led to a global dependency on fossil fuels. About 88% of current energy demand is covered by fossil fuels as an energy source (van der Hoeven, 2015). However, the resulting

impacts on the environment have become one of the most important challenges faced by humankind. A high consumption of fossil fuels affects the environment, mainly from the emission of gases from the combustion process, which releases gases and pollutants such as COx, NOx, SOx, CxHx, soot, ash, droplets of tar and other organic compounds into the atmosphere (Das and Veziroğlu, 2001). These pollutants have a significant impact on the environment and lead to increased concentrations of GHG in the atmosphere, as well as global warming. Aside from environmental impacts, however, fossil fuel reserves are being depleted at a rate which is unsustainable in the long run. Thus, for these two principal reasons (environmental impacts and an uncertain energy supply future), researchers have focused on finding alternative sources of energy for many years. One of these sources is the organic matter present in wastewater, which can be used to produce renewable energy via biological processes.

WWTPs produce two main streams: effluent water (treated wastewater) and sewage sludge. Sewage sludge is rich in organic and inorganic substances from which various materials can be recycled, recovered or reused as secondary sources for many products, including the production of sustainable energy through different technologies (Kim et al., 2015). An AD system is one such technology. It uses sewage sludge as a source to produce biogas, which can then be used on its own or to generate heat and power. Figure (1.1) shows how modern WWTPs integrate energy production from sewage sludge using AD. Using sewage sludge for energy production is an important step towards improving environmental protection, controlling pollution reduction and generating sustainable energy at WWTPs.

Figure 1.1 Anaerobic digestion in a wastewater treatment plant, adapted from (Ferris, 2015).

Biogas is an excellent substitute for conventional fuels and one of the main bioenergy sources that can be produced from organic waste. Many technologies can be used to produce it. AD technology is widely implemented for wastewater treatment, organic mixed solid waste and biogas production (Ferguson et al., 2018). The main advantage of AD is that it is a sustainable, energy-efficient technology for bioenergy (biogas) production (Nishio and Nakashimada, 2007). Typical AD produces biogas which contains, by volume, 50%−70% methane and 30%−50% carbon dioxide, small amounts of hydrogen sulfide (50–2000 ppm), water vapour and various trace hydrocarbons (Bassani et al., 2015, Braun, 2007). Another benefit of biogas is that 1 m^3 of biogas can replace about 0.6 litres of heating oil, which can lead to a decrease in the amount of energy used for heating applications (Baciocchi et al., 2013).

Methane is a natural hydrocarbon containing one carbon atom and four hydrogen atoms; it can be used in a combined heat and power (CHP) engine to produce both heat and electricity (Ayala-Parra et al., 2017). Biogas can be turned into

clean energy by increasing methane content to >90% of the biogas content. This upgraded biogas can be used as vehicle fuel or injected into and transported by the natural gas grid (Deng and Hägg, 2010).

A high-performance AD can be achieved by increasing the net methane yield; many attempts have been made to do so and to optimise AD technology. Co-digestion, feedstock pre-treatment, digestate post-treatment, biogas purification and hydrogen injection have been used to increase methane yield and enhance AD performance; however, many difficulties and challenges remain, especially when these methods are applied to existing AD sites working at a commercial scale.

1.2 Sewage sludge in the UK

Wastewater is produced from homes and municipal, commercial and industrial buildings; more than 12 billion litres of wastewater are produced and treated every day in the UK (Tiseo, 2021). The primary objective of a WWTP is to improve water quality and produce an effluent that meets the corresponding discharge consents to reduce environmental impacts on the receiving water body. As a consequence of the undertaken treatment, a significant amount of sewage sludge is generated.

Before 1998, around 25% of sewage sludge in the UK was either discharged to surface waters or disposed of into the sea (Defra, 2012). However, after the implementation of the EU Urban Waste Water Directive in 1998, these practices were suspended, and alternative methods to treat and dispose of sewage sludge have since been developed. High standards of wastewater treatment to progressively control organic matter, solids, nitrogen and phosphorus were announced in 1998, 2000 and 2005, which have also led to the generation of

much greater quantities of sewage sludge (Defra, 2012). Figure (1.2) shows how the changes in regulations and standards caused changes in sewage sludge reuse and disposal routes.

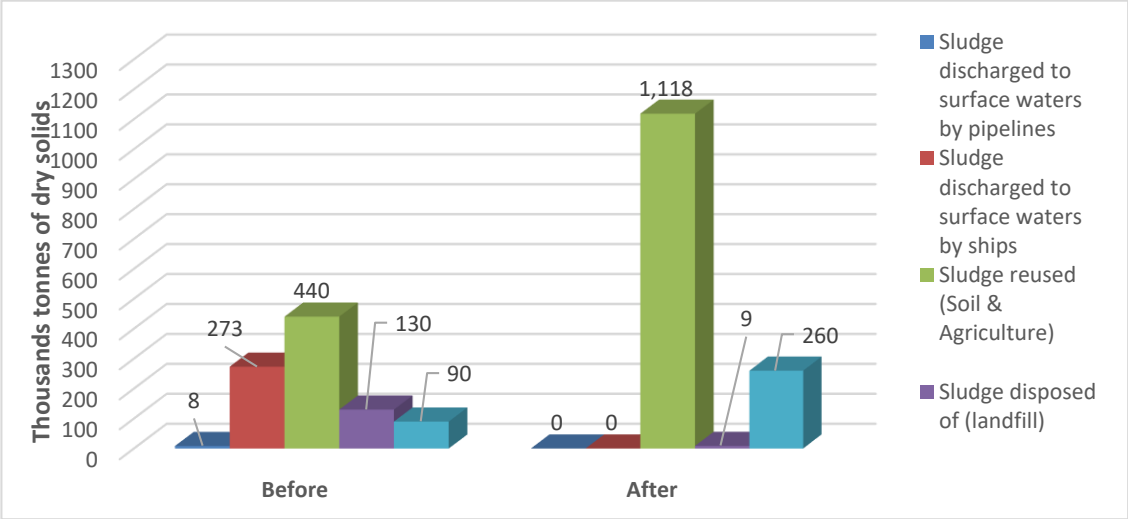


Figure 1.2 Sewage sludge reuse and disposal routes before and after the implementation of the EU Urban Waste Water Directive in 1998, adapted from (Defra, 2012).

About 75% of sewage sludge is now used by AD technology in the UK (Defra, 2012), as AD can reduce residual sewage solids, which need to be disposed of, or generate biogas, which is considered a renewable energy source. Thus, the number of incinerators is decreasing not only due to challenges such as gas emission control and the stabilisation of operation processes but also because of the availability of more stable and sustainable technologies to reuse sewage sludge, such as AD technology.

According to NNFCC (2021), in 2019 there were 642 AD plants in the UK, treating 15.5 million tonnes of waste material annually, 203 of which used waste (sewage sludge, food waste and others). The electricity generated through AD increased from 117 GWh in 2010 to 2,052 GWh in 2016, due to the viability of generating biogas through AD facilities which was later used for bioenergy production, including electricity (Jaganmohan, 2018). Despite the increment in

AD plants and electricity generation, still methane yields are limited and biogas quality cannot go beyond 70% CH_4, without the use of biogas upgrading processes that produces biogas suitable for grid injection or transport use. That limits the use of biogas to in-situ energy production with great inefficiencies and energy losses. Therefore, biogas upgrading method can be assessed to enhance methane yield and improve AD processes. Thus, many studies and experimental works have been conducted to find methods to upgrade biogas by increasing methane yields, reducing carbon dioxide, removing inhibitors and purifying the final biogas. Several upgrading methods have been developed, classified as either ex-situ or in-situ (Suhartini et al., 2014, Bouallagui et al., 2004). Considerable effort is now being made to optimise AD by upgrading biogas, in particular by using hydrogen to convert carbon dioxide to methane during the methanogenesis process. There are three pathways in the methanogenesis stage: the hydrogenotrophic, acetotrophic and methylotrophic (Aryal et al., 2018). To date, the hydrogenotrophic pathway is the most metabolically efficient pathway for upgrading biogas quality and increasing the methane yield (Lever, 2016). However, most studies related to upgrading biogas by hydrogen used sewage sludge as an inoculum only (Luo et al., 2012, Luo and Angelidaki, 2013a, Wang et al., 2013, Xu et al., 2015, Kougias et al., 2017). Moreover, these studies were conducted quite recently (from 2012 to 2017), which indicates that studying biogas upgrading by hydrogen is a relatively new trend, emerging in the past 10 years. Thus, there is still much work to be done, for example in using different hydrogen production methods with less energy consumption. Different methods of hydrogen production, such as the biological methods, need to be applied to AD. The most frequently implemented method for hydrogen production is water electrolysis operated by windmills, but this

method is high in energy consumption; finding another pathway by which to generate hydrogen and use it to increase methane yield will help reduce the cost of this method and open a new way to optimise AD.

1.3 Other opportunities to use sewage sludge

Sewage sludge has the potential to be a sustainable source for hydrogen production through DF processes. DF is a biological process that encourages fermentative bacteria to hydrolyse organic substrates to produce energy carriers such as hydrogen gas. Sewage sludge is an organic-rich feedstock that can be utilised in the DF process for sustainable bio-hydrogen production. In the past 10 years, the DF process has been developed as an interesting pathway for bio-hydrogen production. Different studies have investigated the feasibility of DF processes in producing hydrogen gas, but the use of sewage sludge as feedstock is very limited and faces many challenges; for example, the low carbon content and complexity of the sewage sludge structure makes it difficult for bacteria to utilise it and convert it to hydrogen gas in DF (Xia et al., 2016). Therefore, the characteristics of feedstock are critical to the performance of DF/bio-hydrogen and AD/biogas production. For example, the methane and hydrogen production values presented in this study may differ if a different feedstock is used, such as Faecal sludge, septage waste or organic fraction of municipal solids waste. As different feedstock has different carbon to nitrogen (C/N) ratio which is one of the parameters that has effect on methane and hydrogen production from AD and DF, respectively. For that reason, the type of feedstock should be considered for enhancing the quality and quantity of methane and hydrogen production via those technologies. Moreover, the work described in this thesis gives an idea of bio-hydrogen potential with respect to

relevant carbon content of the used feedstock and provide a solution that can be applied on different waste (i.e sludge comes from other sanitation systems such as septic tanks and pit latrines) for enhancing the carbon content in the feedstock which will led to improve the bio-hydrogen production from DF.

Despite the challenges, sewage sludge is a very promising option for use as a feedstock for bio-hydrogen production via DF. Sewage sludge has the potential to be a sustainable source to promote bio-hydrogen production using the glucose route, which is considered the most favourable and stable route for fermentative bacteria (e.g., *Clostridium* bacteria) (Finlay, 1995). Champagne (2007) reported that an estimated 6.22 Mt/year of glucose can be produced from municipal sludge (387,166 t/year) and livestock manure (177.5 Mt) generated in Canada.

1.4 Research problem statement

Nowadays, municipal wastewater is treated in wastewater treatment plants and during treatment processes, two main products are produced: effluent water (treated wastewater) and sewage sludge. Activated sludge treatment is very commonly implemented in WWTPs and is considered one of the best technologies for biological treatment currently available. The reliance of WWTP on using this technology, as well as population growth, has created environmental challenges as the quantity of sewage sludge continues to rise. Sewage sludge is rich in organic and inorganic substances from which various materials can be recycled, recovered or reused as secondary sources for many products, including the production of sustainable energy through different technologies (Kim et al., 2015). While in the recent years, different technologies have been developed and applied in WWTPs to manage the large quantities of sewage sludge. The main purpose of these technologies is to reduce the overall

volume of sewage sludge and minimise environmental pollution. For example, anaerobic digestion is widely implemented in WWTPs, and sewage sludge management currently relies on this technology. In the UK, about 75% of sewage sludge is now treated with AD technology, because AD can reduce the residual sewage solids which need to be disposed of or generate biogas, which is considered a renewable energy source (Defra, 2012). However, the continuous rise in wastewater volume and ever-tighter environmental restrictions have led to a dramatic increase in the volume of sewage sludge. Also, the reliance of WWTPs on activated sludge as preferred technology for biological wastewater treatment and AD as preferred technology for managing sewage sludge and bioenergy production, necessitate the need of upgrading the existing AD plants. In addition, AD biogas contains about 50%−70% methane and 30%−50% carbon dioxide (Bassani et al., 2015, Braun, 2007). So still this technology contributes to GHG emissions by carbon dioxide production. So, for these reasons there is a need to upgrade the biogas from AD by increasing the methane content and lowering carbon dioxide.

Many studies have investigated methods of upgrading biogas to enhance methane yield. There is potential to upgrade the biogas through hydrogenotrophic pathway by addition of hydrogen gas into AD to be combined with carbon dioxide and produces extra methane gas and, therefore, achieving higher energy output, sewage sludge utilisation and volume reduction (Lever, 2016). However, the source of hydrogen constitutes another challenge; currently, most hydrogen gas is generated by conventional and electrolysis technology, which consumes energy and negatively impacts our environment. Hence, another method must be investigated to produce hydrogen in a more sustainable way, increasing the overall sustainability of using it in AD and encouraging

investors (water companies, farmers, etc.) to upgrade their AD processes. DF is one of the biological methods used to produce hydrogen and may be used as an integral part of AD plants as a source of hydrogen to enhance biogas production. On the other hand, studies such as those conducted by Cheng et al. (2016), Tyagi et al. (2014), Liu et al. (2013) have proven that bio-hydrogen gas can be produced from sewage sludge through DF, although the amount produced is still limited by many factors (such as operational conditions and feedstock complexity). So still there is a need for DF optimisation to enhance the bio-hydrogen production from sewage sludge; however, if DF is optimised and able to produce sufficient amounts of hydrogen, this may create another renewable source (sewage sludge) for bio-hydrogen production, rather than using hydrogen to upgrade AD biogas.

1.5 Research gap statement

This research addresses critical research gaps related to bioenergy production from AD and DF processes aimed at enhancing the overall process of, and optimising methane and hydrogen production for, sustainable bioenergy production at WWTPs. As sewage sludge volume is increasing day by day, due to population growth and ever stricter environmental restrictions on sewage disposal, there is a need to optimise biogas production from AD.

Many studies have investigated methods of upgrading biogas to enhance methane yield, one of the most promising being to optimise the methanogenesis stage at AD by increasing methane production through hydrogenotrophic methanogenesis reactions (hydrotropic pathway) by hydrogen injection. However, this method requires a sustainable supply of hydrogen, and the current hydrogen supply relies on production methods using natural gas decomposition,

petroleum oxidation, coal gasification and water electrolysis. These methods are considered disadvantageous to the whole biogas upgrading approach because they require high energy consumption and are linked to net GHG emissions. Thus, using DF as an alternative for hydrogen production can increase the feasibility of upgrading biogas, making it more attractive for investors and meeting environmental and sustainability targets.

Considering that very limited research has investigated the use of sewage sludge as a substrate for hydrogen production via DF, due to the complexity of sludge contents and the difficulty of reaching stable fermentative bacterial processes under a low C/N ratio (Xia et al., 2016). The C/N ratio is an important parameter characterising DF substrates, as a low C/N ratio is one of the main reasons for low hydrogen production in DF. Mata-Alvarez et al. (2014) reported that the suitable range of C/N ratio for fermentative bacteria is 20–30, while the C/N ratio of sole sludge is usually between 4 and 10. Yang and Wang (2017) summaries and reported the studies that investigated the bio-hydrogen production from sewage sludge. Most of these studies have reported very limited hydrogen production from sewage sludge (including different types: anaerobically digested sludge, waste-activated sludge, primary sludge and thickened sludge). Except one study reported relatively high hydrogen production (28.3 mL/g-VS removed) (Tyagi et al., 2014) and that related of using mixed sludge (municipal solid waste + sewage sludge) which has higher C/N ratio that enhanced the hydrogen production. So, there still need to assess the potential of using sole sewage sludge for bio-hydrogen production. Moreover, there is no study used HSS as a feedstock for DF, so this gap will be addressed in this thesis as this will add more knowledge to the field. Moreover, assessing bio-hydrogen production from this specific feedstock (HSS) will show the potential of using it for bio-hydrogen

production application and upgrading the current biogas production applications (thermal hydrolysis process (THP) + AD). As HSS is the product of (THP) which is widely implemented in WWTPs, especially in Europe, as a pre-treatment for AD.

So, this thesis addresses the following gaps: (a) investigation of the potential of hydrogen production from Hydrolysed sewage sludge; (b) finding solutions to current limited hydrogen yields; and (c) optimising the DF process to enhance hydrogen production.

1.6 Aim, scope and objectives

The overall aim of this study was to optimise bioenergy production processes at WWTPs using sewage sludge as a feedstock for DF processes. In particular, this study aims to assess the potential of using DF (biological technology for bio-hydrogen production) as a hydrogen supply for upgrading AD biogas quality via a hydrotropic pathway (hydrogen injection into AD). This has been planned by studying the potential of methane production from enhanced AD and the potential of hydrogen production from DF. The influence of key process control parameters and different optimising strategies to increase hydrogen yields in DF were investigated at a laboratory scale using real sewage sludge from a local WWTP. Results were used to define potential applications within the wastewater industry.

The research objectives of this project were as follows:

- To determine the current state of knowledge with respect to increasing the methane and hydrogen yield in biogas produced from AD and DF of sewage sludge by carrying out a literature review.

- To evaluate the changes to hydrolysed sewage sludge (HSS) and digestate characteristics over a year.
- To assess the Biochemical methane potential (BMP) of HSS and create a baseline that helps comparison for any works related to upgrading methane yield.
- To assess the impact of different inoculum pre-treatment methods used to enhance hydrogen production through the DF process.
- To assess the conversion path of glucose to hydrogen in DF reactor using HSS as feedstock.
- To assess the Bio-Hydrogen potential (BHP) of HSS and create a baseline that helps comparison for any works related to upgrading hydrogen yield.
- To select and assess a simple and reliable method for measuring glucose concentration in HSS samples.
- To assess glucose production from HSS using the enzymatic hydrolysis (EH) process.
- To evaluate the effect of using enzymes in DF.
- To optimise hydrogen and VFA production by integrating EH with DF.

1.7 Thesis structure

This thesis consists of nine chapters, each of which is briefly described below.

Chapter 1: Introduction

This chapter presents an overview of the bioenergy production process from sewage sludge, from collection from the WWTP to types of treatment (physical, chemical and biological) and, finally, processing via AD for biogas production. It also describes the role of hydrogen gas in upgrading biogas in AD and

conventional and unconventional hydrogen production methods. Moreover, it gives an overview of the bio-hydrogen production from sewage sludge via the DF process, including limitations, influence parameters and optimising methods and presents the research problem, gaps, aim, scope and objectives.

Chapter 2: Literature review

This chapter gives a detailed literature review of biogas production from AD and DF processes and describes the influence of operation parameters/factors and configuration types for both AD and DF reactors. Different biogas upgrading methods, including hydrogen ex-situ/in-situ, are also mentioned. The importance of hydrogen gas and available production methods are reviewed, and the DF process and its reactions are described, along with the influence of operating parameters. In addition, optimising methods to enhance hydrogen production from DF that processes sewage sludge are mentioned.

Chapter 3: Research Methodology and Experimental Design

This chapter describes all the experimental research activities conducted during this project and gives detailed experimental setups and statistical and other analyses. Details of BMP, BHP and EH tests are also described.

Chapter 4 to 7: Results of experimental work

In these chapters, all the results of the experimental work carried out are reported and discussed. Chapter 4 addresses sewage sludge characterisation and biochemical methane potential, after which chapter 5 addresses the investigation of the impact of the inoculum pre-treatment on the performance of DF and BHP of hydrolysed sewage sludge. Then, chapter 6 assesses the EH of sewage sludge as a pre-treatment for DF. Finally, chapter 7 assesses the impact of the EH process in enhancing hydrogen yield from sewage sludge via the DF process.

Chapter 8: General discussion

This chapter discusses and summarises the findings in this research which can be serve as guidance for future research related to bioenergy (methane and hydrogen) production from sewage sludge.

Chapter 9: Conclusions and recommendations

This chapter gives conclusions and recommendations which can be serve as guidance for future research related to bioenergy (methane and hydrogen) production from sewage sludge.

Chapter 2
Literature review

This chapter gives a detailed literature review of biogas production from sewage sludge using AD and DF processes. It also describes key operation parameters and environmental factors, along with process configuration options, for both AD and DF reactors and discusses different biogas upgrading methods, including hydrogen ex-situ/in-situ. The importance of hydrogen gas as an alternative to fossil fuels and the currently available production methods are critically discussed. The DF process and corresponding biochemical reactions are also described in this chapter, along with the influence of operation parameters. In addition, the chapter addresses optimising methods to enhance hydrogen production from DF processing of sewage sludge.

2.1 Sewage sludge

Sewage sludge is a by-product that is generated and accumulates every day in a WWTP. Sewage sludge has different physical conditions, depending on the treatment stage which has produced from it. For example, sewage sludge can be slurry, semi-sold or solid. Two wastewater stages are mainly responsible for generating most of the sewage sludge inside WWTPs, namely primary and secondary treatment stages, and sewage sludge is usually classified in WWTPs as primary or secondary sewage sludge. Primary sewage sludge is the sludge that accumulates due to the physical/chemical activities which occur at this stage (i.e., chemical precipitation, sedimentation and other primary processes), while secondary sewage sludge is generated by biological treatment (i.e., activated sludge). Activated sludge treatment is very commonly implemented in WWTPs and is considered one of the best technologies for biological treatment currently available. WWTPs' reliance on this technology, as well as population growth, has

created environmental challenges as the quantity of sewage sludge continues to rise.

In the UK, more than 12 billion litres of wastewater are produced and treated every day (Tiseo, 2021). After the implementation of the EU Urban Waste Water Directive in 1998, sewage sludge handling management became challenging for WWTPs as it was thereafter illegal to discharge the sewage sludge to surface water (the old disposal method). The continuous rise in wastewater volume and ever-tighter environmental restrictions have led to a dramatic increase in the volume of sewage sludge: according to (Eurostat, 2021), the production of sewage sludge has increased from 5.82 (2007) to 8.0 (2016) Mt of dry solid per year in EU countries and is expected to reach 10 Mt dry solid per year by 2030. Thus, in this situation, managing sewage sludge remains a challenging task.

Therefore, different technologies have been developed and applied in WWTPs to manage large quantities of sewage sludge. The main purpose of these technologies is to reduce the overall volume of sewage sludge and minimise environmental pollution. A series of sewage treatments is commonly used in WWTPs to ease the handling process of sewage sludge. The first treatment is sludge thickening, whose main purpose is to reduce the sewage sludge volume, usually by the use of gravity thickener and dissolved air flotation technologies. The second is sludge digestion, which is considered a biological treatment that has the ability to kill pathogens inside sewage sludge and also generates biogas which can be used later for energy production via CHP. Thirdly, a dewatering process is carried out by sludge-drying beds which, although commonly used, is extremely time-consuming. Therefore, solid–liquid separation and centrifuge technologies are much preferred and can save time. Finally, the product is disposed of, either into landfill, by incineration or for agricultural use as fertiliser.

AD is widely implemented in WWTPs, and sewage sludge management currently relies on this technology. In the UK, about 75% of sewage sludge is now treated with AD technology (Defra, 2012), because AD can reduce the residual sewage solids which need to be disposed of or generate biogas, which is considered a renewable energy source. According to (NNFCC, 2021), there are 642 AD plants in the UK, treating 15.5 million tonnes of waste material annually, 203 of which used waste (sewage sludge, food waste and others) as feedstock. The electricity generated through AD increased from 117 GWh in 2010 to 2,052 GWh in 2016, due to the viability of generating biogas through AD facilities which was used later for bioenergy production, including electricity (Jaganmohan, 2018). These numbers show how WWTPs rely on AD to manage sewage sludge; therefore, the following sections address the AD technology (principle, reactions and influence of operating parameters) and a promising method to upgrade the AD process, in particular increasing the methane yield in the produced biogas to improve its quality (high methane content equivalent to natural gas). It is also necessary to upgrade AD, as this technology still contributes to GHG emissions by carbon dioxide production (30%–40% of biogas is carbon dioxide). This chapter also addresses biological gas upgrading using hydrogen, which is a promising technology in this field which may be the key to reaching a sustainable solution. Also, the DF process (bio-hydrogen production method) is addressed in this chapter as a sustainable and promising method for hydrogen production, especially when sewage sludge is used as feedstock.

2.2 Anaerobic digestion

AD is a natural process which occurs when microorganisms convert organic matter, such as complex carbohydrates, to biogas in the absence of oxygen (i.e.,

in an anaerobic environment); this process occurs naturally in wetlands, landfills and the stomachs of ruminant animals (Novaes, 1986). Biogas is the main product of AD processes and contains about 50%−70% methane and 30%−50% carbon dioxide, with small amounts of hydrogen sulfide (50–2000 ppm), water vapour, oxygen and various trace hydrocarbons (Bassani et al., 2015, Braun, 2007). Digestate is another product of AD that contains bacterial biomass, remaining and partially treated feedstock, and mineralised products, which make it highly rich in nutrients and suitable for use as a fertiliser (Korres et al., 2013).

Different wastewater streams are currently treated by AD, including sewage sludge. The performance of AD reactors depends on the right consortium of microorganisms responsible for converting complex organic molecules, such as lipids, carbohydrates and proteins, to methane and carbon dioxide through several biological steps.

2.2.1 Anaerobic digestion reactions

Several reactions occur inside an AD reactor and can be classified as two types. The first includes physicochemical processes (e.g., liquid–liquid, gas–liquid mass transfer and liquid–solid transformations) and the second involves biochemical reactions (e.g., hydrolysis, acidogenesis, acetogenesis and methanogenesis). The following section explains the biological reactions that occur during AD processes.

2.2.1.1 Biological reactions

Hydrolysis, acidogenesis, acetogenesis and methanogenesis are the four key stages in AD biological reactions. Several groups of bacteria and substrates are involved in these reactions under anaerobic conditions, converting organic material to methane and carbon dioxide. Figure (2.1) shows the four stages of AD and types of bacteria involved.

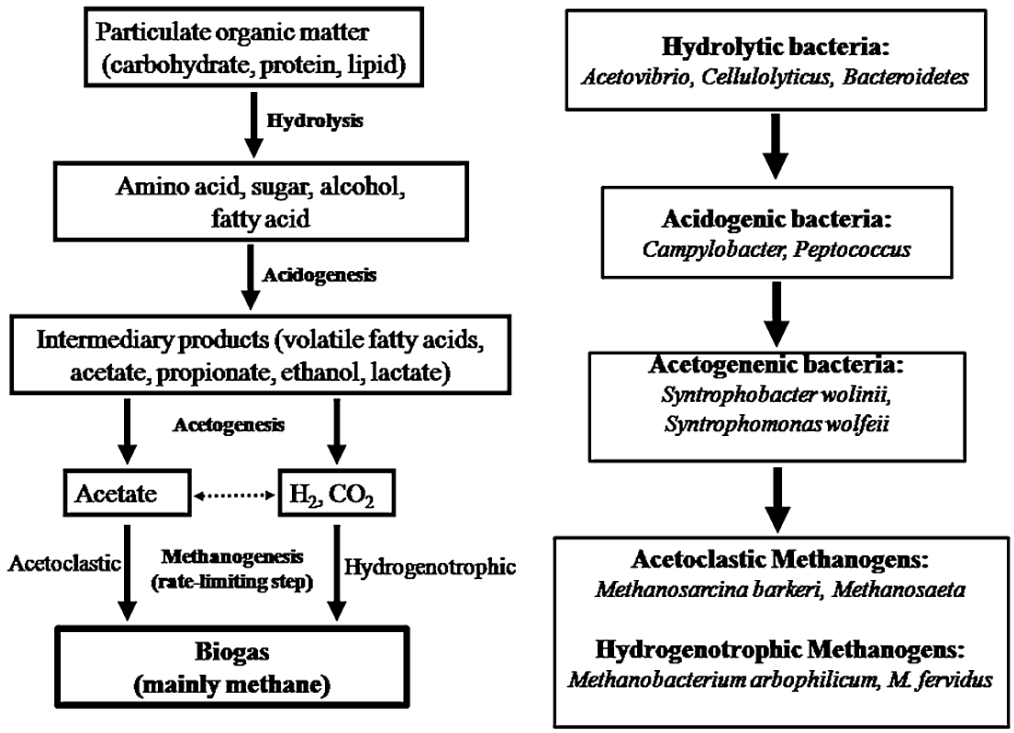


Figure 2.1 Four stages of an anaerobic digester and the bacteria involved in different stages of anaerobic digestion, source (Goswami et al., 2016).

Hydrolysis is the first stage of AD, during which the microorganisms produce different types of enzymes, which break down macromolecules into simple forms. When sewage sludge is fed into an AD reactor, particulate organic matter, such as carbohydrate, protein and lipids, is converted to amino acids, sugars, alcohols and fatty acids. Different microorganisms are active in the hydrolysis stage, for example bacterial groups such as *Bacteroides*, *Clostridium* and *Acetivibrio* (Cirne et al., 2007, Heeg et al., 2014). The hydrolysis reaction is explained in Equation (2.1), where the organic material breaks down into glucose and hydrogen (Kondusamy and Kalamdhad, 2014).

$$nC_6H_{12}O_5 + nH_2O \quad \rightarrow \quad nC_6H_{12}O_6 + nH_2 \qquad (2.1)$$

Acidogenesis is the second stage of AD, in which microbial consortium diversity dramatically increases and reaches a peak. A new group of microorganisms belonging to the *Enterobacterium*, *Acetobacterium* and *Eubacterium* genera become predominant and are involved in both the acidogenesis and hydrolysis

microorganism stage (Schnurer and Jarvis, 2010). These microorganisms are responsible for several fermentation reactions, which convert amino acid, sugar, alcohol and fatty acid molecules into various organic acids (acetic, propionic, butyric, succinic, lactic, etc.) as well as alcohols and ammonia (from amino acids), carbon dioxide and hydrogen (Goswami et al., 2016). Several possible routes for the acidogenesis stage are shown in Equations (2.2–2.5).

The glucose fermentation to ethanol route is as follows:

$$C_6H_{12}O_6 \quad \leftrightarrow \quad 2CH_3CH_2OH \; + \; 2CO_2 \qquad (2.2)$$

while the glucose fermentation to propionate route is:

$$C_6H_{12}O_6 \; + \; 2H_2 \quad \leftrightarrow \quad 2CH_3CH_2COOH \; + \; 2H_2O \qquad (2.3)$$

and the following is the glucose fermentation to acetate route:

$$C_6H_{12}O_6 \quad \leftrightarrow \quad 3CH_3COOH \qquad (2.4)$$

$$C_6H_{12}O_6 \; + \; 2H_2O \quad \leftrightarrow \quad 2CH_3COOH \; + \; 2CO_2 \; + \; 4H_2O \qquad (2.5)$$

Acetogenesis is the third stage in AD, occurring through cooperation between the microorganisms that oxidise organic matter and the methanogens involved in the final stage (methane formation) (Heeg et al., 2014). Various organic acids (acetic, propionic, butyric, succinic, lactic, etc.) are oxidised into simpler forms, such as acetate, hydrogen and carbon dioxide. Genera such as *Syntrophomonas*, *Syntrophus*, *Clostridium* and *Syntrobacter* are active in this stage (McInerney et al., 2008). Examples of acetogenesis reactions are shown in Equations (2.6) and (2.7) (Horan et al., 2011).

The following shows the conversion of butyric acid to acetic acid:

$$CH_3(CH_2)_2COO^- + 2H_2O \leftrightarrow 2CH_3COO^- + 2H_2 + \; H^+$$

$$\Delta G^0 = +48.1 \; kj/mol \qquad (2.6)$$

while the conversion of propionic acid to acetic acid is:

$$CH_3CH_2COO^- + 3H_2O \leftrightarrow 2CH_3COO^- + 3H_2 + \; H^+ + HCO_3^-$$

$$\Delta G^0 = +76.1\ kj/mol \qquad (2.7)$$

Methanogenesis is the final stage in AD, in which the main three pathways occur, as shown in Figure (2.2). The first is methylotrophic methanogenesis (i.e., the production of methane by the decarboxylation of methyl-alcohols/methyl-amine/methyl-sulphides, etc.) (Figure 2.2A). The second is hydrogenotrophic methanogenesis (i.e., the production of methane by the reduction of hydrogen/carbon dioxide) (Figure 2.2B), and the third is acetotrophic methanogenesis (i.e., the production of methane by acetate decarboxylation) (Figure 2.2C) (Goswami et al., 2016).

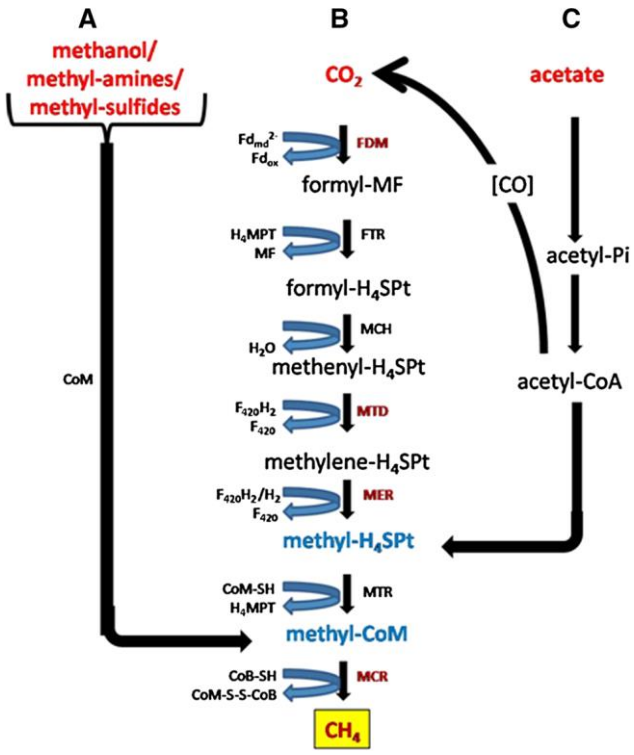


Figure 2.2 The three main pathways in the methanogenesis stage, source (Goswami et al., 2016).

Table 2.1 Methanogenic reactions from typical substrates, source (Pan et al., 2016).

Methanogenic reactions from typical substrates.

Reactions	$\Delta G^{0\prime}$ (kJ/molCH_4)	Microorganisms
I. Hydrogen		
$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	−135	Most methanogens
II. Formate		
$4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O$	−130	Many hydrogenotrophic methanogens
III. Acetate		
$CH_3COOH \rightarrow CH_4 + CO_2$	−33	*Methanosarcina* and *Methanosaeta*

In all these processes, methane is the main product, along with carbon dioxide, hydrogen and a small amount of trace molecules such as hydrogen sulfide. Merlino et al. (2013) reported that up to 70% of methane production comes from acetoclastic methanogenesis reactions during the AD processing of sewage sludge as feedstock. Methanogenic bacteria in the methanogenesis stage occur in two groups. The first is acetotrophic bacteria, responsible for degrading acetate to methane and carbon dioxide; examples of acetotrophic bacteria are *Methanosarcina barkeri*, *Methanonococcus mazei* and *Methanothrix soehngenii* (Schink, 1997). The second is hydrogenotrophic methanogens, which consume hydrogen to produce methane. Equations (2.8) and (2.9) show the acetoclastic methanogenesis and hydrogenotrophic methanogenesis reactions.

The following is the hydrogenotrophic methanogenesis reaction:

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \qquad \Delta G^0 = -130\ kj/mol \qquad (2.8)$$

while the acetoclastic methanogenesis reaction is:

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- \qquad \Delta G^0 = -32.3\ kj/mol \qquad (2.9)$$

Kumaran et al. (2016) reported that around 30% of methane yield is produced via a hydrotropic pathway, which is a rapid process based on its lower Gibbs free energy value. Meanwhile, 70% of methane yield comes via the acetoclastic pathway, even though this reaction is slower than the hydrotropic process.

2.2.2 Anaerobic digester types

Different types of anaerobic digesters can be categorised according to the condition of the feedstock (wet or dry), number of stages (single or multi-chamber/stage), operating temperature (mesophilic or thermophilic) and feeding method (batch or continuous).

2.2.2.1 Feedstock condition

AD feedstock can be dry or wet; dry feedstock has around 15%–40% solids, while wet feedstock has below 15%. Thus, the AD process itself is considered wet or dry depending on the type of feedstock. In dry AD, mechanical sorting and particle size reduction is the minimum pre-treatment for organic waste in a solid form. In wet AD, the process starts with a feedstock that can be transported by pumping as a liquid/slurry, although sometimes the feedstock is pre-treated by thermal hydrolysis to enhance anaerobic biodegradability. The high-moisture feedstock produced on livestock farms and at WWTPs is the most suitable feedstock for wet AD (Defra, 2010b). The recommended solids content for AD feedstock can be determined by the type of AD reactor rather than the source of feedstock: for a complete mix reactor, the recommended solids content is 5%–10%, while for a plug flow reactor it is 11%–14% (Chen and Neibling, 2014). In addition, the European Commission reported different recommended solids content depending on the type of sewage sludge, as shown in Table (2.2).

Table 2.2 Recommended solids content for different types of sewage sludge for AD application, source (European Commission Report, 2001, (Bitton, 2005)).

Type of sewage sludge	Solids content (g/L)
Primary sludge	12
Biological sludge	9
Biological sludge from clarified water	7
Mixed sludge (Primary and biological sludge from clarified water)	10

Digested sludge	30

2.2.2.2 Operating temperature

There are two ranges of operating temperature for AD. The first is mesophilic, with a temperature range of 35 °C–40 °C, and the second is thermophilic, with a temperature range of 55 °C–60 °C. Mesophilic AD is more economical, although thermophilic AD has a faster gas yield, leading to reduced retention time; the latter also has more sterilised digestate but higher energy consumption (Defra, 2010b). Tables (2.3) and (2.4) summarise the advantages and disadvantages of both mesophilic and thermophilic AD in processing sewage sludge.

Table 2.3 Advantages of mesophilic and thermophilic AD, source (Ruffino et al., 2015).

Mesophilic AD	Thermophilic AD
Organic material stabilises during biogas production	Better methane yield and higher biogas production
Reduction in sludge quantity	Shorter hydraulic retention time
Reduction in sludge fertilisation ability	Smaller AD reactor volume
Volume reduction of sludge due to good dewaterability	More pathogen destruction Better sludge dehydration Reduction in foam formation

Table 2.4 Disadvantages of mesophilic and thermophilic AD, source (Ruffino et al., 2015).

Mesophilic AD (Related to the unstabilised sludge)	Thermophilic AD (Related to the mesophilic AD)
Larger AD reactor volume needed and higher investment cost, due to a longer hydraulic retention time	Higher energy demand due to high operation temperature
Low sludge water quality	Low sludge water quality
Fermentation blocking influence of heavy metals	High sensitivity due to temperature fluctuation, more precise temperature regulation is required due to sensitivity to toxic heavy metals

Despite the advantages and disadvantages of mesophilic and thermophilic AD, certain factors can influence which AD operation temperature and digester is determined to be more suitable, including variations of feedstocks and volumes,

capital availability, space availability, existing infrastructure and value of respective outputs (ANDERSONS, 2010). According to (ANDERSONS, 2010), most AD reactors in the UK are operated under mesophilic conditions due to the low energy demand and operation cost.

Moreover, in many WWTPs, sewage sludge is treated by hydrothermal pre-treatment before being fed into an AD reactor. This pre-treatment is carried out in a hydrothermal treatment plant (HTP) and its main purpose is to enhance the solubility and biodegradability of sewage sludge before it goes to AD for biogas production (Wirth et al., 2015, Wang et al., 2010). This pre-treatment is commonly integrated with a mesophilic AD reactor in many WWTPs, including the Esholt WWTP in Bradford, UK.

2.2.2.3 Single- and multi-stage AD

Both single- and multi-stage AD have advantages and disadvantages. Single-stage AD involves a single sealed reactor (tank) where all the AD reactions (biological and physicochemical) occur simultaneously, which can save on footprint and construction costs. However, it produces less biogas than multi-stage AD, because the time of each stage varies (e.g., methanogenesis is slow, while hydrolysis is rapid) and there is a different optimal pH for each stage. Thus, multi-stage AD is generally preferred because it can overcome the disadvantages of single-stage AD.

However, there are also disadvantages to using multi-stage AD, for example the extra cost of constructing more reactors, the difficulty and complexity of maintaining optimal operating conditions for all stages, and the greater amount of space needed for the extra buildings and reactors (Defra, 2010b). As for sewage sludge feedstock, the most common system configuration used is single-

stage AD, especially a continuous stirred tank reactor (CSTR), due to the lower capital cost, the fact that it is easier to control the digestion process and its smaller footprint (Van et al., 2020).

2.2.2.4 Feeding method

The feeding method can be either batch or continuous. A batch AD is a digester which operates when all the feedstock is loaded into it at the same time at the start of the digestion process. In batch operation, the biogas reaches a peak at some point and then decreases as the bacteria consume the feedstock. Batch AD reactors are a good option for laboratory-scale work and research studies, due to the simplicity of their operation and control. Under the other AD type, the feedstock is continuously fed into the AD, and the digested material is continuously removed. Most AD reactors are continuous, because they produce more biogas per unit of feedstock and have lower operating costs (Syrusenergy, 2016).

2.2.3 Key environmental factors controlling anaerobic digestion

The four stages taking place in the anaerobic digester occur under a narrow range of environmental conditions; hence, performance can be affected by changes in key parameters, such as pH, temperature, C/N, solids retention time (SRT) and hydraulic retention time (HRT) (Appels et al., 2008).

2.2.3.1 pH

There is an optimum pH range for each AD stage. For example, methanogenic bacteria can reach their optimum performance within a 6.5–7.2 pH range and are highly sensitive to pH (Boe and Angelidaki, 2006). Acidogenic bacteria have a wide preferred pH range (4.0–8.5) and are less sensitive to pH changes in AD (Hwang et al., 2004). Acidogenesis-stage products can also differ according to the pH value; for example, acetic and propionic acid are produced when the pH

is 8.0 and butyric acid is at pH 5–7 (Boe and Angelidaki, 2006). The main reason for decreasing pH is the production of volatile fatty acids (VFAs) during AD processes; this reduction affects the actions of methanogenic bacteria, and therefore there is a lower methane yield at the end of the AD process.

2.2.3.2 Temperature

The growth rate and metabolism of microorganisms in AD are influenced by temperature, which affects the population dynamics. The temperature also affects the physicochemical properties of the components found in the digestion substrate; acetotrophic methanogens are very sensitive to increasing temperature (Appels et al., 2008). The partial pressure of hydrogen gas in AD can also be affected by temperature, due to its influence on the syntrophic metabolism kinetics.

At higher temperatures, the breakdown of propionate into acetate, carbon dioxide and hydrogen (endergonic reactions) is preferable from an energy perspective, while hydrogenotrophic methanogenesis (exergonic reaction) is less preferable at higher temperatures (Rehm et al., 2000).

There are many advantages to increasing the temperature inside the AD reactor. For example, biological and chemical reaction rates improve, and the solubility of the organic compounds and death rate of pathogens in the thermophilic condition can be increased (Boe and Angelidaki, 2006). However, there are also disadvantages, such as the fact that in thermophilic AD, the increased temperature can cause an increase in the free ammonia fraction, which has an inhibiting effect on microorganisms (Rehm et al., 2000).

Thus, controlling the temperature is very important and sensitive, especially in the thermophilic condition. Maintaining a stable temperature during the digester

operation will minimise the effects of temperature on bacterial activity. This consideration applies to the methanogens, in particular, as it has been reported that changes in temperature in excess of 1 °C/day can cause process failure, and temperatures higher than 0.6 °C/day should be avoided (Turovskiy and Mathai, 2006).

2.2.3.3 Carbon to nitrogen ratio

The C/N ratio is a parameter that indicates the level of nutrients in the AD substrate, and any changes in C/N ratio can affect the AD processes. The total ammonia nitrogen and free ammonia concentration can become low when a high C/N ratio is used; thus, optimising the C/N ratio is an important step in avoiding low biogas production (Mao et al., 2015). Rapid nitrogen degradation (which causes low biogas production) can also occur when a very high C/N ratio is used, leading to an insufficient amount of nitrogen to maintain the biomass cell (Mao et al., 2015). A C/N ratio equal to 25 is the most common ratio used in AD, with the optimal ratio being between 20 and 35 (Zhang et al., 2013, Punal et al., 2000).

Many studies show that adjusting the C/N ratio has an effect on increasing the methane yield, which leads to biogas upgrading. For example, Hills (1979) reported that maximum methane production was achieved by adjusting the C/N ratio for AD which used swine manure as a substrate. Thus, for sewage sludge which has a low C/N ratio (<10), using mixing substrate (sewage sludge and swine manure) can increase the C/N ratio and therefore enhance the biogas production (Mata-Alvarez et al., 2014). Sievers and Brune (1978), Wang et al. (2014) reported that a C/N ratio of 25–30 results in higher biogas production than one of 15. Moreover, with a C/N ratio of 25, a maximum biogas yield of 341 mL/g

of VS added has been achieved from the co-digestion of swine manure and corn straw (Wang et al., 2014).

2.2.3.4 Solids and hydraulic retention time

The average time during which solids stay in the reactor is called the solid retention time (SRT), and the HRT is the average time during which the feedstock is kept inside the reactor. The digestion steps in AD are directly related to SRT as the extent of reactions can be decreased by decreasing the SRT. Therefore, for AD stability and to prevent process failure, it is important to ensure that cell growth compensates the cell removal during the digestate removal process in AD (Turovskiy and Mathai, 2006). The relationship between biogas production and retention time for AD processing of sewage sludge has been studied on a laboratory scale, with results showing the influence of retention time in a CSTR, as described in Table (2.5).

Table 2.5 Relationship between retention time and anaerobic digestion process stability using sewage sludge as feedstock, adapted from (Appels et al., 2008).

Retention time	Result
Less than 5 days	• Insufficient for stable digestion • Increasing in VFA concentration due to a washout of methanogenic bacteria
5–8 days	• VFA concentrations are still relatively high for SRT • Incomplete breakdown of compounds such as lipids
8–10 days	• Low VFA concentrations due to lipid breakdown
More than 10 days	• All sludge compounds are significantly reduced

VFA: volatile fatty acid; **SRT**: solid retention time.

SRT is an important, influential parameter to be considered when designing all anaerobic processes. Figure (2.3) shows the effects of SRT on the digestion level.

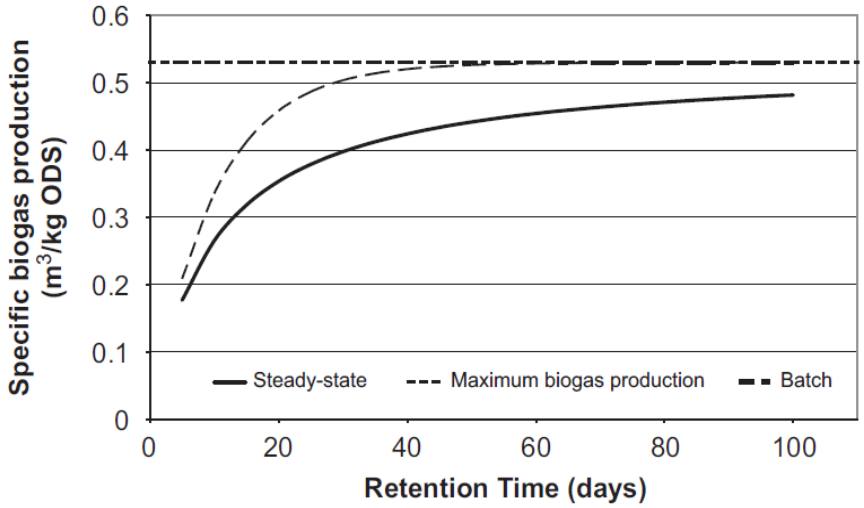


Figure 2.3 The effect of SRT on anaerobic digestion performance, source (Appels et al., 2008).

2.3 Biogas upgrading methods

Biogas is the main product of an anaerobic digester and consists of methane, carbon dioxide, ammonia, water and a small amount of trace components. Biogas production from AD contains 50%−70% methane and 30%−50% carbon dioxide. Biogas has a lower energy value than fossil fuels, due to the high concentration of carbon dioxide and other contaminants (contributing to GHG emissions). It also has a low heat value, around 21.5 MJ/Nm^3, while natural gas has a heat value of around 35.8 MJ/Nm^3 (Sarker et al., 2018). For these reasons, it is necessary to upgrade AD biogas.

Thus, many studies and experimental works have been conducted to find methods to upgrade biogas by increasing methane yields, reducing carbon dioxide, removing inhibitors and purifying the final biogas. However, upgraded biogas specifications need to fall within certain standards, similar to those regarding contaminant levels (Cabbai et al., 2013). For example, an internal combustion engine can be affected when using biogas with a high level of carbon dioxide. Incomplete combustion and poisonous emissions can also occur when water, ammonia, siloxanes and more than 1,000 ppm of halocarbons are

available in the biogas, so these compounds need to be removed (Angelidaki et al., 2005).

The standard composition of natural gas is 80%–96% methane, 2%–3% carbon dioxide, 0.2%–0.5% oxygen, 5 mg/m^3 hydrogen sulfide, 3–20 mg/m^3 ammonia and 5–10 mg/m^3 siloxanes. To achieve and produce biogas within these levels, several upgrading methods have been developed, classified as either ex-situ or in-situ (Suhartini et al., 2014, Bouallagui et al., 2004), as shown in Figure (2.4).

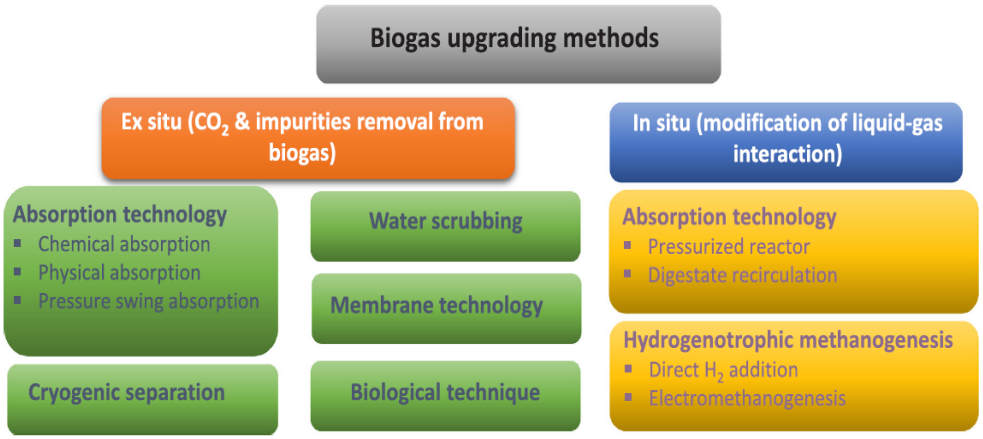


Figure 2.4 Biogas upgrading methods, source (Sarker et al., 2018).

In-situ upgrading methods are used within AD processes as a way to increase methane concentrations in the produced biogas; this increase can be accomplished by adjusting some operation parameters, such as reactor pressure and digestate withdrawal flow rate, and also by adding chemicals (e.g., some salts or additional carbon dioxide) or gases (e.g., hydrogen) (Hayes et al., 1990, Lemmer et al., 2015, Mulat et al., 2017). The different technologies are categorised into four types: pressurised reactors, aerated methanation reactors, digesters enriched with hydrogen and digesters coupled with a bioelectrochemical system. Each of these technologies has weaknesses, either with respect to upgrading biogas to the desired level (e.g., natural gas standard)

or to removal/reduction efficiency for carbon dioxide, hydrogen sulfide and nitrogen. Figure (2.5) shows the types of in-situ biogas upgrading methods.

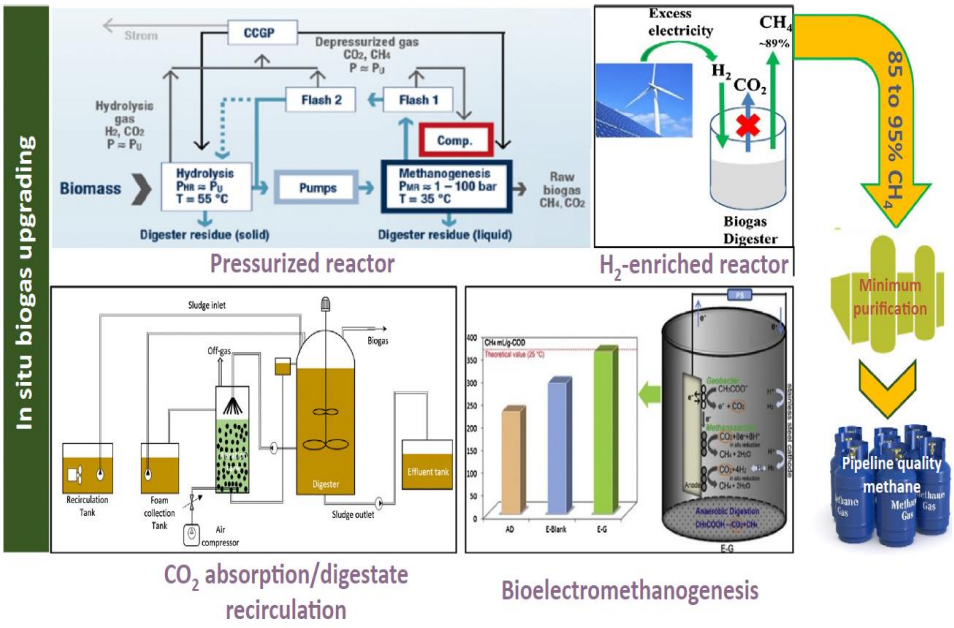


Figure 2.5 In-situ biogas upgrading methods, source (Sarker et al., 2018).

Also, ex-situ upgrading methods are used for biogas upgrading which comes after biogas production from AD and requires downstream biogas processing using methods such as catalytic conversion, absorption and membrane separation (Muñoz and Steinmetz, 2012). Table (2.6) shows the current technologies for ex-situ biogas upgrading.

Table 2.6 Ex-situ biogas upgrading technologies, adapted from (Singhal et al., 2017).

Parameter	Physical scrubbing (water)	Physical scrubbing (organic)	Chemical scrubbing (amine treatment)	Membrane separation	Adsorption (pressure swing adsorption)
CH_4 (%)	96–98	96–99	99	98	98
Water consumption (m^3/Nm^3)	0.00004–0.0004	Not required	Not required	Not required	Not required
Power consumption (kWh/Nm^3)	0.2–0.3	0.2–0.3	0.12–0.14	0.2–0.3	0.2–0.3
Chemicals	Anti-foaming agents (0.00003 kg/Nm^3)	Activated charcoal	Anti-foaming agents (0.00003 kg/Nm^3), amine and activated charcoal	Activated charcoal	Activated charcoal
Heat (kWh/Nm^3)	Not required	Not required	0.55	Not required	Not required
Pre-removal of H_2S	Not required	Required	Required	Required	Required
Limitation nitrogen removal	No nitrogen removal	No nitrogen removal	No nitrogen removal	No nitrogen removal	No nitrogen removal (can be performed using much costlier complex adsorbents)
Limitation oxygen removal	No oxygen removal	No oxygen removal	Damaged by oxygen due to oxidation	Partial removal of oxygen	No oxygen removal (can be performed using much costlier complex adsorbents)

2.3.1 Biogas upgrading by hydrogen injection

AD is a very common and well-implemented technology at different scales (lab, pilot and commercial), although it still has some limitations, such as biogas upgrading expenses and high operating costs (Aryal et al., 2018). To reach a level competitive with other energy technologies, such as traditional fossil fuels, the economic value of AD (i.e., high volume and quality of produced biogas along with low operating and maintenance costs) should be increased by overcoming its weaknesses, thus encouraging investors to increase their use of AD technology (Benjaminsson et al., 2013). The European Biogas Association (EBA) (2020) reported that, in Europe, the number of biomethane (upgraded biogas) plants increased from 483 in 2018 to 728 in 2020, representing a rise of around 51%. As the current production (combined biogas and biomethane) is equivalent to 193 TWh, various studies have calculated that the production of biogas and biomethane will increase to 467 TWh in 2030 and 1,020 TWh by 2050.

Considerable effort is now being made to optimise AD by upgrading biogas, in particular by using hydrogen to convert carbon dioxide to methane during the methanogenesis process. There are three pathways in the methanogenesis stage: the hydrogenotrophic, acetotrophic and methylotrophic (Aryal et al., 2018) as explained in section 2.2.1.1. To date, the hydrogenotrophic pathway is the most metabolically efficient pathway for upgrading biogas and increasing the methane yield (Lever, 2016).

Several techniques have been developed to use hydrogen to increase and enrich the methane in AD biogas; these are classified as either in-situ or ex-situ techniques. However, all such technologies need a source for hydrogen gas.

An in-situ method is where the hydrogen is injected inside the AD, while in the ex-situ method, hydrogen is generated in another reactor and then injected into the AD. Table (2.7) shows a comparison of different in-situ and ex-situ methane upgrades in laboratory-scale reactors. In addition, there are some bottlenecks in using hydrogen, for example its transportation from the point where it is produced to the point where it is consumed. High storage costs are another factor, because hydrogen is a very light gas and can easily leak. Hydrogen also has a lower volumetric energy content (10.88 MJ/m^3) than methane (36 MJ/m^3) (Luo et al., 2012). Hence, it is very important to understand the methods of hydrogen production to find the most economical method with minimum environmental impact.

Table (2.7) shows that using hydrogen to upgrade AD biogas by increasing methane yield is an efficient method. However, most studies listed in Table (2.7) related to upgrading biogas by hydrogen used sewage sludge as an inoculum only. Moreover, these studies were conducted quite recently (from 2012 to 2017), which indicates that studying biogas upgrading by hydrogen is a relatively new trend, emerging in the past 10 years. Thus, there is still much work to be done, for example in using different hydrogen production methods with less energy consumption. Different methods of hydrogen production, such as the biological methods mentioned earlier, need to be applied to AD. The most frequently implemented method for hydrogen production is water electrolysis operated by windmills, but this method is high in energy consumption; finding another pathway by which to generate hydrogen and use it to increase methane yield will help reduce the cost of this method and open a new way to optimise AD.

Table 2.7 Comparison of different in-situ and ex-situ methane upgrades by hydrogen in laboratory-scale reactors, adapted from (Aryal et al., 2018).

	Reactor type	Temperature	Inoculum source	Influent gas	Operation mode	Working volume (L)	Retention time (hr)/ HRT (day)	Mixing speed (rpm)	Methane yield (%)	Reference
In-situ methane upgrading	CSTR	Thermophilic, 55 °C	Digested manure	N.g	Continuous	3.5	14 (day)	300	65 ± 3.3	(Luo et al., 2012)
	CSTR	Thermophilic, 55 °C	AD sludge	Ceramic Column Column	Continuous Continuous Continuous	0.6 0.6 0.6	15 (day) 15 (day) 15 (day)	150 150 300	75 ± 3.4 53 ± 3 68 ± 2.5	(Luo and Angelidaki, 2013a)
	CSTR	Thermophilic, 55 °C	AD sludge	HFM	Continuous	0.6	15 (day)	150	96.1 ± 1.1	(Luo and Angelidaki, 2013b)
	CSTR	Mesophilic, 37 °C	Sewage sludge	HFM with coke oven gas	Continuous	2	10 (day)	200	98.8 ± 0.3	(Wang et al., 2013)
	CSTR	Mesophilic, 35 °C	Anaerobic granules	80%H_2 + 20%CO_2	Batch	0.05	N.g	150	86	(Xu et al., 2015)
	UASB	Thermophilic, 55 °C	Anaerobic granules	N.g	Batch	3.5	20 (day)	200	85.1 ± 3.7	(Bassani et al., 2016)
	CSTR	Mesophilic, 38 °C	Biogas sludge	$H_{2\ \text{pulse injection}}$	Batch	2	20 (day)	1000	100	(Agneessens et al., 2017)
Ex-situ methane upgrading	CSTR	Thermophilic, 55 °C	AD sludge	H_2, CH_4 and CO_2	Continuous	0.6	11–43 (hr)	800	95.4 ± 2.8	(Luo et al., 2012)
	Trickle bed	Mesophilic, 37 °C	Sewage sludge	H_2+CO_2	Batch	26.8	2.25 (day)	N.g	97.9	(Burkhardt and Busch, 2013)
	Two-stage CSTR	Mesophilic, 37 °C Thermophilic, 55 °C	Biogas plants	Biogas plants Biogas plants	Batch Batch	2 2	33 (day) 20 (day)	200 200	88.9 ± 2.4 85.1 ± 3.7	(Bassani et al., 2015)
	Trickle bed reactor	Mesophilic, 37 °C	Mixed anaerobic culture	H_2+CO_2	Continuous	5.8	3.5 (hr)	N.g	96	(Rachbauer et al., 2016)
	CSTR Double UASB in placed in series Bubble column reactor	Thermophilic, 55 °C	Digested biogas plant	62%H_2, 23%CH_4 and 15%CO_2	Continuous Batch Continuous	1.2 1.4 1.2	35 (hr) 15 (hr) 35 (hr)	300 1.2[c] 1.2[c]	79 98 97 - 98	(Kougias et al., 2017)
	UASB	Thermophilic, 55 °C	Biogas plants	H_2, CH_4 and CO_2	Continuous	0.85	15 (hr)	N.g	96.3 ± 0.2	(Bassani et al., 2017)

CSTR: Continuous Sterile Tank Reactor; **UASB:** Upflow Anaerobic Sludge Blanket Digestion; **AD:** Anaerobic Digestion; **(c)** Recirculation in L/h**.; N.g**: Not given.

2.4 Hydrogen production methods

Hydrogen is the energy source of the future. There are many reasons that hydrogen is preferred to other energy sources, such as a high net calorific value (120 MJ/kg) when compared with methane gasoline (50 MJ/kg), ethanol (26.8 MJ/kg) and methanol (19.6 MJ/kg), which gives it the highest energy efficiency (Graboski and McCormick, 1998, Zajic et al., 1978). Hydrogen has a positive impact on the environment, as its combustion does not produce carbon dioxide, which can contribute to reducing GHG from the use of fossil fuels (Łukajtis et al., 2018). Hydrogen is also considered to be competitive against other renewable energy types, such as solar and wind.

Hydrogen can be produced from fossil fuels, water or biomass. Figure (2.6) shows the methods used for hydrogen production from these different sources.

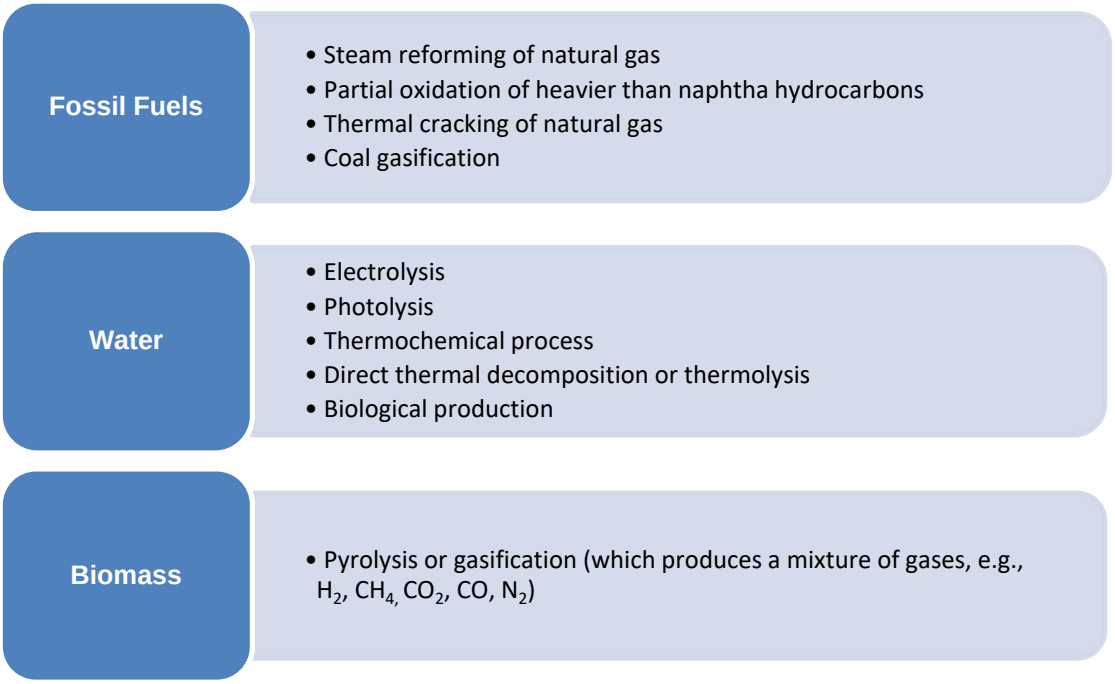


Figure 2.6 Methods used for hydrogen production from different sources, adapted from (Das and Veziroğlu, 2001).

Around 90% of hydrogen is currently produced from light oil fractions, using steam at high temperatures (steam reforming) or by the reactions of natural gas; industrial methods such as coal gasification and the electrolysis of water are also used for hydrogen production (Das and Veziroğlu, 2001). These methods are energy-intensive, because they mainly use fossil fuels as an energy source, and almost all thermochemical and electrochemical hydrogen production methods have high energy consumption and create environmental impacts (Rosen and Scott, 1998, Lodhi, 1987). Despite the advantages of hydrogen as an energy source, the conventionally used methods for hydrogen production, such as natural gas decomposition, petroleum oxidation and coal gasification, have many disadvantages, such as high energy demand and hazardous gas emissions. Operation temperatures for these systems are usually >700 °C (Momirlan and Veziroglu, 2002), and the gases emitted mostly contain oxides of nitrogen, sulphur and carbon along with ashes that contain heavy metals and radioactive substances (Kapdan and Kargi, 2006). Hydrogen production via steam reforming in WWTPs using ammonia and biogas (Grasham et al., 2019, Grasham et al., 2020) can be coupled with carbon dioxide capture and storage to reduce net GHG emissions; however, this process would still rely on the production of high-quality biogas.

Water electrolysis is another available hydrogen production method. In this process, water molecules are split into hydrogen and oxygen; therefore, there are no carbon dioxide emissions if the energy used comes from a non-fossil fuel source. However, it still consumes large amounts of energy, as an electrolyser consumes 39.4-50 kW.h/kg of hydrogen (142–180 MJ/kg) (Luca Bertuccioli, 2014).

Thus, the strong prospect that hydrogen could be used for energy production and the negative impact of current hydrogen gas production methods necessitated research to find other energy-efficient and environmentally friendly hydrogen production methods. Bio-hydrogen production is one of various promising methods based on the use of organic wastes (e.g., food, municipal and agricultural waste) through biological reactions carried out by microorganisms under specific operating conditions (Ghimire et al., 2015).

As Bio-hydrogen production methods have fewer environmental impacts and use less energy (operated under ambient temperatures and pressures), they can lead to the sustainable production of energy resources and, at the same time, contribute to enhancing waste management by using organic waste materials that are usually spread on land or sent to landfill (Benemann, 1997, TANISHO et al., 1983).

There are different types of bio-hydrogen process, such as biophotolysis of water using algae and cyanobacteria. Hydrogen can be produced by the biophotolysis of water, using the same processes found in plants and algal photosynthesis. Photosynthesis consists of two photosynthetic systems operating in series: photosystem II (PSII), considered a water-splitting and oxygen evolving system; and photosystem I (PSI), which generates the reductant used for carbon dioxide reduction (Das and Veziroğlu, 2001). During these two processes, one electron is removed from a water molecule by two photons, and they are used for hydrogen formation or carbon dioxide reduction.

Algae and cyanobacteria can produce hydrogen because of their capacity to excrete hydrogenase enzymes (Benemann, 1997). Hydrogen production by

algae and cyanobacteria has been studied by several researchers. For example Benemann et al. (1973) studied how oxygen influences the water-splitting reaction, Figure (2.7) describes hydrogen production by water biophotolysis. Electrons flow from water to the two photosystems (PSII and PSI), then to the electron carrier ferredoxin (Fd) and, finally, to the hydrogen-evolving enzyme hydrogenase to produce hydrogen as a final product. Benemann et al. (1973) reported that hydrogen production was lower than the carbon dioxide reduction rate, because of the presence of oxygen, which inhibits the hydrogenase activity and leads to a reduction in hydrogen production.

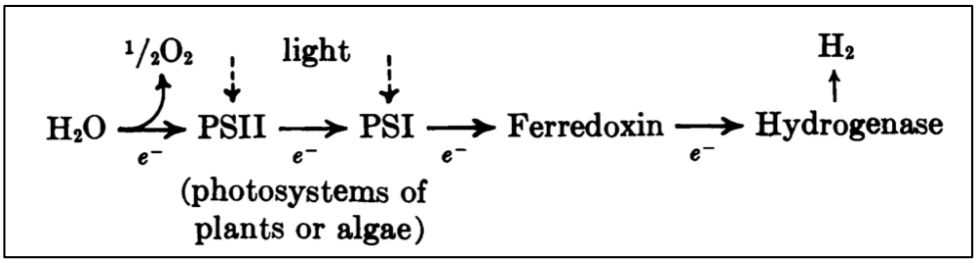


Figure 2.7 Biophotolysis of water using algae and cyanobacteria, source (Benemann et al., 1973).

Green algae are eukaryotic microorganisms that can produce hydrogen under dark, anaerobic conditions, where the hydrogenase enzyme is probably working and produces hydrogen. Cyanobacteria (previously known as blue-green algae) are gram-positive prokaryotic bacteria and also capable of producing hydrogen; they exist in marine environments and different soils (Kaushik, 1998). Cyanobacteria are also called nitrogen-fixing bacteria because they use nitrogenase and hydrogenase for hydrogen production via the biophotolysis of water (Smith et al., 1992). Green algae are better for hydrogen production, because cyanobacteria use more energy-intensive enzymes than Adenosine triphosphate (ATP), requiring nitrogenase for the production of hydrogen (Kumazawa and Mitsui, 1981).

Also, many studies have investigated photodecomposition method (one of biological hydrogen production methods) and the abilities of phototrophic bacteria in utilising the organic compounds for biological hydrogen production (KIM et al., 1981, Fascetti and Todini, 1995, Fascetti et al., 1998). There are four main reasons for using photosynthetic bacteria: first, they have high theoretical conversion yields; second, oxygen activity plays a minimum role (which causes problems in other biological systems, due to oxygen inactivation); third, they have the ability to use a wide spectrum of light; and finally, they have the potential to be involved in the waste treatment process, due to their ability to consume organic substrates.

Equation (2.10) shows the biochemical pathway for the photo fermentation process:

$$(CH_2O)_2 \xrightarrow[\uparrow \text{ATP}]{} \text{Ferredoxin} \xrightarrow[\uparrow \text{ATP}]{} \text{Nitrogenase} \rightarrow H_2 \qquad (2.10)$$

Moreover, photosynthetic bacteria can use a microbial shift reaction for carbon monoxide to produce hydrogen (Uffen, 1976); Equation (2.11) shows this reaction:

$$CO + H_2O \rightarrow CO_2 + H_2 \qquad (2.11)$$

While, fermentative hydrogen production method is considering one of the promising method for bio-hydrogen production as this method has many advantages. For example, an organic substrate for producing the hydrogen can be used constantly throughout the day, and this process has a very high hydrogen evolution rate. TANISHO et al. (1983), Tanisho et al. (1987) reported that fermentative evolution is better than photochemical evolution when

microorganisms are used for hydrogen biomass production. During the fermentation method, fermentative bacteria are encouraged to hydrolyse organic substrates to produce energy carriers such as hydrogen and formate. Equation (2.12) shows the reaction of formate to hydrogen:

$$HCOO^- + H^+ \rightarrow CO_2 + H_2 \qquad \Delta G^0 = -3.4\ \mathrm{kj/mol} \qquad (2.12)$$

One of the main advantages of this method is that it does not require a light source and can easily be adapted to various organic substrates. DF is classified as a fermentation system as it has the ability to produce hydrogen by fermentative bacteria. The following section describes this system in more detail.

2.5 Dark fermentation

DF is among the bio-hydrogen production methods, whereby fermentative bacteria are used to hydrolyse organic substrates to produce energy carriers such as hydrogen gas. One of the main advantages of this method is that it does not require a light source and can easily be adapted to various organic substrates (Nath et al., 2008). DF is sometimes referred to as stressed AD because the biological reactions that occur during it are the same as in the hydrolysis/acidogenesis stage of AD (Antonopoulou et al., 2011).

The DF application for hydrogen production has been assessed over a very short time compared with other technologies (e.g., AD), as the first studies of DF were carried out 20 years ago (according to Scopus). In the last 10 years, researchers have focused on the DF method as it has become interesting for the biological production of hydrogen. Different studies have investigated the feasibility of the DF method in producing hydrogen, considered to be the energy carrier of the future, with many potential applications. The Scopus research engine was used

to explore the research history of and number of publications related to hydrogen production through the DF process. According to Scopus, between 2001 and the time of the search, 111 publications related to applying DF to hydrogen production using sewage sludge were published. Figure (2.8) shows the number of published papers/articles for the different keywords used in Scopus.

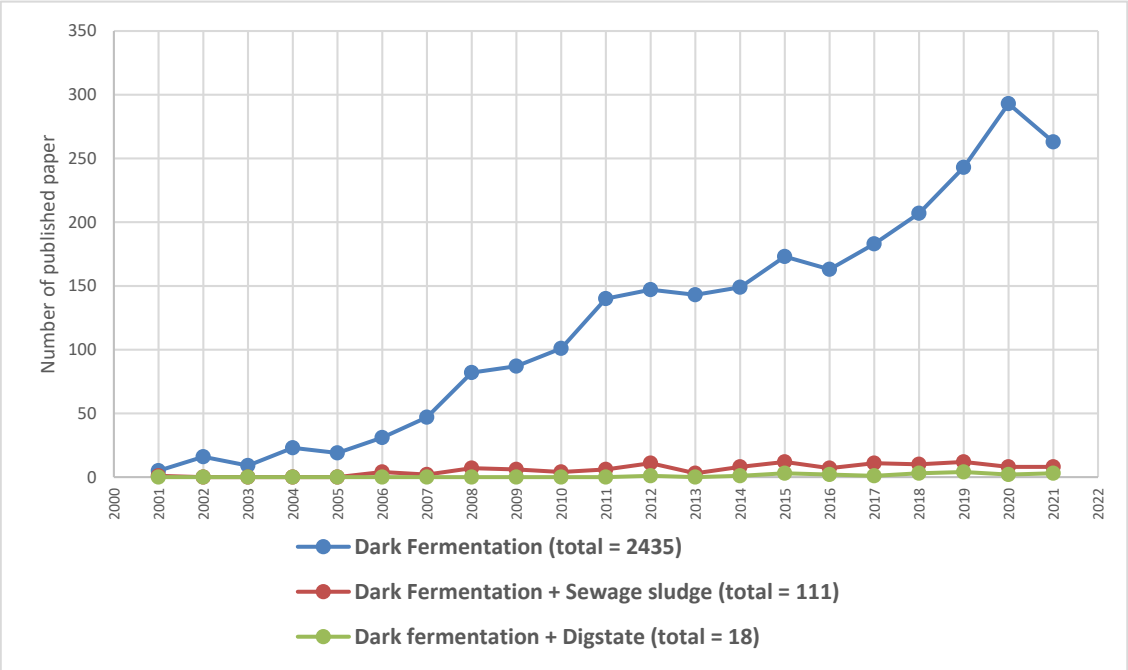


Figure 2.8 Number of published articles on dark fermentation in the past 21 years, source (Scopus).

Meanwhile, the number of publications is increasing, due to the feasibility of this method. Different substrates have been used in this method to encourage the microorganisms, especially fermentative bacteria, to consume the organic matter and transform it into hydrogen gas. Figure (2.9) shows the number of studies which have investigated the different types of substrates in the last 20 years.

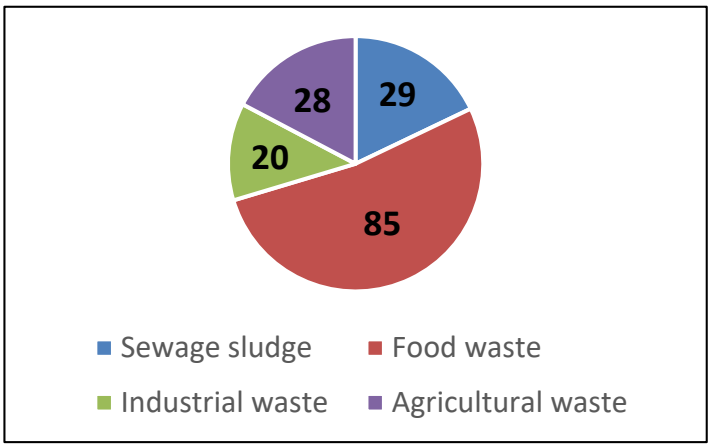


Figure 2.9 Number of studies investigating different types of substrates in dark fermentation, source (Scopus).

2.5.1 Dark fermentation using sewage sludge as substrate

Sewage sludge has the largest volume of all types of organic domestic waste. WWPT managers, engineers and operators face many problems handling the huge amount of sludge produced continuously every day, so using it as a substrate for hydrogen production will reduce the environmental impact as well as the capital costs of handling it (Werther and Ogada, 1999). Sewage sludge contains a high concentration of nutrients and has a high diversity in its microbial community, giving it an advantage in being used as an inoculum source for DF reactors. However, very limited research has investigated sewage sludge as a substrate for hydrogen production in DF reactors. There are many reasons for this, including the complexity of sludge content, as it is difficult for fermentative bacteria to utilise it, and its low C/N ratio (Xia et al., 2016). Table (2.8) shows the limited number of studies which have used raw municipal sludge as a substrate for fermentative bacteria.

Table 2.8 Fermentative hydrogen potential from sewage sludge and digestate, adapted from (Yang and Wang, 2017).

Sludge type	Inoculum	Fermentation conditions	Hydrogen yield	References
ADS	ADS	Batch, 50 °C , Initial pH: 5	0.02 mL/g-TS removed	(Sato et al., 2016)
Mixed sludge	ADS	Batch, 55 °C , Initial pH: 5.5	28.3 mL/g-VS removed	(Tyagi et al., 2014)
WAS	ADS	Batch, 35 °C , Initial pH: 6	17.9 mL/g-VS added	(Cheng et al., 2016)
Primary sludge	ADS	Batch, 37 °C , Initial pH: 5.5	10 mL/g-COD added	(Yu et al., 2013a)
Thickened sludge	ADS	Batch, 37 °C , Initial pH: 8	0.25 mL/g-VSS removed	(Kim et al., 2013)
Thickened sludge	Compost-acclimated sludge	CSTR, 36.5 °C , Initial pH: 6.2-6.5, SRT: 32 hr ORL: 0.3 kg/COD/d	13.7 mL/g-VS added	(Wu and Zhou, 2011)
WAS	None	Batch, 36 °C , Initial pH: 10 Batch, 36 °C , Initial pH: 10.5 Batch, 36 °C , Initial pH: 11 Batch, 36 °C , Initial pH: 11.5 Batch, 36 °C , Initial pH: 12	3.63 mL/g-TS added 6.06 mL/g-TS added 8.08 mL/g-TS added 7.89 mL/g-TS added 6.6 mL/g-TS added	(Cai et al., 2004a)
WAS	Clostridium bifermentans	Batch, 35 °C	6.67 mL/g-COD added 10.9 mL/g-TS added	(Wang et al., 2003)
WAS	None	Batch, 35 °C , Initial pH: 6.67	3.34 mL/g-TS added	(Jan et al., 2007)
WAS	ADS	Batch, 55 °C , Initial pH: 5.7	93 mL/L-Sludge added 12.4 mL/g-TS added 18.6 mL/g-VS added 5.1 mL/g-COD added	(Liu et al., 2013)
WAS	WAS	Batch, 30 °C , Initial pH: 5.5	12.98mL/L-Sludge added 1.41 mL/g-COD added	(Wan et al., 2016)
WAS	None None ADS ADS	Batch, 37 °C , Initial pH: 7 Batch, 37 °C , Initial pH: 11.5 Batch, 37 °C , Initial pH: 5.5 Batch, 37 °C , Initial pH: 5.5	1.21 mL/g-VS added 7.57 mL/g-VS added 7 mL/g-COD added 93 mL/g-VSS added	(Xiao and Liu, 2009)
WAS	None	Batch, 37 °C	5 mL/g-COD removed	(Kotay and Das, 2009)
WAS	WAS	Batch, 35 °C	13.8 mL/g-COD added 20 mL/g-TS added	(Wang et al., 2004)

WAS: Waste-activated Sludge; **ADS**: Anaerobically Digested Sludge; **TS**: total solids; **VS**: volatile solids; **VSS**: volatile suspended solids; **COD**: chemical oxygen demand.

2.5.2 Dark fermentation reactions

During DF, hydrogen is produced by conversion (hydrolysis) of organic substrates (carbohydrates, proteins and lipids) by mixed or pure cultures of microorganisms. This conversion can follow different pathways, each of which has a specific maximum hydrogen production (theoretically). Figure (2.10) shows a series of complex reactions occurring during the hydrogen fermentation process, starting from converting complex organic matter, such as carbohydrates, proteins and lipids in sludge or some other substrate, into soluble

organic matter, such as amino acids, sugars, glycerol and long-chain fatty acids, via hydrolytic bacteria (Yu et al., 2013b).

After sludge hydrolysis, hydrogen producers (microorganisms) consume the sugars derived from the carbohydrates to produce hydrogen. The three pathways for consuming these sugars are as follows. Sugars are degraded into pyruvate through the Embden–Meyerhof–Parnas pathway, when the pyruvate can be decomposed into formate and acetyl-CoA by pyruvate-formate lyase, and then the formate is converted into hydrogen and carbon dioxide; the Nicotinamide adenine dinucleotide (NADH) generated during the glycolysis process is reoxidised to generate hydrogen, probably catalysed by an NADH-dependent Fe–Fe hydrogenase; and sugars are converted into ethanol and VFAs, such as propionic acid, butyrate and valerate, by acidogenic bacteria. Propionic acid, butyrate, valerate and ethanol can be used as substrates for the production of acetic acid and hydrogen by the acetogens (Wang and Wan, 2008). In addition, during sludge hydrogen fermentation, hydrogen consumption pathways have been observed where homoacetogenic bacteria, sulphate-reducing bacteria and hydrogenotrophic methanogens are the main consumers of hydrogen (Yang and Wang, 2017).

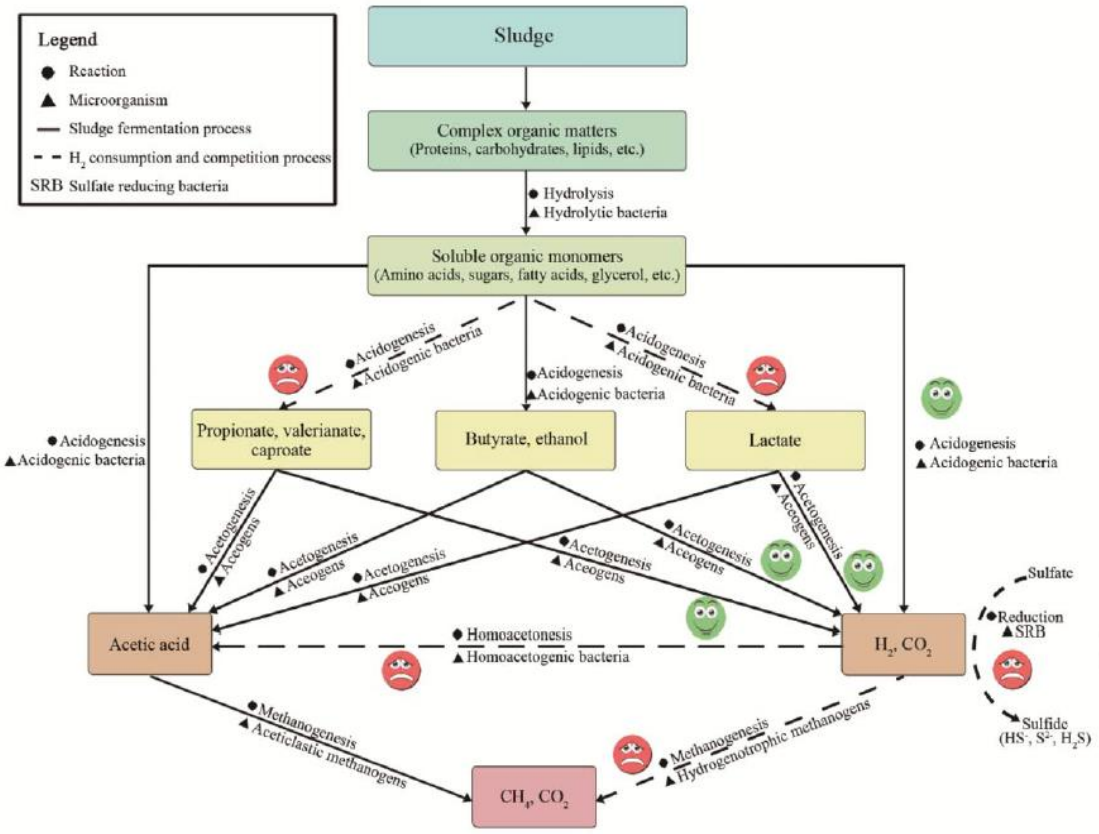


Figure 2.10 Biochemical reactions during the sludge fermentation process, source (Yang and Wang, 2017).

Certain operating parameters have an influence on hydrogen production. Temperature, pH, agitation intensity, retention time, organic loading rate (OLR), the presence of nutrients, inhibitors and inoculum type have major influential effects on the whole process; these operating parameters are related to each other such that changing one parameter may affect another (Wang and Wan, 2009). Given this complexity in the relationship of these influential parameters, more research is needed into the DF method of processing sewage sludge in the production of hydrogen.

2.5.3 Key parameters influencing dark fermentation performance

DF reactors processing mixed substrates such as sewage sludge are considered complex systems in which many factors, such as pH, alkalinity, operation temperature, inoculum pre-treatments and substrate pre-treatments, affect the

metabolic pathways of substrate conversion and microorganism activities (Guo et al., 2010b, Li and Fang, 2007b, Wang and Wan, 2009). In this section, the influence of key process parameters is discussed.

2.5.3.1 pH, alkalinity and operation temperature

The pH, alkalinity and operation temperature have a crucial influence on DF reactions, as optimum metabolic pathways of hydrogen production can be achieved by adjusting and controlling pH. Furthermore, the inhibition of hydrogen-consuming bacteria may occur simultaneously (Hu et al., 2005, Khanal et al., 2004). Luo et al. (2011) reported that methanogenic bacteria were inhibited under pH <6.0 with both mesophilic and thermophilic operation temperature, while homoacetogenic bacteria were inhibited only in DF with an initial pH 5.5 and under thermophilic temperature.

The pH value affects VFAs accumulation; lower pH ranges (4.0–6.0) support butyrate and acetate accumulation, and higher pH ranges (7.0–9.0) support ethanol and propionate accumulation (Hawkes et al., 2007, Pakarinen et al., 2008). Conversely, the pH also influences the diversity of the microbial community and affects hydrogen production; that is, at low pH levels, the dominant species is *Clostridium*, responsible for the production of butyrate, acetate and hydrogen (Hawkes et al., 2007, Temudo et al., 2008). It has been suggested that the optimum pH range, which enhances the hydrogenases (hydrogen producers) in DF, is between 5.0 and 7.0 (Li and Fang, 2007a).

Alkalinity also has an effect on hydrogen production in DF as it acts as a buffer to compensate a drop of pH due to VFAs accumulation. For that reason, the alkalinity in the DF reactor helps to keep the pH within the optimal range required for hydrogen production. Mtui (2009) reported that alkalinity was the most

important parameter affecting hydrogen production. Bina et al. (2019) reported that the optimum starting alkalinity for DF which processed synthetic wastewater as feedstock along with anaerobic sludge as inoculum was 1325 mg/L $CaCO_3$, as this initial alkalinity allowed the highest hydrogen yield (220 mL/d). It should be noted that the range of initial alkalinity tested in this study was between 670 and 2678 mg/L $CaCO_3$.

Moreover, many studies have investigated different ranges of operation temperature, including mesophilic (37 °C), thermophilic (55 °C) and extreme (>65 °C) temperatures to optimise hydrogen production in DF (Shin et al., 2004, Valdez-Vazquez et al., 2005, Kongjan and Angelidaki, 2010). These studies have proved that operation temperature has a crucial impact on hydrogen production as well as VFA conversion pathways. Shin et al. (2004) reported that in DF processing food waste, the hydrogen production was higher under thermophilic than mesophilic temperature and butyrate was the major end-product, while acetate was higher at mesophilic DF. A similar finding was observed by (Valdez-Vazquez et al., 2005), as thermophilic DF had a higher hydrogen yield than mesophilic; however, different dominant VFAs occurred, as butyrate was dominant in mesophilic DF and acetate was dominant in thermophilic DF. Liu et al. (2008) reported similar results, as acetate was the dominant VFA for DF processing household organic waste under an extreme DF temperature (70 °C) and at pH 7.0. In another study that used glucose as substrate, mesophilic DF (37.8 °C) gave the maximum hydrogen yield and production (Wang et al., 2011). The different outcomes of these studies show that not only does operation temperature have an effect on the DF process but substrate type and structure complexity must also be considered when

determining the optimum temperature for optimum DF performance. Guo et al. (2010b), Kongjan and Angelidaki (2010) reported that agricultural waste and food waste need a high operation temperature to achieve a high hydrogen yield, while for DF processing of simple substrates such as glucose, the low temperature (mesophilic) is enough for high hydrogen production (Wang et al., 2011).

2.5.3.2 Carbon to nitrogen ratio

The C/N ratio is an important parameter characterising DF substrates, as a low C/N ratio is one of the main reasons for low hydrogen production in DF. Many studies have shown that enriched substrates (i.e., those with a high C/N ratio, such as food waste and agricultural waste) produce more hydrogen than substrates with a low C/N ratio (e.g., sewage sludge) (Xia et al., 2016). Mata-Alvarez et al. (2014) reported that the suitable range of C/N ratio for fermentative bacteria is 20–30, while the C/N ratio of sole sludge is usually between 4 and 10. For this reason, co-fermentation has been used as a substrate pre-treatment to improve C/N ratio and therefore enhance hydrogen production ((Wu et al., 2016, Xie et al., 2016, Hagos et al., 2017). Table (2.9) shows the relation between C/N ratio and hydrogen production in DF.

Table 2.9 Carbon to nitrogen ratio relation with hydrogen yield.

Inoculum	Substrate	Carbon to nitrogen ratio (C/N)	Hydrogen yield	Reference
Digestate	SS	7.1	17.9 mL/g-VS-added	(Cheng et al., 2016)
Digestate	WAS + FW	33.1	101.1 mL/gVSS-added	(Sreela-Or et al., 2011)
Digestate	PS	4.0	13 mL/gVSS-added	(Zhou et al., 2013)
	FW+PS	26.0	130 mL/gVSS-added	
	FW + WAS	31.0	137 mL/gVSS-added	
	FW+PS+WAS	30.0	165 mL/gVSS-added	
	OFMSW+ GSW	28.8	149.5 mL/gCOD-removed	(Elsamadony and Tawfik, 2015)

Thickener sludge	OFMSW+ GSW+PMS	29.4	157 mL/gCOD-removed

FW: food waste; **WAS**: waste-activated sludge; **SS**: sewage sludge,
PS: primary sludge; **GSW**: gelatine solid waste; **OFMSW**: organic fractions of municipal solid waste
PMS: paperboard mill sludge; **VS**: volatile solids; **VSS**: volatile suspended solids; **COD**: chemical oxygen demand.

2.5.3.3 Inoculum pre-treatment

Inoculum pre-treatment is a very important step in DF that can affect the production of hydrogen. Mixed cultures, such as digestate (from AD), can be used as inoculum in DF and are practical, easy to handle and control, and highly available (Li and Fang, 2007a). However, mixed cultures contain hydrogen-consuming bacteria (methanogens and homoacetogens) and hydrogen-producing bacteria (such as *Clostridium* and *Enterobacter*), and in the DF process, which is operated under anaerobic conditions, the hydrogen-consuming bacteria can easily consume the hydrogen and prevent net hydrogen production (Oh et al., 2003b, Cai et al., 2004a).

Therefore, to inhibit the methanogens' activity, an inoculum pre-treatment is a necessary step in DF. Several studies have investigated different types of inoculum pre-treatment, which are classified into physical, chemical and biological treatment methods (Hu and Chen, 2007b, Zhu and Béland, 2006a, Mu et al., 2007, Mohan et al., 2008b). Physical pre-treatment (heat shock (HST)), freezing-thawing and aeration), chemical pre-treatment (acid shock (AST), basic shock (BST), sodium 2-bromoethanesulfonate or 2-bromoethanesulfonic acid and iodopropane) and combined pre-treatment (e.g., HST followed by BST) are the main pre-treatment methods adopted to inhibit methanogenesis. Table (2.10) shows the different inoculum pre-treatments to enrich hydrogen-producing bacteria and inhibit hydrogen-consuming bacteria in DF.

Table 2.10 Inoculum pre-treatment methods to inhibit hydrogen-consuming bacteria in dark fermentation, adapted from (Ghimire et al., 2015).

Pre-treatment method	Inoculum source	Reference
Heat shock (HST)		
100 °C for 15 min	Anaerobic digested sludge	(Wang and Wan, 2008)
80°C, 90°C and 100°C for 15-30 min	Anaerobic sludge	(Wang et al., 2011)
Heating in boiling water bath for 10-30 min	Anaerobic granular sludge	(Mohammadi et al., 2011)
105 °C for 4h	Anaerobic granular sludge	(Giordano et al., 2011)
Incubation at 90 °C for 1h	Anaerobic granular sludge	(Luo et al., 2010)
100–105 °C in oven for 2h	Cow dung compost	(Fan et al., 2004)
Acid shock (AST)		
pH to 2 for 24h and increasing pH to 5.5 by adding a 2 N NaOH solution	Anaerobic digested sludge	(Mohammadi et al., 2011, Lee et al., 2009)
pH 3 with 2 N HCl for 24h	Anaerobic digested sludge	(Luo et al., 2011)
pH to 3 with1 N HCl for 30 min	Anaerobic digested sludge	(Zhu and Béland, 2006a)
pH 3 with 0.1 N HCl solution for 24h and adjusting back to pH 7	Anaerobic granular sludge	(Hu and Chen, 2007a)
Basic shock (BST)		
pH of the sludge to 3 with 1 mol/L of NaOH for 24h	Anaerobic digested sludge	(Wang and Wan, 2008)
pH 8, 9 and 10 with 1mol/L of NaOH for 3h	Anaerobic sludge	(Wang et al., 2011)
pH 12 with 1 M NaOH for 24h and adjusting back to pH 7 using 1 M HCl	Anaerobic digested sludge	(Sompong et al., 2009)
Load shock (LST)		
Sludge (50 ml) spiked with 40 g of sucrose and acidification for 2 d	Anaerobic granular sludge	(Luo et al., 2010)
Sludge (50 ml) spiked with 500 mL of sucrose (50 g/L) and acidification for 2 d	Anaerobic digested sludge	(Sompong et al., 2009)
Chemical inhibition		
10 mmol of BESA for 30 min and gravity separation for 2h	Anaerobic digested sludge	(Zhu and Béland, 2006a)
0.2 g/l BESA for 24h	Anaerobic granular sludge	(Mohan et al., 2008a)
0.1% (v/v) chloroform for 24h	Anaerobic digested sludge	(Mohammadi et al., 2011)
Aeration		
Aerate with air for 24h	Anaerobic sludge	(Wang and Wan, 2008)
Flushing with air for 30 min	Anaerobic sludge	(Zhu and Béland, 2006a)
Microwave irradiation		
Microwave radiation for 1.5 min	Cow dung compost	(Song et al., 2012)

Several studies have compared the effect of these pre-treatments on increasing hydrogen production but arrived at different conclusions. For example, Zhu and Béland (2006a) studied HST, BST, AST, iodopropane, 2-bromoethanesulfonic acid and aeration. The operation conditions were the same in the two batch tests, and the results show that iodopropane pre-treatment was the best method for the first batch tests (sucrose + digested sludge), while BST was the best in the second batch tests (first batch effluent + sucrose), in terms of inhibiting the methanogens and enriching the hydrogen-producing bacteria. However, Mu et al. (2007) used the same type of inoculum (anaerobic sludge) as (Zhu and Béland, 2006a) and reported that HST was the best method to enrich hydrogen-producing bacteria. Moreover, Cheong et al. (2006) reported that AST was the best method among five pre-treatment methods (dry HST, wet HST, freezing-thawing, sodium 2-bromoethanesulfonate and AST), using cattle manure sludge. (Mohan et al., 2008b) found that of seven pre-treatments, sodium 2-bromoethanesulfonate was the best for hydrogen production, using anaerobic mixed microflora as inoculum. Using sewage sludge as inoculum, (Hu and Chen, 2007b) demonstrated that, of three pre-treatment methods (chloroform, HST and AST), chloroform was the best.

Ghimire et al. (2015) summarised the advantages and disadvantages of different pre-treatment methods and provided a simple assessment of the commonly applied inoculum pre-treatments, as shown in Table (2.11).

Table 2.11 Evaluation of inoculum pre-treatment methods to enhance microorganism activity in DF, adapted from (Ghimire et al., 2015).

Pre-treatment method	Energy Requirement	Chemical Requirement	Economics cost	Scale-up application
Heat shock	* * *	*	* * *	* *
Acid shock	*	* * *	* *	* * *
Chemical	*	* * *	* * *	* *
Aeration	* * *	*	* *	* * *
Load shock	* *	* *	*	* * *

* Low
* * Moderately
* * * High

All these studies and their conflicting results show that there is a need for more investigation and studies to determine which pre-treatment is best for inhibiting hydrogen-consuming bacteria and enriching hydrogen-producing bacteria in a certain type of inoculum, and how it performs depending on the actual substrate. Moreover, research is needed to find a strategy to test types of inoculum and assess the effect of different pre-treatments, as this will help researchers better compare their results.

2.5.3.4 Substrate pre-treatment

Sewage sludge is considered a complex substrate, and the conversion process of this material is limited by biological hydrolysis (Monlau et al., 2013). Therefore, a substrate pre-treatment is essential to enhance hydrogen production from complex substrates. Different substrate pre-treatments have been tested for their ability to enhance the biodegradability of DF feedstock. Figure (2.11) shows these pre-treatments, as many studies reported that they can enhance the degradation of substrate and biogas production in DF (Zhang et al., 2014, Taherzadeh and Karimi, 2008, Mussoline et al., 2013, Kongjan and Angelidaki, 2010, Zhu et al., 2005). Moreover, nutrient formulation is important for the improvement of hydrogen yield (Lin and Lay, 2005), and using a mixed culture is

better than using a pure culture for hydrogen production using sewage sludge as feedstock (Massanet-Nicolau et al., 2008, Kalogo and Bagley, 2008).

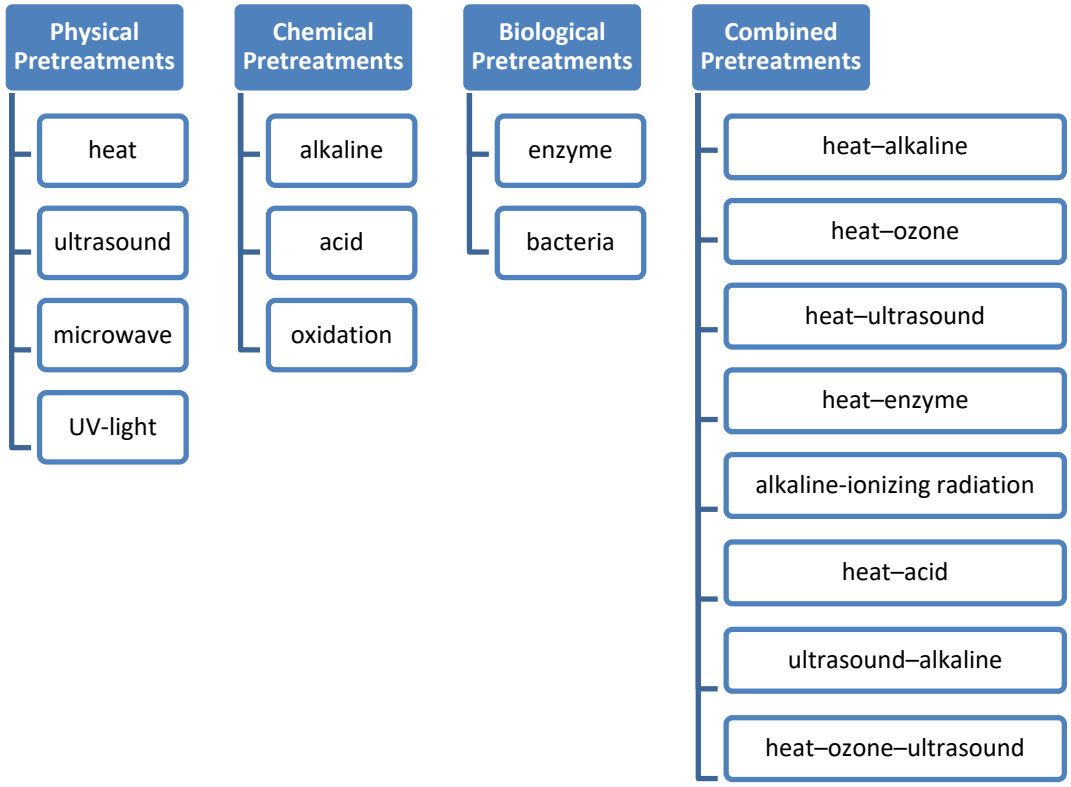


Figure 2.11 Different pre-treatment methods for substrates later used for a dark fermentation reactor, adapted from (Yang and Wang, 2017).

The main idea for substrate pre-treatment is to convert the complex organic matter in substrates (e.g., sewage sludge) into easily biodegradable forms, leading to better consumption by hydrogen-producing bacteria. Many studies have investigated hydrogen production from sugars (simple carbohydrates) (Wang and Wan, 2008, Oh et al., 2003b, Luo et al., 2011). Carbohydrates (sugars) are one of the main fermentable substrates for hydrogen production as they are considered the most favourable substrate for fermentative bacteria (e.g., *Clostridium* bacteria) (Finlay, 1995). Sugars (i.e., glucose) naturally exist in plants and are used extensively in food-processing industrial activities (Fellows,

2009). Therefore, they are not suitable to be used directly as a substrate for hydrogen production through the DF process.

Sewage sludge has the potential to be a sustainable source for glucose production, as it has been reported that an estimated 6.22 Mt/year of sugar can be produced from municipal sludge and livestock manures (Champagne, 2007). Although sewage sludge is processed by AD reactor in WWPTs around the world for methane production, it still offers a large volume of a waste stream rich in organic materials, and from these contents, sugars (i.e., glucose) can be recovered/produced and used as a feedstock for DF for hydrogen production. However, due to the complexity of sewage sludge content, it is difficult for fermentative bacteria to utilise it, and it has a low C/N ratio compared with other types of waste (Xia et al., 2016). Therefore, it is essential to pre-treat sewage sludge to ensure efficient bio-hydrogen production in DF processes. Several studies have reported different pre-treatment methods to enhance hydrogen production from sewage sludge (Yang et al., 2016). Disintegration of sewage sludge is one method that can break down the hard-to-digest macro sewage flocs to easily-digestible micro flocs, as shown in Figure (2.12). As a result, sewage sludge biomass has a suitable fermentable structure that can be easily utilised by fermentative bacteria for hydrogen production.

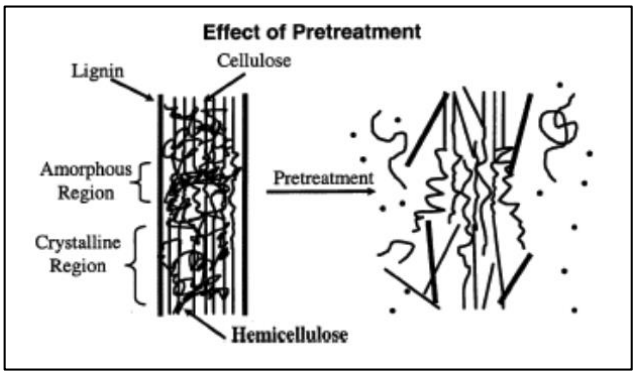


Figure 2.12 Effect of enzymatic hydrolysis in breaking down the complex structure of Lignocellulosic biomass in sewage sludge, adapted from (Hsu et al., 1980).

Disintegration can be achieved by four methods: mechanical, physical, chemical and biological. Enzymatic hydrolysis (EH) method is more favourable than mechanical, chemical and physical pre-treatments, as it is a biological process that requires a lower energy input than other pre-treatments. Very few studies have yet investigated using EH to pre-treat sewage sludge to enhance bio-hydrogen production (Massanet-Nicolau et al., 2008, Parawira, 2012); thus, part of this thesis will address the assessment of the EH process as a pre-treatment of sewage sludge to enhance hydrogen production via DF.

2.6 Research gaps

In summary, there is potential to increase methane yield in the biogas produced in AD processing of sewage sludge. According to this literature review, enhancing methane yield through hydrogenotrophic methanogens is one promising method of upgrading biogas from AD. As this method needs a hydrogen source, implementing the DF process to use sewage sludge for bio-hydrogen production will add benefits (environmental, sustainability-related and economic) to the whole upgrading process, as the available hydrogen production technologies are extensively energy-consuming and have negative impact on the environment.

The hydrolysis process for sewage sludge is a limited step for both the DF and AD systems. More focus on and investigation of the influence of parameters in the DF process, which include inoculum pre-treatments, substrate pre-treatments, pH and inoculum to substrate ratio, will enhance hydrogen production and increase the efficiency of the process, overcoming bottlenecks when moving toward pilot-scale DF implementation.

From a different perspective, optimising DF that processes sewage sludge will draw more attention to the use of sewage sludge as a feedstock for substantial bio-hydrogen production and will thus benefit industry (as an energy source). In addition, using sewage sludge as a feedstock for DF has positive impacts from an environmental perspective, as the volume of sewage sludge disposed of by WWTPs will be reduced. From an economic perspective, meanwhile, handling costs (additional treatment, transfer, etc.) will be reduced.

Therefore, this thesis will assess the potential of producing hydrogen via DF processing of sewage sludge as feedstock and anaerobic digestate as inoculum. Furthermore, several pre-treatments, including inoculum and substrate pre-treatments, will be investigated and assessed to determine whether they enhance hydrogen production. The idea of integrating DF with EH to enhance VFAs and hydrogen production will also be assessed. Overall, this thesis will provide guidance for optimising hydrogen production through DF that processes sewage sludge and fill the gaps addressed in this chapter. Figure (2.13) shows the current application for processing sewage sludge in WWTP and the proposed application, also its summarise the overall research gaps.

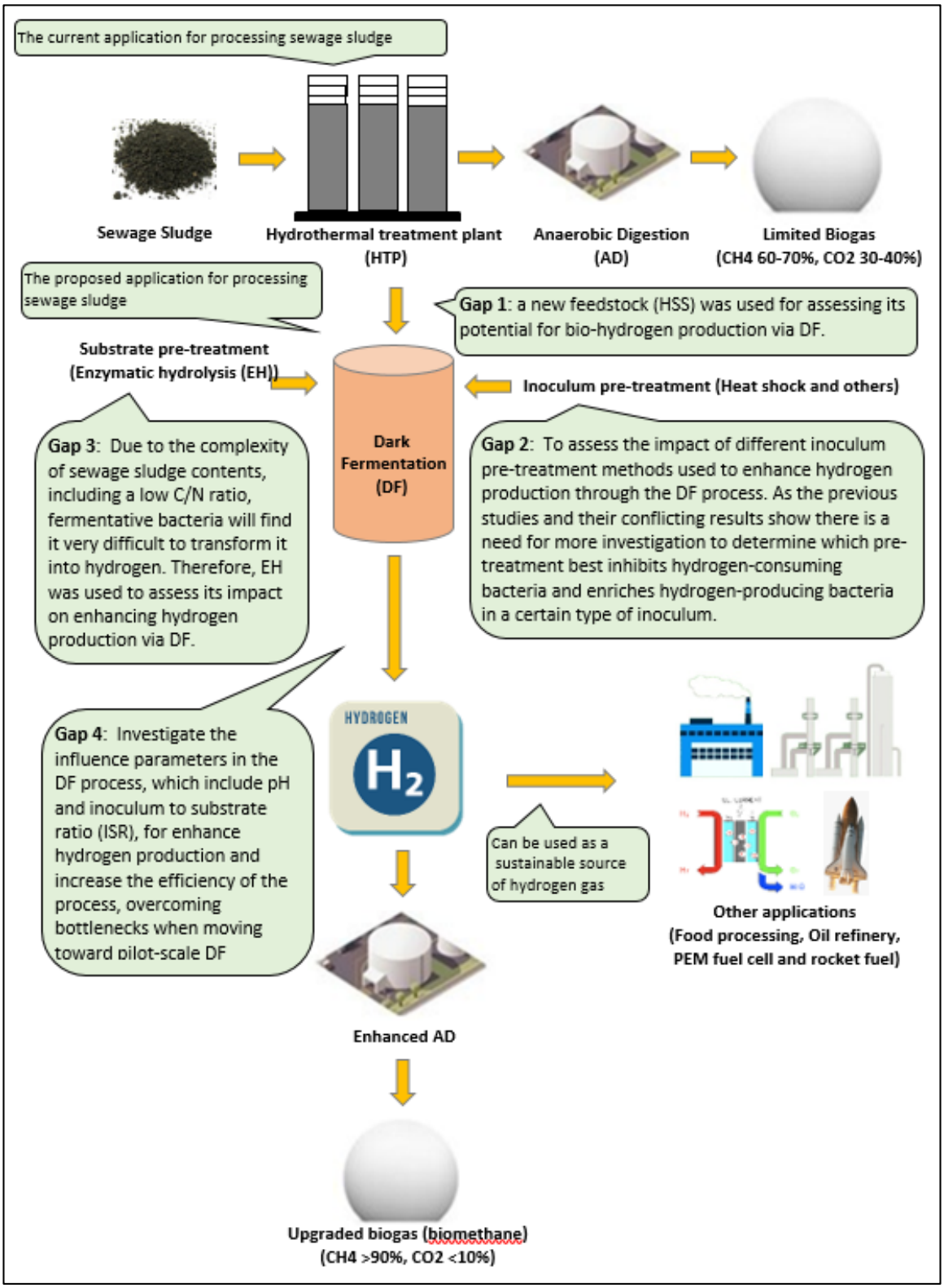


Figure 2.13 Summary of research gaps of the current application of processing sewage sludge for biogas/AD and bio-hydrogen/DF.

Chapter 3
Research Methodology and Experimental Design

This chapter describes all the experimental activities conducted in this research project and presents detailed experimental set-ups as well as the analytical and statistical methods used for sample characterisation and data processing. Details of the biochemical methane potential (BMP), bio-hydrogen potential (BHP) tests and enzymatic hydrolysis (EH) tests are also described.

3.1 Characterisation of hydrolysed sewage sludge and digestate

One of the objectives of this research is to create a characterisation database of hydrolysed sewage sludge (HSS) (used as feedstock in this study) and digestate (used as inoculum in this study) by conducting monthly sampling over one year.

3.1.1 Description of sample collection and processes

Samples were collected from Yorkshire Water's Esholt WWTP, Bradford, UK. Sample collection started in November 2018 and was carried out on a monthly basis. Two type of samples were collected from Esholt: HSS and digestate. Esholt WWTP processes a blended sewage sludge, which is a mix of indigenous primary sludge, imported liquid sludge, thickened secondary surplus activated sludge (SAS) and imported sludge cake. This mix is used to feed the hydrothermal treatment plant (HTP) in Esholt WWTP. The main purpose of hydrothermal treatment is to enhance the solubility and biodegradability of sewage sludge before it goes to AD for biogas production (Wirth et al., 2015, Wang et al., 2010). This pre-treatment is commonly integrated with a mesophilic AD reactor in many WWTPs, including the Esholt WWTP in Bradford, UK. Details

of the operation conditions of HTP are given in Table (3.1). Samples of HSS were collected from the effluent of HTP units.

After hydrothermal treatment, the treated sewage sludge mix is fed to AD reactors for methane production. Digestate samples were collected from the effluent of AD reactors. Figure (3.1) shows the steps for processing blended sewage sludge at Esholt WWTP. Operation conditions for HTP and AD reactors at Esholt WWTP are shown in Table (3.1).

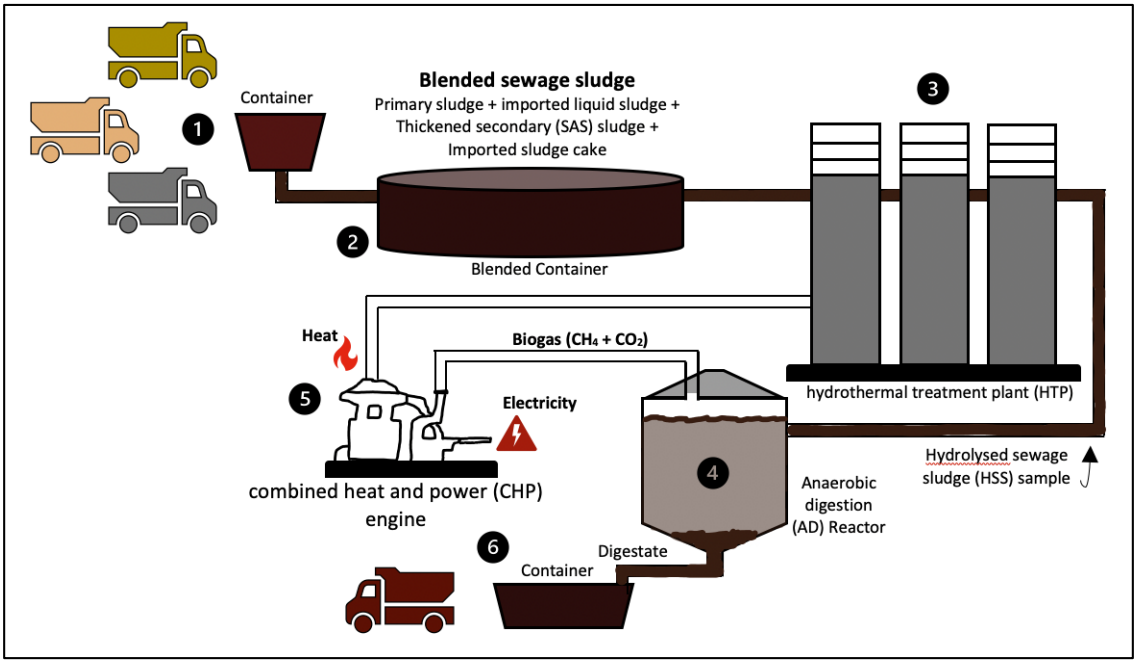


Figure 3.1 Sewage sludge processing at Esholt WWTP, Bradford, UK.

Table 3.1 Details of operation conditions for HTP and AD units at Esholt WWTP.

	Thermal hydrolysis treatment (HTP)	Anaerobic Digestion (AD)
Working temperature	160°C	40°C
Feeding type	Batch	Continuous
Loading rate	Not given	2.3 kg VS/m^3/day
HRT	Not given	10-12 Days
Biogas composition	N/A	40% CO_2 + 60% CH_4

HRT: hydraulic retention time; **N/A**: not applicable.

After HSS and digestate samples were collected from Esholt WWTP, they were transferred to the Public Health Laboratory in the School of Civil Engineering,

University of Leeds. All samples were initially filtered using a 1-mm sieve to remove large particles; then, samples were divided into small containers for easy storage. The sample characterisation was undertaken immediately through different tests carried out in the lab, after which HSS samples were stored in a freezer at -22°C to be used as feedstock for the following experiments. Digestate samples were kept in an incubator at 37°C and fed every week with HSS to keep active the anaerobic consortium of bacteria.

3.1.2 Characterisation

The characterisation of collected samples involved several analytical tests, as described in the following diagram (Figure 3.2).

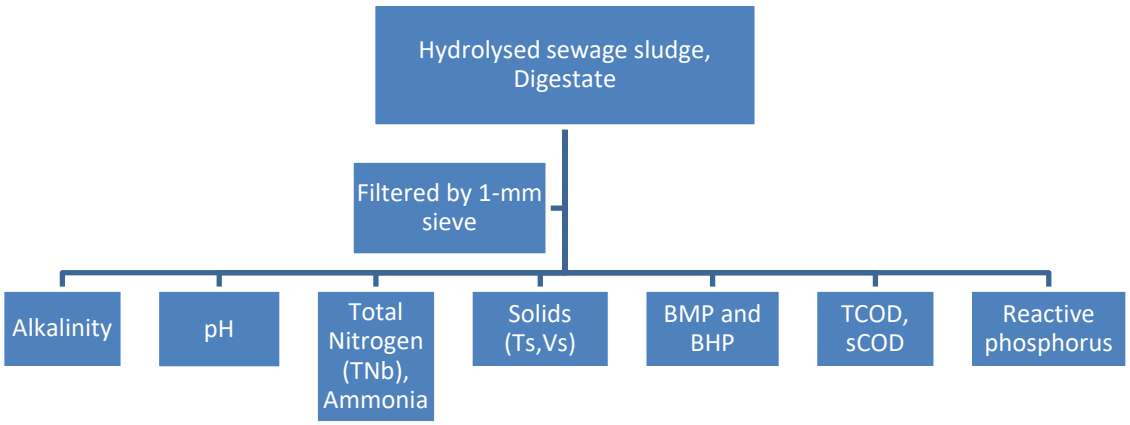
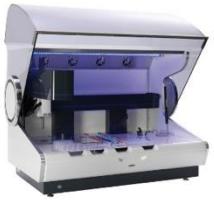


Figure 3.2 Characterisation tests for the samples collected from Esholt, UK.

These analytical tests were selected to evaluate the changes to HSS and digestate characteristics over a year, also to assess the performance of AD plant in Esholt WWTP as these measured parameters have influence on the biogas production and the stability performance of AD reactor. The collected samples were filtered by a 1-mm sieve to remove large particles. A digital HACH HQ40D pH meter and a Mettler-Toledo auto-titrator T50 were used to measure pH and

alkalinity, respectively. Total chemical oxygen demand (TCOD), soluble chemical oxygen demand (sCOD), ammonia and total nitrogen (TNb) were measured by AP3900 Laboratory Robot. Reactive phosphorus was measured by two methods: (1) Shimadzu UV1900 for samples (November 2018 to July 2019) and (2) AP3900 Laboratory Robot for samples (September 2019 to February 2020). Solids (total solids (TS) and volatile solids (VS)) were measured by using the gravimetric method, as described in 2540B and 2540E of standard methods. All tests were conducted in triplicate. Table (3.2) provides more detail of the analytical tests. Also, biochemical methane potential (BMP) and bio-hydrogen potential (BHP) were measured for the collected samples (hydrolysed sewage sludge and digestate).

Table 3.2 Analytical details.

Test	Device	Methodology
pH	Digital HACH HQ40D pH meter	- All the components inside the bottles were transferred to a small tube (50 mL). - Then, the pH electrode was inserted inside the tube to measure the pH directly.
Alkalinity	Mettler-Toledo auto-titrator T50	- A 1-mL sample was taken from the bottle and put into a small cup; 39 mL distilled water (DW) were added. - The device was calibrated and prepared for the test. - The small cup was attached to the device ready for testing.
Total solids (TS)	Weight balance and oven 105 °C	Gravimetric method as described in 2540B* of standard methods. - The empty crucible was weighed. - A small amount of sample was placed inside the crucible, and it was weighed again. - The small crucible was put inside the oven at 105°C for 1 day. - The crucible was taken out of the oven and placed inside the dissector for 1 hour, then weighed.
Volatile solids (VS)	Weight balance and furnace 550 °C	Gravimetric method as described in 2540E* of standard methods. - After the crucible was weighed for TS, it was placed inside the furnace at 550°C for 2 hours. - The crucible was taken out of the furnace and placed inside the dissector for 1 hour, then weighed.
Total chemical oxygen demand (TCOD)	AP3900 Laboratory Robot	- The sample was prepared by specific diluted factor to be within the range of measurement. - The sample was placed inside the device, which was calibrated and programmed to measure specific parameters. - The COD kit (LCK 514: COD cuvette test 1000–2000 mg/L O2) was placed inside the device at a specific location. - The device then started to measure COD automatically.
Soluble chemical oxygen demand (sCOD)		- The sample was centrifuged by using centrifuge Eppendorf 5810 at 4,000 rpm for 5 min to separate the solid and liquid. - The liquid sample was extracted and prepared by specific diluted factor to be within the range of measurement. - The sample was placed inside the device, which was calibrated and programmed to measure specific parameters. - The COD kit (LCK 514: COD cuvette test 100–2000 mg/L O_2) was placed inside the device at a specific location. - The device then started to measure COD automatically.
Ammonia		- The sample was prepared by specific diluted factor to be within the range of measurement. - The sample was placed inside the device, which was calibrated and programmed to measure specific parameters. - The ammonia kit (LCK302: Ammonium cuvette test 47–130 mg/L NH_4-N) was placed inside the device at a specific location. - The device then started to measure ammonia automatically.

Total nitrogen (TNb)		- The sample was prepared by specific diluted factor to be within the range of measurement. - The sample was placed inside the device, which was calibrated and programmed to measure specific parameters. - The total nitrogen kit (APC338: TNb, Total Nitrogen cuvette test, 20–100 mg/L) was placed inside the device at a specific location. - The device then started to measure TNb automatically.
Reactive phosphorus	Shimadzu UV1900 	- All the equipment was acid-washed with 1M hydrochloric acid solution. - Four reagents (ammonium molybdate solution, potassium antimonyl tartrate solution, 0.1M ascorbic acid solution and 2.5M sulphuric acid) were prepared by specific procedures. - The 100 mL combined reagent was prepared with the following composition: 50 mL of 2.5M sulphuric acid; 5 mL of potassium antimonyl tartrate solution; 15 mL of ammonium molybdate solution; 30 mL of ascorbic acid solution. - The sample was prepared by specific diluted factor to be within the range of measurement. - The standardisation test was carried out before the samples were tested. - Standard phosphate solution was prepared for the standardisation test, and the device was set up for a specific phosphate concentration before the standardisation test. - The sample was tested after the standardisation test was finished.

*(APHA, 1998).

3.2 Process monitoring and analysis

Several items of equipment and methods were used to monitor the tests conducted in this research. This section describes in detail all tests and equipment used during this research.

3.2.1 Gas analysis

The volume of gas produced from BMP and batch BHP tests were measured by using the water displacement method (Figure 3.3). The biogas composition – hydrogen, carbon dioxide and methane – was measured by gas chromatography (GC – Agilent 7890A) with a thermal conductivity detector (TCD). The GC–TCD was fitted with a Carboxen 1010 PLOT column with the following dimensions: length 30m, diameter 0.53mm and film thickness 30µm. The inlet and oven temperatures were 200°C and 230°C, respectively, and argon was used as the carrier gas at 3mL/min. The GC was calibrated with three standard gas mixtures – 20%-O_2:80%-N_2; 50%-CH_4:3%-H_2:47%-N_2; and 10%-CO_2:90%-N_2 – at predetermined intervals. Gas samples were manually injected using 200µl injection volume.

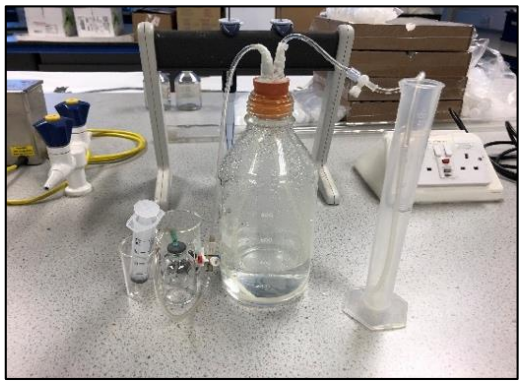


Figure 3.3 Water displacement set-up to measure gas production.

3.2.2 Liquid analysis

The liquid samples produced from BMP, BHP and EH tests were characterised using the following methods and equipment. The pH was measured by a digital pH meter (HACH HQ40D) and alkalinity by a Mettler-Toledo auto-titrator T50 (more details are given in Table (3.2)). VFA concentration was measured by gas chromatography (GC - Agilent 7890A) with a flame ionisation detector (FID). The GC-FID was fitted with a DB-FFAP column with the following dimensions: length 30m, diameter 0.32mm and film thickness 0.5µm. The GC-FID was operated at 150°C inlet temperature and 200°C oven temperature with helium as carrier gas (10 mL/min). Liquid samples were injected by an autosampler at 10µl injection volume. The GC was calibrated with a SUPELCO Volatile Acid Standard Mix, which contains acetic-, propionic-, iso-butyric-, butyric-, iso-valeric-, valeric-, iso-caproic-, caproic- and heptanoic- acids. The liquid samples for VFA analysis were prepared by lowering the pH to 2.00–2.20 with phosphoric acid and allowing to rest for 30 min. Samples were then centrifuged using a Pico 21 Centrifuge at 14,000 rpm for 5 min. The supernatant was then collected and sieved through a 0.2-µm filter before injection for analysis in the GC-FID equipment.

Pyruvate, formate and ethanol were measured by high-performance liquid chromatography (HPLC – Thermo Ultimate 3000) with photodiode array detection (PDA detector) and Supelcogel™ C-610H (6% crosslinked) column. The HPLC equipment was operated at 0.5 mL/min flow rate, 10 mL sample injection volume, 30°C column temperature, and a mobile phase of 0.1% of phosphoric acid in distilled water. High purity standards were used for calibration.

3.3 Biochemical methane potential

The BMP test is a well-known analytical technique that has been used to assess the potential production of biogas production, especially methane gas, from organic waste samples and is a common test used for the characterisation of sewage sludge (Remigi and Buckley, 2006). The BMP test was used to assess the methane potential of the samples collected from Esholt WWTP. HSS was used for biogas production through experimental batch BMP tests. The operation temperature of the BMP reactor was adjusted to 37°C (mesophilic condition) using a water bath, as a replicate of the AD units at Esholt WWTP. Knowing the amount of methane that can be produced from HSS is essential for optimising the AD process.

3.3.1 Theoretical maximum methane potential

The theoretical maximum methane potential (TMP) of AD feedstock (e.g., sewage sludge) can be calculated by using well-known models such as the Buswell equation and Dulong formula. These are two of the models most commonly used to calculate TMP values, which are based on the biochemical or elemental composition of organic waste in sewage sludge, as well as estimations based on the total oxygen demand (Nielfa et al., 2015). Complete degradation of organic contents in processed biomass is assumed in TMP calculations without counting the internal enzymatic reactions in AD processes. Therefore, TMP values are usually higher than the figures reported from BMP experimental tests (Defra, 2010a, Nielfa et al., 2015). The TMP values for the processed HSS samples were thus estimated according to the Buswell equation (see Equation (3.1), (Kong et al., 2016):

$$TMP\ (mLCH_4/g\ VS) = \frac{22.4 \times 1000 \times (\frac{c}{2} + \frac{h}{8} - \frac{o}{4} - \frac{3n}{8})}{12c + h + 16o + 14n} \qquad (3.1)$$

Where the letters c, h, o and n represent the subscripts of the corresponding elements (carbon, hydrogen, oxygen and nitrogen) in the empirical formula of the biomass, determined as follows:

$$Subscripts\ (c, h, n, or\ o) = \frac{Element\ (C,H,N\ or\ O)}{Element's molar\ weight} \qquad (3.2)$$

Carbon (C), hydrogen (H), oxygen (O) and nitrogen (N) were measured by elemental analysis as samples were dried at 40°C and then ground to powder. After that, samples were wrapped in aluminium foil and stored in a dissector until they were tested using a FLASH 2000 Elemental Analyzer. Materials' degradability potential can also be estimated by calculating TMP. As shown in Equation (3.3), the anaerobic biodegradability (BD) of biomass can be calculated by dividing BMP experimental over TMP (Raposo et al., 2011) as BD can also be used as an indication of optimising the AD process:

$$BD_{CH4}(\%) = \frac{BMP_{exp}}{TMP} \times 100 \qquad (3.3)$$

3.3.2 BMP test set-up

The experimental set-up for the BMP test entailed the use of Wheaton glass bottles and a water bath, as shown in Figure (3.4). The Wheaton bottles were sealed with a rubber cap and an aluminium seal to prevent biogas leakage.

Figure 3.4 BMP equipment: A 160-mL Wheaton bottle (left) and water bath for temperature control (right) were used during the BMP test.

The inoculum-to-substrate ratio (ISR) 1:1 was used to set up the BMP test. The following diagram (Figure 3.5) shows the details of the BMP test set-up.

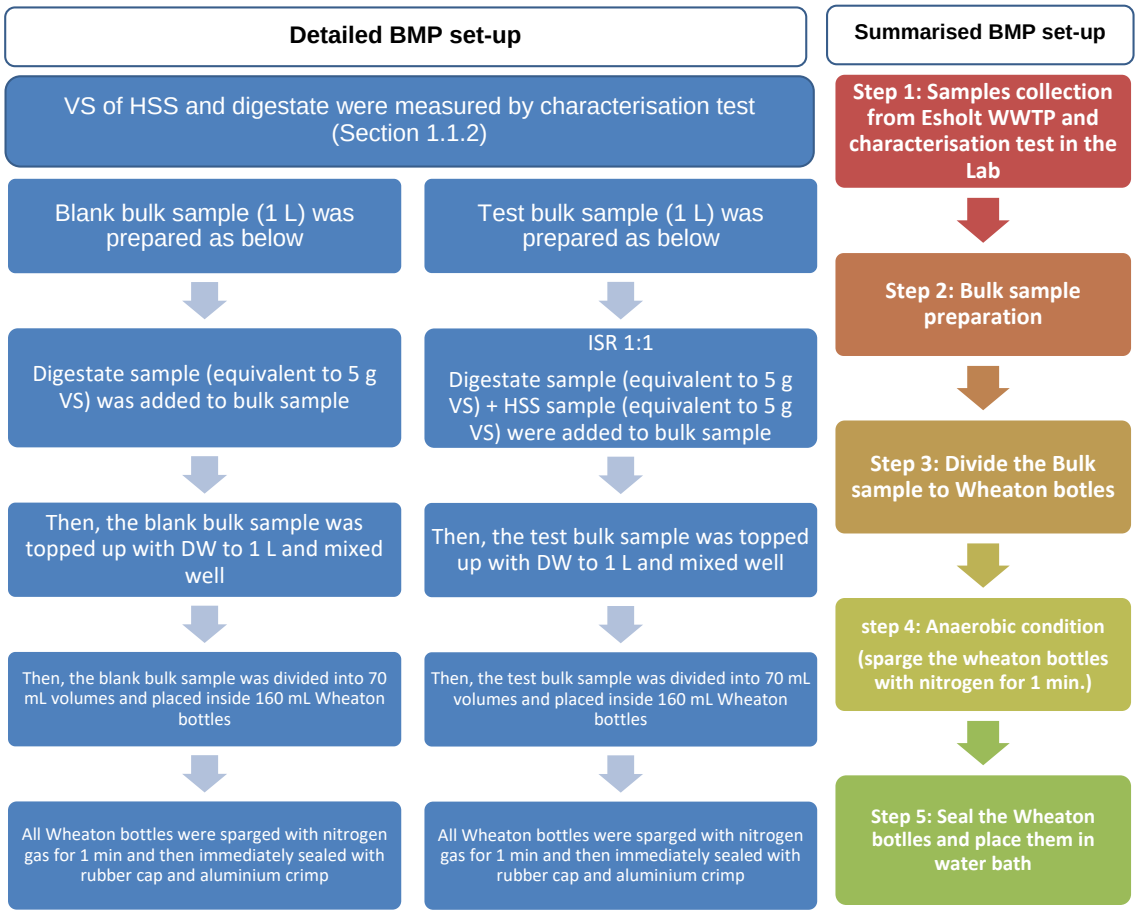


Figure 3.5 Details of BMP test set-up.

All the bottles were treated by nitrogen gas (sparged through the solution for 1 min) to ensure that the BMP bottles were in anaerobic condition, after which they

were placed in the water bath at 37°C (mesophilic condition). The bottles were tagged by day number and as either blank or test. On Days 2, 4, 8, 15, 22 and 28, four bottles (two blank and two test, as duplicates) were taken out of the water bath and tested by the analysis tests shown in Section (3.2). Table (3.3) shows the testing day during the 28 days of BMP.

Table 3.3 Sample collection and characterisation during 28-day BMP test.

Parameter/Day	0	2	4	8	15	22	28
pH	x	x	x	x	x	x	x
Alkalinity	x	x	x	x	x	x	x
TS	x	x	x	x	x	x	x
VS	x	x	x	x	x	x	x
TCOD	x	x	x	x	x	x	x
sCOD	x	x	x	x	x	x	x
Ammonia	x	x	x	x	x	x	x
Total nitrogen	x	x	x	x	x	x	x
R. phosphorus	x	x	x	x	x	x	x
VFAs	x	x	x	x	x	x	x
Biogas volume		x	x	x	x	x	x
Biogas composition		x	x	x	x	x	x

TCOD: total chemical oxygen demand; **sCOD**: soluble chemical oxygen demand; **TS**: total solids; **VS**: Volatile solids; **VFAs**: Volatile Fatty Acids.

3.3.3 Data processing and statistical analysis

3.3.3.1 Biogas conversions to standard temperature and pressure (STP)

The ideal gas law was employed to convert all gas volumes recorded to STP:

$$The\ ideal\ gas\ law\ is\ given\ as\ PV\ =\ nRT$$

$$Where\ P\ =\ pressure\ of\ the\ gas\ (atm);\ V\ =\ volume\ of\ the\ gas\ (L);$$

$$n\ =\ number\ of\ moles;$$

$$R\ =\ universal\ gas\ constant\ given\ as\ 0.08206\ L\ atm\ K^{-1}\ mol^{-1}$$

$$and\ T\ =\ temperature\ (Kelvin)$$

$$At\ STP, 1\ mole\ of\ a\ gas\ occupies\ 22.4\ L,$$

$$Hence, at\ the\ operating\ temperature\ (37℃, 310K),$$

$$1\ mole\ of\ gas\ will\ occupy\ \frac{310 \times 22.4}{273} = 25.44\ L$$

$$If\ 1\ mole\ occupies\ 25.44\ L, n\ number\ of\ moles\ of\ measured\ volume = \frac{measured\ volume\ (L) \times 1\ mol}{25.44\ L}$$

$$Where\ number\ of\ mols, n\ (mol) = \frac{mass\ of\ gas, m\ (g)}{Molar\ mass, M\ (g/mol)}$$

The individual masses were then calculated using their respective densities at STP:

$$mass\ of gas\ at\ STP, m\ (g) = n\ at\ STP\ (mol) \times M\ (g/mol)$$

The ideal gas law was rearranged to estimate the pressure of the respective gases (P_g):

$$P_g = \frac{n_g \times R \times T_g}{V_g}$$

The combined gas law was then used to estimate the volumes of the respective gases at STP:

$$Combined\ gas\ law: \frac{P_1 \times V_1}{T_1} = \frac{P_2 \times V_2}{T_2}$$

Replacing the left operand as the measured gas parameters and the right operand as the parameters of the gas at STP, the volume of the gas at STP (V_s) becomes:

$$V_s = \frac{P_g \times V_g \times T_s}{P_s \times T_g}$$

3.3.3.2 Process kinetics

Process kinetics is an important tool that helps to assess the BMP result. Origin 2018b graphical and statistics software was used to analyse and fit the data. The Modified Gompertz (MGompertz) model (as shown in Equation (3.4)) was used to fit the biogas results, as this model is extensively used for batch experiments

that have a growth rate, such as the BMP batch experiment (Pagliaccia et al., 2016, Zwietering et al., 1990). The MGompertz model is preferred over the first order kinetics model as it can provide more information, such as daily maximum specific methane yield and lag time, which is used to assess the efficiency of AD:

$$y = A.exp\left\{-exp\left[\frac{\mu_m.e}{A}(\lambda - t) + 1\right]\right\} \qquad (3.4)$$

$where; (y)\ is\ cumulativemethaneyield(mLCH_4/gVS),$

$(A)\ is\ maximum\ methane\ yield\ (mLCH_4/g\ VS)\ at\ time\ t.$

$(\mu_m)\ is\ maximum\ specifiv\ methane\ yield\ per\ day\ ((mmLCH_4/g\ VS)/day).$

$(\lambda)\ is\ lag\ time\ (day)\ and\ (e)\ is\ exp(1).$

The k-value is a BMP kinetic rate constant (also called the hydrolysis rate) and is widely used to determine the optimal condition of AD in terms of design and operation. A high k-value is an indication of a fast hydrolysis process in a BMP test (Ortega-Martinez et al., 2016). The k-value was calculated by the standard Gompertz model in Origin 2018b software.

3.4 Bio-hydrogen potential

The BHP test has been used to assess the potential hydrogen production from a DF reactor that processes either sole substrate (e.g., glucose) or complex substrate (e.g., food, municipal and agricultural waste). This section is divided into three parts, each of which represents a chapter in this thesis.

3.4.1 BHP – inoculum pre-treatments

An inoculum pre-treatment is a very important step for DF and can affect BHP. Mixed cultures, such as digestate (from AD), can be used as inoculum in DF because they are easier to handle and control and highly available (Li et al., 2007). However, mixed cultures contain both hydrogen-consuming bacteria

(methanogens and homoacetogens) and hydrogen-producing bacteria (such as *Clostridium* and *Enterobacter*). In the DF process, which is operated under anaerobic conditions, the hydrogen-consuming bacteria can easily consume the hydrogen and inhibit hydrogen production (Oh et al., 2003b, Cai et al., 2004b). Therefore, to inhibit the methanogens' activity, an inoculum pre-treatment is a necessary step in DF. Several studies have investigated different types of inoculum pre-treatment, which are classified into physical, chemical and biological treatment methods (Hu and Chen, 2007b, Zhu and Béland, 2006a, Mu et al., 2007, Mohan et al., 2008b).

There is no universal pre-treatment method to inhibit hydrogen-consuming bacteria; thus, different outcomes have been reported for DF experiments (Chaganti et al., 2012, Cai et al., 2009, Liu et al., 2009, Pendyala et al., 2012, Argun and Kargi, 2009). The selection of pre-treatment methods was based on simplicity, frequency of use, availability of resources and potential for scale-up. Therefore, the following three pre-treatment methods to inactivate hydrogen-consuming bacteria were chosen (Wang and Wan, 2008):

(i) Acid shock pre-treatment (AST) was performed by adjusting the pH of the digestate to pH 3 using 1 M HCl and storing it in the fridge at 4°C for 24 hr. After 24 hr, the pH was returned to pH 7 using 1 M NaOH.

(ii) Basic shock pre-treatment (BST) was performed by adjusting the pH of the digestate to pH 10 using 1 M NaOH and storing it in the fridge at 4°C for 24hr. After 24 hr, the pH was returned to pH 7 using 1 M HCl.

(iii) Heat shock pre-treatment (HST) was conducted by heating the digestate for 20 min at 115°C (approx. 1.5 bar), using a standard autoclave.

The effectiveness of each pre-treatment method was tested by conducting batch BHP tests with the pre-treated digestate as inoculum and glucose (D-glucose) as the sole carbon source (substrate). The standard substrate, glucose, was chosen for the BHP experiments because it is a simple carbon source, easy to digest, and would easily allow comparison of the different pre-treatment methods based on the differences in process stability and ultimate H_2 yields. The BHP tests were set up using 160-mL Wheaton bottles as fermentative reactors with 70 mL working volume (Figure 3.6). The reactors were sparged with nitrogen gas for 1 min and then immediately sealed with a rubber cap and an aluminium crimp. The BHP tests included one control (untreated digestate as inoculum and glucose as substrate) and three tests (treated digestate (including HST, AST and BST) as inoculum and glucose as substrate).

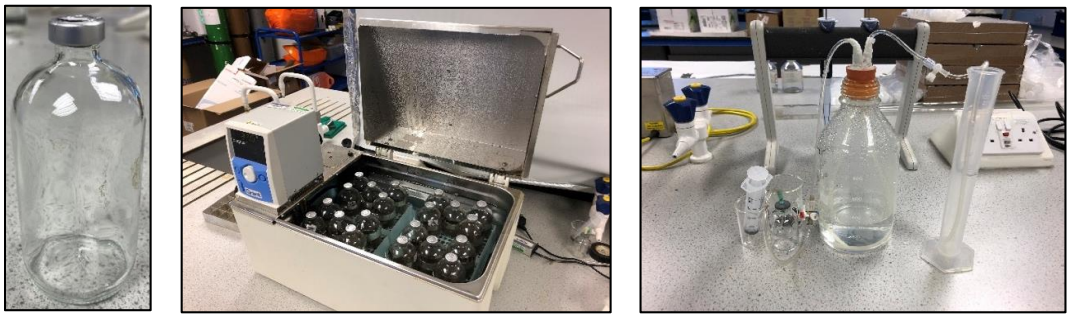


Figure 3.6 BHP equipment and material (a) Wheaton bottle (160 mL), (b) Water bath 37°C, (c) Water displacement device.

All tests were conducted in triplicate with (ISR 1:1 , 5 g VS inoculum to 5 g VS glucose) at 37°C. Initial pH was adjusted to 5.5 by using 1M NaOH and 1M HCL,

this being the optimum pH for DF processing glucose (Li et al., 2007). Several items of equipment and methods were used to calculate the parameters shown in Table (3.4) during the five-day BHP test.

Table 3.4 Testing points during the 5-day BHP test.

Parameter/Day	0	1	2	3	4
pH	x	x	x	x	x
Alkalinity	x	x	x	x	x
TS	x				x
VS	x				x
VFAs	x	x	x	x	x
Biogas volume		x	x	x	x
Biogas composition		x	x	x	x

TS: total solids; **VS**: Volatile solids; **VFAs**: Volatile fatty acids.

Further investigations were carried out by BHP-processing glucose for hydrogen and VFAs production. First, another HST for inoculum was tested as an alternative to HST, carried out using autoclave equipment (as mentioned earlier). Wang and Wan (2008) reported using HST with a furnace at 100°C for 15 min, and Luo et al. (2011) reported using a furnace at 100°C for 60 min for HST; both succeeded in inhibiting hydrogen-consuming bacteria and had the maximum hydrogen yield of all the inoculum pre-treatments. Therefore, another HST was used (105°C for 60 min) as an alternative to HST by autoclave (115°C for 20 min). Second, another BHP test was carried out by studying the effect of initial pH (5.5 or 7.0) on hydrogen yield and VFA production. Finally, further BHP tests were carried out to assess the impact of the ISR where different ISR ratios were used (1:1 and 2:1). The set-up for these BHP tests was similar to the BHP test for pre-treatment assessment.

3.4.2 BHP of hydrolysed sewage sludge

In the work described in this section, the collected HSS sample from Esholt WWTP was used as substrate for BHP for hydrogen production. The BHP of HSS was conducted in four sequential stages (BHP series batches), each lasting five days. This method, called the enrichment method, is used to enrich the population of hydrogen-producing bacteria from previously pre-treated digestate samples in order to produce a better inoculum for processing HSS (complex organic material) and therefore enhance hydrogen production (De Gioannis et al., 2013b, Show et al., 2012, Wong et al., 2014). At the end of the first stage, the resulting biomass was collected from the small reactors (160-mL Wheaton bottles, Figure (3.6a) in Section 3.4.1 and treated again with HST (using an autoclave), after which it was used as inoculum to seed another series of small reactors for the second stage of the BHP test. The same procedure was repeated for stages three and four. In every stage, all reactors were fed with the same ISR of 1:1, by adding 5 g VS of inoculum to 5 g VS of HSS. All batch tests were operated at 37°C and with an initial pH of 5.5. BHP control, which has glucose as substrate, and treated digestate (HST by autoclave) was used to ensure the utilisation efficiency of the inoculum. All the reactors were sparged with nitrogen gas for 1 min and then immediately sealed with a rubber cap and an aluminium crimp. All BHP tests were conducted in triplicate.

Before each stage, characterisation of inoculum (digestate) and substrate (HSS) was conducted, and the VS was used to set up BHP reactors. Several items of equipment and methods were used to calculate the parameters shown in Table (3.5) during the BHP test.

Table 3.5 Testing points during the BHP test.

Parameter/Day	0	1	2	3	4
pH	x	x	x	x	x
Alkalinity	x	x	x	x	x
Ts	x				x
Vs	x				x
VFAs	x	x	x	x	x
Biogas volume		x	x	x	x
Biogas composition		x	x	x	x

TS: total solids; **VS**: Volatile solids; **VFAs**: Volatile fatty acids.

3.4.3 BHP of mixed substrates (hydrolysed sewage sludge + enzyme)

In the work described in this section, the effectiveness of integrating the EH process within the DF process was tested by conducting batch BHP tests with the pre-treated digestate as inoculum and HSS with cellulase enzyme as mixed substrate. The inoculum was treated by HST (details in Section 3.4.1). Cellulase enzyme dosage was selected according to the results presented in Chapter 6 (more details in Section 3.5.2) and after conducting several BHP (EH+DF) trials. The BHP was set up using 160-mL Wheaton bottles as fermentative reactors with 70 mL working volume, as shown in (Figure 3.6, a-c) in Section 3.4.1. VS was measured for treated inoculum and HSS prior to the set-up of BHP.

The BHP set-up was based on an ISR of 1:1 (5 g VS inoculum: 5 g VS substrate) without counting the enzyme added, as this was later removed from the results when hydrogen yield was calculated to make comparison easier. All bulk samples were adjusted to initial pH 7.0 using 1 M HCl (initial pH was changed from 5.5 to 7.0 based on the results presented in Section 5.3.3.5 in Chapter 5). Then, they were divided into small Wheaton bottles with a working volume of 70 mL, sparged with nitrogen for 1 min each and sealed with a rubber cap and an

aluminium crimp. All bottles were placed in an incubator at 37°C. BHP Blank, Control 1 and Control 2 were in duplicate, while BHP Test was in triplicate. Table (3.6) shows the details for each BHP reactor.

Table 3.6 Set-up details for each BHP reactor.

	Unit	Blank	Control 1	Control 2	Test
Treated inoculum	VS (g)	5	5	5	5
	mL/L	160	160	160	160
Glucose[a]	VS (g)		5		
	g/L		5		
Enzyme[b]	mL/L			10	10
HSS	VS (g)				5
	mL/L				90

(a) D-Glucose Powder; **(b)** Cellulase blend enzyme.
VS: Volatile solids; **HSS**: hydrolysed sewage sludge.

Three modifications were made to optimise the BHP (HSS + enzyme), as the results were not at the expected level. The three modifications were as follows:

1. BHP operation time was extended to eight days instead of five days to complete the DF reactions, as there was a lag time in the first BHP trial which meant the DF processes did not complete and the hydrogen production was therefore limited.
2. An alternative HST method (furnace: 105°C for 60 min) was used to prepare the inoculum for the optimised BHP. The results showed that some of the hydrogen-producing bacteria were de-activated by HST (autoclave: 115°C for 20 min), possibly due to overheating.
3. The ISR was changed from 1:1 to 2:1 to increase the amount of bacteria in the optimised BHP test, which may have resolved the limited hydrogen production seen in the first BHP trial.

Other than these three modifications, the experiment details remained as described earlier in this section. Moreover, VS was measured for treated inoculum and HSS prior to the set-up of BHP. Four bulk samples of volume 1 litre (except Test which had 1.5 litres) were prepared for this experiment; Table (3.7) shows the details of each BHP reactor.

Table 3.7 Set-up details for each BHP reactor.

	Unit	Blank	Control 1	Control 2	Test
Treated inoculum	VS (g)	10	10	10	10
	mL/L	360	360	360	360
Glucose[a]	VS (g)		5		
	g/L		5		
Enzyme[b]	mL/L			10	10
HSS	VS (g)				5
	mL/L				90

(a) D-Glucose Powder; **(b)** Cellulase blend enzyme.
VS: Volatile solids; **HSS**: hydrolysed sewage sludge.

BHP set-up was based on an ISR of 2:1 (10 g VS inoculum: 5 g VS substrate). All bulk samples were adjusted to an initial pH 7.0 using 1 M HCl, then divided into small Wheaton bottles with a working volume of 70 mL, sparged with nitrogen for 1 min each and sealed with a rubber cap and an aluminium crimp. All bottles were placed in an incubator at 37°C. BHP Blank, Control 1 and Control 2 were in duplicate, while the BHP Test was in triplicate.

3.4.4 Statistical and kinetic analysis

Minitab 18 statistical software was used to run correlation analyses between hydrogen yield and VFAs (acetate and butyrate) using a confidence level of $\alpha = 0.05$. Further, for kinetics analysis, the MGompertz model was used to fit the result of hydrogen production in the BHP test (more details about MGompertz are given in Section 3.3.3.2) and calculate hydrogen production potential, maximum hydrogen production rate, R^2 value and lag phase.

3.5 Enzymatic hydrolysis

Due to the complexity of sewage sludge structure, it is difficult for fermentative bacteria to utilise it, as shown in Chapter 5 of this thesis. Moreover, given the low C/N ratio (Xia et al., 2016), a pre-treatment of sewage sludge is an essential step to ensure efficient bio-hydrogen production in the DF process. Several studies have reported different substrate pre-treatment methods to enhance hydrogen production from sewage sludge (Yang et al., 2016). Disintegration of sewage sludge is one method that can break down the hard-to-digest macro sewage flocs to more easily digestible micro flocs. As a result, sewage sludge biomass will have a suitable fermentable structure that can be easily utilised by fermentative bacteria for hydrogen production. Disintegration can be achieved by four methods: mechanical, physical, chemical and biological, with EH more favourable than mechanical, chemical or physical pre-treatments. Not only is it a biological process that requires a lower energy input than the other pre-treatments, but its ability to reduce sludge volume and improve hydrogen production from sewage sludge has been proved (Massanet-Nicolau et al., 2008, Parawira, 2012). This section is divided into two parts, each of which describes the experimental design details given in Chapter 6, ‘research works’.

3.5.1 Glucose measurement method

The aim of the work described in this section was to select and assess a reliable method for glucose concentration measurement in a sewage sludge sample. Several methods were used to determine the glucose concentration in a solution. High performance liquid chromatography (HPLC) is frequently used to determine the glucose concentration in a solution; unfortunately, however, due to Covid-19 restrictions and university closure, HPLC was not available. Extensive research

to find a suitable and reliable glucose measurement method was carried out, and Benedict's quantitative method was selected for use as the glucose measurement in EH testing in this research.

Benedict's method has been widely used in the laboratory to detect reducing-sugars (e.g. glucose) in a solution. In the work described in this section, Benedict's quantitative method was selected and used to detect and quantify glucose in a solution that has sewage sludge. Benedict's quantitative reagent can help to determine and quantify the glucose concentration in a solution by changing from a deep-blue colour (no glucose content) to colours ranging from mid-blue (traceable glucose) to very light blue (high glucose content). The experimental set-up was divided into two parts to achieve the aim of this section, as described below.

3.5.1.1 Determining the optimum wavelength for Benedict's quantitative reagent

Benedict's quantitative reagent (purchased from the Fisher company) has a deep-blue colour and depends on the absorbance value to determine the glucose concentration in a solution. It was critical to find the optimum wavelength, especially when Benedict's reagent was mixed with HSS, as the colour changes to a range of different blue-green colours, which can affect the absorbance reading. Spectrophotometry (Shimadzu UV1900) was used to measure the absorbance value at wavelengths between 620 nm and 840 nm to find the optimum one for different sample compositions, as shown in Table (3.8). Total volume and composition have an effect on the final colour; hence, prepared samples were divided into two groups: group A with 6 mL and group B with 7 mL

(total volume). Different volumes were used for each substance to cover the possible scenarios that can happen during an EH test.

Table 3.8 Composition details for different samples for optimum wavelength test.

Group	ID	1% glucose solution	Distilled water	HSS	Benedict's quantitative reagent	Total volume	Expected glucose concentration added
		mL	mL	mL	mL	mL	mg/mL of 1% glucose solution
A	1	1	0	0	5	6	10
	2	0.5	0.5	0	5	6	5
	3	0.1	0.9	0	5	6	1
	4	0	1	0	5	6	0
B	1	0	2	0	5	7	0
	2	1	1	0	5	7	10
	3	1	0	1	5	7	10

HSS: hydrolysed sewage sludge
1% glucose standard solution: (10 g of D-glucose powder in 1 litre of DW).

After sample preparation, the following procedures were carried out to calculate the absorbance value for each wavelength:

1- All test tubes were mixed well and placed in hot blocks for 25 min at 80°C to complete the reaction. Then, they were removed from the hot blocks and placed in a tube holder and allowed to cool to room temperature.
2- All the samples were centrifuged using Eppendorf 5810 at 4000 rpm for 10 min and then filtered by 0.45-µm syringe filter.
3- The absorbance for all samples was measured against a blank sample (D.W) (Abs = 0) using Shimadzu UV1900 at different wavelengths between 620 nm and 840 nm.

3.5.1.2 Standard and modified glucose curve

A glucose standard curve is essential for calculating the glucose concentration in a solution as it explains the relation between glucose concentration and the absorbance value of the sample at optimum wavelength. Determining glucose

concentration by Benedict's quantitative method requires the absorbance value to be measured, after which the glucose concentration must be found using the glucose standard curve. To maximise the accuracy of Benedict's method, a modified glucose curve was calculated as Benedict's colour is affected by HSS, changing to a range of colours between blue and blue-green. Two TCOD of HSS (5 and 10 g TCOD/L) were used to calculate different modified glucose curves. All curves (standard and modified) were set up to cover range 0–10 mg glucose/mL of the sample and were calculated by the following procedures:

1- Seven glass tubes (50 mL) were used to prepare seven test samples as shown in Tables (3.9) and (3.10); the two experiments were run separately but followed the same procedures.

2- The seven tubes contained the following:

Table 3.9 Set-up details for determining standard glucose curve.

Glucose standard curve (0–10 mg/mL)	**ID**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
	mg/mL	10	8	6	4	2	1	0
Glucose solution (1%)	mL	1	0.8	0.6	0.4	0.2	0.1	0
Distilled water	mL	0	0.2	0.4	0.6	0.8	0.9	1
Benedict's reagent	mL	5	5	5	5	5	5	5
Total solution volume	mL	6	6	6	6	6	6	6

1% glucose solution: (10 g of D-glucose powder in 1 litre DW).

Table 3.10 Set-up details to determine modified glucose curve.

Modified glucose curve (0–10 mg/mL)	**ID**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
	mg/mL	10	8	6	4	2	1	0
Glucose solution (1%)	mL	1	0.8	0.6	0.4	0.2	0.1	0
HSS (5 or 10 g TCOD/L)	mL	1	1	1	1	1	1	1
Distilled water	mL	0	0.2	0.4	0.6	0.8	0.9	1
Benedict's reagent	mL	5	5	5	5	5	5	5
Total solution volume	mL	7	7	7	7	7	7	7

1% glucose solution: (10 g of D-glucose powder in 1 litre DW).
HSS: hydrolysed sewage sludge

3- All tubes were mixed well and placed in hot blocks for 25 min at 80°C to complete the reaction.
4- Then, the tubes were removed from the hot blocks, placed in the tube holder and allowed to cool to room temperature.
5- All the samples were centrifuged using Eppendorf 5810 at 4000 rpm for 10 min and then filtered by 0.45-µm syringe filter.
6- The absorbance of samples was measured against a blank sample (just distilled water, Abs = 0) at wavelength 740 nm (optimum wavelength from the results presented in Section 3.5.1.1).

3.5.2 Enzymatic treatment of hydrolysed sewage sludge

Cellulase enzyme blend was purchased from the Sigma-Aldrich company and used for the EH test. Cellulase enzyme is well known in enzymatic treatment applications as it is used as a pre-treatment for lignocellulosic biomass materials (such as sewage sludge) to degrade cellulose material to fermentable sugars such as glucose. The cellulase enzyme blend contains cellulases, ß-glucosidases and hemicellulose. Two concentrations of HSS (5g and 10g of TCOD /L) with different enzyme dosages (1–7 mL) were used to assess the effect of enzyme dosage on glucose production during the EH process. The set-up details for EH test are as follows (the following procedures were carried out for each enzyme dosage in triplicate):

1- The TCOD of HSS was measured by Hach DR3900 using COD kit (LCK 514) and Hach LT200 equipment.

2- A bulk sample of 1 litre was prepared by adding a specific volume of HSS equivalent to (TCOD 5 or 10 g/L) and topped up with DW to 1 litre (total volume).

3- The bulk sample was divided into 250-mL portions and placed in four Duran 500-mL bottles. The four bottles were used to set up the experiment as one control and three tests.

4- Another three blank reactors (250 mL) with only enzyme and DW were prepared to remove any interference of enzyme on glucose measurement and production.

5- Cellulase enzyme (from Sigma-Aldrich) was used to inoculate only the test bottles.

6- The details of the set-up are shown in Table (3.11).

Table 3.11 Details of set-up of EH test (Test A and B are in triplicate).

	Unit	Blank	Control A	Control B	Test A	Test B
HSS (TCOD)	g/L	None	5	10	5	10
Enzyme (Cellulase blend)	mL	1-7	None	None	1-7	1-7
Working volume	mL	250	250	250	250	250
Operation time	hrs.	4	4	4	4	4

HSS: hydrolysed sewage sludge; **TCOD**: total chemical oxygen demand.

7- All the bottles were placed in a Multitron incubator at 37°C and 150 rpm for one day before the experiment started (due to limited laboratory working time under Covid-19 restrictions).

8- After 24 hrs, the test bottles were inoculated with a specific volume of cellulase enzyme, as shown in Table (3.11) above.

9- Samples were taken from the bottles at a specific time to measure pH, glucose concentration, TCOD and sCOD. Table (3.12) shows the sampling time and testing.

Table 3.12 Testing points during EH test.

Parameter/Time	9:30	10:30	11:30	12:30	13:30
Sample test size (mL)	15	8	8	15	8
pH	x	x	x	x	x
TCOD	x				
sCOD	x			x	
Glucose concentration	x	x	x	x	x

TCOD: total chemical oxygen demand; **sCOD**: soluble chemical oxygen demand.

10- Benedict's quantitative method was used to measure glucose concentration during the EH test.

Chapter 4
Characterisation of Hydrolysed Sewage Sludge and Digestate Samples

4.1 Introduction

AD is a natural process which occurs when microorganisms convert organic matter, such as complex carbohydrates, to biogas in the absence of oxygen. AD depends on microorganisms responsible for converting complex carbohydrates, such as lipids, carbohydrates and proteins, to methane and carbon dioxide through several biological processes. These biological processes are classified according to four stages of biological reactions (hydrolysis, acidogenesis, acetogenesis and methanogenesis). It is known that biogas (50%−70% methane and 30%−50% carbon dioxide) production is related to the methanogenic process, which is limited by the hydrolysis rate of organic matter of sewage sludge. Thus, many studies have focused on improving AD biogas by enhancing the hydrolysis rate and methane yield (Hindle, 2013, Abelleira-Pereira et al., 2015).

Many pre-treatments have been used to improve the hydrolysis stage in AD, such as ultrasound, thermal, ozone oxidation, alkaline, mechanical and enzymatic pre-treatments (Elliott and Mahmood, 2007). Most of these aim to enhance biogas production by enhancing the solubility and biodegradability of sewage sludge. The thermal hydrolysis process (THP) is widely implemented in WWTPs, especially in Europe, as a pre-treatment for AD. During this treatment, sewage sludge is treated by heat and pressure (usually 170°C and 7 bar for 30 min) (Shana et al., 2013). The use of THP has proved that it can enhance the hydrolysis stage in AD, which leads to pathogen reduction, high solubilisation,

good dewaterability and increased biogas production (Wirth et al., 2015, Wang et al., 2010). Many studies have reported that using THP to treat sewage sludge prior to AD enhances the biogas yield. Pérez-Elvira et al. (2010) reported a 40% increment in biogas yield compared to conventional AD when THP was used, while Donoso-Bravo et al. (2011) reported that a 55% higher yield of biogas was achieved when THP was used with AD. The sewage sludge processed by THP is called HSS.

Beside AD pre-treatments, characterisation of AD feedstock is one important test that helps monitor the performance of the AD reactor in WWTP. In order to prevent any failure in the AD reactor, continuous controlling of feedstock characterisation should be carried out. Furthermore, characterisation can be used as an evaluation tool to assess the performance of AD pre-treatment processes. In addition, the continuous long-running operation of an AD reactor poses many challenges for WWTP operators, as a low yield of the organic dry solids' degradation efficiency and methane production can occur during AD operation (Appels et al., 2011, Hindle, 2013). Thus, for many reasons, the characterisation of AD feedstock should be carried out on a weekly, monthly and yearly basis to ensure high-quality control of AD performance.

This chapter aims to characterise the AD feedstock (HSS) collected from an actual pilot AD reactor in Esholt WWTP, Bradford, UK, over a year. As in this research, HSS was used as feedstock for all the tests conducted, including BMP (biogas production), BHP (bio-hydrogen production) and EH (enhancing glucose content). Also, such characterisation will help to identify and understand the changes which occur in HSS over a year and set a baseline of current methane

potential of HSS which can be used for comparison in any works related to upgrading methane yield in future.

4.2 Objectives of this chapter

- To evaluate the changes to HSS and digestate characteristics over a year.
- To assess the BMP of HSS and create a baseline that helps comparison for any works related to upgrading methane yield in future.

4.3 Physicochemical characterisation of HSS and digestate samples

4.3.1 Materials and methods

4.3.1.1 Sampling source, processing and storage.

Two type of samples were collected from Esholt: HSS and digestate. Esholt WWTP processes a blended sewage sludge, which is a mix of indigenous primary sludge, imported liquid sludge, thickened secondary surplus activated sludge (SAS) and imported sludge cake. This mix is used to feed the hydrothermal treatment plant (HTP) in Esholt WWTP. The main purpose of hydrothermal treatment is to enhance the solubility and biodegradability of sewage sludge before it goes to AD for biogas production (Wirth et al., 2015, Wang et al., 2010). The reasons behind selecting HSS is because this research aims to improve the quality of biogas that generated from the existing/current biogas production application in WWTP and HTP is commonly integrated with a mesophilic AD reactor in many WWTPs, including the Esholt WWTP in Bradford, UK. After hydrothermal treatment, the treated sewage sludge mix is fed to AD

reactors for methane production. Digestate samples were collected from the effluent of AD reactors and used as inoculum for BMP and BHP tests. Ten samples of HSS (AD feed and effluent of the HTP- after step 3 in Figure (4.1)) and 11 samples of AD digestate (after step 4 in Figure (4.1)) were collected from November 2018 to February 2020. The characterisation tests were carried out in triplicate. Figure (4.1) shows the steps for processing blended sewage sludge at Esholt WWTP.

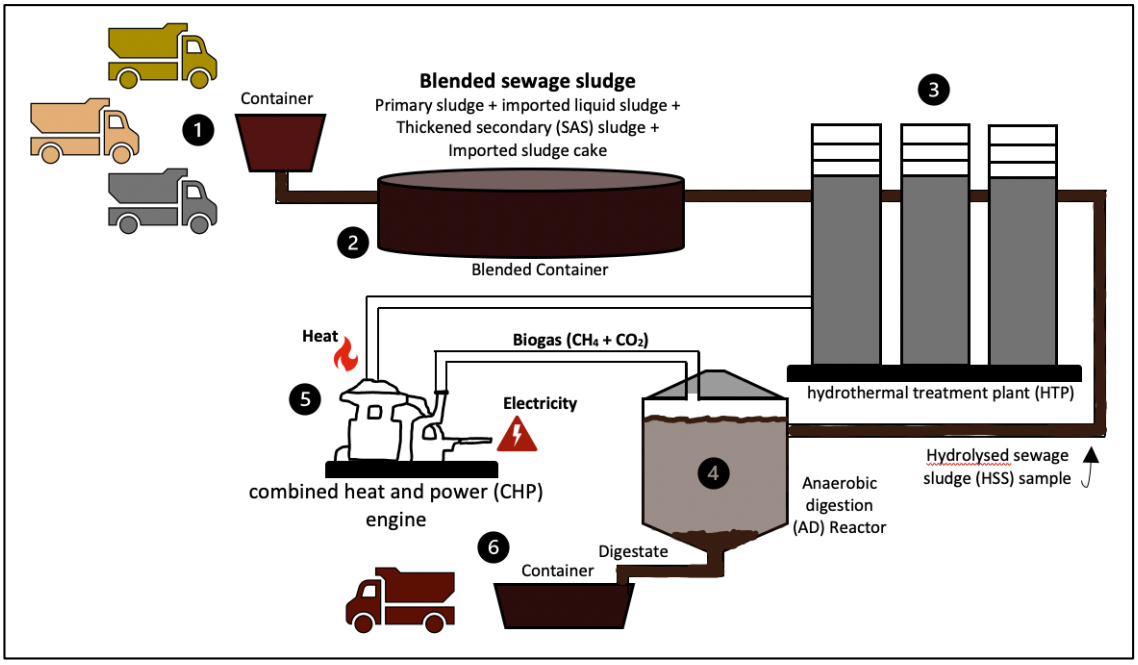


Figure 4.1 Sewage sludge processing at Esholt WWTP, Bradford, UK.

Samples were transferred to the lab and filtered using a 1-mm sieve to remove large particles, after which they were divided into small containers for easy storage. The sample characterisation was proceeded with immediately by carrying out different tests in the lab; then, the HSS was stored in a freezer at -22°C to be used for the following experiments, while the digestate was kept in the incubator at 37°C and fed every week with HSS to keep the bacteria active.

4.3.1.2 Analytical tests

The characterisation of the collected samples involved several tests, namely pH, alkalinity, TCOD, sCOD, ammonia and total nitrogen (TNb) and reactive phosphorus. More details of the methodology of analytical tests are given in Section 3.1.2 (Chapter 3).

4.3.2 Results and discussion

Several analytical tests were carried out for the collected samples (HSS and digestate) from Esholt WWTP over a year on a monthly basis. Table (4.1) shows the results of the sample characterisation.

4.3.2.1 pH

The pH of HSS samples fluctuated between 5.27 and 6.58 for the period during which samples were collected, as shown in Table (4.1). This change is related to the amount of VFAs, ammonia nitrogen and amino acids that were generated during THP (Aragón-Briceño et al., 2017). For digestate samples, the pH ranged between 7.28 and 8.12, which is slightly higher than the optimum pH range of methanogenic bacteria activity (6.5–7.2) (Boe and Angelidaki, 2006) and the typical pH range of digestate sludge (6.5–7.5) (Tchobanoglous et al., 2003).

4.3.2.2 Soluble chemical oxygen demand

Enhancing the solubility and biodegradability of sewage sludge is the main reason for THP. The results of the characterisation tests show the impact of THP on increasing the sCOD of HSS, as shown in Table (4.1). sCOD was within the range of 14,820–33,140 mg/L, which is higher than the values of untreated sewage sludge reported in other studies (500 mg/L (Babu et al., 2021), 1,843 mg/L (Aragón-Briceño et al., 2017) and 2,860 mg/L (Appels et al., 2011)). This result indicates that the HTP in Esholt WWTP is working efficiently and achieving

the purpose of this treatment. Moreover, Pérez-Elvira et al. (2010) reported that sCOD increased fourfold when THP (170°C, 30 min) was conducted, as sCOD for untreated sewage sludge was 2,860 mg/L and HSS was 7,325 mg/L. In addition, Aragón-Briceño et al. (2017) reported that THP increased the sCOD content in digestate massively, as the sCOD for untreated digestate was 1,843 mg/L, while at THP (160°C, 5 bar, 30 min), it was 12,642 mg/L, THP (220°C, 35 bar, 30 min) it was 12,992 mg/L, and at THP (250°C, 40 bar, 30 min) it was 12,164 mg/L.

The degradation of sCOD in the AD reactor at Esholt was calculated from the results shown in Table (4.1). A comparison between HSS and digestate shows that the average reduction of sCOD was equal to 76.9% for a range between 68.2% and 84.2%.

4.3.2.3 Total solids and volatile solids

The results in Table (4.1) show that the TS of the HSS samples fluctuated between 33.68 and 122.29 g/L. This wide range of TS values was due to the application of a dilution process in Esholt WWTP, as there are sometimes blockages in the pipeline that feed the AD reactor which require the injection of water into the pipeline. In digestate samples, meanwhile, the TS value was within the range of 31.78–57.43 g/L. These results are in line with the recommended TS content in AD digestate (30 g/L) (Bitton, 2005). As for VS, a comparison between HSS and digestate shows that AD in Esholt had an average reduction of VS equal to 54.5% (range between 34.42% and 72.06%). Pérez-Elvira et al. (2010) reported that the VS removal was enhanced in an AD reactor which processes HSS, as the VS was 50% for THP+AD and 36% for AD processing raw sewage sludge.

Table 4.1 Physicochemical characterisation of HSS and digestate samples collected from Esholt WWTP (Bradford, UK)

Hydrolysed Sewage Sludge													
Parameter		**unit**	**Nov-18**	**Feb-19***	**Mar-19**	**Jun-19**	**Jul-19**	**Sep-19**	**Oct-19**	**Nov-19**	**Dec-19**	**Jan-20**	**Feb-20**
pH	**Average of triplicate**	-	5.48	5.77	5.59	5.27	5.29	No samples due to the shutdown of the HTP in the Esholt plant for maintenance	6.36	6.09	6.58	5.81	5.94
Alkalinity		mg $CaCO_3$/L	2,397	843	2,637	2,198	2,126		1,869	2,013	1,331	1,043	1,543
Total Solid (TS)		%	9.45	3.37	12.07	12.23	9.48		9.09	10.84	6.43	7.00	8.19
Total Solid (TS)		g/L	94.50	33.68	120.71	122.29	94.80		90.88	108.37	64.30	70.01	81.91
Volatile Solid (VS)		%	7.27	2.60	9.07	8.27	6.88		5.80	6.95	4.33	5.10	5.37
Volatile Solid (VS)		g/L	72.70	26.04	90.71	82.69	68.80		57.99	69.48	43.32	51.04	53.68
Moisture Content		%	90.55	96.63	87.93	87.77	90.52		90.91	89.16	93.57	93.00	91.81
Ammonia		mg/L	818	252	898	844	982		721	646	301	431	608
TCOD		mg/L	35,240	21,100	92,240	92,200	92,600		84,600	72,200	58,380	57,100	73,560
sCOD		mg/L	31,050	9,835	22,815	33,140	30,720		18,750	21,030	14,820	18,540	20,390
Total Nitrogen (TNb)		mg/L	2,914	1,405	4,320	3,300	3,912		2,450	1,188	1,002	1,212	1,998
R. Phosphurus		mg P/L	502	299	816	1,075	742		416	278	96	392	573

* The results of this sample were affected by the dilution process at Esholt WWTP due to a blockage problem in the feeding pipe and cooling unit located before the AD, as operators needed to dilute the effluent from the HTP to solve this problem.
The results in this table are the average value of the triplicate tests.
TCOD: total chemical oxygen demand
sCOD: soluble chemical oxygen demand

Digestate													
Parameter		**unit**	**Nov-18**	**Feb-19**	**Mar-19**	**Jun-19**	**Jul-19**	**Sep-19**	**Oct-19**	**Nov-19**	**Dec-19**	**Jan-20**	**Feb-20**
pH	**Average of triplicate**	-	7.28	7.61	7.59	7.62	7.79	7.96	8.12	7.93	7.99	7.85	7.66
Alkalinity		mg $CaCO_3$/L	7,572	6,273	6,891	6,938	6,762	6,118	5,448	5,905	6,059	4,934	6,022
Total Solid (TS)		%	5.59	3.96	4.20	5.23	5.35	4.06	5.13	5.74	5.30	3.18	5.31
Total Solid (TS)		g/L	55.90	39.61	41.96	52.29	53.50	40.58	51.30	57.43	52.96	31.78	53.06
Volatile Solid (VS)		%	3.42	2.50	2.53	3.02	3.22	2.37	2.82	3.14	2.84	1.91	2.93
Volatile Solid (VS)		g/L	34.20	25.03	25.34	30.24	32.19	23.75	28.16	31.41	28.41	19.14	29.25
Moisture Content		%	94.41	96.04	95.80	94.77	94.65	95.94	94.87	94.26	94.70	96.82	94.69
Ammonia		mg/L	1759	1689	1581	1798	1984	1687	1347	1592	1198	1297	1555
TCOD		mg/L	58,200	44,120	42,460	52,120	53,760	39,080	48,980	53,960	46,840	29,740	49,240
sCOD		mg/L	6,820	4,500	4,730	5,235	6,530	5,510	4,155	4,940	4,720	4,345	5,540
Total Nitrogen (TNb)		mg/L	3,160	2,612	2,228	2,240	3,364	2,358	2,574	1,850	1,120	1,558	2,318
R. Phosphurus		mg P/L	1,056	809	910	1,085	1,008	457	381	138	51	260	441

TCOD: total chemical oxygen demand; **sCOD**: soluble chemical oxygen demand.
The results in this table are the average value of the triplicate tests.

4.4 Biochemical methane potential

In this section, a BMP experiment was used to assess the methane potential of the samples collected from Esholt. HSS was used for biogas production through a batch BMP experiment. Knowing the amount of methane that can be produced from HSS is part of the characterisation and essential for the optimisation of AD processes.

4.4.1 Materials and methods

4.4.1.1 Substrate and inoculum sources

The HSS collected from Esholt WWTP was used as substrate for a BMP batch test, while the digestate from the AD reactor in Esholt was used as inoculum for the BMP test. Sample collection processes are described in Section 3.1.1 (Chapter 3).

4.4.1.2 Analytical tests used to monitor BMP experiments

Several tests were carried out during the batch BMP experiments. Seven testing points were selected to monitor the 28-day BMP test. Table (4.2) summarises the tests conducted on sacrificial bottles. More details of BMP process monitoring and analysis are given in Section 3.2 (Chapter 3).

Table 4.2 Sample collection and characterisation during 28-day BMP test.

Parameter/Day	0	2	4	8	15	22	28
pH	x	x	x	x	x	x	x
Alkalinity	x	x	x	x	x	x	x
TS	x	x	x	x	x	x	x
VS	x	x	x	x	x	x	x
TCOD	x	x	x	x	x	x	x

sCOD	x	x	x	x	x	x	x
Ammonia	x	x	x	x	x	x	x
Total nitrogen	x	x	x	x	x	x	x
R. phosphorus	x	x	x	x	x	x	x
VFAs	x	x	x	x	x	x	x
Biogas volume		x	x	x	x	x	x
Biogas composition		x	x	x	x	x	x

TCOD: total chemical oxygen demand; **sCOD**: soluble chemical oxygen demand; **TS**: total solids; **VS**: Volatile solids; **VFAs**: Volatile Fatty Acids.

4.4.1.3 Biogas volume conversion to standard temperature and pressure

After monitoring biogas production during the BMP test, a series of calculations was used to report methane and carbon dioxide yields at STP conditions by using the ideal gas law, which is in line with most published works, which report biogas yields at 0°C and 1 Atm (STP conditions). The details of data processing and statistical analysis are given in Section 3.3.3 (Chapter 3).

4.4.2 BMP test set-up

Wheaton glass bottles were used to set up the BMP test. The ISR ratio of 1:1 was used in the BMP test. The operation temperature was set at 37°C (mesophilic condition) and maintained by water bath. All the bottles were treated by nitrogen gas, which was sparged through the solution for 1 min to ensure that the BMP bottles were in anaerobic condition. A rubber cap and an aluminium crimp were used to prevent biogas leakage. On each testing day, there were four bottles (two blank and two test, as duplicates). Set-up details are given in Section 3.3.2 (Chapter 3).

4.4.3 Results and discussion

4.4.3.1 Biogas production

HSS (feedstock) was utilised by the consortium of microorganisms present in digestate samples (inoculum) used for the BMP test. As a result of this biological process under anaerobic conditions, biogas started to accumulate in the headspace inside the 160-mL Wheaton bottles. Gas analysis results show that biogas samples consisted of methane, nitrogen and carbon dioxide, where methane and carbon dioxide were from the digestion process and nitrogen was from the sparged process in the set-up phase used to remove oxygen inside the bottles. Maximum methane composition reached 65% of biogas, which is similar to the AD reactor at Esholt WWTP. Figure (4.2) shows the biogas composition during the BMP test. Kim et al. (2015) reported that THP treatment of sewage sludge had a positive impact on increasing methane composition in the produced biogas: methane composition was 50% for untreated sewage sludge, while after THP was applied, it increased to 63.4% at THP 180°C and 58.8% at THP 210°C. These methane percentages are in line with the results of the BMP test of HSS in this chapter.

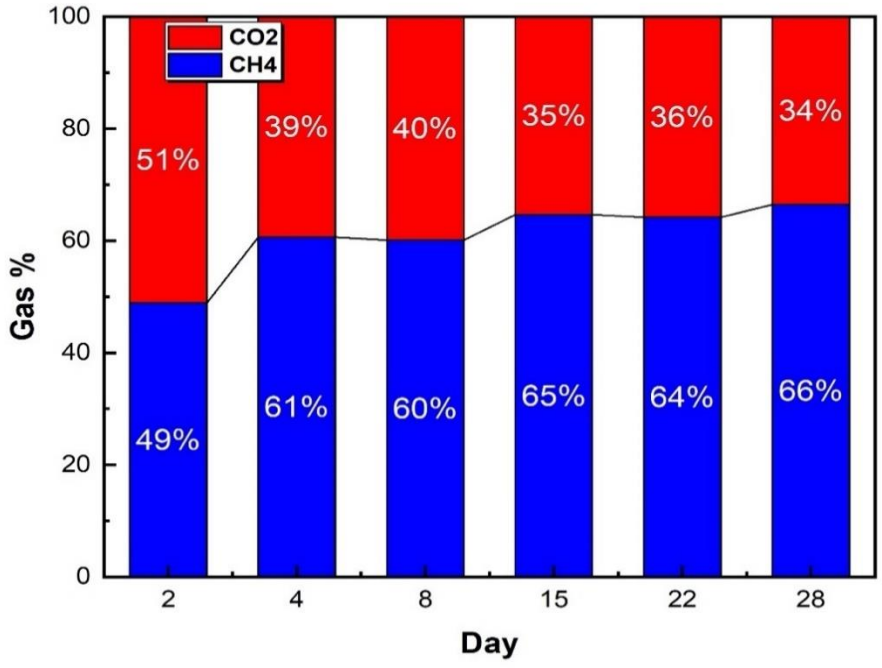


Figure 4.2 Biogas composition at each sampling point during 28-day BMP test.

Maximum methane yield was achieved on Day 28 of the BMP test. Methane yield figures were calculated and reported at STP conditions by using the ideal gas law (more details are given in Section 3.3.3.1 in Chapter 3). Figure (4.3) shows the methane and carbon dioxide yield under STP conditions (0°C (273.15K, 32°F) and 1 atm). The maximum cumulative methane yield was achieved on Day 28 (249.59 NmL-CH_4 / gVS-added).

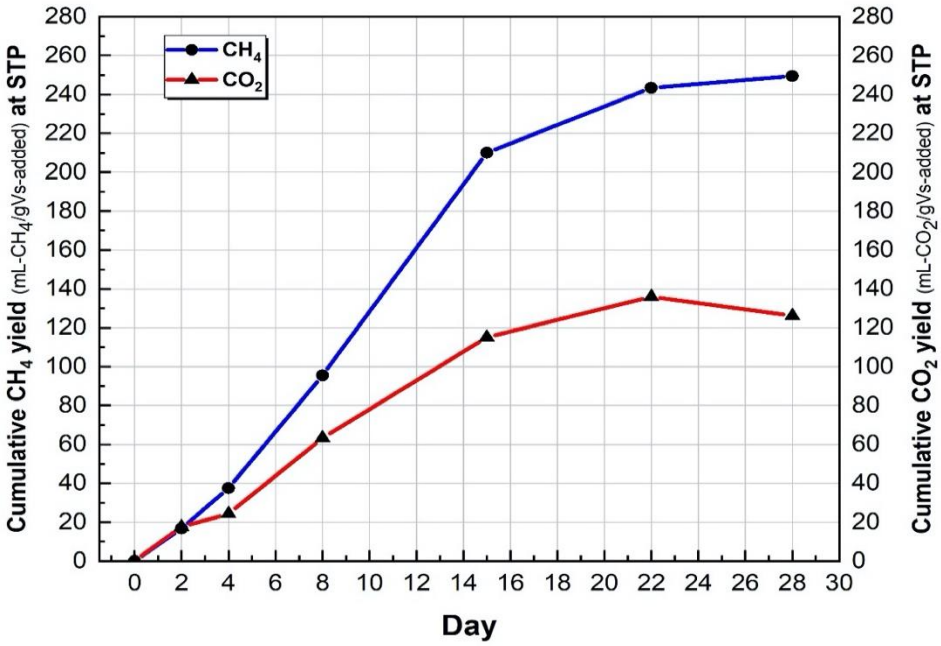


Figure 4.3 Cumulative methane and CO2 yield at STP during 28-day BMP test (average of two replicates).

Similar results were reported by (Park et al., 2021, Kim et al., 2015), who tested sewage sludge at various temperatures (100–300°C) and found that THP treatment enhanced the methane production in the BMP test. Table (4.3) shows a comparison between methane yield for untreated sewage sludge and HSS. The results in Table (4.3) demonstrate the impact of THP on methane production. HTP enhanced the biodegradability of sewage sludge and, as a result, the methane yield was enhanced. Despite the methane yield, the cumulative CO_2

reached its maximum on Day 22 (135.94 NmL-CO_2 / gVS-added) and then slightly decreased (to 126.14 NmL-CO_2 / gVS-added), due to progressive methane production, as shown in Figure (4.3).

Table 4.3 Comparison of methane yield of untreated sewage sludge and HSS.

Pre-treatment	Methane yield (mL-CH4 / gVS-added)	Reference
Untreated sewage sludge	142.7	
THP 150°C	210.0	(Kim et al., 2015)
THP 180°C	222.0	
Untreated sewage sludge	140.0	
THP 150°C	180.0	(Park et al., 2021)
THP 175°C	205.0	
THP 160°C	283.4	This study

THP: Thermal hydrolysis process.

4.4.3.2 Process kinetics

Process kinetics and biodegradability analysis are important tools that help to assess BMP results from batch experiments. Origin 2018 graphical and statistics software was used for data analysis and modelling. The MGompertz model was used to fit the biogas results, as this model is extensively used for batch experiments that have a growth rate (Pagliaccia et al., 2016, Zwietering et al., 1990). More details are given in Section 3.3.3.2 in Chapter 3. Figure (4.4) shows the fitting curve for methane yield.

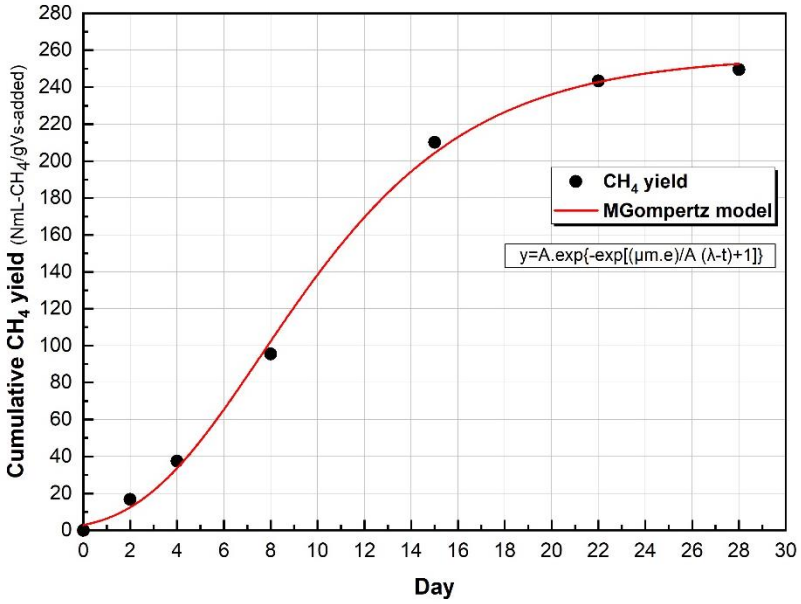


Figure 4.4 MGompertz model fitting for methane yield during 28-day BMP test .

Elemental composition analysis (Carbon, Oxygen, Hydrogen and Nitrogen) of HSS samples was carried out as part of the characterisation tests. Theoretical methane potential (TMP) was calculated using Buswell equation, which is based on the result of elemental composition analysis of HSS samples. The results of the elemental analysis of HSS and untreated sewage sludge are presented in Table (4.4).

Table 4.4 Elemental composition analysis for hydrolysed sewage sludge.

Element	HSS	Untreated sewage sludge		HSS
	(This study)	(Bitton, 2005)	(Lim and Wi, 2003)	(Moon et al., 2015)
Carbon (C)	34.3%	51.0%	40.1%	56.6%
Hydrogen (H)	4.4%	7.4%	5.4%	8.3%
Nitrogen (N)	3.7%	7.1%	1.8%	9.4%
Oxygen (O)	57.3%	33.0%	11.7%	24.7%
Sulphur (S)	0 %	1.5%	1.9%	1.1%

HSS: hydrolysed sewage sludge.

Table (4.5) shows that the TMP in BMP processing HSS was 219.19 mL-CH_4/gVS-added. Biodegradability percentage (BD) was calculated using the

TMP and experimental methane potential (BMP_{exp}). BD is defined as the amount of organic matter that can be degraded during the BMP test (Aragon Briceño, 2018). The results show that BD is more than 100%, as shown in Table (4.5). Usually, the BD is less than 100% as the BMP_{exp} mostly becomes lower than the TMP value. However, in this BMP test the BMP_{exp} was 249.59 NmL-CH_4/gVS-added, while the TMP was 219.19 mL-CH_4/gVS-added, which is lower than the BMP_{exp}. Similar results were reported by (Aragon Briceño, 2018, Okoro-Shekwaga, 2019), where BD was more than 100% and TMP was less then BMP_{exp} in some BMP tests.

R^2 value is an important tool that shows how much the fitting curve fits the experimental data. In this BMP experiment, the R^2 value was 0.998, which is very close to 1. The k-value is a BMP kinetic rate constant (also called hydrolysis rate), which is widely used to determine the optimal condition of AD in terms of design and operation. A high k-value is an indication of a fast hydrolysis process in a BMP test (Ortega-Martinez et al., 2016). In this experiment, the k-value was 0.198, which is slightly higher than (Ortega-Martinez et al., 2016), where the k-value was 0.1 for the BMP of thermally treated sewage sludge. The k-value was calculated by standard Gompertz model in Origin 2018b software. The short lag phase (0.36 day^{-1}), as shown in Table (4.5), indicates the ability of the inoculum to digest the HSS in a short time (fast adaption of inoculum to HSS). Ortega-Martinez et al. (2016) reported that the lag time was between 1.5 days for untreated sewage sludge and one day for thermally treated sewage sludge due to the effect of thermal treatment, which makes sewage sludge more degradable (Shana et al., 2013).

Table 4.5 Process kinetics and biodegradability.

	units	value
Theoretical methane potential (TMP)	mL-CH_4 / g VS-added	219.19
Experimental methane potential (BMP_{exp})	NmL-CH_4 / g VS-added	249.59
R^2		0.998
k-value	Day-1	0.198
Lag phase	Day	0.36
Biodegradability percentage	%	113.9

TMP is calculated based on Buswell equation: $(TMP\ (mLCH_4/g\ \ VS) = \frac{22.4 \times 1000 \times (\frac{c}{2}+\frac{h}{8}-\frac{o}{4}-\frac{3n}{8})}{12c+h+16o+14n}$

R^2, Lag phase are calculated based on MGompertz model: $y = A.exp\left\{-exp\left[\frac{\mu_m.e}{A}(\lambda - t) + 1\right]\right\}$

k-value is calculated based on standard Gompertz model in Origin 2018b software.

Biodegradability percentage is calculated based on $BD_{CH4}(\%) = \frac{BMP_{exp}}{TMP} \times 100$

4.4.3.3 Volatile fatty acids

VFAs play an essential role in the methane formation process; however, they need to be controlled and monitored because their accumulation could inhibit the abilities of syntrophic acetogens and methanogenic bacteria to consume VFAs and produce methane and carbon dioxide (Zhang et al., 2014). Studies show that the use of high organic loads leads to high concentrations of VFAs, a drop in pH values and AD process failure (Komemoto et al., 2009, Palacio-Barco et al., 2010). In this BMP test, total VFAs were 178 mg/L on Day 0, rapidly increasing to 554 mg/L on Day 2, as shown in Figure (4.5). If the VFA accumulation continues at that point, it might result in a drop in pH and, possibly, AD failure; however, in this experiment there was no accumulation in VFAs after Day 4, and the AD remained stable. The VFA concentration reached its maximum value (610 mg/L) on Day 4, then started to drop and was entirely consumed by the bacteria by Day 28. That is, the reported value of -8.05 mg/L is due to the subtraction of blank values from the actual test, which shows that the AD was stable and the conversion of VFAs to methane was good. Figure

(4.5) shows the total amount of VFAs, and Figure (4.6) shows the concentration of different fatty acids on each sampling day of the BMP test. Figure (4.7) shows the percentage of different fatty acids during the 28-day BMP test.

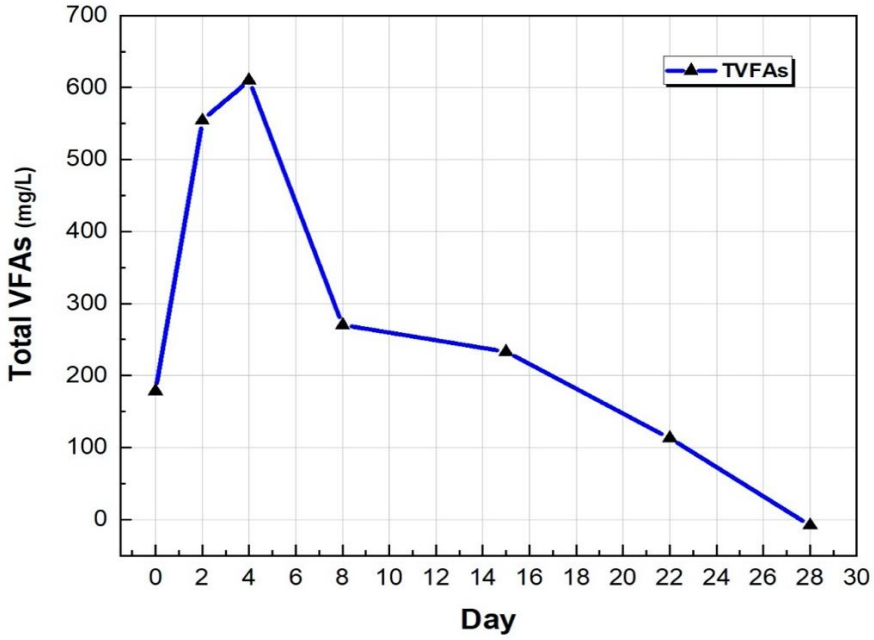


Figure 4.5 Total VFA concentration during 28-day BMP test (average of two replicates).

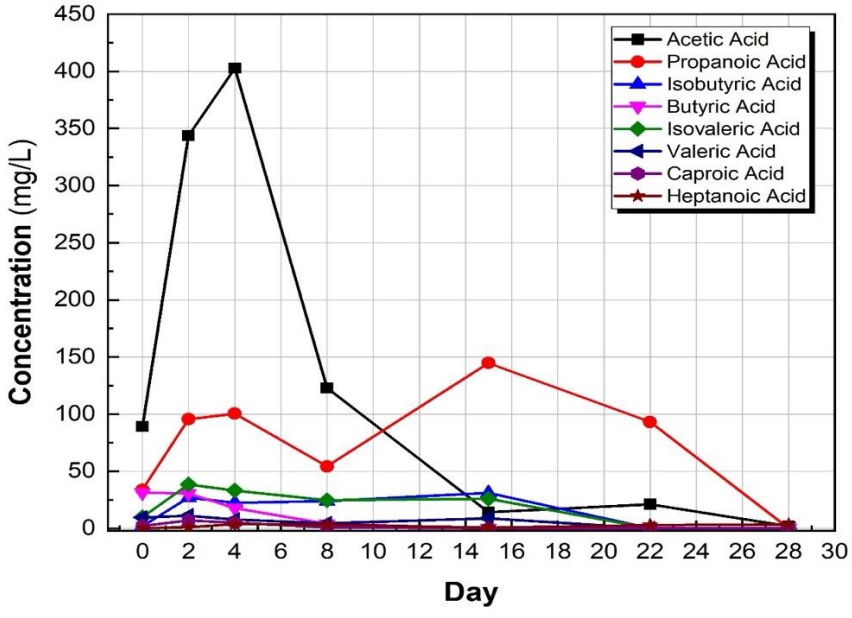


Figure 4.6 Individual VFA concentrations during 28-day BMP test (average of two replicates).

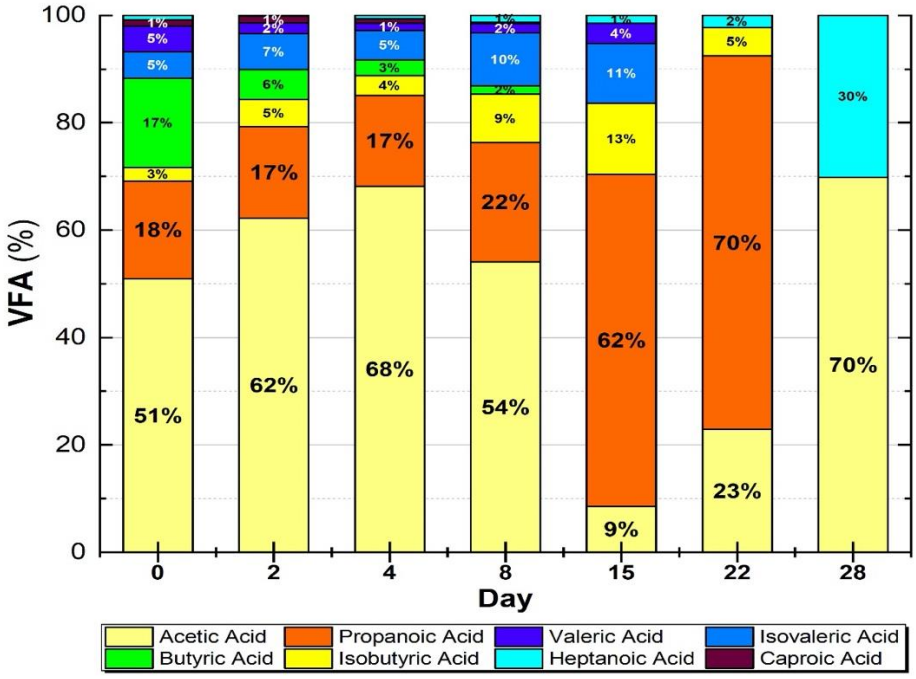


Figure 4.7 Percentage of different VFAs during 28-day BMP test.

Acetic acid and propionic acid were the main acids seen during the experiment and play a major role in methane formation (Zhang et al., 2014). During the BMP test, acetic acid was dominant on Days 0, 2, 4 and 8. On Day 15, there was a high concentration of propionic acid, as shown in Figure (4.7). One important indication of biogas and VFA production inhibition which should be calculated during a BMP is the ratio of propionic acid/acetic acid (P/A). Table (4.6) and Figure (4.8) shows that the P/A ratio was 0.38, 0.28, 0.25 and 0.44 on Days 0, 2, 4 and 8, which is less than 1.4 mg/L. This is favourable for methane production and the stability of the AD system (Buyukkamaci and Filibeli, 2004, Marchaim and Krause, 1993). However, the P/A was higher than 1.4 on Day 15 (10.18) and Day 22 (4.40). This may inhibit/decrease methane production rate; however, given the BD (113.9%) and the fact that the acetic acid reached its lowest value (14.22 mg/L) on Day 15, it is unlikely that methane production was inhibited or affected by the increase in the P/A ratio.

Table 4.6 Propionic Acid/Acetic Acid Ratio during 28-day BMP test .

Day	Propionic Acid (mg/L)	Acetic Acid (mg/L)	Propionic Acid/ Acetic Acid (P/A)
0	33.76	89.13	0.38
2	95.58	343.73	0.28
4	100.48	402.61	0.25
8	54.27	122.97	0.44
15	144.69	14.22	10.18
22	93.18	21.19	4.40
28	0	1.98	0.00

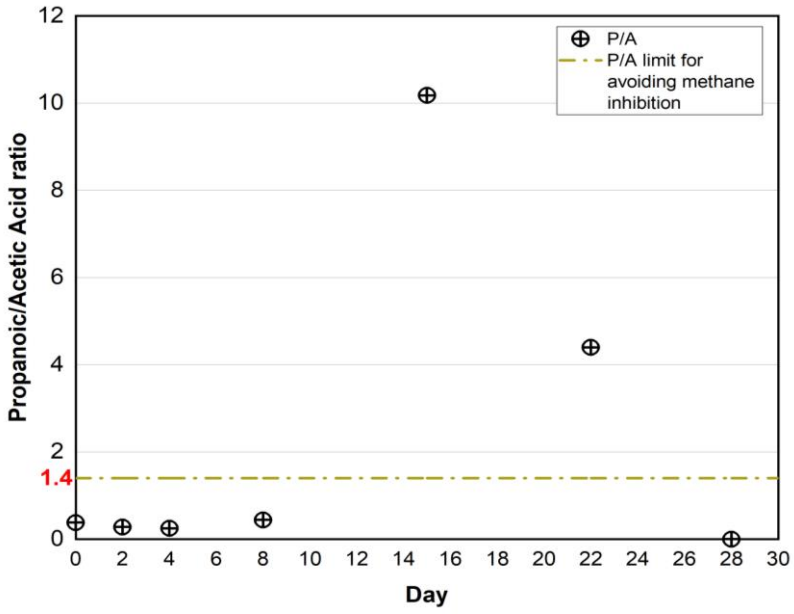


Figure 4.8 Propionic Acid/Acetic Acid Ratio at testing point during 28-day BMP test .

4.4.3.4 pH and alkalinity

The pH is an important parameter used to monitor and control AD reactors. A pH range of 6.8–7.4 is suitable for stable AD (Kumaran et al., 2016). In this BMP experiment, the pH value for the test (HSS + inoculum) was between 6.86 and 7.63, which is similar to the preferred range of AD stability. As shown in Figure (4.9), the pH of the blank (only inoculum) remained stable until Day 4, then slightly decreased to 8.10 on Day 8 and 8.05 on Day 15 and reached its minimum value on Day 28 (7.93). In contrast, there was a sharp decrease in pH for the tests from Day 0 (7.63) to Day 15 (6.78), due to the accumulation and production

of VFAs during this period. It then started to increase from Day 15 and reached 6.86 on Day 28. The effect of pH is crucial in AD. As the pH value for this BMP experiment shows, having a pH within the desired range for AD can enhance the VFA consumption (as shown in Figures 4.5 and 4.6) and optimise the methane production (BD 113.9%).

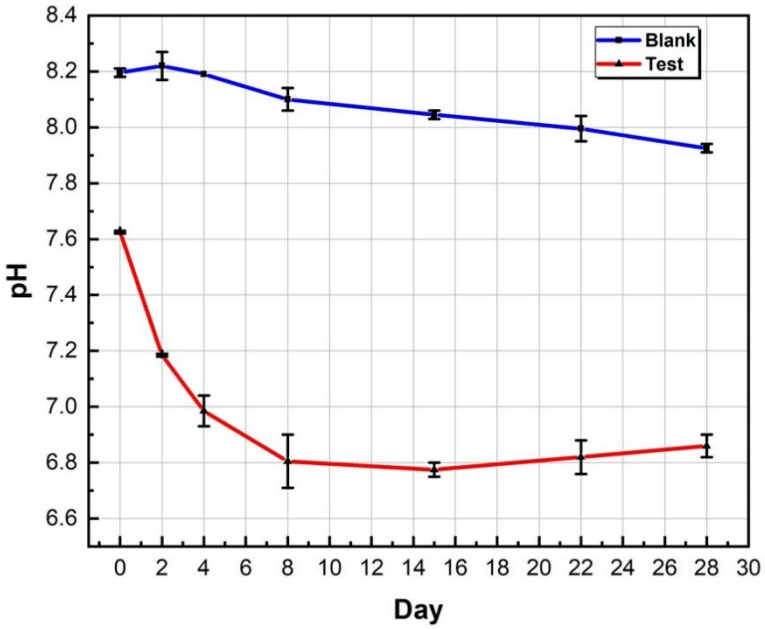


Figure 4.9 pH curves for Test and Blank during 28-day BMP test (average value of two replicates with max/min bar).

Figure (4.10) shows the alkalinity curve for both Blank and Test. Despite the drop in pH Test during the BMP test, the alkalinity curve shows a good buffering for pH drop, as it was 1375 mg$CaCO_3$/L at Day 0 and 1954 mg$CaCO_3$/L at the lowest pH (6.83), Day 15. Another explanation for the rise in Test alkalinity is that the production of carbon dioxide can affect the alkalinity value as carbon dioxide is part of the carbonate/bicarbonate equilibrium. In addition, this buffering of pH kept the pH inside the reactor in the preferred range, which did not inhibit the methane production, as the low buffering and continuity of alkalinity reduction

inside the AD reactor can lead to inhibition of methane production and VFA accumulation (Appels et al., 2008).

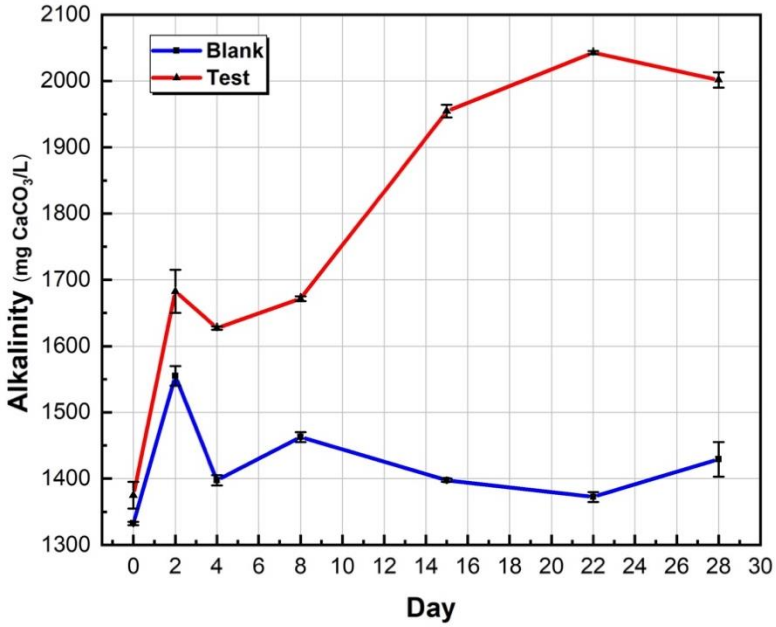


Figure 4.10 Alkalinity curves for Test and Blank during 28-day BMP test (average value of two replicates with max/min bar).

4.4.3.5 Total and soluble chemical oxygen demand

TCOD and sCOD are good indicators for anaerobic metabolic activities in AD. In the first of the four AD stages (hydrolysis), the substrate contents are hydrolysed into TCOD by microorganisms, after which the sCOD is converted to VFAs, which is consumed again by methanogenesis, resulting in methane production at the end. Hence, a high reduction of sCOD is a good indicator of efficient AD processes. Figure (4.11) demonstrates the good condition of microorganisms in consuming the soluble organic matter, as sCOD started at 1719 mg/L, followed by a sharp decrease to reach 375 mg/L on Day 28, with a 78.2% reduction in sCOD. As regards TCOD concentration, this started with 9190 mg/L and decreased to 5910 mg/L on Day 15. Thereafter, the TCOD concretion fluctuated between 6360 and 6980 mg/L due to the increase of total solids.

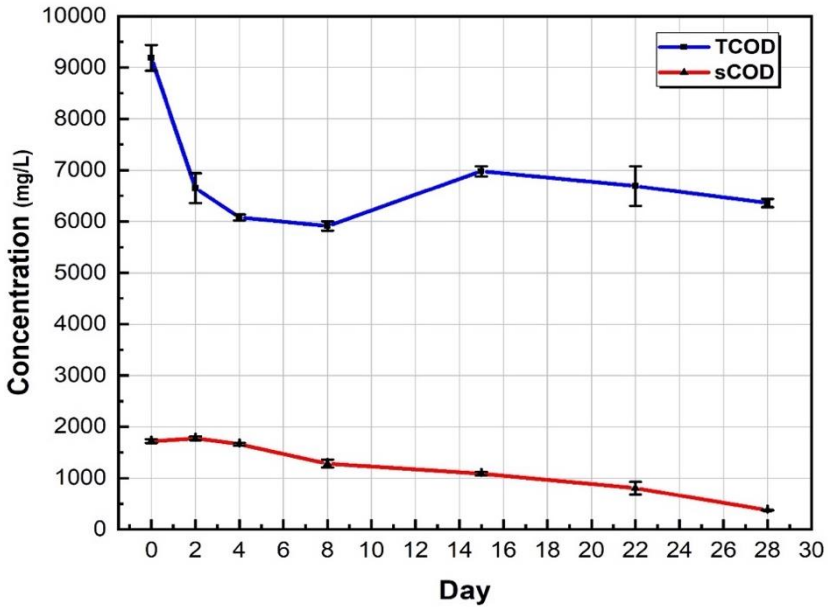


Figure 4.11 TCOD and sCOD during the 28-day BMP test (average value of two replicates with max/min bar).

4.4.3.6 Ammonia, Total Nitrogen and Reactive phosphorus

Ammonia is one of the inhibitor factor that affect the methane formation process in AD. Figure (4.12) shows that there was sharp increase in ammonia concentration from day 0 to day 4 and this cause a delay in methane formation process, however from day 4 to 22, ammonia concentration was fluctuated and reached the maximum at day 28 (187.75 mg/L). Total Nitrogen concentration started at 236 mg/L and then was fluctuated between 123 mg/L at day 4 and 183 mg/L at day 28 with a maximum value at day 15 (368 mg/L). Reactive phosphorus concentration started at 46.95 mg/L and reached the maximum value at day 15 (107.4 mg/L) and then remain stable until the end of the experiment.

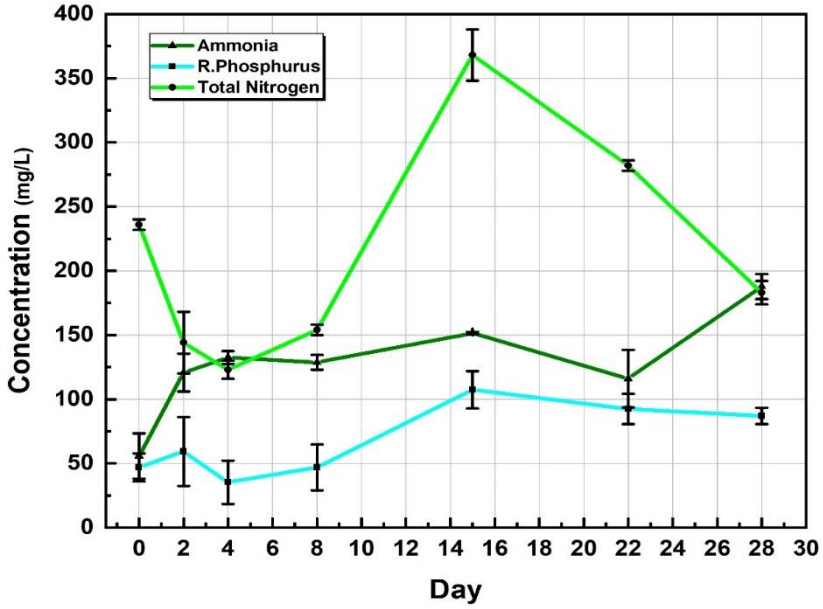


Figure 4.12 Ammonia, Total Nitrogen and Reactive phosphorus during 28-day BMP test (average value of two replicates with max/min bar).

4.5 Conclusion

In the work described in this chapter, the collected samples (HSS and digestate) from Esholt (a WWTP in Bradford) were assessed by several characterisation tests. The changes in the characteristics of the collected samples were assessed and observed over one year of collection and testing, which helped to assess the performance of HTP and AD reactors at the Esholt WWTP. Moreover, the work described explains the fluctuation that can occur in HTP performance and how this can affect AD performance in terms of process stability and high biogas production. Additional analysis was carried out by BMP test as part of the characterisation. The results help to create a baseline for comparison with the results presented in the following chapters and show the methane potential of the current biogas production applications (THP+AD). Therefore, alternative

biogas upgrading methods can be assessed to enhance methane yield and improve AD processes.

Chapter 5
Impact of Inoculum Pre-Treatment on the Performance of Dark Fermentation and Bio-Hydrogen Potential of Hydrolysed Sewage Sludge

5.1 Introduction

Under DF, a method of bio-hydrogen production, fermentative bacteria are used to hydrolyse organic substrates to produce energy carriers such as hydrogen gas and formate. Among the main advantages of this method are that it does not require an oxygen supply or a light source, and it can easily be adapted to various organic substrates (Nath et al., 2008). DF is sometimes referred to as stressed AD because the biological reactions that occur during DF are the same as those in the hydrolysis/acidogenesis stage of AD (Antonopoulou et al., 2011). According to the literature review presented in Chapter 2, DF is an interesting method of bio-hydrogen production. Different studies have investigated the feasibility of the DF method in producing hydrogen, and it has been considered as an energy carrier and a future fuel for many applications.

During DF, hydrogen is produced by the conversion (hydrolysis) of organic substrates (carbohydrates, proteins and lipids) using mixed or pure cultures of microorganisms. This conversion can follow different pathways, with the associated production of VFAs, and each conversion pathway has, theoretically, a specific maximum hydrogen production. Therefore, knowing the concentration of VFAs will play a huge role in identifying the pathway and conversion efficiency required to enhance hydrogen production through DF.

Certain operating parameters influence hydrogen production. Temperature, pH, agitation intensity, retention time, organic loading rate (OLR), the presence of

nutrients, inhibitors and inoculum type have major influential effects on the whole process; these operating parameters are related to each other such that changing one parameter may affect another (Wang and Wan, 2009). Given the complexity in the relationship of these influential parameters, it is necessary to identify approaches to optimising DF for hydrogen production.

In this research, HSS and digestate were used as feedstock and inoculum, respectively, in DF for bio-hydrogen production. The selection of the right inoculum is a very important step in DF, as it can affect the production of hydrogen. Mixed cultures, such as digestate (from AD), can be used as inoculum in DF because they are easier to handle and control and highly available (Li et al., 2007). However, mixed cultures contain both hydrogen-consuming bacteria (methanogens and homoacetogens) and hydrogen-producing bacteria (such as *Clostridium* and *Enterobacter*). In the DF process, which is operated under anaerobic conditions, the hydrogen-consuming bacteria can easily consume the hydrogen and inhibit hydrogen production (Oh et al., 2003b, Cai et al., 2004a). Therefore, to inhibit methanogenic activity, the selected inoculum is often pre-treated, and this pre-treatment process is a necessary step in DF.

Several studies have investigated different types of inoculum pre-treatment, which are classified into physical, chemical and biological treatment methods (Hu and Chen, 2007b, Zhu and Béland, 2006a, Mu et al., 2007, Mohan et al., 2008b). Physical pre-treatment (HST, freezing–thawing and aeration), chemical pre-treatment (AST, BST, sodium 2-bromoethanesulfonate or 2-bromoethanesulfonic acid and iodopropane) and combined pre-treatment (e.g., combined AST and BST) are the main pre-treatment methods adopted to inhibit methanogenesis. Several studies have compared the effect of these pre-

treatments on increasing hydrogen production but arrived at different conclusions, as shown in the literature review (Section 2.5.3.3 in Chapter 2).

All these studies and their conflicting results show there is a need for more investigation to determine which pre-treatment best inhibits hydrogen-consuming bacteria and enriches hydrogen-producing bacteria in a certain type of inoculum. The aims of this chapter are therefore to address the necessity of inoculum pre-treatment for hydrogen production, determine how to assess and select the best pre-treatment for any inoculum used in future studies, and assess the hydrogen potential of sewage sludge.

5.2 Objectives of chapter

- To assess the impact of different inoculum pre-treatment methods used to enhance hydrogen production through the DF process.
- To assess the conversion path of glucose to hydrogen in a DF reactor using HSS as feedstock.
- To assess the BHP of HSS and create a baseline that helps comparison for any works related to upgrading hydrogen yield.

5.3 Inoculum pre-treatment

5.3.1 Materials and methods

5.3.1.1 Inoculum source and quality assessment

The inoculum used for the batch BHP test was prepared from the digestate of an anaerobic digester processing sewage sludge at Yorkshire Water's Esholt WWTP, Bradford, UK. The sample collection and processes are described in Section 3.1.1 (Chapter 3). The digestate was incubated at 37°C and fed with HSS once a week to keep the microorganisms active. D-glucose powder

obtained from Sigma-Aldrich UK (≥99.5% purity) was used as a standard substrate for BHP tests to assess the quality and suitability of the inoculum for hydrogen production.

5.3.1.2 Inoculum pre-treatment methods

There is no universal pre-treatment method to inhibit hydrogen-consuming bacteria; thus, different methods are reported in the literature for conducting DF experiments (Chaganti et al., 2012, Cai et al., 2009, Liu et al., 2009, Pendyala et al., 2012, Argun and Kargi, 2009). The selection of pre-treatment methods used for this research work considered criteria such as simplicity, previous reports in the literature (a method commonly used), practical access to reagents and equipment and scale-up potential. Therefore, the three following pre-treatment methods for inactivating hydrogen-consuming bacteria were chosen (Wang and Wan, 2008):

(i) AST was performed by adjusting the pH of the digestate to pH 3 using 1 M HCl and storing it in the fridge at 4°C for 24 hr. After 24 hr, the pH was adjusted to pH 7 using 1 M NaOH.

(ii) BST was performed by adjusting the pH of the digestate to pH 10 using 1 M NaOH and storing it in the fridge at 4°C for 24 hr. After 24 hr, the pH was adjusted to pH 7 using 1 M HCl.

(iii) HST was conducted by heating the digestate for 20 min at 115°C using a standard autoclave at approximately 1.5 bar.

5.3.1.3 Analytical tests

Several analytical tests were carried out during batch BHP experiments. Five testing points were selected to monitor the five-day BHP test. Table (5.1)

summarises the tests conducted on sacrificial bottles. The BHP process monitoring and analysis are reported on in more detail in Section 3.4.1 (Chapter 3).

Table 5.1 Sampling points and analysis conducted to monitor five-day BHP tests.

Parameter/Day	0	1	2	3	4
pH	x	x	x	x	x
Alkalinity	x	x	x	x	x
TS	x				x
VS	x				x
VFAs	x	x	x	x	x
Biogas volume		x	x	x	x
Biogas composition		x	x	x	x

TS: total solids; **VS**: volatile solids; **VFAs**: volatile fatty acids.

5.3.1.4 Statistical analysis

Minitab 18 statistical software was used to run correlation analyses between hydrogen yield and VFAs (acetate and butyrate) using a confidence level of α = 0.05. More details are reported in Section 3.4.4 (Chapter 3).

5.3.2 Experimental set-up

The effectiveness of each pre-treatment method was tested by conducting batch BHP tests with the pre-treated digestate as inoculum and glucose (D-glucose) as the sole carbon source (substrate). The standard substrate, glucose, was chosen for the BHP experiments because it is a simple carbon source and easy to digest, and it allows comparison of the different pre-treatment methods based on the differences in process stability and ultimate hydrogen yields.

Wheaton glass bottles were used to set up the BHP tests. An ISR of 1:1 was used in all BHP tests. The operation temperature was set at 37°C (mesophilic conditions) and maintained constant by using a water bath. All bottles were

degassed by nitrogen gas injection, which was sparged through the solution for 1 min to insure that BHP bottles were in fully anaerobic condition. A rubber cap and an aluminium crimp were used to prevent hydrogen/biogas leakage. The working volume was 70 mL for all bottles. The BHP set-up details are described in Section 3.4.1 (Chapter 3). Table (5.2) shows the characterisation of all reactors on Day 0.

Table 5.2 Characterisation results of all reactors on Day 0 of BHP tests.

		Control	Test I	Test II	Test III
Inoculum (Digestate)		Untreated	Heat shock (HST)	Acid shock (AST)	Basic shock (BST)
Substrate		glucose	glucose	glucose	glucose
pH	-	5.5	5.5	5.5	5.5
Alkalinity	mg CaCO3/L	287 (3)	249 (6)	200 (13)	255 (9)
TS	%	1.23 (0.09)	1.40 (0.10)	1.12 (0.28)	1.15 (0.11)
VS	%	0.88 (0.04)	1.00 (0.06)	0.80 (0.31)	0.82 (0.11)

TS: total solids; **VS**: volatile solids.
(STD: standard deviation from the mean (n = 3)).

5.3.3 Results and discussion

5.3.3.1 Hydrogen yields

Cumulative hydrogen yield for the control and three test conditions is shown in Figure (5.1). The three test conditions (HST, AST and BST) produced high amounts of hydrogen gas during the five-day DF process (BHP test), and no methane gas was detected in any of the reactors, including the control (untreated inoculum). As the inoculum for this experiment was collected from an AD reactor processing HSS, it might be that fermentative bacteria were dominant. Wang and Wan (2008) reported a similar phenomenon, that is, that no methane was detected for untreated inoculum (digestated sludge) with glucose as substrate. Moreover, (Luo et al., 2011) used the same type of inoculum and substrate and

concluded that, besides inoculum pre-treatment, the fermentation condition (mesophilic and low pH (5.5)) also impacts methanogenesis inhibition. Contrariwise, (Hu and Chen, 2007a) used raw sewage sludge and methanogenic granules as inoculum and glucose as substrate, and (Chen et al., 2002) used sludge collected from a drying bed as inoculum and glucose as substrate; both reported detectable methane in biogas from the untreated inoculum during DF.

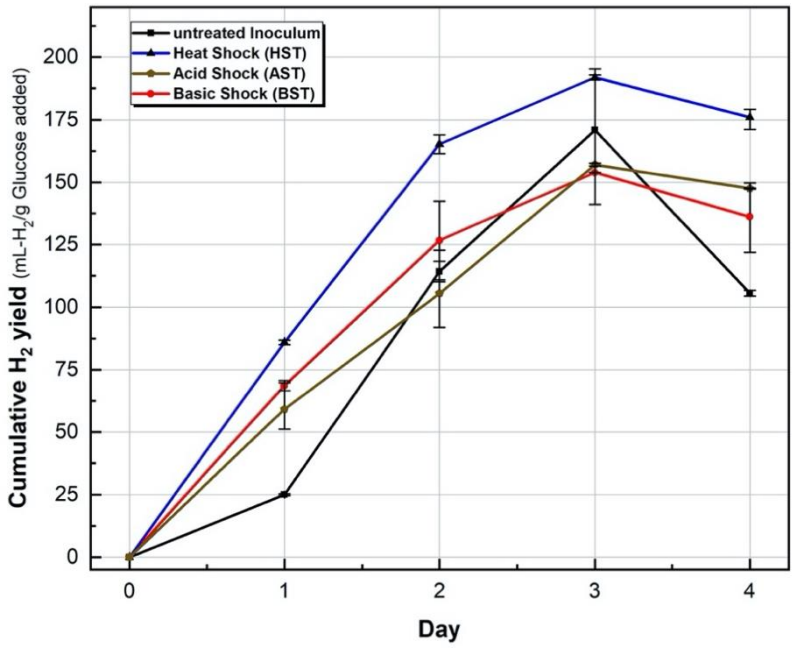


Figure 5.1 Cumulative hydrogen production for untreated and treated inoculum during 5-day BHP tests (average value of triplicate with max/min bar).

However, of the three pre-treatments and untreated inoculum investigated in the present study, HST achieved the maximum cumulative hydrogen production (191.8 mL-H_2/gVS-added), which is slightly lower than figures reported by (Wang and Wan, 2008) (221 mL-H_2/gVS-added) but higher than those reported by (Luo et al., 2011) (155 mL-H_2/gVS-added), using HST of similar inoculum type, substrate and operation conditions (batch, temperature and pH), as shown in Table (5.3). One possible reason for the different figures is the different heat shock temperatures and retention times used in these studies: 100°C for 15 min by (Wang and Wan, 2008) and 100°C for 60 min by (Luo et al., 2011), while in

the present study the HST was 115°C for 20 min, which may have influenced the level of inhibition of the hydrogen-producing bacteria (Hu and Chen, 2007a, Zhu and Béland, 2006a). The control (untreated inoculum) reached 170.91 mL-H_2/gVS-added, which is higher than the 50 mL-H_2/gVS added reported by (Wang and Wan, 2008) and 156 mL-H_2/gVS added reported by (Luo et al., 2011). This increase might imply that the fermentative bacteria were dominant in the inoculum used in the present study and that AST and BST do not make a major difference. Although BST showed a faster production rate in the first two days, and both types of pre-treatment (AST and BST) reached 154-157 mL-H_2/gVS-added on Day 3.

Table 5.3 Comparison of different inoculum pre-treatment methods in BHP experiments.

Inoculum, substrate	Temperature and operation system	Pre-treatment	Initial pH	Hydrogen yield (mL-H_2/g Glucose added)	Reference
Digested sludge	Mesophilic (35-37°C)	Untreated	5.5	153	a
			7.0	157	a
			7.0	66	c
			5.5	171 (27.5)	This study
		HST	5.5	155	a
			7.0	141	a
			6.2	120	b
			7.0	221	c
			5.5	192 (1.4)	This study
Glucose	Batch	BST	7.0	136	c
			5.5	157 (4.9)	This study
		AST	5.5	154	a
			7.0	145	a
			7.0	99	c
			5.5	157 (0.8)	This study

(a) (Luo et al., 2011): hydrogen yield is from the fifth batch
(b) (Oh et al., 2003a) and **(c)** (Wang and Wan, 2008)
HST: heat shock, **BST**: basic shock and **AST**: acid shock
(STD: standard deviation from the mean (n = 3)).

A comparison between untreated inoculum, HST, AST and BST might imply that the BST and AST did not provide long-term inhibition of hydrogen-consuming bacteria, which led to no additional impact on hydrogen production, in contrast to HST. In agreement with (Wang and Wan, 2008), the BHP test with HST pre-treatment had the highest hydrogen yield and production rate: Day 1 (85.93),

Day 2 (79.30), Day 3 (26.61) and Day 4 (-15.85) (- value because of hydrogen consumption this day) mL-H_2/gVS-added (at daily bases), as shown in Figure (5.1). In all conditions, however, hydrogen production peaked by Day 3, followed by an observed decline in the hydrogen yield by Day 4. This decrease might be because of the bio-conversion of hydrogen gas to acetic acid through homoacetogenesis under anaerobic condition (Akutsu et al., 2009, Zhao et al., 2015), considering that homoacetogenic bacteria are spore-forming bacteria that can survive even after harsh pre-treatments while temporarily de-activated (Valdez-Vazquez et al., 2009). This reduction in hydrogen gas on Day 4 indicates the time needed to reach maximum hydrogen production for the specific type of inoculum and substrate used in this study: sewage digestate and glucose, respectively.

A simple energy balance was calculated based on the energy consumption of using an autoclave for HST pre-treatment and the estimated energy production from hydrogen yield in BHP_{HST}. Table (5.4) shows the energy balance for the best inoculum pre-treatments (HST) according to hydrogen yield in Figure (5.1). The results show that the net energy was positive over 1-year of DF operation, which indicates that using an autoclave for HST is feasible from an energy production perspective. However, more energy can be produced by using the effluent of DF (reached by VFAs) in AD for biogas production, improving the net energy and making the overall process more feasible and attractive. More investigation is needed to optimise the hydrogen production for high/positive net energy production. And this can be achieved using another heating instrument such as a furnace with lower energy consumption than an autoclave. Also,

testing different periods of HST (less than 20 min) may improve the net energy production.

Table 5.4 Energy balance of BHP with HST pre-treatment based on 1 Kg substrate and 1 year of operation.

Energy consumption (E*c*)	Energy production (E*p*)	Net energy (E*p* – E*c*)
Kwh/Kg inoculum.year	Kwh/Kg substrate.year	Kwh
23.7[a]	42.3[b]	+18.64

(a) Ec was calculated according to the energy consumption of heating the inoculum (digestate) (HST pretreatment) from its initial temperature (37°C) to the final temperature (115°C). A water heating calculator (Darcy, 2022), was used to calculate (**Ec**) with the assumption that the density of inoculum is equal to the density of water because of the inoculum VS value (30 g/L).
(b) E*p* was calculated according to the maximum hydrogen yield (191.8 mL-H_2/g glucose-added) and the energy production of hydrogen gas (33.6 Kwh/kg of hydrogen, (Molloy, 2019)).

5.3.3.2 Volatile fatty acids and by-products of glucose transformation

VFA analysis provides an understanding of the predominant glucose conversion pathways in the present study. The conversion pathway can affect the hydrogen yield from the BHP test. For example, 1 mole of glucose could theoretically yield 12 moles of hydrogen, as shown in Equation (5.1). However, if the reaction follows the acetate pathway, only 4 moles of hydrogen would be produced, as shown in Equation (5.2) (De Gioannis et al., 2013a), while the butyrate pathway can produce only 2 moles of hydrogen, as shown in Equation (5.3).

$$C_6H_{12}O_6 + 6H_2O \rightarrow 12H_2 + 6CO_2 \quad (5.1)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 6CO_2 + 2CH_3COOH \quad (5.2)$$

$$C_6H_{12}O_6 \rightarrow 2H_2 + 2CO_2 + CH_3CH_2CH_2COOH \quad (5.3)$$

Moreover, the lactic and ethanol pathways (as shown in Figure (5.2)) yield no hydrogen, while the propionate pathway consumes hydrogen (Guo et al., 2010a). Figure (5.2) shows conversion routes for glucose in DF processes. Therefore, the concentration of VFAs at the end of a DF process can be used as an

indication of the conversion pathway and the maximum theoretical hydrogen production that could be achieved.

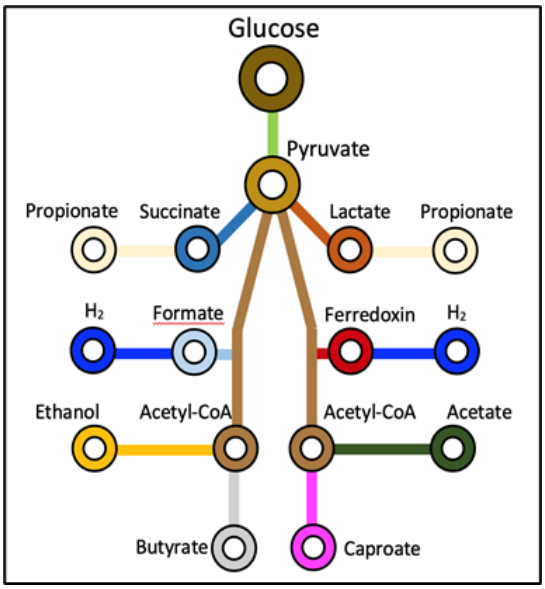


Figure 5.2 Conversion routes for glucose in dark fermentation processes. Adapted from (Kim et al., 2004).

In the present study, the butyrate conversion route was the predominant one, followed by acetate accumulation, as shown in Figure (5.3, a-d), in which case Equations (5.2) and (5.3) would best describe the expected hydrogen yield from the BHP. The prevalence of butyrate and acetate production might be due to the low pH ranges (between 4.2 and 4.6) observed for all conditions (see Section 5.3.3.3). Several studies have reported that the accumulation of butyric acid and acetic acid can be related to low pH ranges (between 4.5 and 6.0), while ethanol and propionate can accumulate in DF reactors that have neutral (7.0) pH or higher (Hawkes et al., 2007, Kim et al., 2004, Pakarinen et al., 2008).

For all conditions, butyrate peaked on Day 3, with a concentration of 1490 mg/L, 1446 mg/L, 1388 mg/L and 1012 mg/L in the untreated, HST, BST and AST reactors, respectively, while acetic acid had a lower accumulation.

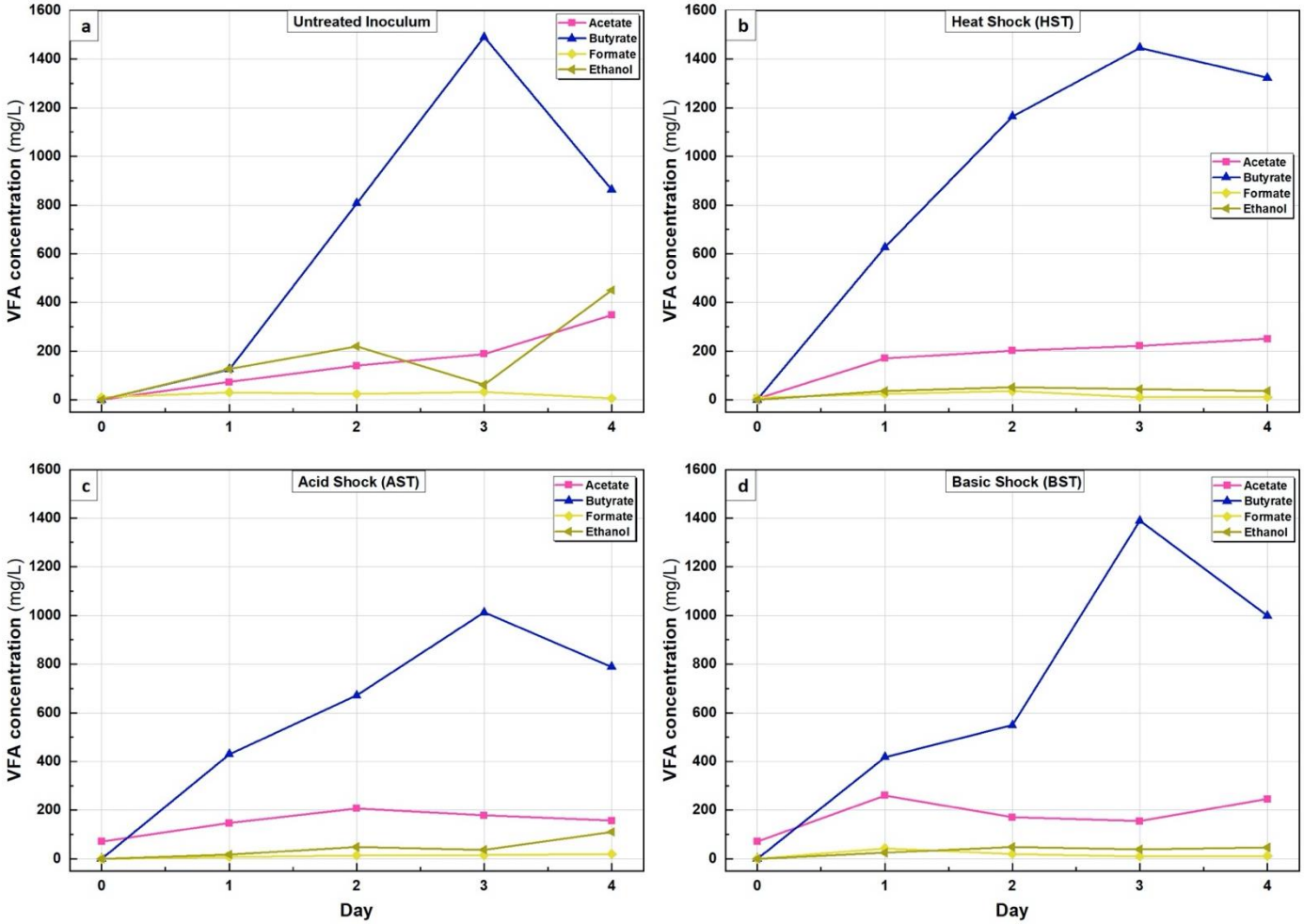


Figure 5.3 VFA accumulation during 5-day BHP tests (a) untreated inoculum, (b) HST, (c) AST and (d) BST.

The low accumulation of acetic acid in comparison to butyric acid in the treated and untreated inoculum had a negative impact on hydrogen production by reducing and consuming the hydrogen in the reactors between Day 3 and Day 4, as shown in Table (5.5).

The decline in butyrate between Day 3 and Day 4 might be due to the re-activation of the acetogens and, consequently, the use of butyrate as a substrate for the production of acetic acid and hydrogen (Yang and Wang, 2017). However, the corresponding decline in the hydrogen yield between Day 3 and Day 4 (Section 5.3.3.1) indicates a simultaneous bio-conversion of hydrogen gas to acetic acid by homoacetogenesis reaction, which is a possible hydrogen sink under anaerobic condition (Zhao et al., 2015). According to Table (5.5), a relationship between the level of acetate increments and the percentage reduction in hydrogen gas between day 3 and day 4. For example, the highest increment in acetate was observed with the untreated inoculum, which also had the highest reduction in hydrogen, and in AST, which had the lowest acetate increment and the smallest percentage of hydrogen reduction. Similar results regarding the negative impact of acetic acid accumulation on hydrogen production have been reported by (Luo et al., 2011, Wang and Wan, 2008, Zhu and Béland, 2006b, Hu and Chen, 2007a).

Table 5.5 The effect of acetic acid on hydrogen production during 5-day BHP tests.

Pre-treatment	Acetic acid concentration (mg/L)			Maximum hydrogen yield	Decrease in hydrogen production (%) at Day 4
	Day 3	Day 4	Increment[a]	(mL-H_2/g Glucose added)	
Untreated	189 (38.2)	348 (43.9)	159	171 (27.5)	38%
HST	222 (51.6)	251 (7.1)	29	192 (1.4)	8%
AST	178 (15.5)	157 (17.4)	-21	154 (0.9)	6%
BST	155 (22.7)	246 (83.7)	91	157 (4.9)	13%

HST: heat shock; **BST**: basic shock; **AST**: acid shock.
(a) difference between Day 3 and Day 4.
(STD: standard deviation from the mean (n = 3)).

The decrease in hydrogen production when pre-treated inoculum was used was lower than when untreated inoculum was used, indicating the effect of inoculum pre-treatment on sustaining the inactivation of hydrogen consumers during the DF process. Moreover, this drop in hydrogen yield on Day 4 indicates the point at which the pre-treated inoculum should be added to the reactor media (applicable to batch DF) to sustain the inhibition of the hydrogen-consuming bacteria.

5.3.3.3 pH and alkalinity

The pH and alkalinity have a crucial influence on the reactions occurring during DF. The pH value affects VFA accumulation; lower pH ranges (4.00–6.0) support butyrate and acetate accumulation, and higher pH ranges (7.0–9.0) support ethanol and propionate accumulation (Hawkes et al., 2007, Pakarinen et al., 2008). Conversely, the pH also influences the diversity of the microbial community and, effectively, hydrogen production; that is, at low pH levels, the dominant species is *Clostridium*, which is responsible for the production of butyrate, acetate and hydrogen (Hawkes et al., 2007, Temudo et al., 2008). The optimum pH range, which enhances the hydrogenases (hydrogen producers) in DF, has been suggested to be pH 5.0–pH 7.0 (Li and Fang, 2007a).

Alkalinity also has an effect on hydrogen production in DF, as the VFA accumulation results in a drop in pH, and alkalinity helps to buffer the pH within the optimal range of hydrogen production in DF. Mtui (2009) reported that alkalinity was the most important parameter affecting hydrogen production. Bina et al. (2019) reported that the optimum initial alkalinity for DF that allowed the

highest hydrogen yield (220 mL/d) was 1325 mg/L $CaCO_3$ for initial alkalinity tested between 670 and 2678 mg/L $CaCO_3$.

In this study, the initial pH was adjusted to 5.5 for both control (untreated inoculum) and tests (treated inoculum), which is within the recommended pH level mentioned earlier. The production of VFA by Day 1 resulted in a decline of pH in the control and test reactors (Figure 5.4a); moreover, HST, which had the highest VFA production on Day 1 (829 mg/L), also had the smallest pH value (4.3) on Day 1. However, the VFA composition of HST was predominantly butyrate and acetate; hence, a higher hydrogen yield was obtained from it (see Section 5.3.3.1). Nonetheless, the final pH for the control and all tests was 4.2–4.6. Similarly, a final pH of 4.6 was reported by (Zhang et al., 2005) for batch BHP tests.

The starting alkalinity (Day 0) was 200–287 mg $CaCO_3$/L for control and the three tests (Figure 5.4b). Like the pH, VFA production led to the consumption of the alkalinity (Figure 5.4b), and as VFA accumulation progressed through time, a continuous decline in the alkalinity was observed for all conditions. However, HST and BST showed better alkalinity recovery potentials, consequently providing pH buffering, which allowed slightly higher pH levels under these treatment conditions. Although the starting alkalinity levels in this study were lower than levels reported in other studies, the production of hydrogen for all conditions in this study demonstrates that hydrogen can still be produced during DF under very low alkalinity.

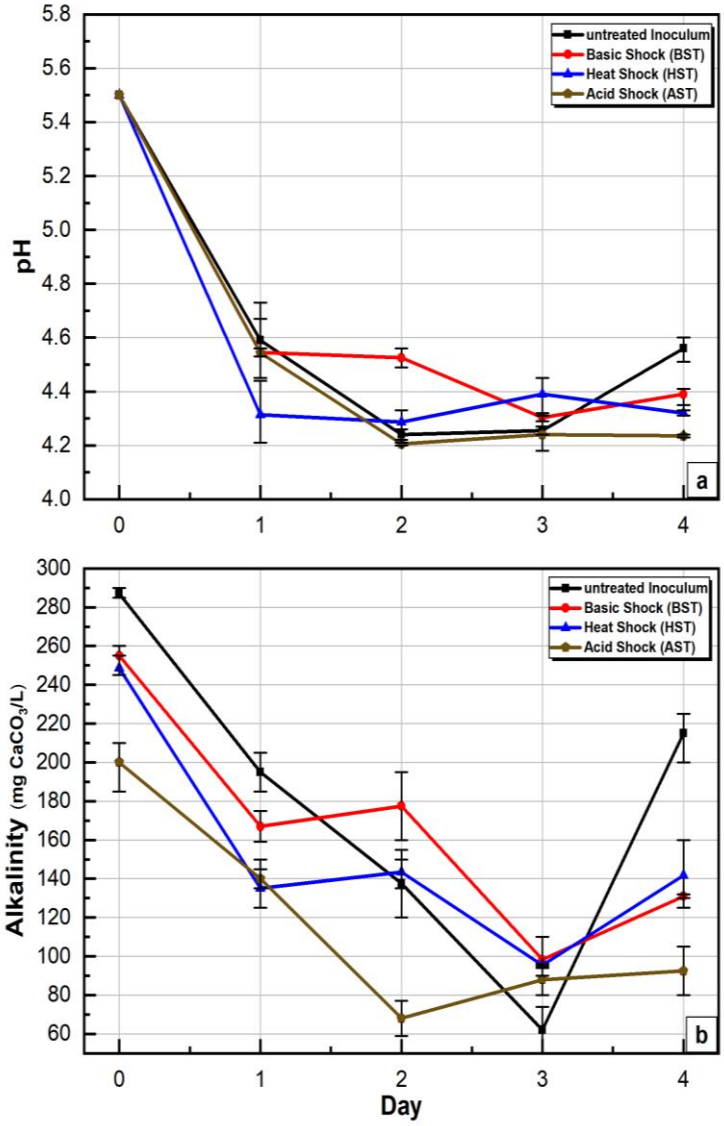


Figure 5.4 The behaviour curve of (a) pH and (b) alkalinity during 5-day BHP tests (average value of triplicate with max/min bar).

5.3.3.4 Process kinetics

The fitting was run for the period of hydrogen accumulation from Day 0 until Day 3, when hydrogen reached its peak, as this model (MGompertz) is extensively used for batch experiments that have growth rate without gas consumption (Pagliaccia et al., 2016, Zwietering et al., 1990, Cai et al., 2004a).

Theoretical hydrogen potential (BHP_{th}) can be determined by either Equation (5.2) or (5.3) (Section 5.3.3.2), which are based on the butyrate or acetate conversion pathway. Table (5.6) shows the comparison between experimental

hydrogen potential (BHP_{exp}) and BHP_{th} according to the dominant conversion pathway, which is the butyrate pathway, as shown in Figure (5.3, a-d).

Table 5.6 Comparison between experimental hydrogen potential (BHP_{exp}) and theoretical hydrogen potential (BHP_{th}).

Conversion pathway	Pre-treatment	Theoretical hydrogen yield potential (BHP_{th})	Experimental hydrogen yield potential (BHP_{exp})	(BHP_{exp}/BHP_{th})
		mole-H_2 /mole glucose added		%
Butyrate	Untreated	2.00	1.37 (0.22)	68.5
	HST		1.54 (0.01)	77.0
	AST		1.26 (0.01)	63.0
	BST		1.24 (0.00)	62.0

HST: heat shock; **BST**: basic shock; **AST**: acid shock.
(STD: standard deviation from the mean (n = 3)).

As butyrate conversion was dominant from Day 0 until Day 3, as shown in Table (5.6), the highest percentage of (BHP_{exp}/ BHP_{th}) was for HST (77%), while the others (untreated, AST and BST) had 63%, 62% and 68.5%, respectively. The HST is the most suitable pre-treatment for this type of inoculum and substrate in terms of maximising hydrogen production and inhibiting hydrogen-consuming bacteria (homoacetogenic and methanogens).

Table (5.7) shows the process kinetics for untreated and treated inoculum. HST has the highest hydrogen production potential (***P***) and maximum hydrogen production rate ($\boldsymbol{R_m}$). The long lag phase for the untreated inoculum (19.54 hours), compared with the treated inoculum (4.25–6.64 hours), shows the positive impact of pre-treatment on enhancing a rapid production of hydrogen after set-up, hence shortening the time needed to reach maximum hydrogen during the DF process. The R^2 value, as shown in Table (5.7), ranged from 0.987–0.999, which demonstrates that the MGompertz model provided a good fit to the data. These results also show that there is a relationship between lag time and VFA accumulation, especially the dominant VFA which, in this study,

was butyric acid. As shown in Figure (5.3a), butyrate slowly increased during the first 24 hours and reached 125 mg/L, while for the treated inoculum, there was a fast rate of accumulation of butyrate, as shown in Figure (5.3, b-d). The increment was around 3.5-fold for AST and BST and fivefold for HST.

Table 5.7 Process kinetics for untreated and treated inoculum during 5-day BHP tests.

Pre-treatment	P	R_m	λ	R^2
	(mL)	(mL/h)	(h)	
untreated	178.8	4.31	19.54	0.997
HST	197.2	4.90	6.64	0.999
AST	195.5	2.60	4.25	0.987
BST	159.8	3.53	5.16	0.998

HST: Heat shock, **BST**: Basic shock and **AST**: acid shock

The statistical analysis presented in Table (5.8) shows the correlation relationship between hydrogen yield, butyrate and acetate obtained from Minitab 18.

Table 5.8 Correlation relationship between hydrogen, butyrate and acetate.

Pre-treatment	Correlation (Hydrogen-Acetate)	Correlation (Hydrogen-Butyrate)	n
Untreated	0.594*	0.939**	14
HST	0.936**	0.996**	15
AST	0.821**	0.991**	13
BST	0.417	0.973**	13

HST: heat shock, BST: basic shock and AST: acid shock
* significant correlation with 0.05 > *p*-value > 0.01
** significant correlation with 0.01 > *p*-value

As mentioned earlier, the acetate pathway has higher hydrogen production potential than the butyrate pathway (De Gioannis et al., 2013a). Table (5.8) shows that the correlation analysis is consistent with hydrogen yield. HST had the highest hydrogen yield and a significant correlation with both acetate and butyrate, while untreated and BST had a lower hydrogen yield and a significant correlation with butyrate only. However, AST shows that even if the correlation

is significant between hydrogen and both VFAs, the hydrogen yield may be smaller. A similar result was reported by (Cai et al., 2004a), as BST had a higher hydrogen yield than untreated inoculum because of the conversion type: there was a significant correlation between hydrogen yield and both acetate and butyrate in BST, while in untreated inoculum the significant correlation was only between hydrogen and butyrate.

5.3.3.5 Effect of different HST pre-treatment methods on hydrogen yield in the BHP test

The results presented in Section 5.3.3.1 show that HST of inoculum produced the maximum hydrogen yield among all the pre-treatments. This section further investigates the use of the same principle of HST for inoculum but with a different methodology. Wang and Wan (2008) reported using HST with a furnace at 100°C for 15 min, and Luo et al. (2011) reported using a furnace at 100°C for 60 min for HST. Both groups succeeded in inhibiting hydrogen-consuming bacteria and found this pre-treatment produced a larger hydrogen yield than any of the others. Therefore, in this section another HST was used (furnace at 105°C for 60 min (HST_{105})) as an alternative to HST by autoclave (115°C for 20 min (HST_{115})), which was used in Section 5.3.3.1.

Figure (5.5) shows that, for BHP with an initial pH 5.5, HST_{115} had a lower hydrogen yield (187 mL/g glucose added) than HST_{105} (202 mL/g glucose added). One plausible reason for this slight change in hydrogen yield is that HST by autoclave may cause de-activation of (i.e., it kills) some of the hydrogen-producing bacteria, which reduces the amount of active hydrogen-producing bacteria. Wang and Wan (2008) reported that hydrogen production reached (221 mL-H_2/gVS-added) with HST (100°C for 15 min) which is higher than both HST_{105}

and HST_{115} in this study. For BHP at initial pH 7.0, the hydrogen yield for both methods was almost equal (213 and 211 mL/g glucose added for HST_{115} and HST_{105}, respectively) as shown in Figure (5.5).

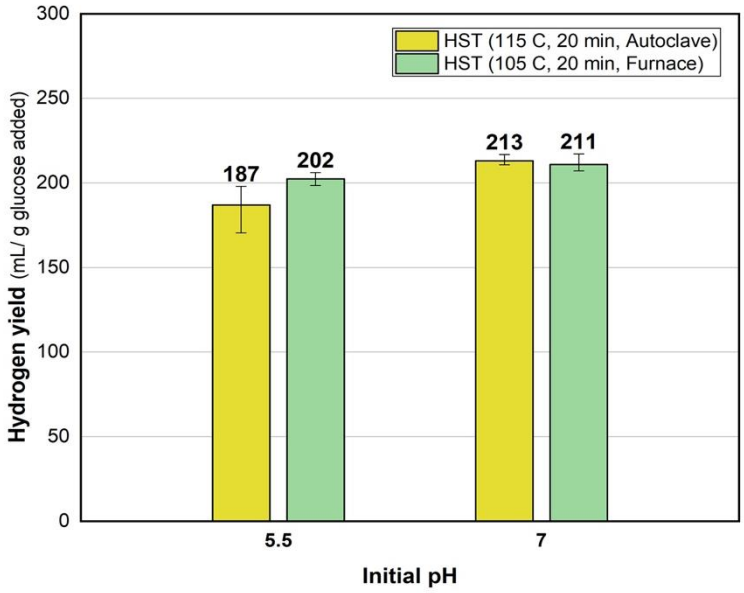


Figure 5.5 Impact of different HST methods on hydrogen yield with different initial pH (hydrogen yield value is for Day 4, at the end of the BHP experiment) (average value of triplicate with max/min bar).

5.3.3.6 Effect of initial pH and inoculum to substrate ratio

For better assessment, more investigations were carried out by studying the effect of initial pH on hydrogen yield and VFA production. Figure (5.6, a-d) shows that changing the initial pH from 5.5 to 7.0 resulted in higher hydrogen and VFA production: for pH 7.0, the hydrogen yield was higher (206 mL/g glucose added, Figure 5.6b) than for pH 5.5 (191 mL/g glucose added, Figure 5.6a). Furthermore, the VFA production curves shown in Figure (5.6, c-d) explain why an initial pH 7.0 was better, as butyric acid (butyrate) dramatically increased and reached 5937 mg/L on Day 3, as shown in Figure (5.6d), while in an initial pH 5.5, the maximum value was 1446 mg/L, as shown in Figure (5.6c). For acetic acid (acetate), the concentration was 1316 mg/L for pH 7.0 and 250 mg/L for pH

5.5, which increased twofold. These data indicate that starting BHP with pH 7.0 enhanced both hydrogen yield and VFA production.

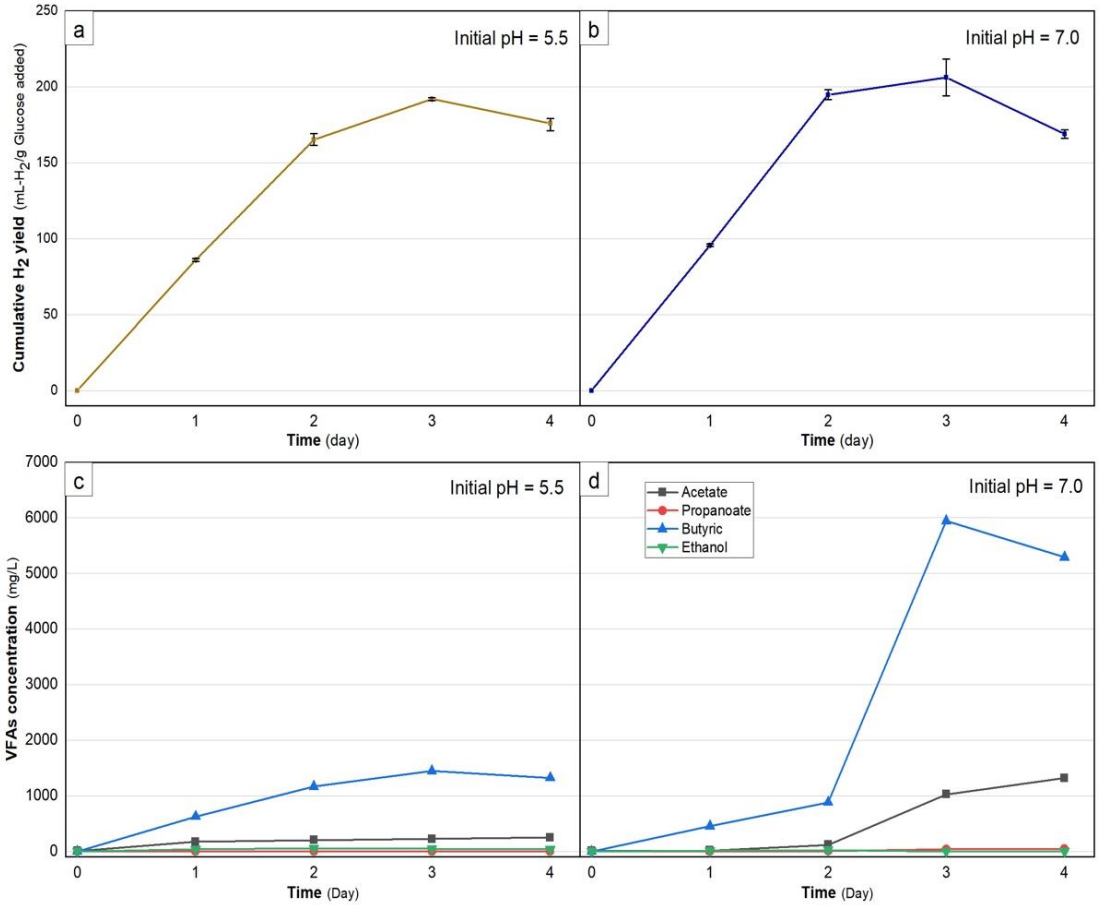


Figure 5.6 Impact of initial pH on hydrogen yield and VFA production during 5-day BHP tests, (average value of triplicate with max/min bar).

Further BHP tests were carried out to assess the impact of the ISR. Two different ISR were used: 1:1, 5g VS treated inoculum by HST with 5g VS glucose as substrate; and 2:1, 10g VS treated inoculum by HST with 5g VS glucose as substrate. Initial pH was adjusted to 7.0 for both BHP tests.

Figure (5.7,a-b) shows that $ISR_{1:1}$ achieved a higher hydrogen yield (206 mL/g glucose added) than $ISR_{2:1}$ (169 mL/g glucose added) because in $ISR_{1:1}$, most of the glucose conversion was toward hydrogen production rather than VFA (acetate and butyrate) production. Figure (5.7c) shows there was a lag in the

production of both acetate and butyrate during the first two days, while the hydrogen was dramatically increasing. After Day 2, butyrate production saw a huge jump, from 882 to 5937 mg/L, while acetate similarly rose from 115 to 1316 mg/L, and there was only a slight increase in hydrogen yield (Figure 5.7a). This pattern indicates that the glucose conversion after Day 2 shifted toward VFA production. In contrast, for $ISR_{2:1}$, there was no lag in VFA production as the butyrate and acetate immediately saw a very high production rate: butyrate reached 6020 mg/L on Day 1, and acetate reached 3066 on Day 2, as shown in Figure (5.7d). This high production of VFAs may affect the hydrogen production shown in Figure (5.7b), indicating that most of the glucose conversion was toward VFA production rather than hydrogen production. Therefore, according to the results presented in this section, pH 7.0 was selected to be used for the BHP tests described in the following chapters.

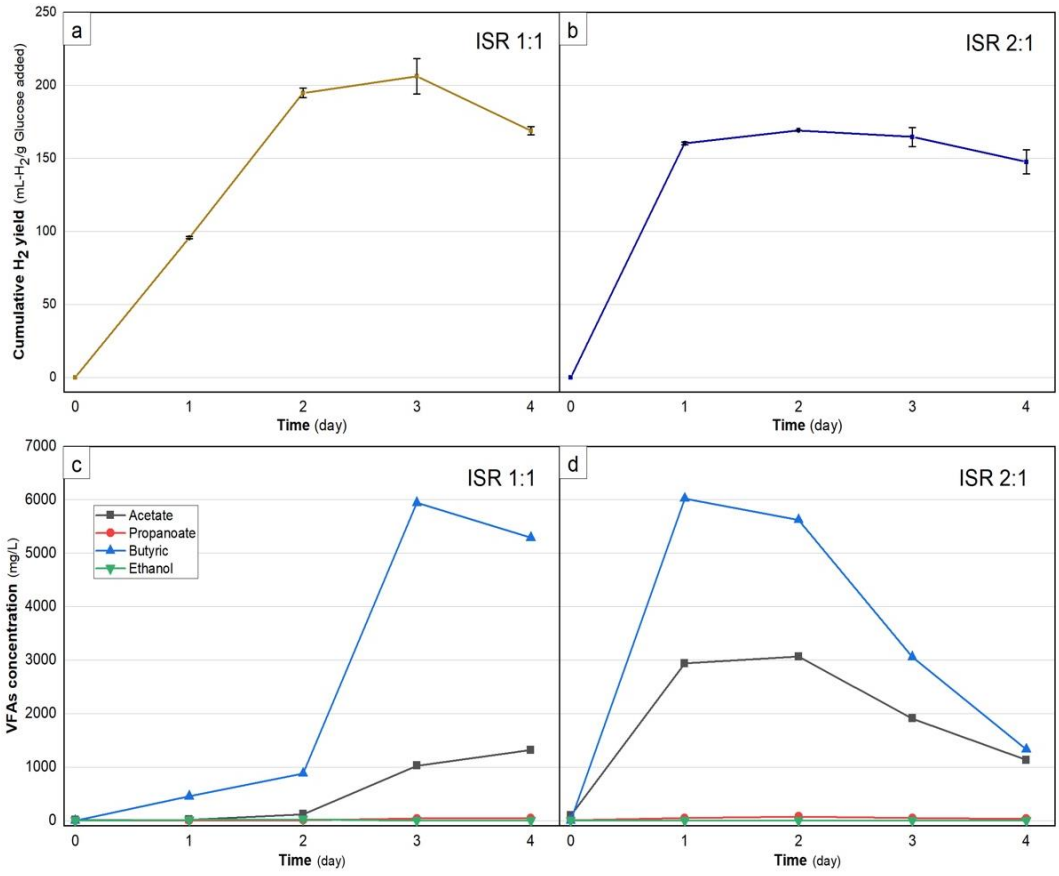


Figure 5.7 Impact of ISR on hydrogen yield and VFAs production during 5-day BHP tests (with max/min bars; all values are average of triplicate).

5.4 Bio-hydrogen potential of hydrolysed sewage sludge

Different substrates have been used in DF to acclimatise microorganisms, especially fermentative bacteria, to consume specific organic feedstocks and re-direct their transformation to hydrogen gas production. Sewage sludge contains a high concentration of nutrients and a highly diverse microbial community, giving it an advantage as an inoculum source for DF. Unfortunately, very limited research has investigated sewage sludge as a substrate for hydrogen production in DF due to its apparent low hydrogen production efficiency. There are many reasons for this; for example, the complexity of sludge content makes it difficult for fermentative bacteria to metabolise it, and it has a low C/N ratio (Xia et al., 2016). As a result, most previous studies have focused on using sewage sludge pre-treatment (Yang et al., 2016) or co-fermentation with other carbon-rich substrates to increase the C/N ratio (Wu et al., 2016, Xie et al., 2016) and modifying the operation process (pH, retention time and OLR, temperature and nutrients) (Wang and Wan, 2009) to enhance hydrogen production through the DF process. Therefore, this section will assess the BHP for HSS, as the results will be used later for comparison with hydrogen production when pre-treatment is applied to HSS (substrate pre-treatment (Chapter 6)).

5.4.1 Materials and methods

5.4.1.1 Substrate and inoculum source

The collected HSS from Esholt WWTP was used as substrate for a BHP batch test, and digestate from the AD reactor in Esholt was used as inoculum. The sample collection and processes are described in Section 3.1.1 in Chapter 3.

5.4.1.2 Inoculum pre-treatment method

As a result of the work described in Section 1.3 (pre-treatment methods), HST was selected as the best pre-treatment method for the inoculum (digestate) and was therefore used in this experiment. Details of HST are given in Section 5.3.1.2 in this chapter.

5.4.1.3 Analytical tests

Several tests were carried out during the batch BHP experiment. Five testing points were selected to monitor the five-day BHP test. Table (5.9) summarises the tests conducted on sacrificial bottles. The BHP process monitoring and analysis are described in more detail in Section 3.4.1 in Chapter 3.

Table 5.9 Testing points during the 5-day BHP test.

Parameter/Day	0	1	2	3	4
pH	x	x	x	x	x
Alkalinity	x	x	x	x	x
TS	x				x
VS	x				x
VFAs	x	x	x	x	x
Biogas volume		x	x	x	x
Biogas composition		x	x	x	x

TS: total solids; **VS**: volatile solids; **VFAs**: volatile fatty acids.

5.4.2 Experimental set-up

The HSS samples collected from Esholt WWTP were used as substrate for BHP for hydrogen production. The BHP of HSS was conducted in four sequential stages (BHP series batches), each one lasting five days. This step was conducted to sequentially enrich the population of hydrogen-producing bacteria from previously pre-treated digestate samples and, hence, produce a better inoculum for processing HSS (complex organic material) and enhancing

hydrogen production (De Gioannis et al., 2013b, Show et al., 2012, Wong et al., 2014).

Wheaton glass bottles were used to set up the BHP test. An ISR of 1:1 was used in the BHP test. The operation temperature was 37°C (mesophilic condition), maintained by water bath. All the bottles were purged with nitrogen gas, which was sparged through the solution for 1 min to ensure that the BHP bottles were under anaerobic conditions. A rubber cap and an aluminium crimp were used to prevent biogas leakage. The working volume was 70 mL for all bottles. The BHP set-up is described in detail in Section 3.4.2 in Chapter 3. Table (5.10) shows the characterisation of all reactors on Day 0.

Table 5.10 Characterisation results of all reactors on Day 0.

		1st Stage	2nd Stage	3rd Stage	4th Stage
Inoculum		Digestate	Biomass from 1st stage	Biomass from 2nd stage	Biomass from 3rd stage
Pre-treatment		Heat shock (HST), (115 °C for 20 min)			
Substrate		HSS	HSS	HSS	HSS
pH	-	5.5	5.5	5.5	5.5
Alkalinity	mg $CaCO_3$/L	451 (9)	250 (10)	430 (10)	350 (5)
TS	%	1.55 (0.03)	1.41 (0.01)	1.41 (0.05)	1.49 (0.06)
VS	%	1.00 (0.02)	0.99 (0)	0.96 (0.04)	1.05 (0.04)

TS: total solids, **VS**: volatile solids
HSS: hydrolysed sewage sludge, **HST**: heat shock pre-treatment.
(STD: standard deviation from the mean (n = 3)).

5.4.3 Results and discussion

5.4.3.1 Hydrogen yields and volatile fatty acid degradation

The cumulative hydrogen yield from the control (glucose substrate) and test (hydrolysed sewage sludge substrate) for four sequential stages is shown in Figures (5.8) and (5.9), respectively. VFA accumulation for the same experiment is shown in Figures (5.10) and (5.11), respectively. There was no methane

detection for either control or test, and all tests used the same inoculum, which was pre-treated by HST. The results of hydrogen production from glucose using treated inoculum in this experiment (183.7 mL-H_2 / gVS-added) are similar to those reported in Section 5.3.3.1. Hydrogen production for stages 2 and 4 (135 and 100 mL-H_2 / gVS-added, respectively) were lower than for stage 1, with the difference being the inoculum used (digestate for the first stage, biomass from the first stage for the second stage, and biomass from the third stage for the fourth stage). Unfortunately, there are no data to report from the control in the third stage due to experiment set-up errors. There was high acetic acid production during the first 24 hrs in stages 2 and 4, and, as a result, some of hydrogen was consumed by homoacetogenic bacteria. However, after 24 hrs, the fermentative bacteria took over the digestion process, as shown in Figure (5.10), where the acetic acids drop to 71 mg/L and 80 mg/L for the second and fourth stage, respectively (Akutsu et al., 2009, Zhao et al., 2015) (Wang and Wan, 2008). Although the inoculum was treated with HST, which was the best pre-treatment for inhibiting homoacetogenic bacteria, as discussed in Section 5.3 of this chapter, homoacetogenic were nonetheless the dominant bacteria during the first 24 hrs for the BHP control.

In contrast to the BHP control, very low hydrogen production was detected in the BHP test, and this result was predicted because the complexity of HSS makes it difficult to bacteria to utilize it. Moreover, several studies have reported very limited hydrogen production from sewage sludge due to the complexity of sludge contents and the difficulty of reaching stable fermentative bacterial processes under a low C/N ratio (Xia et al., 2016). Yang and Wang (2017) summaries and reported the studies that investigated the bio-hydrogen production from sewage

sludge, as shown in Table (5.11). Most of these studies have reported very limited hydrogen production from sewage sludge (including different types: anaerobically digested sludge, waste-activated sludge, primary sludge and thickened sludge). Except one study reported relatively high hydrogen production (28.3 mL/g-VS removed) (Tyagi et al., 2014) and that related of using mixed sludge (municipal solid waste + sewage sludge) which has higher C/N ratio that enhanced the hydrogen production. Despite the unit deference of the reported hydrogen yields in Table (5.11), its clearly, raw sludge performed relatively low hydrogen yields compared with other feedstock such as glucose (as shown in Section 5.3.3.1), and several studies even observed that almost no hydrogen was generated during raw sludge fermentation (Xiao and Liu, 2009, Lee et al., 2014).

Table 5.11 Hydrogen potential from sewage sludge and digestate.

Sludge type	Inoculum	Fermentation conditions	Hydrogen yield	References
ADS	ADS	Batch, 50°C , Initial pH: 5	0.02 mL/g-TSremoved	(Sato et al., 2016)
Mixed sludge	ADS	Batch, 55°C , Initial pH: 5.5	28.3 mL/g-VSremoved	(Tyagi et al., 2014)
WAS	ADS	Batch, 35°C , Initial pH: 6	17.9 mL/g-VSadded	(Cheng et al., 2016)
Primary sludge	ADS	Batch, 37°C , Initial pH: 5.5	10 mL/g-CODadded	(Yu et al., 2013a)
Thickened sludge	ADS	Batch, 37°C , Initial pH: 8	0.25 mL/g-VSSremoved	(Kim et al., 2013)
WAS	None	Batch, 35°C , Initial pH: 6.7	3.34 mL/g-TSadded	(Jan et al., 2007)
WAS	None	Batch, 37°C	5 mL/g-CODremoved	(Kotay and Das, 2009)
WAS	None	Batch, 37°C , Initial pH: 7	1.21 mL/g-VSadded	(Xiao and Liu, 2009)
WAS	ADS	Batch, 37°C , Initial pH: 5.5	7 mL/g-CODadded	(Xiao and Liu, 2009)
WAS	WAS	Batch, 30°C , Initial pH: 5.5	12.98 mL/L-Sludge added 1.41 mL/g-CODadded	(Wan et al., 2016)
WAS	WAS	Batch, 35°C	13.8 mL/g-CODadded 20 mL/g-TSadded	(Wang et al., 2004)
WAS	ADS	Batch, 55°C , Initial pH: 5.7	12.4 mL/g-TSadded 18.6 mL/g-VSadded 5.1 mL/g-CODadded	(Liu et al., 2013)

The maximum yield of BHP processing glucose was (191.8 mL-H_2/g VSadded) (section 5.3). For comparison, the maximum yield was converted to different units (191.8 mL-H_2/g TSadded and 179.8 mL-H_2/g CODadded) due to the difficulties of converting the reported yields in this table to constant units (data limitation).
WAS: waste-activated sludge; **ADS**: anaerobically digested sludge.

Table source: (Yang and Wang, 2017).

Inoculum enrichment through a series of BHP tests is one method to enhance hydrogen production and can produce a better inoculum for processing HSS (a complex organic material) (De Gioannis et al., 2013b, Show et al., 2012, Wong et al., 2014). Figure (5.9) shows there was no hydrogen in the first stage, while in the second stage there was low hydrogen production, reaching 4.18 mL-H_2 / gVS-added. It seems that the enrichment method had a positive impact, as the second-stage inoculum had less difficulty using HSS, which led to the production of some hydrogen gas. It was assumed that more hydrogen should produce in stages 3 and 4, as a result of the enrichment method; But, the hydrogen production was less than stage 2 and the maximum hydrogen production was 1 and 1.1 mL-H_2/gVS-added for the third and fourth stages, respectively. One possible reason was the accumulation of propanoic acid in the third and fourth stages, as shown in Figure (5.11). This accumulation may be a result of propionate conversion pathway, which is considered a hydrogen-consuming pathway (Guo et al., 2010a), while there was no accumulation of propanoic acid in the second stage, which led to higher hydrogen production. Kim et al. (2004) reported a positive relationship between hydrogen production and acetate and butyrate but a negative relationship between propionate and hydrogen production. Another possible reason is the acetic acid accumulation shown in Figure (5.11), where the maximum acetic acid for stage 2 was 173 mg/L, while for stages 3 and 4, it was 341 mg/L (twofold) and 250 mg/L (1.4-fold), respectively. This accumulation of acetic acid may be a result of the bio-conversion of hydrogen to acetic acid by homoacetogenic bacteria (Akutsu et al., 2009, Zhao et al., 2015).

A prediction of higher hydrogen production for the collected HSS than that achieved in other studies (shown in Table 5.11) was made before this experiment was conducted, as this HSS was treated by HTP at Esholt WWTP, Bradford, UK. It is known that this treatment can enhance the solubility and biodegradability of sewage sludge, which leads to higher utilisation by microorganisms than of untreated sewage sludge (Wirth et al., 2015, Wang et al., 2010). However, unfortunately, the result shows that very limited hydrogen was achieved for HSS nonetheless, while there was a high hydrogen production from the same inoculum with a different substrate (glucose). This finding indicates the need to apply one of the pre-treatments to HSS to enhance hydrogen production.

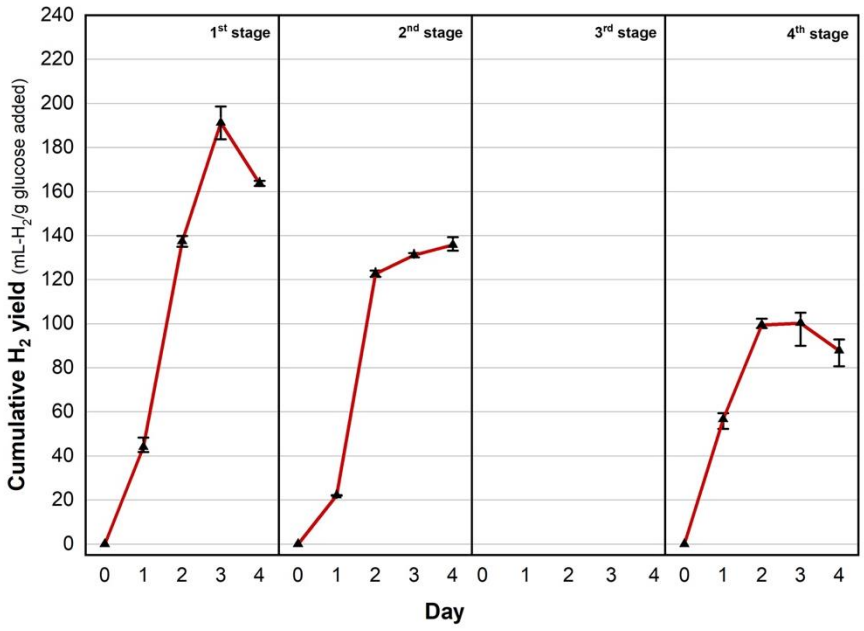


Figure 5.8 Cumulative hydrogen yield for control (glucose substrate) during 5-day BHP test, (missing data: there was no control in stage 3 due to an experiment set-up fault) (with max/min bars; all values are average of triplicate).

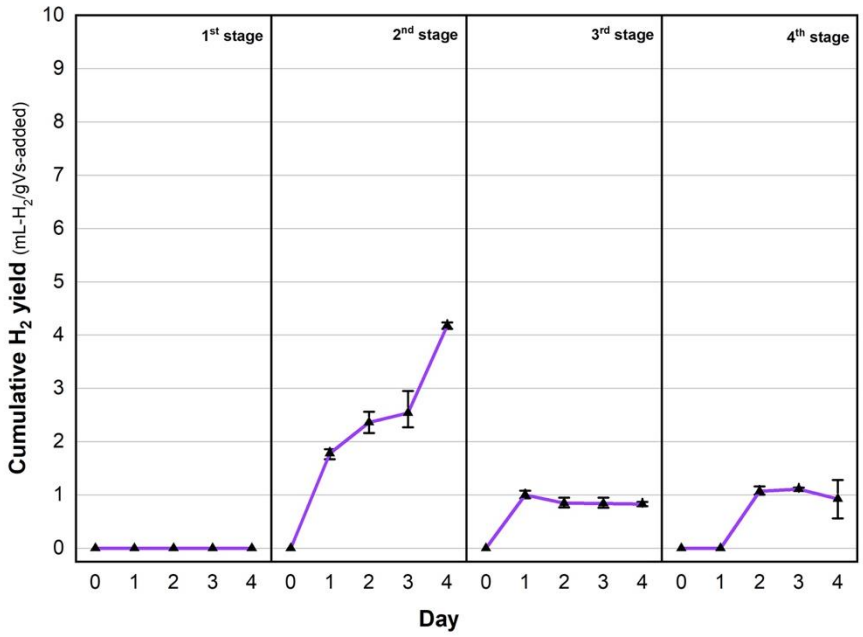


Figure 5.9 Cumulative hydrogen yield for Test (HSS substrate) during 5-day BHP test (with max/min bars; all values are average of triplicate).

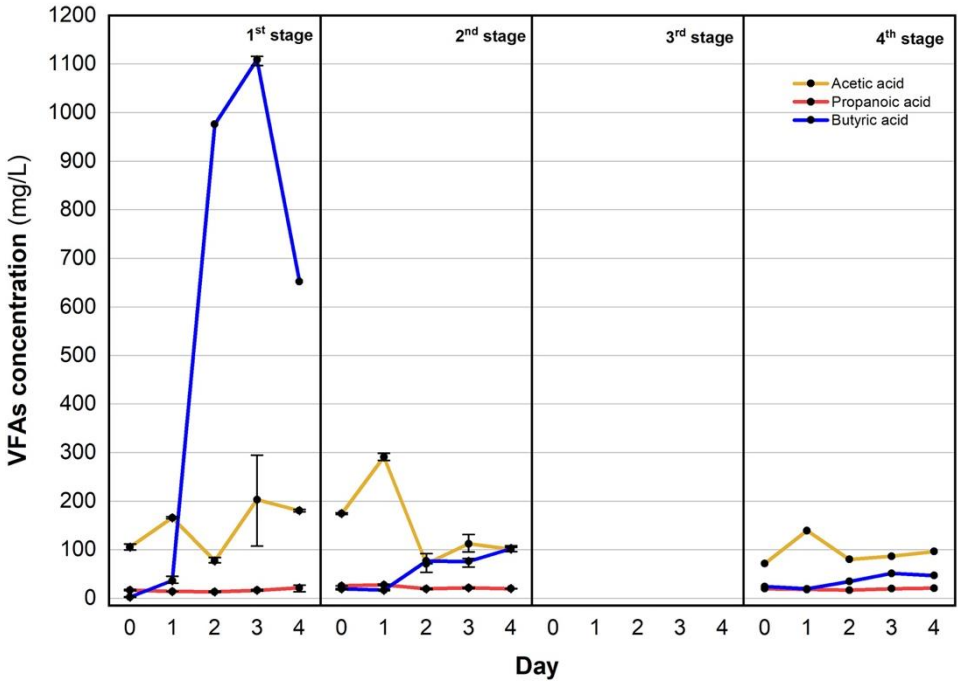


Figure 5.10 VFA accumulation for control (glucose substrate) during 5-day BHP test, (missing data: there was no control in stage 3 due to an experiment set-up fault) (with max/min bars; all values are average of triplicate).

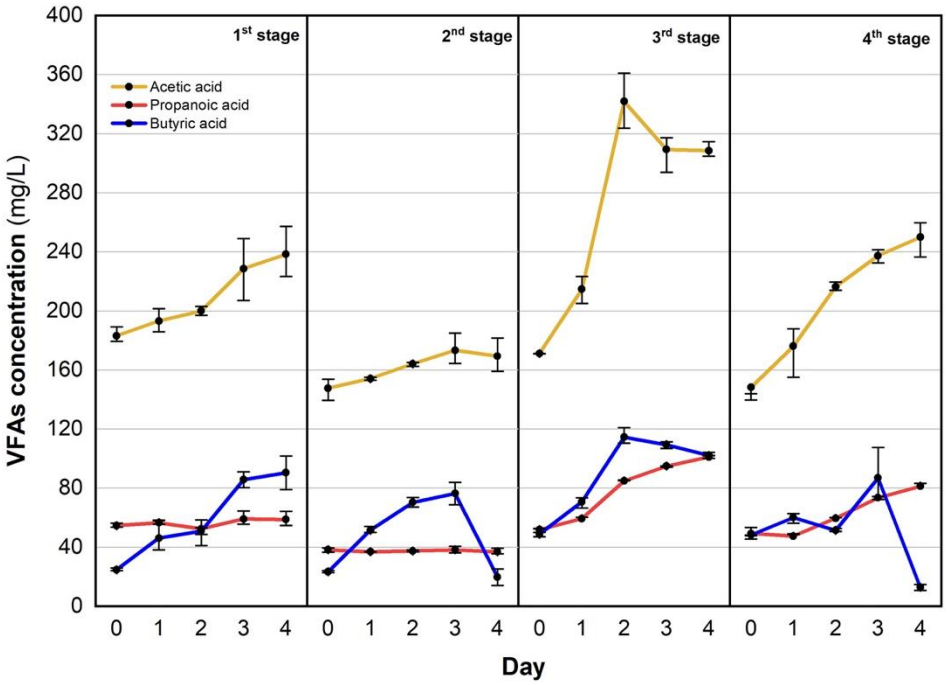


Figure 5.11 VFA accumulation for Test (HSS substrate) during 5-day BHP test (with max/min bars; all values are average of triplicate).

5.4.3.2 pH, alkalinity and C/N ratio

The pH and alkalinity have a crucial influence on the reactions occurring during DF. The pH value has an effect on the VFA accumulation, as for low pH (4.00–6.0) the butyrate-acetate accumulates, while, on the other hand, the ethanol and propionate accumulate at pH 7.0–9.0 (Hawkes et al., 2007, Pakarinen et al., 2008). In this experiment, the increase in pH in four stages favoured the acetate conversion pathway, as shown in Figure (5.12). Yin and Wang (2016) reported that acetate pathway fermentation was dominant during BHP with a pH higher than 5.0, as high acetate concentration was observed compare it other VFAs concentration. It is well known that the pH drops during the BHP test as a result of VFA accumulation in the reactor; however, low accumulation of VFAs and low hydrogen yield are correlated with pH behaviour.

On the other hand, alkalinity also has an effect on hydrogen production in DF (Ağdağ and Sponza, 2005, Valdez-Vazquez et al., 2005), with Mtui (2009)

reporting that it is the most important parameter affecting hydrogen production. In this experiment, the alkalinity curve is in line with the pH, as there was a low increment in both between stages 2 and 4, as shown in Figure (5.13), but there was a different scenario in stage 1, where the extreme drop in alkalinity led to a failure in buffering for pH, which may inhibit the fermentation process. Bina et al. (2019) reported that biological activity of fermentation bacteria (hydrogenase) can be affected by alkalinity and therefore affect hydrogen production.

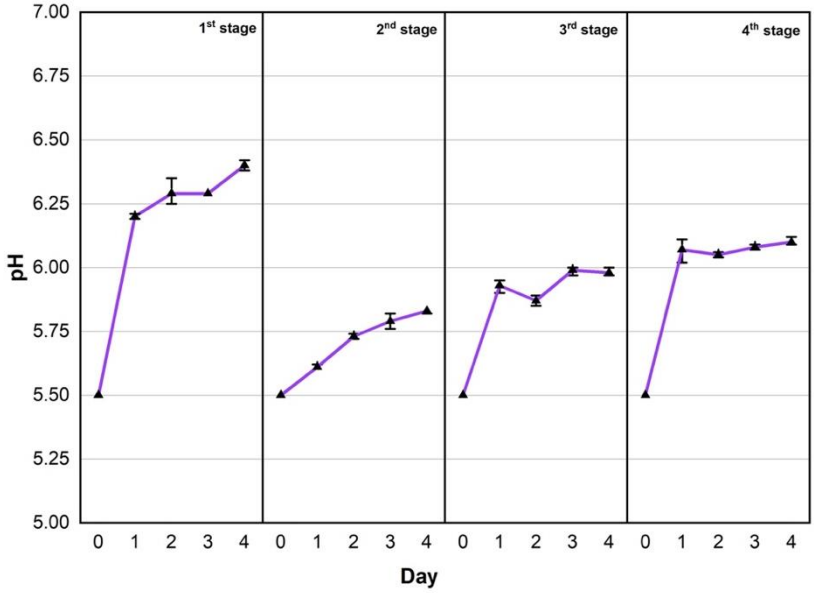


Figure 5.12 pH curve for Test (HSS substrate) during 5-day BHP test (with max/min bars; all values are average of triplicate).

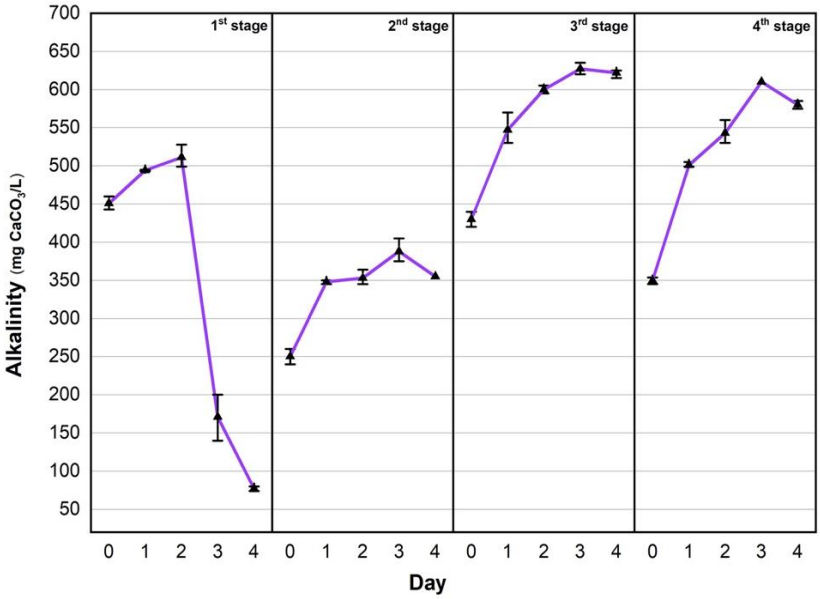


Figure 5.13 Alkalinity curve for Test (HSS substrate) during 5-day BHP test (with max/min bars; all values are average of triplicate).

The C/N ratio is one of the reasons for low hydrogen production, as many studies have shown that enriched (high-C/N) substrate has a higher hydrogen production than that with a low C/N ratio (e.g., sole sewage sludge, as used in this study) (Xia et al., 2016). Mata-Alvarez et al. (2014) reported that the suitable range of C/N for fermentative bacteria is 20–30, while the C/N of sole sludge is usually 4–10. For this reason, co-fermentation was used as one of the pre-treatments for the substrate to improve the C/N and therefore enhance hydrogen production (Wu et al., 2016, Xie et al., 2016, Hagos et al., 2017). As the BHP test consisted of both HSS and digestate, the C/N ratio calculated in this study was 8.54, while for control (glucose + digestate), it was 23.3, which is another reason for the low hydrogen production in the BHP test. Table (5.12) shows the relation between C/N ratio and hydrogen production in DF.

Table 5.12 C/N ratio relation with hydrogen yield.

Inoculum	substrate	Carbon to nitrogen ratio (C/N)	Hydrogen yield	Reference
Digestate	SS	7.1	17.9 mL/g-VS-added	(Cheng et al., 2016)
Digestate	WAS + FW	33.1	101.1 mL/gVSS-added	(Sreela-Or et al., 2011)
Digestate	PS	4.0	13 mL/gVSS-added	(Zhou et al., 2013)
	FW+PS	26.0	130 mL/gVSS-added	
	FW + WAS	31.0	137 mL/gVSS-added	
	FW+PS+WAS	30.0	165 mL/gVSS-added	
Thickener sludge	OFMSW+ GSW	28.8	149.5 mL/gCOD-removed	(Elsamadony and Tawfik, 2015)
	OFMSW+ GSW+PMS	29.4	157 mL/gCOD-removed	
Digestate	HSS	8.5	4.18 mL/gVS-added	This study
	Glucose	23.3	183.7 mL/gVS-added	

HSS: hydrolysed sewage sludge; **FW**: food waste; **WAS**: waste-activated sludge; **SS**: sewage sludge; **PS**: primary sludge; **GSW**: gelatin solid waste; **OFMSW**: organic fractions of municipal solid waste; **PMS**: paperboard mill sludge.

5.5 Conclusions

The study described in Section 5.3 demonstrated that an inoculum pre-treatment is an essential step toward enhancing hydrogen production in the DF process. HST was the best pre-treatment for hydrogen production and the stability of DF, while AST and BST made no difference to BHP performance. The highest cumulative hydrogen production was achieved with HST, which could be attributed to the longer inhibition of hydrogen consumers. In terms of VFA analysis, the dominant conversion pathway for glucose in this study was the butyrate pathway for untreated and treated inoculum. This study also shows that there is a relationship between lag time and VFA accumulation as lag time of DF processes, might be used as an indicator of how efficient the pre-treatment on inhibiting hydrogen consuming bacteria.

However, fermentation operation conditions, such as temperature, alkalinity, initial pH and ISR, have a crucial impact. Hence, without selecting the proper conditions for the inoculum and substrate types, a high re-activation of hydrogen-consuming bacteria (acetobacteria or methanogens) will occur. Moreover, this study demonstrated that hydrogen can still be produced during DF under very low alkalinity. The chapter also assessed the impact of initial pH and ISR on hydrogen and VFA production in DF, as the conversion pathway for glucose or other substrates can be changed by changing these operation parameters. Therefore, it is very important to control the operating conditions to ensure the best VFA route to gain maximum hydrogen production from DF.

The hydrogen production values in this study may differ when a different substrate, such as food waste, sewage sludge or agricultural waste, is used. To create a guideline procedure for all DF experiments in future, therefore, the first step is to understand the capability of using inoculum (sole or complex) for

hydrogen production and how it may react with different pre-treatment methods (HST, BST and AST). Moreover, it is necessary to understand which pre-treatments lead to maximum hydrogen production and ensure maximum time for inhibiting the activity of hydrogen consuming bacteria.

This chapter also assessed the BHP of HSS, which will help to create a baseline for comparison with the result chapters presented hereafter. The study described in Section 5.4 shows that very limited hydrogen was achieved for HSS, while a high amount of hydrogen was produced by the same inoculum but with a different substrate (glucose; Section 5.3). This finding indicates that it is necessary to apply one of the substrate pre-treatments to HSS to enhance hydrogen production. Overall, the main target is to find an efficient and economical biological hydrogen production method, conditional on using available renewable resources, such as sewage sludge.

Chapter 6
Enzymatic hydrolysis of sewage sludge as a pre-treatment for dark fermentation

6.1 Introduction

It is considered more sustainable to produce hydrogen via biological processes than conventional processes such as natural gas decomposition, petroleum oxidation and coal gasification. As biological processes do not require the same fossil-fuel energy inputs, they emit much less carbon dioxide overall. Bio-hydrogen production can contribute to the net reduction of GHG emissions (Łukajtis et al., 2018) and provide an alternative and more sustainable option to waste management without any dependency on carbon energy sources. Indeed, bio-hydrogen production can utilise a wide range of substrates and requires relatively low-cost operation conditions, such as ambient temperature, atmospheric pressure and no need for external energy (Singh et al., 2015, Kapdan and Kargi, 2006).

DF is one of several methods used for bio-hydrogen production, whereby fermentative bacteria are used to hydrolyse organic substrates to produce hydrogen gas. One of the main fermentable substrates for hydrogen production is carbohydrates (sugars), as they are considered the most favourable substrate for fermentative bacteria (e.g., *Clostridium* bacteria) (Finlay, 1995). Several studies (including the results presented in Chapter 5 of this thesis) have reported high hydrogen production in DF processes using sugars such as glucose as substrate (Luo et al., 2011, Oh et al., 2003a, Wang and Wan, 2008). Sugars naturally exist in plants, and they are used extensively in food processing industrial activities (Fellows, 2009). Although biohydrogen can be produced from

plant-based sugars using DF processes, this practice would directly compete with food production, just as current biodiesel and bioethanol production competes with that of soy beans and sugar cane. Therefore, the direct use of plant-based sugars as a substrate for hydrogen production via DF processes is not advised.

As mentioned earlier, DF has the ability to utilise organic waste for hydrogen production. Sewage sludge has the potential to be a sustainable source for glucose production as an intermediate product to support hydrogen production: Champagne (2007) reported that 6.22 Mt/yr of sugar can be produced from municipal sludge and livestock manures generated in Canada. Although the current routes for sewage sludge processing at WWTWs use AD, there is still an opportunity to move towards sugar production to support biohydrogen production.

In this research, HSS (collected from Esholt WWTP) was used as feedstock in DF for bio-hydrogen production. Due to the complexity of HSS contents, including a low C/N ratio, fermentative bacteria will find it very difficult to transform it into hydrogen (Xia et al., 2016), as demonstrated in the research results reported in Chapter 5. In order to overcome this hurdle, the pre-treatment of HSS is an essential step towards efficient bio-hydrogen production in the DF process. Several studies have reported different pre-treatment methods to enhance hydrogen production from sewage sludge (Yang et al., 2016). Disintegration of sewage sludge is among the methods that can break down the hard-to-digest macro sewage flocs to more easily digestible micro flocs. As a result, pre-treated sewage sludge biomass will have a suitable fermentable structure that can be easily utilised by fermentative bacteria for hydrogen

production. Disintegration can be achieved by four methods: mechanical, physical, chemical and biological (EH). However, EH is preferred to mechanical, chemical and physical pre-treatments, as it requires lower energy inputs than the other three. Moreover, its ability to reduce sludge volume and improve hydrogen production from sewage sludge has been proven (Massanet-Nicolau et al., 2008, Parawira, 2012). Therefore, in this chapter, EH treatment was used to treat the HSS.

This chapter aims to determine the potential of increasing glucose (a favourable substrate for fermentative bacteria) content in HSS by the EH process.

6.2 Objectives of chapter

- To select and assess a simple and reliable method for measuring glucose concentration in HSS samples.
- To assess glucose production from HSS using the EH process.

6.3 Glucose production from sewage sludge via enzymatic hydrolysis

The aim of the work described in this section was to break down the complex organics in HSS samples by using enzymes through the EH process, as EH has the ability to transform HSS content to suitable fermentable organics (e.g., glucose) that can be easily utilised by hydrogen-producing bacteria.

6.3.1 Materials and methods

6.3.1.1 Feedstock source and enzyme

The feedstock (HSS) used for the EH test was collected from the effluent of the HTP at Yorkshire Water's Esholt WWTW, Bradford, UK. Sample collection and processes are described in Section 3.1.1 (Chapter 3). Cellulase, enzyme blend

was purchased from Sigma-Aldrich and used for the EH test. Cellulase enzyme is well known in enzymatic treatment applications as it is used as a pre-treatment for lignocellulosic biomass materials (such as sewage sludge) to degrade cellulose material to fermentable sugars such as glucose (Champagne and Li, 2009, Ferreira Filho et al., 2020, Ishak et al., 2022). More details of Cellulase enzyme are given in Section 3.5.2 (Chapter 3).

6.3.1.2 Analytical tests

6.3.1.2.1 Glucose analysis

The main objective of the work described in this chapter was to release glucose from HSS through EH. Several methods were used to determine the glucose concentration in a solution. Benedict's quantitative method was selected to measure sugars (glucose) from EH experiments. Details of Benedict's quantitative method are reported in Section 3.5.1 (Chapter 3).

6.3.1.2.2 Liquid analysis

TCOD, sCOD and pH were measured during the EH test. Full details are reported in Table (3.2) (Chapter 3).

6.3.2 Experimental set-up

The experimental set-up was divided into four parts, as follows.

6.3.2.1 Part 1- Determining the optimum wavelength for Benedict's quantitative reagent

Benedict's quantitative reagent is blue in colour. The method is based on changes in light absorbance induced by the concentration of sugars in process samples. It was critical to find the optimum wavelength of the light beam that would produce the maximum light absorbance in HSS samples processed with Benedict's reagent, as their background colour induced changes in the reagent

within the blue–green colour range, which had an effect on absorbance readings, as reported in the results section.

The EH test was carried out by using HSS. The optimum wavelength test was conducted using solutions with different compositions, including Benedict's reagent, distilled water (DW), glucose solution and HSS. The absorbance of test samples was measured using a UV-Vis spectrophotometer (Shimadzu UV1900). Wavelengths between 620 nm and 840 nm were used to find the optimum wavelength from the tested samples (see Table (6.1)). Total mix volume and composition affect the final colour of the tested samples and, hence, its absorbance. For this reason, the prepared samples were divided into two groups: (a) Group A samples had 6 mL volume, and (b) Group B samples had 7 mL volume. Different volumes of a 1% glucose solution were used for each tested sample to cover the possible range of sugar concentrations that can be expected during an EH test. Table (6.1) gives more details about this experimental set-up, and full set-up details are also reported in Section 3.5.1.1 (Chapter 3).

Table 6.1 Composition details for different samples for the optimum wavelength test.

Group	ID	1% glucose solution	Distilled water	HSS	Benedict's quantitative reagent	Total volume	Expected glucose concentration added
		mL	mL	mL	mL	mL	mg/mL of 1% glucose solution
A	1	1	0	0	5	6	10
	2	0.5	0.5	0	5	6	5
	3	0.1	0.9	0	5	6	1
	4	0	1	0	5	6	0
B	1	0	2	0	5	7	0
	2	1	1	0	5	7	10
	3	1	0	1	5	7	10

HSS: hydrolysed sewage sludge
1% glucose standard solution: (10 g of D-glucose powder in 1 litre of DW).

6.3.2.2 Part 2- glucose standard curve

The glucose standard curve is essential for calculating an unknown glucose concentration in a solution, especially if the method includes measuring the absorbance. In this experiment, Benedict's quantitative reagent (Fisher Chemical) was used as a detector of glucose in a solution, as described in Chapter 3. The glucose standard curve ranged between 0 and 10 mg glucose/mL. The absorbance of samples was measured against a blank sample (just DW, Abs = 0) at wavelength 740 nm (the optimum wavelength from the results reported in Part 1). Table (6.2) gives more details of this experimental set-up, and full set-up details are reported in Section 3.5.1.2 (Chapter 3).

Table 6.2 Set-up details for determining glucose standard curve.

Glucose standard curve (0-10 mg/mL)	**ID**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
	mg/mL	10	8	6	4	2	1	0
Glucose solution (1%)	mL	1	0.8	0.6	0.4	0.2	0.1	0
Distilled water	mL	0	0.2	0.4	0.6	0.8	0.9	1
Benedict's reagent	mL	5	5	5	5	5	5	5
Total solution volume	mL	6	6	6	6	6	6	6

1% glucose standard solution: (10 g of D-glucose powder in 1 litre of DW).

6.3.2.3 Part 3- Modified glucose curve

Modified glucose curve was also calculated to maximise the accuracy of Benedict's method of measuring glucose concentration in a solution that contains HSS. The idea is that, prior to the EH test being conducted, a modified glucose curve is created each time for specific HSS. Benedict's method depends on the absorbance, and different TCOD concentrations of HSS have different effects on Benedict's colour (which ranges from blue to blue–green). The procedure described in Section 6.3.2.2 was carried out for two HSS TCOD concentrations (5 and 10 g TCOD/L); Table (6.3) gives more detail of this experimental set-up, and full set-up details are reported in Section 3.5.1.2 (Chapter 3).

Table 6.3 Set-up details for determining modified glucose curve.

Modified glucose curve (0-10 mg/mL)	ID	1	2	3	4	5	6	7
	mg/mL	10	8	6	4	2	1	0
Glucose solution (1%)	mL	1	0.8	0.6	0.4	0.2	0.1	0
HSS (5 or 10 g TCOD/L)	mL	1	1	1	1	1	1	1
Distilled water	mL	0	0.2	0.4	0.6	0.8	0.9	1
Benedict's reagent	mL	5	5	5	5	5	5	5
Total solution volume	mL	7	7	7	7	7	7	7

1% glucose standard solution: (10 g of D-glucose powder in 1 litre of DW).
HSS: hydrolysed sewage sludge

6.3.2.4 Part 4- Enzymatic hydrolysis test

Cellulase, enzyme blend from Sigma-Aldrich was used in EH for glucose production from HSS. Two concentrations of HSS (5g and 10g of TCOD/L), with different enzyme dosages between 1 and 7 mL, were used to assess the effect of enzyme dosage on glucose production during the EH process. Table (6.4) shows the set-up details of this experiment, while Table (6.5) summarises the tests conducted to monitor and analyse the EH processes carried out during this experiment. Full details are reported in Section 3.5.2 (Chapter 3).

Table 6.4 EH experiment set-up details (tests A and B are in triplicate).

	Unit	Blank	Control A	Control B	Test A	Test B
HSS (TCOD)	g/L	None	5	10	5	10
Enzyme (Cellulase blend)	mL	1-7	None	None	1-7	1-7
Working volume	mL	250	250	250	250	250
Operation time	hrs.	4	4	4	4	4

HSS: hydrolysed sewage sludge

Table 6.5 Testing points during the EH test.

Parameter/Time	9:30	10:30	11:30	12:30	13:30
Sample test size (mL)	15	8	8	15	8
pH	x	x	x	x	x

TCOD	x				
sCOD	x			x	
Glucose concentration	x	x	x	x	x

TCOD: total chemical oxygen demand; **sCOD**: soluble chemical oxygen demand.

6.3.3 Results and discussion

6.3.3.1 Determining the optimum wavelength during Benedict's test

Benedict's method is a test that can determine the presence of reducing sugars (including glucose) in a solution by several chemical reactions that affect the absorbance of a sample, usually measured using a spectrophotometer device. There are two types of Benedict's reagent. The qualitative reagent confirms the presence or absence of reducing sugars in a solution by changing the colour of the solution from deep-blue to one ranging from green (traceable reducing sugars) to brick-red (high reducing sugars). The quantitative reagent, on the other hand, can help determine and quantify the glucose concentration in a solution by changing the deep-blue colour (no glucose) to a range of colours between mid-blue (traceable glucose) and very light blue (high glucose).

The first step in Benedict's test is the chemical reaction between the solution and the reagent; the second is to measure the absorbance of the new solution colour with a spectrophotometer device (Shimadzu UV-1900 was used in this research). The final step is to determine the glucose concentration for the sample by using a glucose standard curve.

Each colour has a specific wavelength which exhibits the greatest absorbance; therefore, the wavelength range of 620–840 was selected (according to the deep-blue colour of quantitative Benedict's reagent) and used to determine the optimum wavelength for quantitative Benedict's reagent. Figure (6.1) shows that

the optimum wavelength for different samples was 740 nm, as seven samples were prepared to cover the different scenarios that can occur during Benedict's test. A similar result was reported by (Hernández-López et al., 2020), who tested a wide range of wavelengths (490–890 nm) and also reported 740 nm as the optimum wavelength for Benedict's reagent.

This chapter assesses the glucose production during EH test processing HSS as feedstock. And because of the selection of Benedict's quantitative test as a tool for detecting the glucose content in HSS sample. Finding the optimum wavelength should be the first step for more accurate results. As, the absorbance value will be affected when adding a coloured substance, such as HSS (which is yellow-brown in colour).

Figure (6.1) shows that the absorbance value changed when the wavelength changed, but the 740 nm wavelength had the maximum absorbance value of all the samples (A1–A4 and B1–B3), although the glucose concentration in the samples varied. The composition of the samples consists of Benedict's reagent (B) 5 mL and one or all of the following: DW, HSS and glucose 1% solution (G) (10 g glucose in 1 Litre of DW), as shown in Table (6.1) in Section 6.3.2.1. The total volume of the sample (6 or 7 mL) also has an impact on the absorbance value, as shown in Figure (6.1), where sample B1 (5 mL of B + 2 mL of DW) had a lower absorbance value (2.872) than sample A4 (5 mL of B + 1 mL of DW) (3.353). Thus, increasing the total volume of the sample by 1 mL DW had a negative impact on the absorbance value: the solution's colour changed, becoming lighter than the B1 sample. Therefore, the final volume of the sample has an effect on the final concentration of glucose due to the relevant dilution.

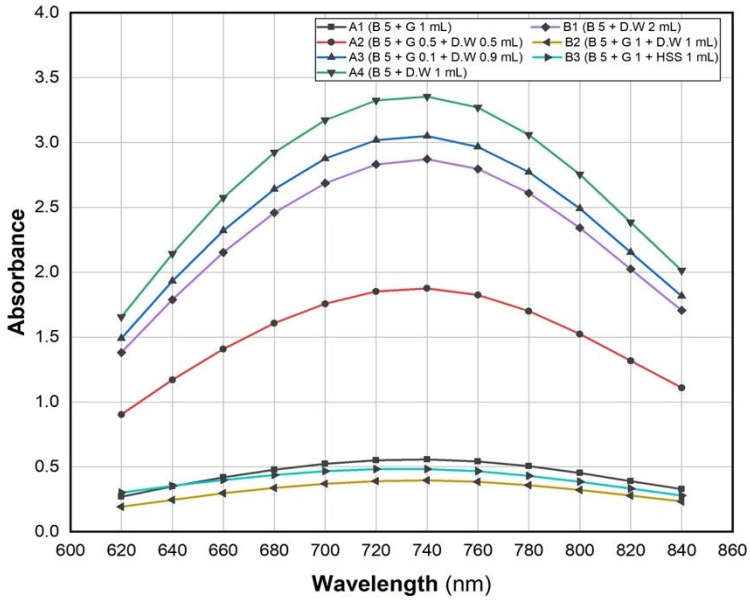


Figure 6.1 Optimum wavelength for different sample compositions during Benedict's test (B: Benedict's reagent, G: 1% glucose solution, HSS: hydrolysed sewage sludge and DW: distilled water) (the absorbance is the average value of the duplicate).

Moreover, the more G a sample contains, the lighter in colour the solution becomes after the reaction is complete, as shown in Figure (6.1), where a comparison between samples A1–A4 (total volume = 6 mL) shows the impact of G amount in the solution on the B colour. The A4 sample, which had no G in it, had the maximum absorbance of all four samples, while the A1 sample, with 1 mL of G, had the lowest absorbance value because the deep-blue colour disappeared due to the reaction between B and G.

Figure (6.2) also shows the effect of adding other substances to the solution (such as HSS or DW) on the B colour. HSS and DW had a negative impact by decreasing the absorbance value, which affected the result of the test. Furthermore, the comparison between B2 and B3 samples in Figure (6.2) shows that adding a coloured substance, such as HSS (which is yellow-brown in colour), can affect the result of Benedict's test, which will affect the glucose concentration result when the absorbance value is used in a glucose standard curve.

Therefore, testing the absorbance for a range of samples with different compositions is an essential step during Benedict's quantitative test.

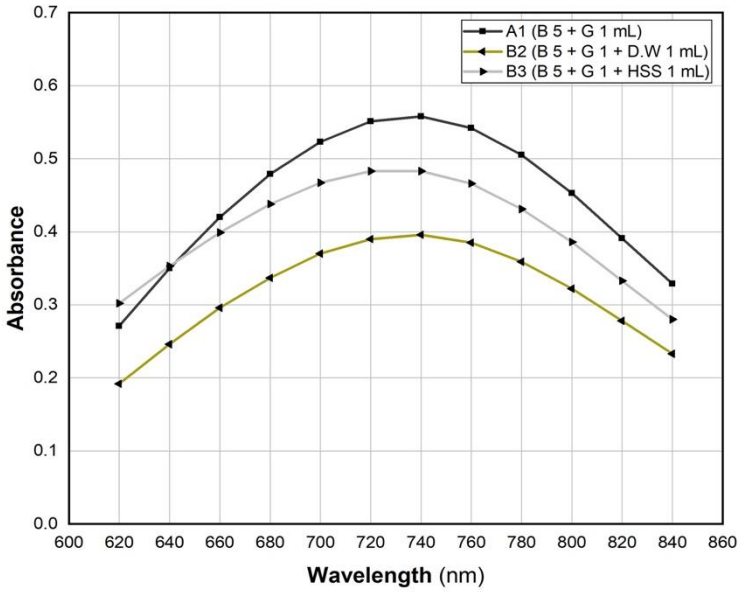


Figure 6.2 Optimum wavelength: the effect of adding HSS or DW to the absorbance value of B colour, during Benedict's test (B: Benedict's reagent, G: 1% glucose solution, HSS: hydrolysed sewage sludge and DW: distilled water) (the absorbance is the average value of the duplicate).

6.3.3.2 Glucose standard curve

After measuring the absorbance of the unknown sample concentration, the next step is to create the standard curve for the known sample concentration. For example, for a glucose standard curve, a series of dilutions for 1% glucose solution (G) (10g in 1 Litre of DW) was prepared, as shown in Table (6.2) in Section 6.3.2.2. After the reaction between these samples and Benedict's reagent was complete, the absorbance was measured at the optimum wavelength (740 nm). Figure (6.3) shows the negative linear relationship between the absorbance and glucose concentration: the highest absorbance is for the sample without glucose content (deep-blue colour), while the lowest absorbance is for the sample with 1 mL of 1% G (very light-blue colour). Linear regression fitting was applied using OriginPro 2018b software, and the R^2 = 0.9995, which is considered an excellent fitting to the test results.

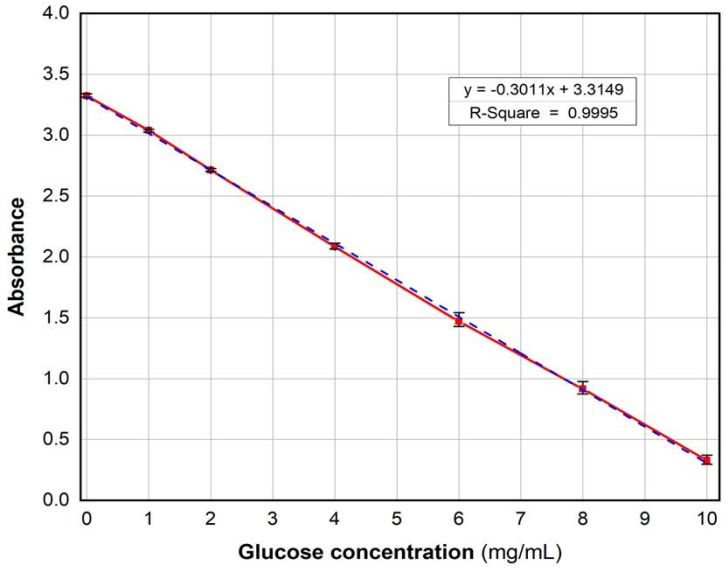


Figure 6.3 Glucose standard curve with fitting curve at the optimum wavelength 740 nm (average value of triplicate with max/min bar)**.**

6.3.3.3 Modified glucose curve

Adding a coloured substance, such as HSS (which is yellow-brown in colour), can affect the result of Benedict's test. Therefore, the glucose concentration result will be affected when the absorbance value is used in the glucose standard curve. Thus, creating a modified glucose curve may enable glucose concentration to be determined more accurately when using Benedict's method. Two concentrations were used for HSS (5 and 10 g of TCOD/L) to create modified glucose standard curves, as shown in Figures (6.4 and 6.5), both of which have high R^2. These modified curves can be used later on in EH experiments to determine the glucose concentration in a solution that has the same HSS concentration. To ensure accurate results, it is recommended that a new modified glucose curve be created for any specific HSS TCOD concentration prior to an EH experiment.

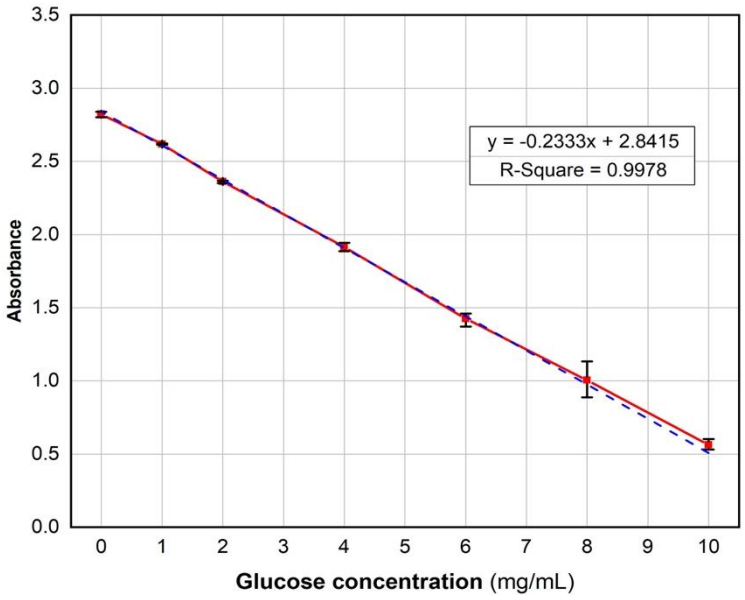


Figure 6.4 Modified glucose curve: TCOD of HSS = 5 g/L, with fitting curve at the optimum wavelength 740 nm (average value of triplicate with max/min bar)**.**

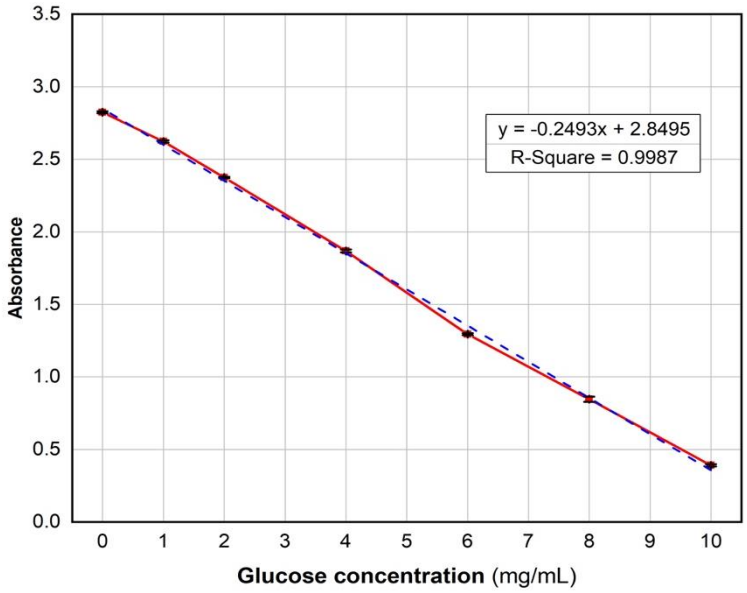


Figure 6.5 Modified glucose curve: TCOD of HSS = 10 g/L, with fitting curve at the optimum wavelength 740 nm (average value of triplicate with max/min bar)**.**

6.3.3.4 Enzymatic hydrolysis test

6.3.3.4.1 Sugar content in cellulase enzyme

Enzyme has the ability to disintegrate hard-to-digest macro sewage flocs to more easily digestible micro flocs in the EH process under specific operation

conditions. Some commercial enzymes have glucose in their content. In this research, cellulase, enzyme blend (from Sigma-Aldrich) was used for the EH test, and the results shows that sugars (e.g., glucose) make up 26% (average value) of the enzyme, as shown in Table (6.6).

Table 6.6 Sugar content in Cellulase enzyme.

Enzyme[a] dose (mL) /250 mL of D.W	Unit	1	2	3	4	5	6	7
Sugar concentration	mg/L	1364	2474	3788	4837	6328	7312	8196
Sugar mass/250 mL	Mg	341	619	947	1209	1582	1828	2049
Sugar	%	28	26	26	25	26	25	24

[a] Cellulase, enzyme blend (commercial enzyme purchased from Sigma-Aldrich)

Figure (6.6) shows sugar concentrations at each enzyme dosage. All reactors were inoculated with a specific enzyme dosage (mL) at the start of the experiment (time (T) 0 hour), and the curves show a maximum sugar concentration that remains relatively constant after T 1 hour of operation, as there were no bacteria to consume the sugar and transfer it to gases and VFAs. Figure (6.7) shows the linear relationship between sugar concentration and enzyme dosage with a fitting curve with an R^2 value of 0.999.

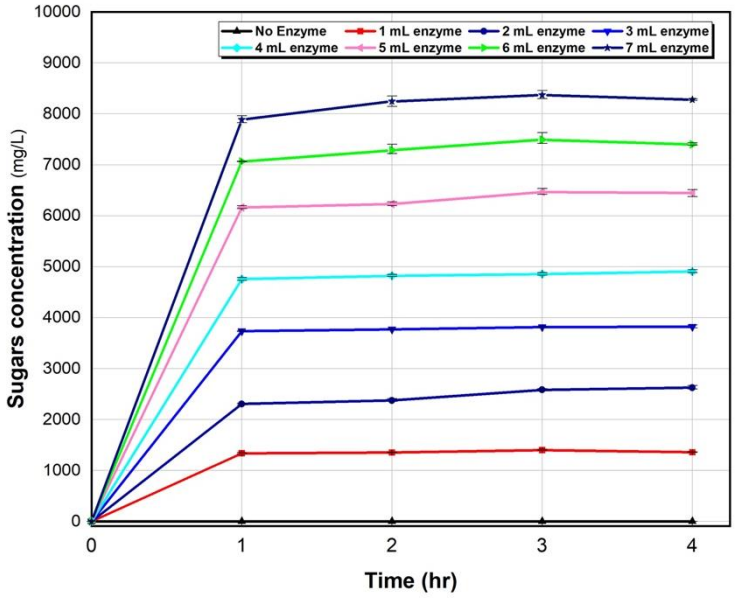


Figure 6.6 Sugar concentration for cellulase enzyme vs time (average value of triplicate with max/min bar)**.**

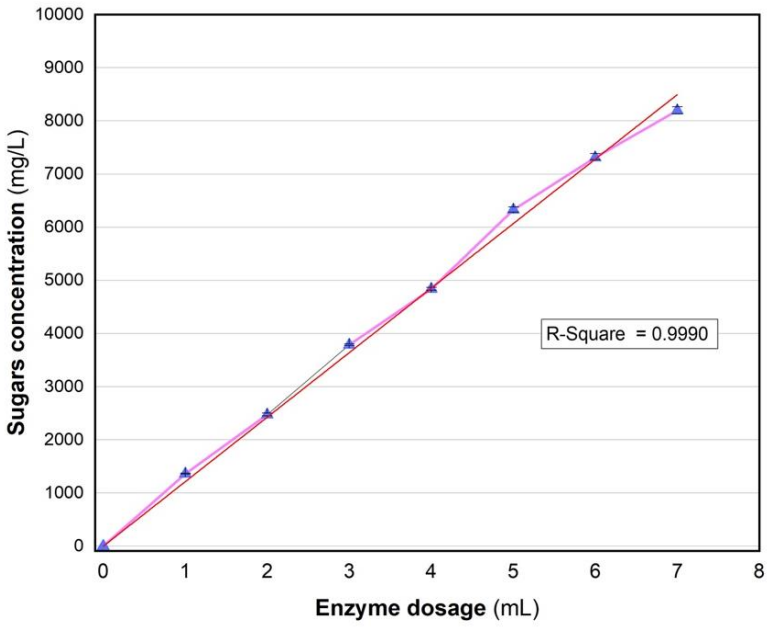


Figure 6.7 Sugar concentration vs cellulase enzyme dosage with fitting curve (average value of triplicate with max/min bar)**.**

6.3.3.4.2 Glucose production

EH is a biological process that uses enzyme to convert lignocellulosic biomass material to fermentable sugars. Sewage sludge is considered a lignocellulosic biomass feedstock, as cellulose is one of the main components in its complex

structure. In this research, cellulase, enzyme blend was used to convert cellulose in HSS to fermentable sugars, such as glucose, which can be used later for hydrogen production via DF.

Seven different enzyme dosages (1–7 mL in 250 mL DW) were used to find the optimum enzyme dosage for maximum glucose production from HSS. More details of experimental set-up and method are described in Section (3.5.2) in Chapter 3. Figure (6.8) describes the physico-chemical and biochemical processes occurring in EH test. HSS was mixed with Cellulase enzyme for assessing the impact of enzyme treatment on increasing glucose content in HSS.

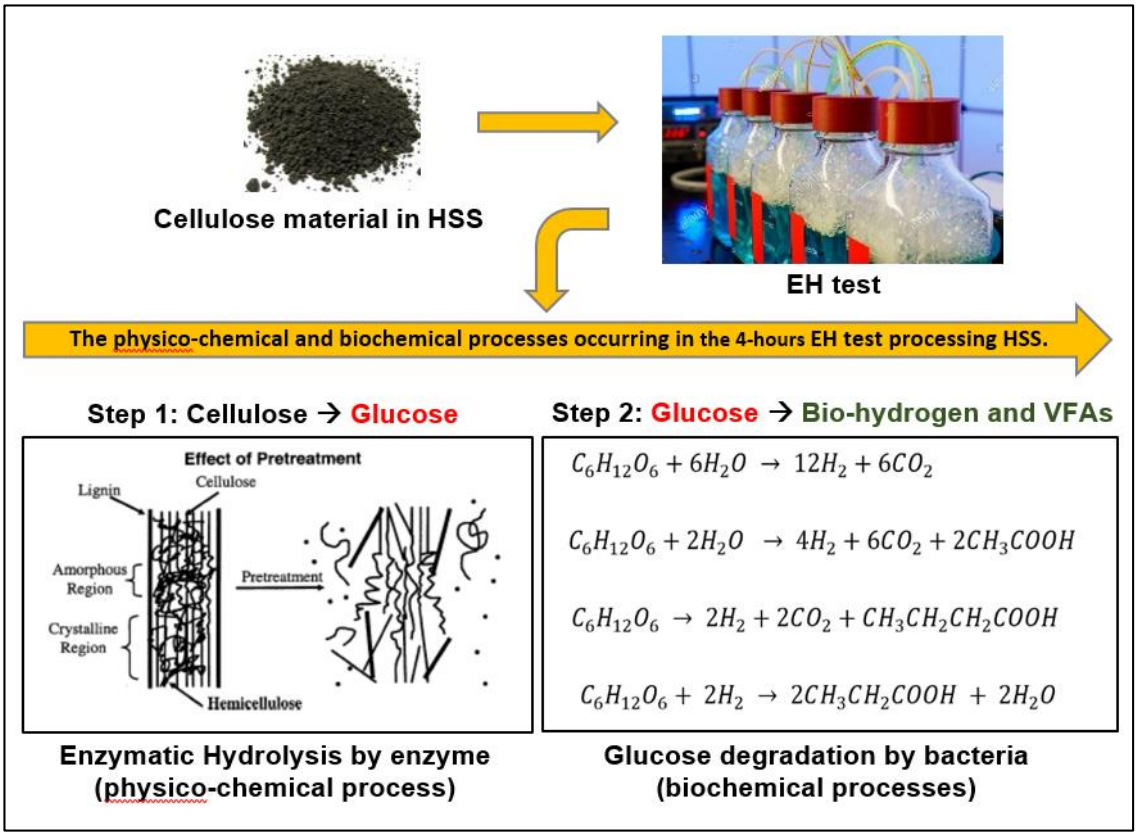


Figure 6.8 The physico-chemical and biochemical processes occurring in the EH test processing HSS.

Figure (6.9) shows that cellulase, enzyme blend was able to release glucose from HSS. EH (physico-chemical process) is a rapid process, as four hours of operation time was enough to reach the maximum glucose production. Despite

the fluctuation of glucose concentration between one and four hours, the maximum glucose concentration occurred at T 1 hour, as shown in Figure (6.9). On the one hand, this rapid reaction is advantageous for the EH process, as it takes only a short time to convert some of the sewage flocs (cellulose) to glucose, which benefits bio-fuel industries. On the other hand, however, it is difficult to maintain the glucose in the reactor for a long time, as it is highly likely to be consumed by bacteria that exist in HSS and converted to VFAs and/or biogas (biochemical process) (which may explain the fluctuation in glucose concentration after T 1 hour shown in Figure (6.9)).

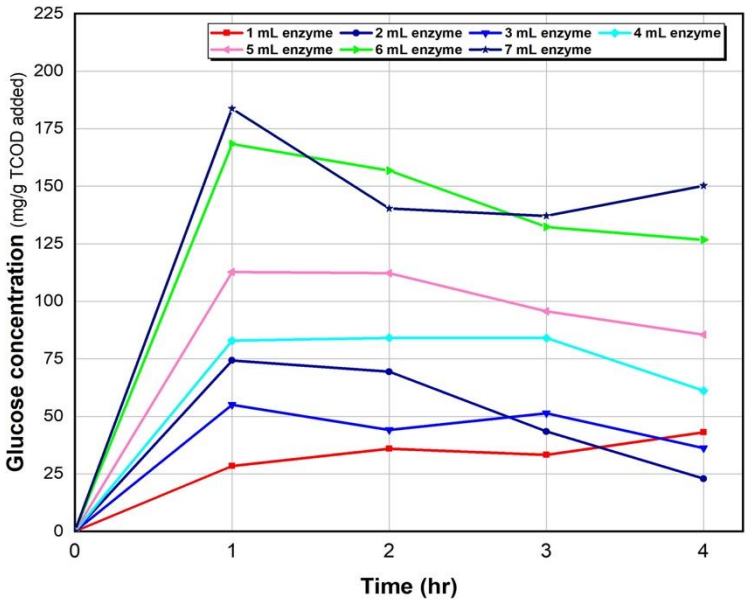


Figure 6.9 Glucose production via the enzymatic hydrolysis process for HSS (without blank: enzyme only) (average of triplicate)**.**

Several parameters can influence enzyme activity and hydrolysis rate in EH. For example, operation temperature and pH are important to maximise glucose production in EH, as enzymes become active in a certain range of temperature and pH. For Cellulase, enzyme blend, the optimal operation temperature is between 45°C and 55°C (Gregg and Saddler, 1996), while the optimal range for

pH is 4.5–5.0 (Janssen et al., 2002, Wilkins et al., 2005). In this experiment, 37°C was used for EH, as this was the temperature used for DF for hydrogen production, as presented in the following chapter. It is challenging to find the optimal temperature for a DF reactor that has enzymes, as the optimal temperature for enzymes is 45–55°C, while most DF reactors operate at 37°C, as this is considered the optimal temperature for hydrogen production. Although some DF reactors operate under thermophilic range (50–65°C), this is not appropriate for commercial-scale production as it will consume more energy.

Also, mixing speed influences enzyme activity. Champagne and Li (2009) reported that excessive mixing speeds (>200 rpm) decrease the extent of hydrolysis because the enzyme activity is lowered. Therefore, mixing speed (150 rpm) was selected for the EH test in this study. More investigation needs to assess the impact of mixing speed on enhancing the glucose production from sewage sludge.

Figure (6.10) shows the pH behaviour during the EH experiment. At a lower substrate concentration (HSS 5g TCOD/L), the enzyme affected pH: immediately after the inoculation process (after T 0 hour), the pH dropped and then started to recover until T 3 hour). Thereafter, the pH started to drop again due to the accumulation of VFAs, as there were some bacteria in the HSS that started to consume glucose and transfer it to VFAs. As shown in Figure (6.11), the inoculation process for different enzyme dosages did not have a big effect on the pH condition, as the substrate concentration was higher (HSS 10g TCOD/L), which helps to overcome the effect of enzyme pH. For enzyme dosages of 1–3 mL, there was a slight increase in pH, while an enzyme dosage of 4 mL did not change the pH, and enzyme dosages of 5–7 mL caused a slight decrease in the pH. Moreover, the same drop in pH happened again after T 3 hour, as the pH started to drop due to the activity of bacteria in degrading the produced glucose.

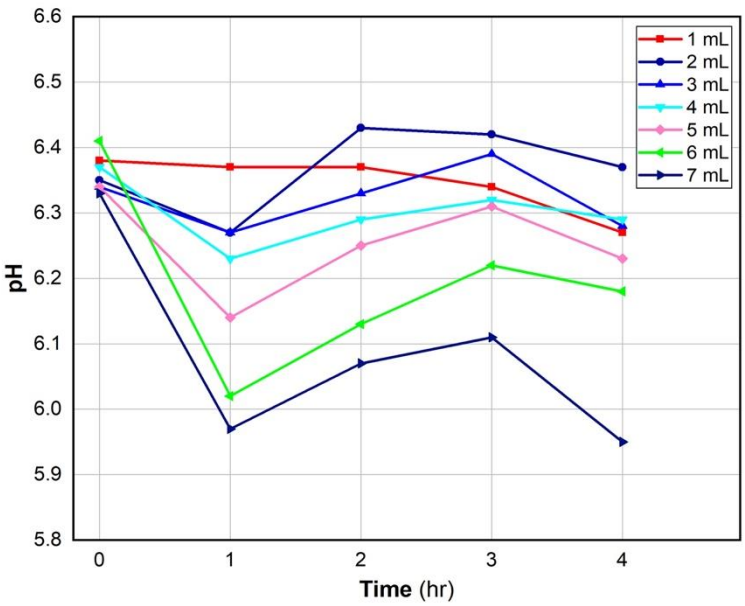


Figure 6.10 pH curve for HSS 5g TCOD/L during EH test (average of triplicate).

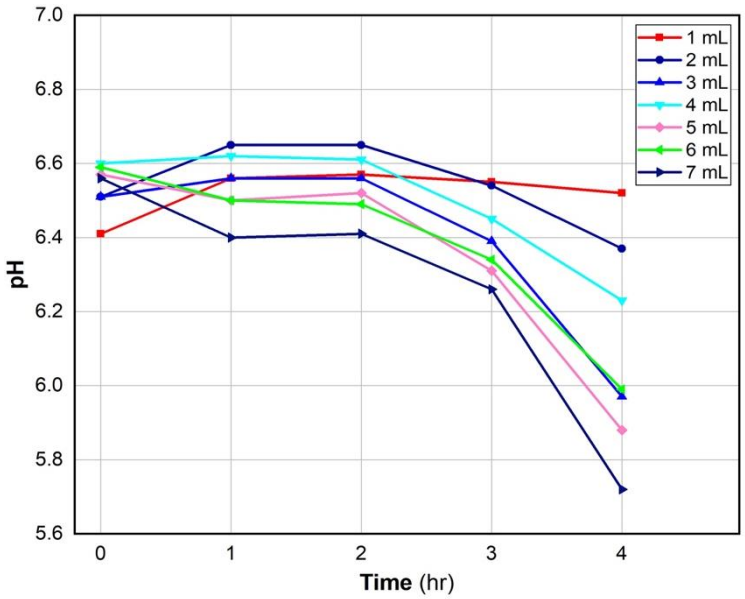


Figure 6.11 pH curve for HSS 10g TCOD/L during EH test (average of triplicate).

Glucose is the product of the saccharification process for cellulose material (Li, 2008). Most studies have reported glucose yield according to cellulose content in the substrate; in this chapter, however, the glucose yield is reported according to TCOD.

The glucose content for untreated HSS was zero, as shown in Figures (6.4 and 6.5), where 5g and 10g TCOD HSS were added to Benedict's reagent. The results for both show zero interaction between HSS and Benedict's reagent, which confirms the absence of glucose in the HSS. The reason for this is that the glucose is part of cellulose, which is a linear polymer of cellobiose (glucose-glucose dimer) (Li, 2008). Hamelinck et al. (2005) reported that the difficulty in breaking this polymer is due to the rigidity derived from the orientation of the linkages and hydrogen bonding in cellulose. Therefore, glucose cannot react with Benedict's reagent unless the cellulose is treated by the EH process.

Figure (6.12) shows that treating HSS by the EH process results in the breakdown of cellulose material in HSS; therefore, glucose starts to be released

and detected by Benedict's test. The glucose yield and enzyme dosage were calculating according to TCOD added (5g or 10g/L) of HSS. It was necessary to remove the glucose content in Cellulase enzyme, by conducting an EH experiment for the blank reactor with only enzyme and DW, as shown in Table (6.4) in Section 6.3.2.4, as it was difficult to remove it physically.

After removing the blank value (glucose yield from the reactor that had only enzyme and DW) from the test value (HSS + enzyme + DW), the result in Figure (6.12) shows the effect of EH on glucose production. The fitting curve, with an R^2 value of 0.969, shows the linear relationship between enzyme dosage and glucose yield: the more enzyme used, the more glucose was produced.

The fluctuation in glucose yield curve is due to the influence parameter that affects the EH process and enzyme activity such as substrate concentration. Also, due to the activity of bacteria (that exist in HSS) in degrading the produced glucose.

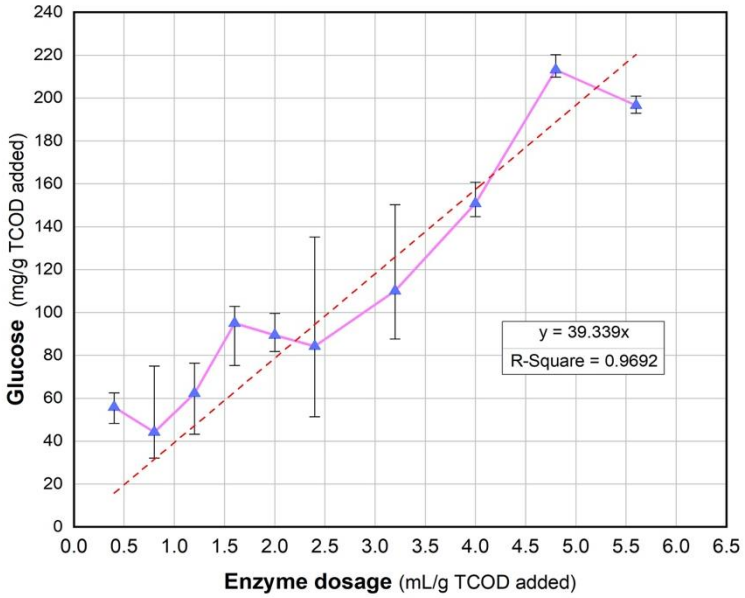


Figure 6.12 Glucose yield vs enzyme dosage (without blank: enzyme only) during EH test (average of triplicate).

As shown in Figure (6.13), the lower HSS concentration (5g TCOD/L) had a higher glucose yield than 10g TCOD/L for enzyme dosage (2–7 mL). The maximum percentage increment was 84% for both enzyme 2 mL and 5 mL at HSS 5g TCOD/L. These results agree with the findings of (Li, 2008), who reported a 50% increase in glucose yield when the substrate concentration (newspapers and scrap paper) was reduced from 15 g/L to 5 g/L using cellulase enzyme. Moreover, there was an increase in glucose yield for different substrates when the substrate concentration decreased, 43.6% (carrot peeling), 35% (potato peeling), 24.6% (grass) and other substrate. Similar results were reported by (Cheung and Anderson, 1997), who enhanced glucose yields by using a low substrate concentration and concluded that a high substrate concertation can inhibit the EH process, although this inhibition is subject to the ratio of total substrate to total enzyme used (Huang and Penner, 1991, Penner and Liaw, 1994).

Figure (6.13) shows the optimum dose reached for 6 mL enzyme added to a solution containing 5g TCOD/L, with a sugar yield of 213 mg/g TCOD. However, more research is needed to find the optimum HSS concentration to maximise glucose production using cellulase enzyme.

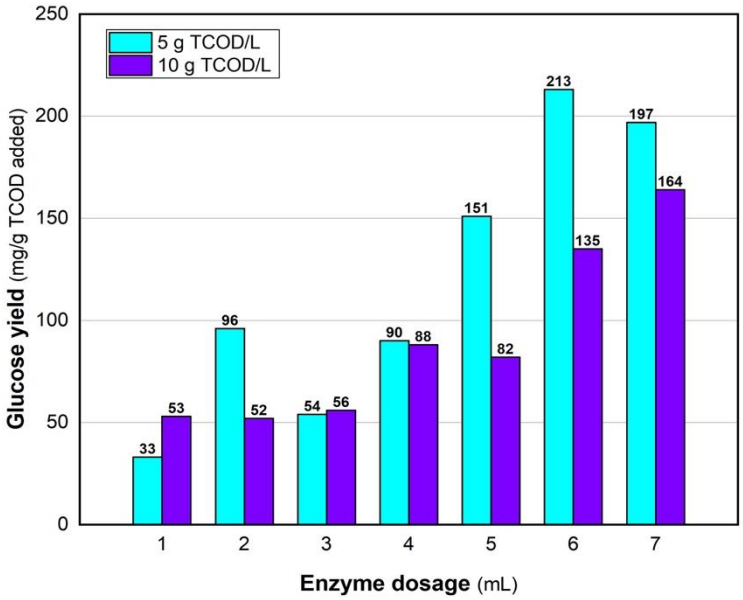


Figure 6.13 Substrate concentration (HSS) effect on glucose yield during EH test, (without blank: enzyme only) (average of triplicate).

6.3.4 Conclusions

Benedict's method has been widely used in laboratories to detect sugars (e.g., glucose) in a solution. This chapter assessed Benedict's quantitative method to detect and quantify glucose content in a solution that contains HSS. With some modifications (finding the optimum wavelength for a mixed sample and modified glucose curve), Benedict's quantitative method can be more reliable and more accurate than the original Benedict's method, for measuring glucose concentration in HSS samples.

In the work described in this chapter, the final volume of the sample has an effect on the final concentration of glucose, due to the relevant dilution. Therefore, finding the optimum wavelength should be the first step in any future works that use Benedict's method, as this will give more accurate results. Moreover, creating a modified glucose curve is another approach to ensure accurate

glucose concentration measurements in an EH test. The work described in this chapter demonstrates the effect on HSS of Benedict’s reagent colours and how HSS can negatively impact glucose measurement results.

Using the EH process as pre-treatment for HSS enhanced its glucose content and converted some macro sewage flocs to easily digestible micro flocs (glucose). Therefore, the substrate will be better and more easily digested by bacteria in a DF reactor, which will lead to enhanced production of hydrogen and VFAs.

More research needs to be done to find the optimum enzyme dosage, initial substrate concentration and operation temperature (especially when an enzyme is used inside a DF reactor).

Chapter 7
Impact of enzymatic hydrolysis of sewage sludge on hydrogen yield via dark fermentation

7.1 Introduction

Sewage sludge is processed for methane production in AD reactors at WWTPs around the world. It is produced in large quantities and is rich in biodegradable organic materials, and from these contents, sugars (e.g., glucose) can be produced, recovered and used as a substrate to support hydrogen production through the DF process.

Due to the complexity of the content of sewage sludge and its low C/N ratio, it is difficult for fermentative bacteria to utilise it, as shown in Chapter 5 and (Xia et al., 2016). Therefore, sewage sludge pre-treatment is an essential step to ensure efficient bio-hydrogen production in DF. The results presented in Chapter 6 prove that EH treatment can convert some of the hard-to-digest macro sewage flocs (i.e., cellulose material and bacterial biomass) to small molecules with the potential to be converted into sugars (e.g., glucose). This pre-treated HSS should have the potential for high hydrogen production due to the increased glucose content, as glucose is the most favourable substrate for fermentative bacteria (e.g., *Clostridium* bacteria) (Finlay, 1995).

Many studies have investigated the use of EH as a pre-treatment for sewage sludge prior to DF (Massanet-Nicolau et al., 2008) or AD (to enhance methane production through increasing the biodegradability of sewage sludge) (Agabo-Garcia et al., 2019). This chapter investigates the potential integration of EH with DF as another approach to enhancing hydrogen production, as this may provide a solution for upgrading the existing DF reactors in biofuel industries.

The aim of this chapter is to assess the potential of hydrogen production from HSS using an integrated EH and DF process to overcome the very limited hydrogen production reported in Chapter 5 (4.18 mL-H_2 / gVS-added).

7.2 Objectives of this chapter

- To evaluate the effect of using enzymes in DF.
- To optimise hydrogen and VFA production by integrating EH with DF.

7.3 Bio-hydrogen potential of mixed substrate (enzyme + hydrolysed sewage sludge)

7.3.1 Materials and methods

7.3.1.1 Inoculum and feedstock source

The HSS collected from Esholt WWTP was used as substrate for the BHP batch test, and digestate from the AD reactor in Esholt was used as inoculum. The sample collection and processes are described in Section 3.1.1 (Chapter 3).

7.3.1.2 Enzyme and D-glucose

D-glucose powder (≥99.5% purity) and cellulase, enzyme blend were purchased from Sigma-Aldrich for use in the BHP tests. More details of cellulase enzyme are given in Section 3.5.2 (Chapter 3).

7.3.1.3 Inoculum pre-treatment method

As a result of the work presented in Section 5.3 (Chapter 5), HST was selected as the best pre-treatment method for the inoculum (digestate) and was therefore used in this experiment. The HST was conducted by heating the digestate for 20 min at 115°C (approx. 1.5 bar), using a standard autoclave.

7.3.1.4 Analytical tests

Several tests were carried out during the BHP experiments. Five sampling points were selected to monitor the five-day BHP tests. Table (7.1) summarises the tests conducted on sacrificial bottles. More details of the BHP process monitoring and analysis are given in Table (3.2) and Section 3.4.1 (Chapter 3).

Table 7.1 Sampling points and tests conducted during five-day BHP experiments.

Parameter/Day	0	1	2	3	4
pH	x	x	x	x	x
Alkalinity	x	x	x	x	x
TS	x				x
VS TCOD, sCOD	x				x
VFAs	x	x	x	x	x
Biogas volume		x	x	x	x
Biogas composition		x	x	x	x

TS: total solids; **VS**: volatile solids; **TCOD**: total chemical oxygen demand; **sCOD**: soluble chemical oxygen demand; **VFAs**: volatile fatty acids.

7.3.2 Experimental set-up

The effectiveness of integrating EH within DF processes was tested by conducting batch BHP tests using digestate treated by HST as inoculum and HSS with cellulase enzyme as mixed substrate. The cellulase enzyme dosage was selected according to the results reported in Chapter 6 and after several EH+DF trials were conducted.

Volatile solid concentration was measured for the treated inoculum and HSS prior to the set-up of BHP. Wheaton bottles (160 mL) were used as fermentative reactors, with a 70 mL working volume. The BHP set-up was based on an ISR of 1:1 (5 g VS inoculum : 5 g VS substrate). Four bulk samples of volume 1 Litre were prepared for this experiment. Table (7.2) shows the details of each BHP reactor.

Table 7.2 Experimental set-up used for BHP reactors.

	Unit	Blank	Control 1	Control 2	Test
Treated inoculum	VS (g)	5	5	5	5
	mL/L	160	160	160	160
Glucose[a]	VS (g)		5		
	g/L		5		
Enzyme[b]	mL/L			10	10
HSS	VS (g)				5
	mL/L				90

(a) D-Glucose Powder
(b) cellulase, blend enzyme

All bulk samples were adjusted to an initial pH of 7.0 using 1 M HCl. All bottles were sparged with nitrogen gas for 1 min each and sealed with a rubber cap and an aluminium crimp. All bottles were placed in an incubator at 37°C. BHP Blank (inoculum), Control 1 (inoculum + glucose) and Control 2 (inoculum + enzyme) were run in duplicate, while BHP Test (inoculum, HSS and enzyme) was conducted in triplicate. Table (7.3) shows the characterisation of all reactors on Day 0.

Table 7.3 Characterisation results of all reactors on Day 0.

		Blank	Control 1	Control 2	Test
Inoculum (Digestate)		HST	HST	HST	HST
Substrate		-	Glucose	Enzyme	HSS + Enzyme
pH	-	7.0	7.0	7.0	7.0
Alkalinity	mg CaCO3/L	616 (6.2)	610 (12.7)	661 (12.0)	1049 (22.4)
TS	%	0.99 (0.01)	1.41 (0.01)	1.56 (0.00)	2.10 (0.02)
VS	%	0.55 (0.00)	0.95 (0.00)	1.09 (0.00)	1.39 (0.01)
TCOD	mg/L	9030 (71)	15050 (212)	16130 (42)	22627 (81)
sCOD	mg/L	998 (32)	6420 (71)	7820 (85)	9547 (102)

TS: total solids, **VS**: volatile solids, **TCOD**: total chemical oxygen demand,
sCOD: soluble chemical oxygen demand.
HST: heat shock pre-treatment (20 min at 115°C (approx. 1.5 bar) using a standard autoclave).
(STD: standard deviation from the mean (n = 3)).

7.3.3 Results and discussion

7.3.3.1 Hydrogen yield

Hydrogen and carbon dioxide gases were observed in all BHP tests (Control 1, Control 2 and Test) except the BHP Blank (which only contained treated inoculum). Moreover, there was no methane production in any of the BHP tests because of inoculum pre-treatments (HST for 20 min at 115°C). As shown in Chapter 5, HST can inhibit methanogenic bacteria from converting VFAs to methane gas. Four BHP tests were conducted at the same time to evaluate the effect of using cellulase enzyme with HSS in DF processes to enhance hydrogen production. As shown in Chapter 5, very limited hydrogen was produced from HSS; therefore, an EH pre-treatment for HSS was carried out (as shown in Chapter 6) to overcome the limited hydrogen production in BHP by breaking down the complex structure of HSS and releasing glucose.

Figure (7.1) shows the hydrogen and carbon dioxide during the BHP tests. The gas yields were reported by gram of glucose added, as shown in Table (7.2) earlier, while 26% of cellulase enzyme content is sugar (as shown in Section 6.3.3.5.1 in Chapter 6), and the glucose production from mixed substrate (cellulase enzyme + HSS) in the EH process (according to the equation in Figure (6.11) in Chapter 6) was considered for gas yield calculations.

The hydrogen production in BHP Control 1 (treated inoculum + glucose), Figure (7.1a), indicates that hydrogen-producing bacteria were active, while the hydrogen-consuming bacteria were inactive as a result of the inoculum HST prior to the BHP set-up. Similarly to the results reported in Chapter 5, a maximum hydrogen yield of 206 mL-H_2/g glucose added was achieved in BHP Control 1 on Day 3. In BHP Control 2 (treated inoculum + enzyme), Figure (7.1b), the

hydrogen yield was higher than in Control 1, as maximum hydrogen was 302 mL-H_2/g glucose added on Day 4. Although the amount of glucose in Control 2 (3.12 g/L) was lower than in Control 1 (5 g/L), the hydrogen yield in Control 2 was higher, which can be related to the conversion pathway of glucose, as TVFA production was higher in BHP Control 2 than Control 1. Table (7.4) shows the acetate and butyrate production during BHP Control 1 and 2.

Table 7.4 Acetate and butyrate production on Day 4 in BHP Control 1 and 2.

BHP reactor (substrate)	Amount of glucose inside the reactor	Acetate concentration (percentage of TVFAs) on Day 4	Butyrate concentration (percentage of TVFAs) on Day 4	TVFAs on Day 4
	g/L	mg/L (%)	mg/L (%)	mg/L
Control 1 (glucose)	5	1316 (9.8)	5286 (79.5)	6645
Control 2 (Cellulase enzyme)	3.12[a]	2741 (33.7)	5369 (65.9)	8144

(a) calculated on the basis that 26% of cellulase enzyme is glucose, as per the results in Chapter 6.
TVFAs: total volatile fatty acids.

Butyrate was dominant in Control 1 (79.5% of TVFAs on Day 4), which indicates that the butyrate conversion pathway was dominant. This led to less hydrogen being produced than in Control 2, as 1 mole glucose gives 2 mole hydrogen, theoretically, in the butyrate pathway (De Gioannis et al., 2013a). BHP Control 2 had more TVFAs than Control 1 and double the amount of acetate on Day 4, indicating that some of the glucose was converting through the acetate pathway, which led to the production of more hydrogen than Control 1. Theoretically, in the acetate conversion pathway, 1 mole glucose gives 4 mole hydrogen (De Gioannis et al., 2013a).

The hydrogen production in BHP Test (Figure 7.1c) was lower than in Control 2, which was the opposite of what was expected. As BHP Test had both enzyme and HSS, more glucose should have been available for bacteria to convert to

hydrogen. The glucose content in BHP Test was 3.51 g/L (3.12 g from 10 mL cellulase enzyme + 0.39 g from EH of HSS), while that in BHP Control 2 was 3.12 g/L, so more hydrogen should have been produced in the Test than Control 2, or at least the same amount.

Figure (7.2) shows the gas yield in BHP Test after removing the gas yield from cellulase enzyme. The negative gas yield indicates that the mixed substrate (enzyme + HSS) did not enhance hydrogen production. Thus, there remains a need to optimise BHP for mixed substrate, especially as the results presented in Chapter 6 show that EH had an enhanced glucose content in HSS, and hydrogen production should therefore have been enhanced in BHP Test.

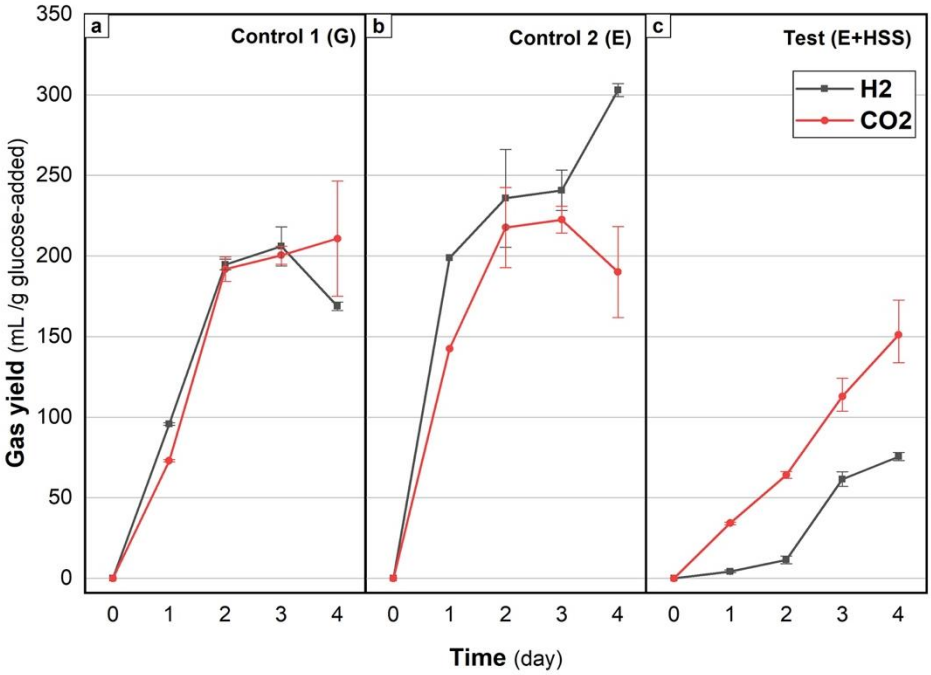


Figure 7.1 Gas yield in BHP tests (a) Control 1: inoculum + glucose, (b) Control 2: inoculum + enzyme and (c) Test: inoculum + enzyme + hydrolysed sewage sludge (HSS) (average value of triplicate with max/min bar).

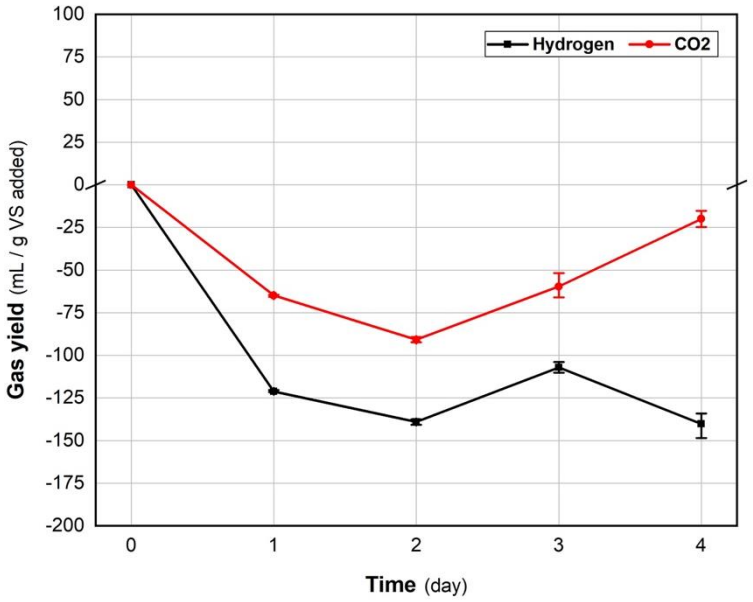


Figure 7.2 Gas yield in BHP Test after subtract the gas yield of BHP Control 2 (average value of triplicate with max/min bar).

7.3.3.2 Volatile fatty acid production

VFA analysis provides an understanding of the predominant glucose conversion pathways. The conversion pathway can affect the hydrogen yield in a BHP test. For example, 1 mole of glucose could theoretically yield 12 moles of hydrogen, as shown in Equation (7.1); however, if the reaction follows the acetate pathway, only 4 moles of hydrogen would be produced, as shown in Equation (7.2) (De Gioannis et al., 2013a), while the butyrate pathway can produce only 2 moles of hydrogen, as shown in Equation (7.3).

$$C_6H_{12}O_6 + 6H_2O \rightarrow 12H_2 + 6CO_2 \quad (7.1)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 6CO_2 + 2CH_3COOH \quad (7.2)$$

$$C_6H_{12}O_6 \rightarrow 2H_2 + 2CO_2 + CH_3CH_2CH_2COOH \quad (7.3)$$

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \quad (7.4)$$

Moreover, the lactic and ethanol pathways yield no hydrogen, while the propionate pathway consumes hydrogen (Equation 7.4) (Guo et al., 2010a, Vavilin et al., 1995).

VFA production, accumulation and consumption for the four BHPs (Blank, Control 1, Control 2 and Test) are represented in Figure (7.3,a–d). Very limited VFA production was observed in the Blank, as shown in Figure (7.3a), as the maximum accumulation of TVFAs was 1056 mg/L on Day 3 because there was no substrate in this reactor, while the other BHPs had a substrate. In contrast, very high VFA accumulation and production was observed in the other BHPs (Control 1, Control 2 and Test). TVFAs for Control 1 reached their maximum on Day 3 (6998 mg/L), as shown in Figure (7.3b), while the maximum TVFAs for Control 2 and Test were at day 4 (8144 mg/L and 8721 mg/L, respectively).

Similar VFA accumulation and consumption behaviour for the BHP with glucose as substrate (results presented in Chapter 5) were shown in BHP Control 1: butyrate conversion was the dominant glucose conversion pathway, and the maximum concentration was on Day 3, followed by a decline in butyrate between Day 3 and Day 4. This decline might be due to the re-activation of the acetogens and consequent utilisation of butyrate as a substrate for the production of acetic acid and hydrogen (Yang and Wang, 2017). However, the corresponding decline in the hydrogen yield between Day 3 and Day 4 (Figure 7.1a in Section 7.3.3.1) would indicate a simultaneous bio-conversion of hydrogen gas to acetic acid by homoacetogenesis reaction, which is a possible hydrogen sink under anaerobic condition (Zhao et al., 2015).

In BHP Control 2 (Figure 7.3c), during the first two days butyrate was the most dominant of the VFAs, which indicates the butyrate conversion pathway, as described in Equation (7.3). After Day 2, however, acetate started to accumulate and increased dramatically. The glucose conversion pathway changed and was divided between the acetate and butyrate pathways (Equations (7.2) and (7.3), respectively), which led to higher hydrogen production in Control 2 than Control 1, as shown in Figure (7.1b) in Section 7.3.3.1. A similar VFA accumulation behaviour was seen in Control 2 as that in BHP Test, except for the accumulation of propanoate. As mentioned earlier, the propionate pathway consumes hydrogen (Guo et al., 2010a), which, with the low butyrate accumulation during the first two days (Figure 7.3d), was the reason for the low hydrogen production in BHP Test even though it was expected that BHP Test would produce more hydrogen than Control 2. The glucose content in BHP Test was 3.51 g/L (3.12 g from 10 mL cellulase enzyme + 0.39 g from EH of HSS), while that in Control 2 was 3.12 g/L.

Although hydrogen production is associated with VFA accumulation, the low VFA production in Control 1 and Control 2 during the first two days did not affect the hydrogen production, as shown in (Figure (7.1,a–b) in Section 7.3.3.1. A comparison between the results presented in Chapter 5 (Section 5.3.3.1 and 5.3.3.2) and those from Control 1 can explain why this low VFA production did not negatively impact the hydrogen production.

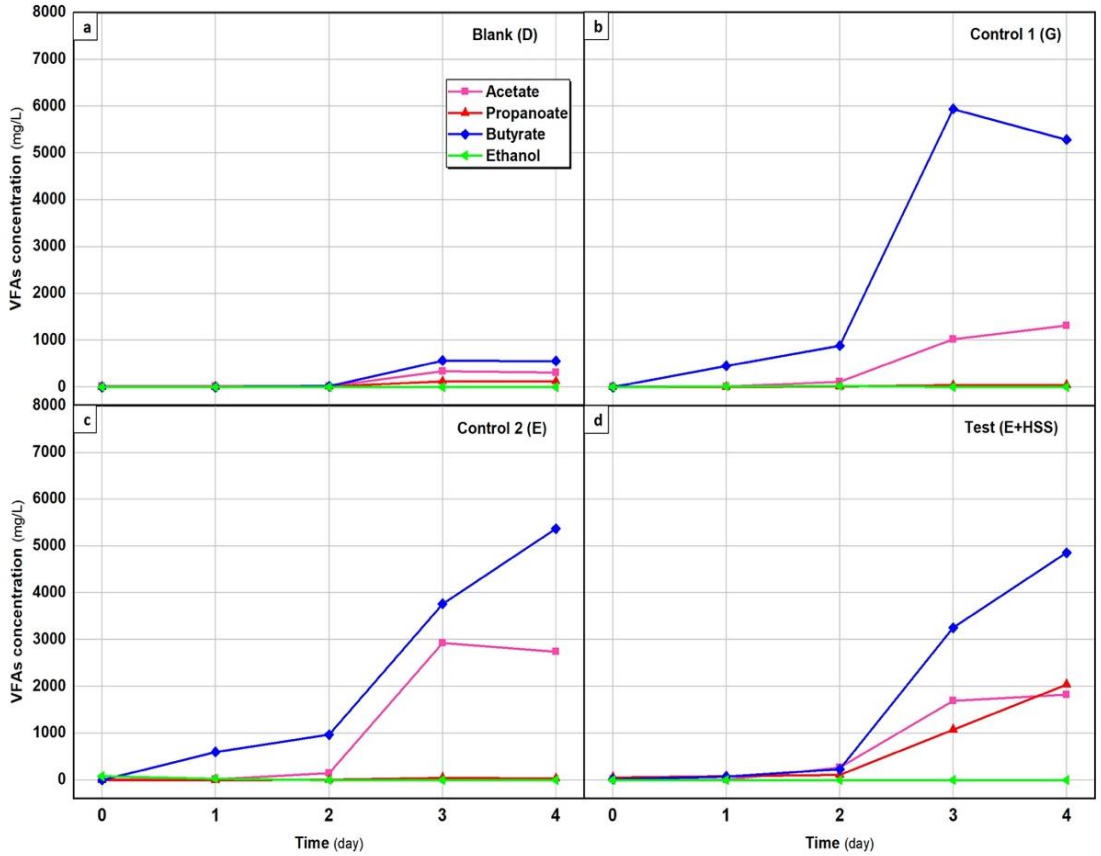


Figure 7.3 VFA production in BHP tests, (a) Blank: inoculum, (b) Control 1: inoculum + glucose, (c) Control 2: inoculum + enzyme and (d) Test: inoculum + enzyme + hydrolysed sewage sludge (HSS) (average value of triplicate).

Table (7.5) shows that a similar hydrogen production was achieved (191 and 194 mL/g glucose added, for $BHP_{chapter\ 5}$ and $BHP_{Control\ 1}$, respectively) with only butyrate accumulation (882 and 1446 mg/L, for $BHP_{chapter\ 5}$ and $BHP_{Control\ 1}$, respectively), as the dominant glucose conversion was the butyrate pathway. This indicates that the low butyrate accumulation – compared to 5937 mg/L on Day 3 in Control 1 – did not negatively impact hydrogen production. Moreover, in Control 2 (Table 7.5), the hydrogen yield reached 235 mL/g glucose added with butyrate accumulation (969 mg/L).

The comparison between Control 2 and Test in Table (7.5) shows that the very limited hydrogen in Test was a consequence of a lag in the accumulation of

butyrate. Even after reaching the same butyrate and TVFA concentration as Control 2 on Day 3, the hydrogen yield in Test remained very low compared with the 240 mL/g glucose added in Control 2. Thus, increasing the operation time of BHP Test should be considered to complete the DF processes and prevent any lag in VFA production. Table (7.5) shows that hydrogen yield is associated with VFA accumulation at certain concentrations, and high VFA accumulation may not necessarily enhance hydrogen production. On the contrary, it can be an indication that DF processes are shifting toward VFA production instead of hydrogen production.

Table 7.5 The relationship between hydrogen and butyrate production in determining the by-products of BHP.

Day	Hydrogen yield	Butyrate concentration	TVFAs	Glucose conversion
	mL/g glucose added	mg/L	mg/L	Toward VFA or H_2 production
BHP Chapter 5				
0-2	165	1167	1367	H_2 production
2-3	191	1446	1670	H_2 production
Increment	(26)	(279)	(303)	
BHP Control 1				
0-2	194	882	1028	H_2 production
2-3	206	5937	6997	VFA production
Increment	(12)	(5055)	(5969)	
BHP Control 2				
0-2	235	969	1122	H_2 production
2-3	240	3761	6730	VFA production
Increment	(5)	(2792)	(5608)	
BHP Test				
0-2	13	230	603	unknown
2-3	57	3252	6015	VFA production
Increment	(44)	(3022)	(5412)	

BHP: batch bio-H_2 potential test, **TVFAs**: total volatile fatty acids.
BHP chapter 5: inoculum + glucose, **Blank**: inoculum, **Control 1:** inoculum + glucose, **Control 2:** inoculum + enzyme, **Test:** inoculum + enzyme + hydrolysed sewage sludge (the results are average value of triplicate).

7.3.3.3 pH and alkalinity behaviour

The pH and alkalinity have a crucial influence on the reactions occurring during DF. The pH value affects VFA accumulation; lower pH ranges (4.0–6.0) support butyrate and acetate accumulation, and higher pH ranges (7.0–9.0) support

ethanol and propionate accumulation (Hawkes et al., 2007, Pakarinen et al., 2008). Conversely, the pH also influences the diversity of the microbial community and, effectively, hydrogen production; that is, at low pH levels the dominant species is *Clostridium*, which is responsible for the production of butyrate, acetate and hydrogen (Hawkes et al., 2007, Temudo et al., 2008). The optimum pH range to enhance the hydrogenases (hydrogen producers) in DF has been suggested to be between pH 5.0 and pH 7.0 (Li and Fang, 2007a).

pH behaviour for the four BHPs are shown in Figure (7.4). As Blank had no substrate and limited VFA accumulation, the pH was in a steady state. In contrast, the other BHPs (Control 1, Control 2 and Test) had a dramatic drop in pH from 7.0 to 4.68–4.78 on Day 1, due to VFA accumulation, especially butyric acid. After Day 1, the pH in Control 1 and Control 2 remained stable within the range of 4.5–5.0, which enhanced VFA production, as shown in Figures (7.3b) and (7-3c) in Section 7.3.3.2, as this range supports acetate and butyrate production.

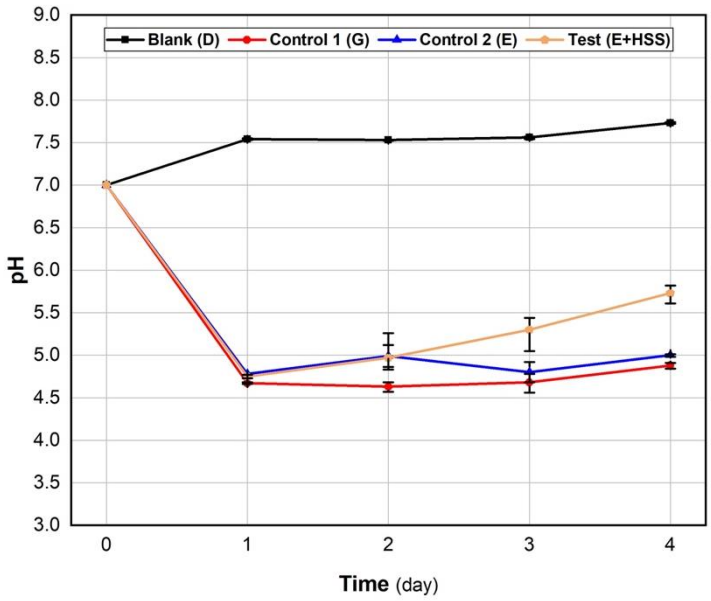


Figure 7.4 pH curve for BHP tests, (Blank: inoculum, Control 1: inoculum + glucose, Control 2: inoculum + enzyme and Test: inoculum + enzyme + hydrolysed sewage sludge (HSS)), (average value of triplicate with max/min bar).

Moreover, starting BHP with 7.0 instead of 5.5 enhanced VFA production in all BHPs, and, at the same time, the hydrogen yield reached the same level in Control 1 and was higher in Control 2. A low hydrogen yield was observed in Test because of the lag time, and no hydrogen gas was observed in Blank for both pH 5.5 and 7.0. Table (7.6) shows the effect of changing initial pH on enhancing VFA production during the BHP test.

Table 7.6 Effect of pH set-up on VFA production in BHP tests.

pH set-up	**Blank** (inoculum)	**Control 1** (inoculum + glucose)	**Control 2** (inoculum + enzyme)	**Test** (inoculum + enzyme + HSS)
	TVFAs (mg/L) on Day 4			
pH 5.5[a]	140 (20)	1187 (218)	1966 (66)	662 (47)
pH 7.0	1021 (429)	6661 (584)	8079 (283)	8936 (469)
Increment (%)	629	461	311	1250

(a) Results obtained from BHP trials with the same experimental set-up (Section 7.3.2), except the starting pH was 5.5 instead of 7.0.
HSS: hydrolysed sewage sludge.
(STD: standard deviation from the mean (n = 3))

Alkalinity also has an effect on hydrogen production in DF, as the VFA accumulation results in a drop in pH. Alkalinity helps to buffer the pH within the optimal range of hydrogen production in DF. Mtui (2009) reported that alkalinity was the most important parameter affecting hydrogen production. Bina et al. (2019) reported that the optimum initial alkalinity for DF that allowed the highest hydrogen yield (220 mL/d) was 1325 mg/L $CaCO_3$, for initial alkalinity ranging between 670 and 2678 mg/L $CaCO_3$.

Figure (7.5) shows the alkalinity behaviour during the BHP tests. The initial alkalinity was in the range of 610–661 mg $CaCO_3$/L for Blank, Control 1 and Control 2, while Test started with 1049 mg $CaCO_3$/L, as adding HSS (organic load) increased the initial alkalinity in Test (Rangela et al., 2020). As VFAs started to accumulate in Control 1 and Control 2, the alkalinity was successful in

buffering the pH and keeping it within the optimum range for hydrogen production. However, the alkalinity behaviour in Test was different to the other BHPs. It started with 1049 mg $CaCO_3$/L (Day 0), then dropped to 503 mg $CaCO_3$/L (Day 1), then dramatically increased from Day 1 to Day 4 and reached 1021 mg $CaCO_3$/L at the end of BHP. This recovery or increase in alkalinity may be due to the production of carbon dioxide along with limited hydrogen production (as shown in Figure (7.1c)), as in a sealed reactor some of the carbon dioxide may be dissolved in water and produce bicarbonate, which can elevate the alkalinity value.

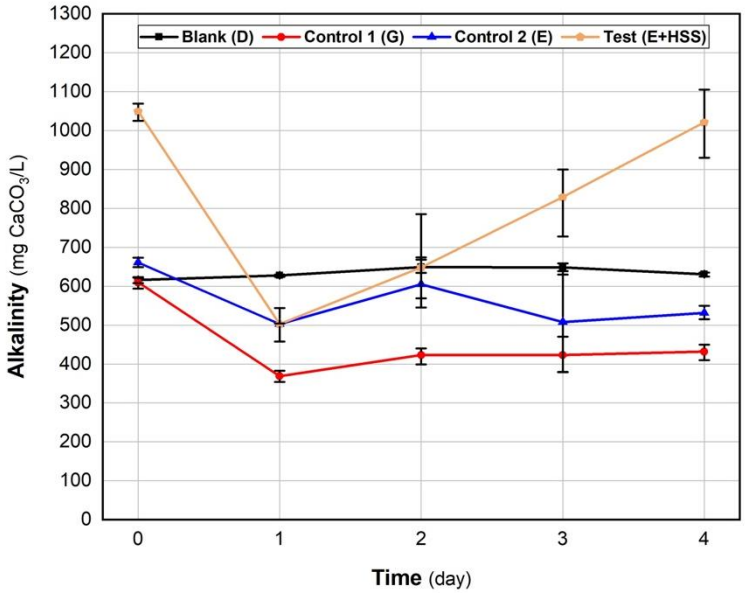


Figure 7.5 Alkalinity curve for BHP tests, (Blank: inoculum, Control 1: inoculum + glucose, Control 2: inoculum + enzyme and Test: inoculum + enzyme + hydrolysed sewage sludge (HSS)), (average value of triplicate with max/min bar).

7.3.4 Conclusion

It has been proved that EH treatment can increase the glucose content in HSS by converting the hard-to-digest macro sewage flocs (cellulose material) to more easily digestible micro flocs (glucose) with the aid of cellulase enzyme. Still, Section 1.3 shows the need to modify the BHP to overcome the limited hydrogen production for mixed substrate (enzyme and HSS). Increasing the operation time

of BHP should be considered to avoid a lag time in overall DF processes; using a different ISR may also enhance hydrogen production. The following section will therefore address these modifications.

7.4 Optimising DF processing mixed substrate (enzyme + sewage sludge)

7.4.1 Materials and methods

7.4.1.1 Inoculum and feedstock source

The collected HSS from Esholt was used as substrate for a BHP batch test, and digestate from the AD reactor in Esholt was used as inoculum. The sample collection and processes are described in Section 3.1.1 (Chapter 3).

7.4.1.2 Enzyme and D-glucose

D-glucose powder (≥99.5% purity) and cellulase, enzyme blend purchased from Sigma-Aldrich were used in the BHP tests. More details of cellulase enzyme are given in Section 3.5.2 (Chapter 3).

7.4.1.3 Inoculum pre-treatment method

HST was conducted for the digestate by heating it for 60 min at 105°C using a furnace. From several pre-treatment trials, the hydrogen-producing bacteria were more sensitive to HST by autoclave so became inactive, which led to inefficient substrate utilisation and hydrogen/VFA production. Moreover, the results presented in Section 5.3.3.5 in Chapter 5 show no difference between the two in terms of hydrogen yield amount.

7.4.1.4 Analytical methods

Several tests were carried out during the Batch BHP experiment. Table (7.7) summarises the tests conducted on sacrificial bottles. More details of BHP

process monitoring and analysis are given in Table (3.2) and Section 3.4.1 (Chapter 3).

Table 7.7 Testing points during BHP test.

Parameter/Day	0	1	2	3	4	7	8
pH	x	x	x	x	x	x	x
Alkalinity	x	x	x	x	x	x	x
Ts	x						x
Vs	x						x
TCOD, sCOD	x						x
VFA	x	x	x	x	x	x	x
Biogas volume		x	x	x	x	x	x
Biogas composition		x	x	x	x	x	x

TS: total solids, **VS**: volatile solids, **TCOD**: total chemical oxygen demand, **sCOD**: soluble chemical oxygen demand, **VFAs**: volatile fatty acids

7.4.2 Experimental set-up

Three modifications were made to the experimental set-up in Section 7.3.2, as follows:

1. BHP operation time was extended to eight days from five days to complete the DF reactions, as there was a lag time in the BHP Test (Section 7.3 in this chapter) which affected the completion of DF processes. Therefore, the hydrogen yield was lower than BHP Control 2, while the opposite was expected due to the higher glucose content in Test than Control 2.
2. An alternative HST method (furnace: 105°C for 60 min) was used to prepare the inoculum for BHP, as the results showed that some hydrogen-producing bacteria were affected by HST (autoclave: 115°C for 20 min).

3. The ISR was changed to 2:1 from 1:1 (Section 7.3 in this chapter) to increase the amount of bacteria in BHP, which might resolve the limited hydrogen production in Test.

Other than these three modifications, the experiment details remained as described in Section 7.3.2 in this chapter. Table (7.8) shows the details of each BHP reactor.

Table 7.8 Set-up details for each BHP reactor.

	unit	Blank	Control 1	Control 2	Test
Treated inoculum	VS (g)	10	10	10	10
	mL/L	360	360	360	360
Glucose[a]	VS (g)		5		
	g/L		5		
Enzyme[b]	mL/L			10	10
HSS	VS (g)				5
	mL/L				90

(a) D-Glucose powder
(b) cellulase, blend enzyme

The BHP set-up was based on ISR 2:1 (10 g VS inoculum : 5 g VS substrate) without counting the enzyme added, as this was later removed from the results when the hydrogen yield was calculated to make it easier to compare the results presented in this chapter and those in Chapter 5. Initial pH was adjusted to 7.0 using 1 M HCl. Wheaton bottles with a working volume of 70 mL were used, sparged with nitrogen for 1 min each and sealed with a rubber cap and an aluminium crimp. All bottles were placed in an incubator at 37°C. BHP Blank, Control 1 and Control 2 were in duplicate, while BHP Test was in triplicate. Table (7.9) shows the characterisation of all reactors on Day 0.

Table 7.9 Characterisation results of all reactors on Day 0.

		Blank	Control 1	Control 2	Test
Inoculum (digestate)		HST	HST	HST	HST
substrate		-	Glucose	Enzyme	HSS + Enzyme
pH	-	7.0	7.0	7.0	7.0
Alkalinity	mg CaCO3/L	1969 (20.3)	1934 (39.5)	1871 (8.1)	2421 (50.4)
TS	%	1.75 (0.02)	2.13 (0.04)	2.25 (0.01)	3.04 (0.01)
VS	%	1.01 (0.52)	1.22 (0.01)	1.21 (0.32)	1.75 (0.23)
TCOD	mg/L	17300 (849)	22240 (707)	24200 (113)	33467 (250)
sCOD	mg/L	2009 (11)	7145 (219)	8310 (71)	11013 (99)

TS: total solids, **VS**: volatile solids, **TCOD**: total chemical oxygen demand,
sCOD: soluble chemical oxygen demand
HST: heat shock pre-treatment (furnace: 60 min at 105°C).
(STD: standard deviation from the mean (n = 3))

7.4.3 Results and discussion

7.4.3.1 Gas yield

ISR is one of the influence parameters in the DF process and needs to be adjusted for optimal hydrogen production. It is very difficult to determine the value of ISR, as a very wide range of substrates has been tested for hydrogen production through DF (e.g., food, municipal and agricultural waste). In addition, inoculum type (pure or mixed culture) can affect the optimal value of ISR. In general, however, studies have reported that cell deaths may occur in DF due to a high ISR, which can cause excessive competition between microorganisms. A low ISR, on the other hand, may result in a delay in hydrogen production (long operation time) in DF, while an increase in biomass concentration may lead to an excessive proliferation of microorganisms (Alavi-Borazjani et al., 2021, Martinez-Burgos et al., 2020, Reddy et al., 2017).

The initial ISR used in the BHP mixed substrate (enzyme + HSS) was 1:1 (5 g VS inoculum: 5 g VS HSS) (Section 7.3 in this chapter). The results showed a lower hydrogen yield in BHP Test than in BHP Control 2. Therefore, in the work

described in this section, the ISR was changed to 2:1 (10 g VS inoculum: 5 g VS HSS). Figure (7.6) shows that increasing the ISR enhanced the hydrogen production in BHP Test. A comparison between hydrogen yield in $BHP_{ISR\ 1:1}$ and $BHP_{ISR\ 2:1}$ shows an increment each day during the first four days as follows: 369% on Day 1, 564% on Day 2, 65% on Day 3 and 34% on Day 4. Note that the glucose content in Test was 3.51 g/L (Note that the glucose production from mixed substrate (cellulase enzyme + HSS) in the EH process (according to the equation in Figure (6.12) in Chapter 6) was considered for gas yield calculations).

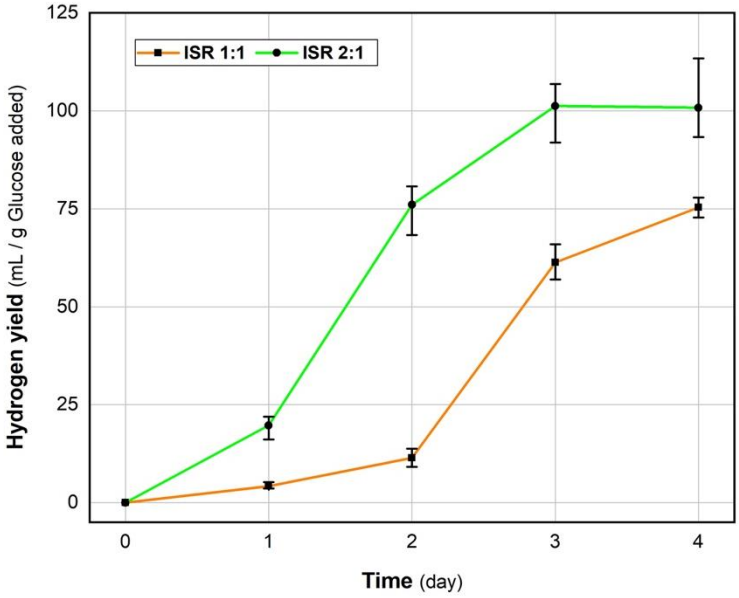


Figure 7.6 Impact of inoculum to substrate ratio (ISR) on hydrogen yield in BHP test processing HSS, (ISR 1:1 (5 g VS inoculum: 5 g VS HSS) and ISR 2:1 (10 g VS inoculum: 5 g VS HSS)), (average value of triplicate with max/min bar).

Table (7.10) shows the process kinetics for hydrogen yield in $BHP_{ISR\ 1:1}$ and $BHP_{ISR\ 2:1}$ calculated by Origin 2018b software. The fitting was run for the period of hydrogen accumulation from Day 0 until Day 4, when hydrogen reached its peak, as this model (MGompertz) is extensively used for batch experiments that have growth rate without gas consumption (Pagliaccia et al., 2016, Zwietering et al., 1990, Cai et al., 2004a).

$BHP_{ISR\ 2:1}$ had higher hydrogen production potential (***P***) and maximum hydrogen production rate ($\boldsymbol{R_m}$) than $BHP_{ISR\ 1:1}$. The long lag phase for the $BHP_{ISR\ 1:1}$ (43.98 hours) compared with the $BHP_{ISR\ 2:1}$ (17.31 hours) shows the positive impact of increasing ISR on enhancing the hydrogen production rate and minimising the lag time in BHP test. The R^2 value, as shown in Table (7.10), ranged from 0.996–0.999, which demonstrates that the MGompertz model provided a good fit to the data.

Table 7.10 Process kinetics for BHP Test (inoculum + enzyme + HSS).

Pre-treatment	***P***	$\boldsymbol{R_m}$	$\boldsymbol{\lambda}$	$\mathbf{R^2}$
	(mL)	(mL/h)	(h)	
ISR 1:1[a]	77.6	2.49	43.98	0.996
ISR 2:1	103.7	2.75	17.31	0.999

(a) Results from Section 7.3; **ISR**: inoculum to substrate ratio
ISR 1:1 (5 g VS inoculum: 5 g VS HSS) and ISR 2:1 (10 g VS inoculum: 5 g VS HSS).

Increasing ISR in BHP had a positive impact on hydrogen yield per gram glucose added. As BHP Control 2 (enzyme + inoculum) had hydrogen production, removing the yield values of hydrogen and carbon dioxide from BHP Test was necessary to eliminate any interference with gas yield data. Figure (7.7,a-b) shows the gas yield (hydrogen and carbon dioxide) per gram volatile solids (VS) added. The negative gas yield value in Figure (7.7,a-b) indicates that gas yield was higher in Control 2 than in Test ($BHP_{ISR\ 1:1}$, Figure 7.7a), where the hydrogen yield in Test was lower than Control 2. In contrast, at $BHP_{ISR\ 2:1}$ the hydrogen yield was negative during the first two days only but after that became positive, as the hydrogen yield in Test was higher than in Control 2 at that point. Theoretically, it was expected that the hydrogen yield in Test would be higher than in Control 2, as the glucose content in Test was 3.51 g/L (3.12 g from 10

mL cellulase enzyme + 0.39 g from EH of HSS), while that in Control 2 was 3.12 g/L.

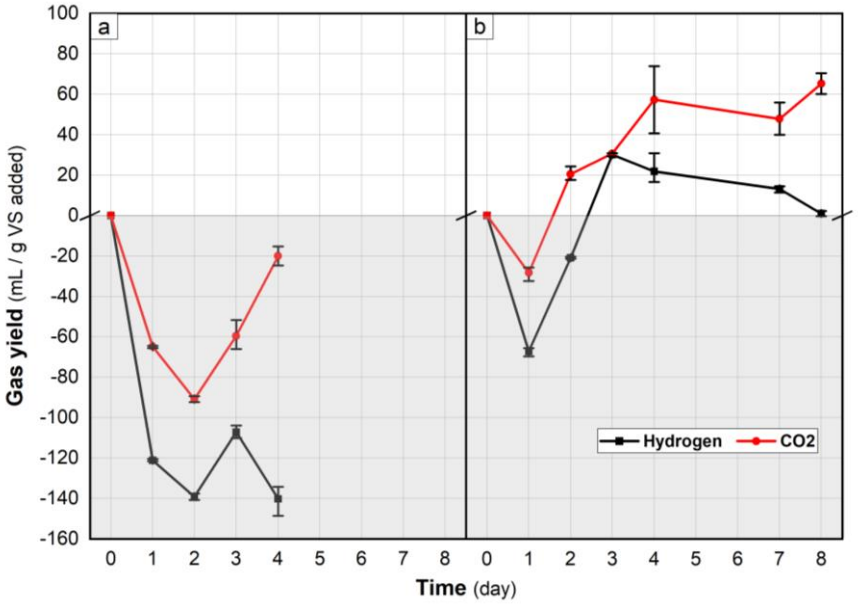


Figure 7.7 Gas yield in BHP Test after subtract the gas yield of BHP Control 2, (a) BHP Test: ISR 1:1 (5 g VS inoculum: 5 g VS HSS) and (b) BHP Test: ISR 2:1 (10 g VS inoculum: 5 g VS HSS), (average value of triplicate with max/min bar).

7.4.3.2 Volatile fatty acid production

In this chapter, a mixed substrate (enzyme + HSS) was used in BHP to enhance the limited hydrogen production from HSS. The complex structure of HSS prevents the bacteria from utilising the organic material and converting it to VFAs and biogas. However, the results from the first trials of mixed substrate (Section 7.3) show that Test (inoculum + enzyme + HSS) had a lower hydrogen yield than Control 1 (inoculum + glucose) and Control 2 (inoculum + enzyme). Given the lag time for VFA production (around two days), lower acetate accumulation and accumulation of propanoate were the obvious reasons for the low hydrogen yield in BHP Test. Therefore, three modifications to BHP were proposed and carried out as a solution to enhance hydrogen yield in BHP Test.

Figure (7.8,a-b) shows the TVFA accumulation and consumption in four BHPs (Blank, Control 1, Control 2 and Test) at different ISR. The lag time was reduced

significantly for all $BHP_{ISR\ 2:1}$ (Figure 7.8b), as the maximum amount of TVFAs in Control 1 and Control 2 was seen on Day 1, while in $BHP_{ISR\ 1:1}$ (Figure 7.8a), the maximum amount of TVFAs was seen on Day 3. Moreover, the lag time was reduced in Test $_{ISR\ 2:1}$. In addition, the TVFAs were enhanced in Control $1_{ISR\ 2:1}$ (from 7255 to 9098 mg/L), while a slight increase in TVFAs was seen in Control $2_{ISR\ 2:1}$ (from 9135 to 9338 mg/L). However, a comparison of TVFA curves for $Test_{ISR\ 1:1}$ and $Test_{ISR\ 2:1}$ shows that there was an incomplete process in $Test_{ISR\ 1:1}$ due to the lag time, and it was unknown whether the TVFAs were able to become higher/lower than or equal to TVFAs in $Test_{ISR\ 2:1}$. Therefore, extending the BHP operation time from five to eight days was a good method of ensuring a complete reaction and giving a better understanding of VFA accumulation and consumption during BHP.

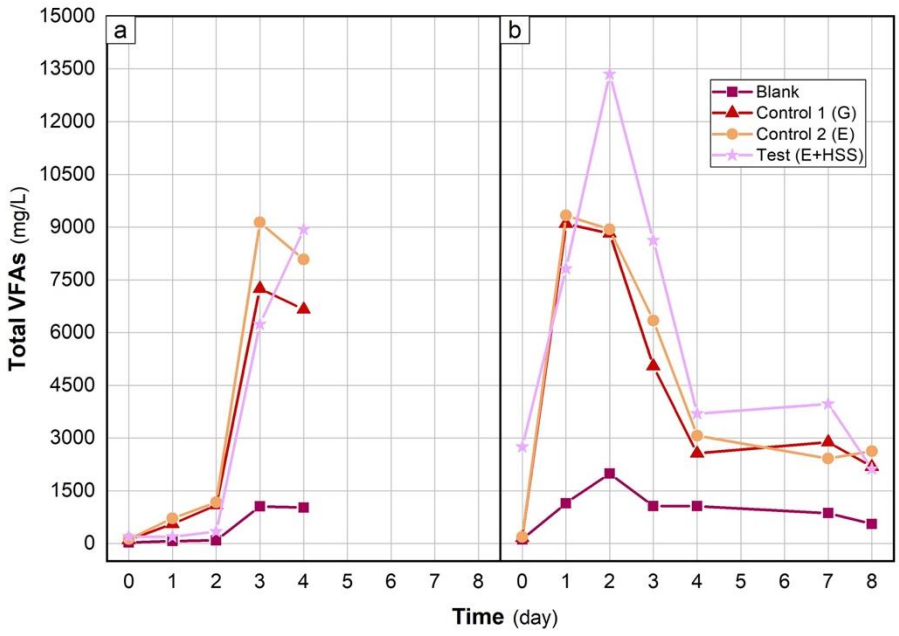


Figure 7.8 TVFA accumulation and consumption during BHP tests, (a) ISR 1:1 (5 g VS inoculum: 5 g VS HSS) and (b) ISR 2:1 (10 g VS inoculum: 5 g VS HSS), (Blank: inoculum, Control 1: inoculum + glucose, Control 2: inoculum + enzyme and Test: inoculum + enzyme + hydrolysed sewage sludge (HSS)), (average value of triplicate).

Table (7.11) shows the conversion percentage of TVFAs produced during $BHP_{ISR\ 2:1}$. Most of the accumulated VFAs in all BHP were converted to biogas

(hydrogen and carbon dioxide) except Blank (carbon dioxide only). Test had the highest conversion percentage (84.1%) of maximum TVFAs on Day 8, but the absence of lag time accelerated the VFA production and, therefore, most of the accumulated VFAs were converted to biogas on Day 4. As shown in Table (7.11), there was a slight increase in conversion percentage between Day 4 and Day 8.

Table 7.11 Conversion percentage of TVFAs during BHPs with ISR 2:1 (10 g VS inoculum: 5 g VS HSS).

	Maximum TVFAs		TVFAs on Day 4	Conversion percentage	TVFAs on Day 8	Conversion percentage
	mg/L	At day	mg/L	% of Max.	mg/L	% of Max.
Blank	1993	2	1064	46.6	558	72.0
Control 1	9098	1	2563	71.8	2182	76.0
Control 2	9338	1	3069	67.1	2624	71.9
Test	13351	2	3690	72.4	2124	84.1

TVFAs: total volatile fatty acids.
Blank: inoculum; **Control 1:** inoculum + glucose; **Control 2:** inoculum + enzyme; **Test:** inoculum + enzyme + hydrolysed sewage sludge (HSS).

Despite the lag time and TVFA production in $BHP_{ISR\ 1:1}$, there was a change in the glucose conversion pathway in $BHP_{ISR\ 2:1}$, and, as a result, hydrogen yield was enhanced. Figure (7.9, a-b) shows the VFA accumulation during BHP $Test_{ISR\ 1:1}$ and $Test_{ISR\ 2:1}$. It seems that glucose conversion in $Test_{ISR\ 1:1}$ (Figure 7.9a) was toward butyrate conversion pathway as the dominant VFA was butyrate, while in $Test_{ISR\ 2:1}$ (Figure 7.9b) the conversion was divided between the acetate and butyrate pathways, with more acetate accumulation. This change in glucose conversion pathway enhanced the hydrogen production in $Test_{ISR\ 2:1}$, as the acetate pathway yields 4 mole hydrogen from 1 mole glucose (Equation 7.2, Section 7.3.3.2), while the butyrate pathway yields 2 mole hydrogen per 1 mole glucose (Equation 7.3, Section 7.3.3.2). In addition, the lower accumulation of propanoate in $Test_{ISR\ 2:1}$ (Figure 7.9b) had a positive effect

on hydrogen production, as the propanoate pathway consumes hydrogen (Equation 7.4, Section 7.3.3.2). Therefore, the hydrogen yield was greater in $Test_{ISR\ 2:1}$ than $Test_{ISR\ 1:1}$, as shown in Figures (7.6 and 7.7b).

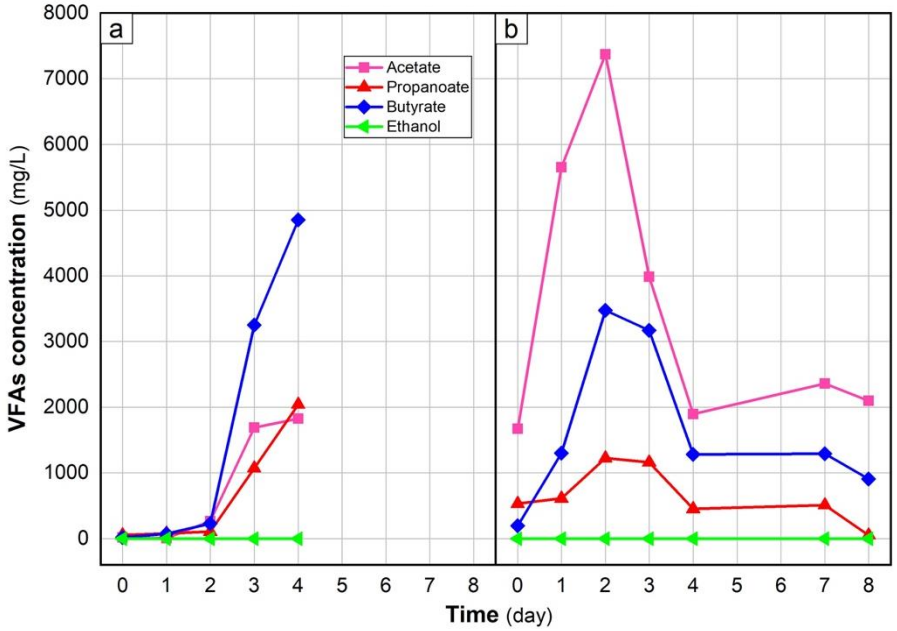


Figure 7.9 VFA production in BHP Test, (a) ISR 1:1 (5 g VS inoculum: 5 g VS HSS) and (b) ISR 2:1 (10 g VS inoculum: 5 g VS HSS), (average value of triplicate).

7.4.3.3 pH and alkalinity behaviour

pH has the role of determining the pathway type of glucose conversion (Hawkes et al., 2007, Temudo et al., 2008). As the existence of cellulase enzyme inside the DF reactor enhanced/released glucose content in the HSS (results presented in Chapter 6) after the EH process, the main substrate for hydrogen-producing bacteria is glucose. In the work described in this section, with all the modifications to the experimental set-up (Section 7.4.2), the pH behaviour for all BHP tests was similar to the pH behaviour described in Section 7.3. Figure (7.10,a-b) shows the similarity of pH behaviours between the two sets of BHP tests. With an initial pH 7.0 and HST pre-treatment for the inoculum, the dominant active bacteria were the hydrogen-producing bacteria (i.e., *Clostridium*). The VFA production during the first two days indicates the type of active bacteria. As shown in Figure

(7.9b), acetate and butyrate were the dominant VFAs during BHP, indicating the existence of hydrogen-producing bacteria (i.e., *Clostridium*). Studies have reported that the *Clostridium* species is responsible for the production of butyrate, acetate and hydrogen (Hawkes et al., 2007, Temudo et al., 2008). In addition, lower pH ranges (4.0–6.0) support butyrate and acetate accumulation, while higher pH ranges (7.0–9.0) support ethanol and propionate accumulation (Hawkes et al., 2007, Pakarinen et al., 2008). As shown in this experiment (Figure 7.10b), the pH for all BHPs except BHP_{Blank} dropped after Day 1, due to the high VFA accumulation, and stayed within the range of 5.0–5.5 for the rest of the BHP experiment. Thus, hydrogen and VFA production was enhanced as the pH of BHPs was within the optimum range for VFAs (acetate and butyrate specifically) and hydrogen production. Li and Fang (2007a) reported that the optimum pH range for hydrogenases (hydrogen producers) in DF was between pH 5.0 and pH 7.0.

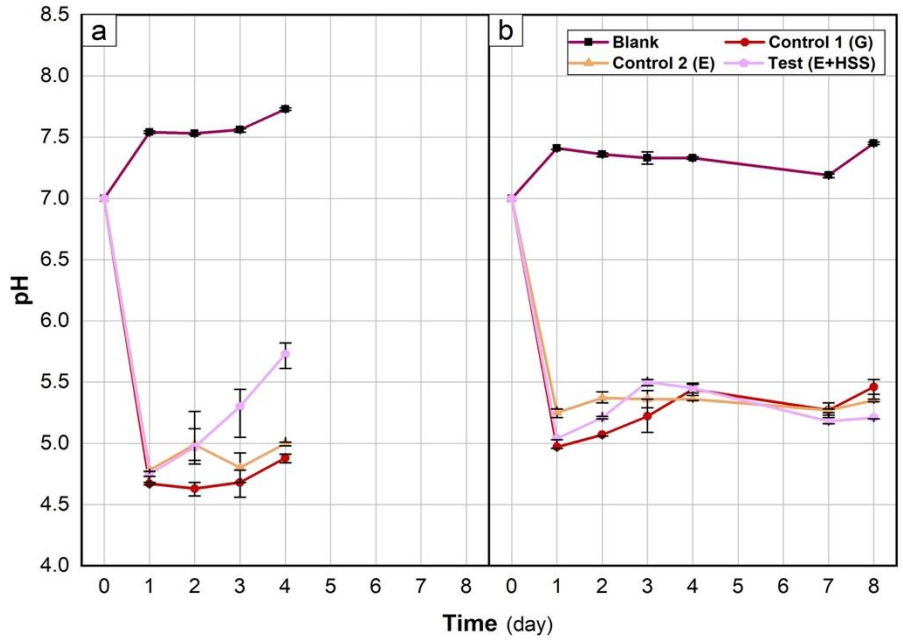


Figure 7.10 pH curve for BHP tests, (a) ISR 1:1 (5 g VS inoculum: 5 g VS HSS) and (b) ISR 2:1 (10 g VS inoculum: 5 g VS HSS), (Blank: inoculum, Control 1: inoculum + glucose, Control 2: inoculum + enzyme and Test: inoculum + enzyme + hydrolysed sewage sludge (HSS)), (average value of triplicate with max/min bar).

Alkalinity also plays a role in maintaining the stability of the DF process by buffering the pH drops due to VFA accumulation. Changing the ISR in this set of BHPs increased the initial alkalinity for all BHPs (Blank, Control 1, Control 2 and Test), as the inoculum was twice (10 g VS) the amount of that in the work described in Section 7.3 (5g VS). Despite the increase in the initial alkalinity, a similar alkalinity behaviour was seen in this set of BHPs as in the BHPs described in Section 7.3. Figure (7.11,a-b) shows that the alkalinity behaviour for the two sets of BHPs sharply dropped during the first day as a result of VFA accumulation, but increasing alkalinity after Day 1 buffered the pH and kept the BHPs within the optimum range of hydrogen production.

In the work described in this section, all BHPs started with alkalinity ranging between 1869 and 2421 mg/L $CaCO_3$. However, on Day 1 the alkalinity dropped in all BHPs except Blank. The alkalinity was within the range of 977–1328 mg/L $CaCO_3$, then increased until Day 4, when the hydrogen production reached the maximum yield (as shown in Figure 7.6). Among these BHPs, Test had the highest increment after the drop on Day 1, then it increased and stayed within the range of 1974–2139 mg/L $CaCO_3$ from Day 3 to Day 8. A comparison between $Test_{ISR\ 1:1}$ (Figure 7.11a) and $Test_{ISR\ 2:1}$ (Figure 7.11b) shows the positive impact of higher alkalinity in BHP, as high alkalinity may have given $Test_{ISR\ 2:1}$ the advantage to reach a higher hydrogen yield than $Test_{ISR\ 1:1}$ (as shown in Figure 7.6). Still, more investigation is needed to test different ranges of initial alkalinity in DF processing of mixed substrate (enzyme + HSS) to maximise hydrogen yield.

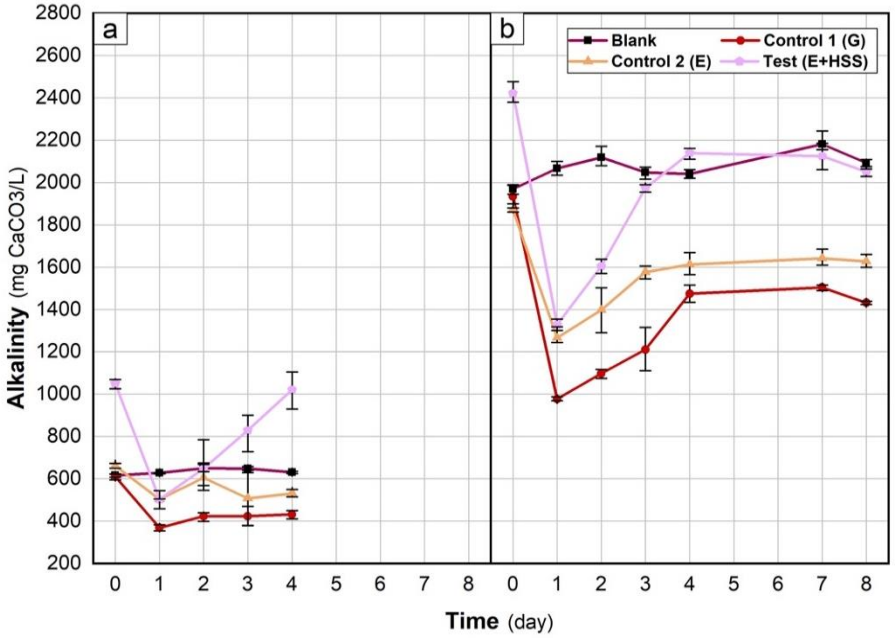


Figure 7.11 Alkalinity curve for BHP tests, (a) ISR 1:1 (5 g VS inoculum: 5 g VS HSS) and (b) ISR 2:1 (10 g VS inoculum: 5 g VS HSS), (Blank: inoculum, Control 1: inoculum + glucose, Control 2: inoculum + enzyme and Test: inoculum + enzyme + hydrolysed sewage sludge (HSS)), (average value of triplicate with max/min bar).

7.4.4 Conclusion

Applying some modifications to ISR, operation time and HST method enhanced hydrogen and VFA production in BHP. The work described in this section demonstrates the positive impact of increasing ISR on resolving the lag time observed in the results described in Section (7.3). Moreover, increasing the operation time of BHP gives a better understanding of VFA accumulation and consumption. Therefore, these modifications should be considered when carrying out BHP on sewage sludge.

7.5 Overall impact of enzyme on DF processing of HSS

Comparing the results presented in Chapter 5 (testing BHP of HSS, Section 5.4) and this chapter (using mixed substrate: HSS + enzyme, Section 7.4) shows the impact of using enzyme in DF processing of HSS. As shown in Figure (7.12),

both hydrogen and carbon dioxide yield were enhanced when the enzyme was used in BHP; in this regard, the results presented in Chapter 6 show that EH succeeded in breaking down and enhancing glucose content in HSS. Therefore, the HSS became more favourable and easily digestible for hydrogen-producing bacteria.

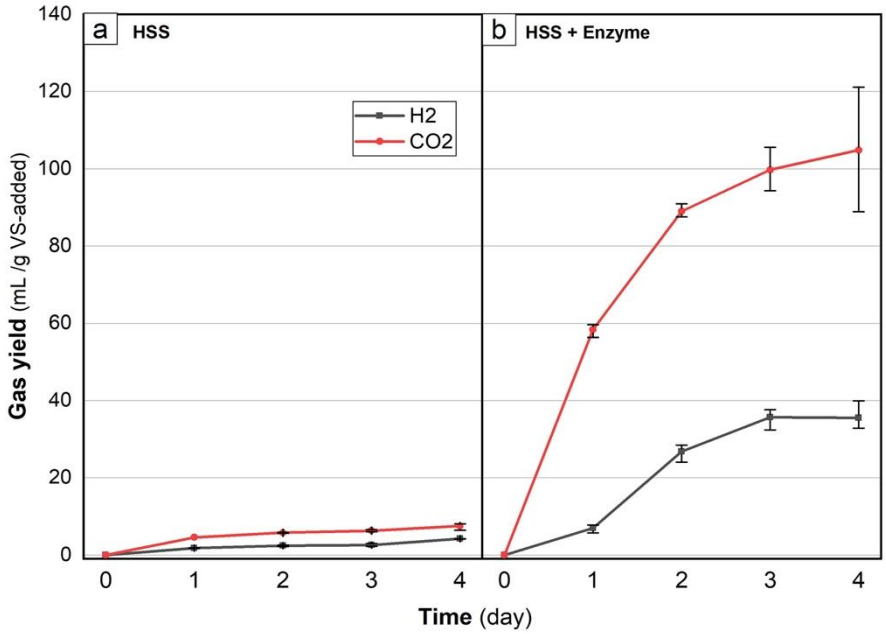


Figure 7.12 Gas yield in BHP, (a) HSS and inoculum with ISR 1:1 (5 g VS inoculum: 5 g VS HSS) and (b) HSS + enzyme + inoculum with ISR 2:1 (10 g VS inoculum: 5 g VS HSS), (average value of triplicate with max/min bar).

Beside biogas production, VFA production/accumulation was massively enhanced when enzyme was used in BHP, as shown in Figure (7.13). The acetate and butyrate were the dominant acids, indicating the increase in glucose content by EH, as these VFAs are the main products of glucose conversion in DF processes. Overall, therefore, both glucose concentration and VFA production were enhanced when BHP processed mixed substrate (enzyme and HSS), and, thus, hydrogen yield was enhanced.

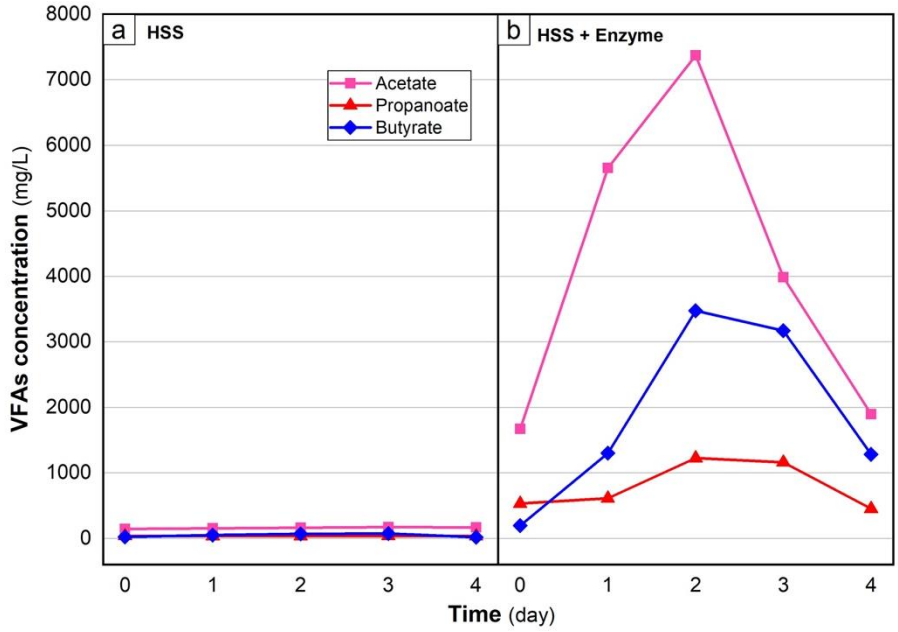


Figure 7.13 VFA accumulation and consumption during BHP, (a) HSS and inoculum with ISR 1:1 (5 g VS inoculum: 5 g VS HSS) and (b) HSS + enzyme + inoculum with ISR 2:1 (10 g VS inoculum: 5 g VS HSS), (average value of triplicate).

7.6 Overall conclusion

The limited hydrogen production from HSS in DF can be resolved by the aid of enzyme. This chapter has proved the positive impact of EH in enhancing the biodegradability and digestibility of HSS, as both hydrogen and VFA production improved. The findings in this chapter show that using a mixed substrate (enzyme + HSS) in BHP and applying the modifications enhanced the overall DF process and that the hydrogen was enhanced along with VFA production, accumulation and consumption. These findings show the potential of integrating the two processes (DF and EH) in one system (reactor). Doing so will make the method more efficient than other methods, which use separate reactors, because the footprint of the reactor at commercial scale will be reduced. Beside biogas production, VFA production/ accumulation was massively enhanced when enzyme was used in the BHP, as acetate and butyrate were the dominant acids. This indicates the increase in glucose content due to using EH, as these

VFAs are the main products of glucose conversion in the DF process. Overall, therefore, BHP processing of mixed substrate (enzyme and HSS) enhanced both glucose concentration and VFA production and, thus, hydrogen yield was enhanced. However, the limited research related to hydrogen production from mixed substrate (enzyme and sewage sludge) and the positive results presented in this chapter show that more investigation and assessment is required to find the optimum enzyme dosage and operation condition to optimise hydrogen production via DF.

Chapter 8
General discussion

This chapter discusses and summarises the findings in this research, which can serve as guidance for future research related to bioenergy (methane and hydrogen) production from sewage sludge.

8.1 The future of sewage sludge management

Sewage sludge has the potential to be a sustainable resource for many applications, such as biogas production. Methane gas has been massively produced from AD plants processing sewage sludge. Still, AD operators in most AD plants cannot increase the methane content in the generated biogas as the maximum percentage reached to date is approximately 70% (Bassani et al., 2015, Braun, 2007). The findings reported in Chapter 4 show that the calculated methane potential (based on laboratory experiments) is parallel with those reported by the Esholt WWTP, which reveal that methane content is about 60% of generated biogas. Thus, implementing an efficient upgrading biogas method in a commercial AD plant will increase the methane yield in biogas, leading to high utilisation and volume reduction of sewage sludge. In addition, it could achieve higher net energy production than the current process can achieve.

One of the conclusions from the literature review reported in Chapter 2 is that biogas upgrade (increased methane yield) through the hydrogenotrophic pathway can be considered the most metabolically efficient pathway for upgrading biogas quality and increasing methane yields (Lever, 2016). This approach includes the addition of hydrogen gas into AD to be combined with carbon dioxide and produces extra methane gas; as a result, methane quality

and yield increase. Many studies have reported that this upgrading method has a positive impact on biogas production (Luo and Angelidaki, 2013b, Wang et al., 2013, Xu et al., 2015, Agneessens et al., 2017, Kougias et al., 2017). This alternative uses an external hydrogen source, which usually comes from conventional fossil-fuel-dependent hydrogen production methods which have a negative impact due to high GHG emissions (e.g., water hydrolysis, steam reforming). Therefore, this thesis investigates bio-hydrogen production from HSS as an alternative to conventional methods to be integrated in biogas upgrading and reduce the overall environmental impacts attributed to hydrogen production. Many studies have reported high biological hydrogen production from other feedstock, such as food waste (Shin et al., 2004, Chu et al., 2008, Pagliaccia et al., 2016), while very limited studies have used HSS as feedstock for bio-hydrogen production. Thus, successfully producing high amount of hydrogen gas from DF may create other pathways by which AD technology can be optimised and integrated with DF technology for increasing methane content in AD biogas's. Furthermore, by overcoming the limitations of upscaling DF technology at a commercial scale, the technology may become a good alternative to or complementary method of conventional production technologies that use fossil fuel for hydrogen generation. Moreover, our environment will be enhanced and the use of hydrogen instead of fossil fuel as a source of energy for industrial applications will be facilitated.

8.2 Hydrogen potential from sewage sludge

In the future, hydrogen may become the best alternative to fossil fuel as a source of energy for many applications. There are many reasons that hydrogen is

preferred to other energy sources, such as a high net calorific value (hydrogen 120 MJ/kg) compared with methane gasoline (50 MJ/kg), ethanol (26.8 MJ/kg) and methanol (19.6 MJ/kg), which gives hydrogen the highest energy efficiency (Graboski and McCormick, 1998, Zajic et al., 1978). Hydrogen also has a positive impact on the environment, as the combustion of hydrogen does not produce carbon dioxide, which can improve the climate by reducing GHG emissions (Łukajtis et al., 2018). In addition, hydrogen is considered to be competitive with other renewable energy types, such as solar and wind (Zajic et al., 1978). Therefore, hydrogen has a good chance of being one of the main energy vectors in the future, and the inefficiencies of current production methods (conventional and electrolysis methods) require further research into other energy-efficient and environmentally friendly routes for hydrogen generation, such as bio-hydrogen production. Thus, this thesis assessed the hydrogen potential from HSS via the DF process. DF is a method of bio-hydrogen production and one promising method of hydrogen production from different types of waste (e.g., food, municipal and agricultural waste) through biological reactions carried out by microorganisms under specific operating conditions (Ghimire et al., 2015).

However, the findings presented in this thesis show that a very limited amount of hydrogen can be produced from HSS via DF, although the HSS used as feedstock went through hydrothermal pre-treatment by HTP at Esholt WWTP, Bradford, UK. This pre-treatment has a positive impact on enhancing the solubility and biodegradability of sewage sludge before it goes to AD for biogas production (Wirth et al., 2015, Wang et al., 2010). Still, when it was used as substrate for DF experiments in this thesis, very limited hydrogen was produced. There are many reasons for that; for example, the complex structure of HSS

renders it difficult for bacteria to use and convert to hydrogen. The C/N ratio is another reason for low hydrogen production, as many studies shows that enriched (high-C/N) substrate has a higher hydrogen production than that with a low C/N ratio (e.g., sole HSS, as in this study) (Xia et al., 2016). Mata-Alvarez et al. (2014) reported that the suitable range of C/N for fermentative bacteria is between 20 and 30, while the C/N of sole sewage sludge is usually between 4 and 10. In this research, the C/N for HSS was 8.54, and these findings are in parallel with other studies that have reported very limited hydrogen production due to a low C/N ratio (Cheng et al., 2016, Sreela-Or et al., 2011). For this reason, co-fermentation is among the methods used as a pre-treatment for substrate to improve C/N and therefore enhance hydrogen production (Wu et al., 2016, Xie et al., 2016, Hagos et al., 2017). In this thesis, however, we decided to assess the use of the EH process for HSS alone, without mixing it with other types of waste (co-fermentation). The findings from the literature review show that, on the one hand, EH has a positive impact on increasing the C/N ratio in food waste or other feedstock and thus leads to enhanced hydrogen production in DF (Han et al., 2016). On the other hand, EH is more favourable than mechanical, chemical and physical pre-treatments, as it is a biological process that requires a lower energy input than other pre-treatments. Moreover, many studies have reported that EH has the ability to reduce sludge volume and improve hydrogen production from sewage sludge (Massanet-Nicolau et al., 2008, Parawira, 2012).

Inoculum pre-treatment is an essential treatment in DF. The findings presented in Chapter 5 in this thesis show how important it is to pre-treat the inoculum before conducting any DF experiment, as this will deactivate the hydrogen-

consuming bacteria and, as a result, hydrogen production will be enhanced. The findings in the literature review also show how studies have reached conflicting results when assessing the best pre-treatment methods, which justifies the need for more investigation to determine which pre-treatment is best for inhibiting hydrogen-consuming bacteria and enriching hydrogen-producing bacteria in a certain type of inoculum and substrate. Therefore, one of the objectives of this thesis was to address the necessity of inoculum pre-treatment for hydrogen production and create a guideline for assessing and selecting the best pre-treatment for any inoculum and substrate used in future studies. The findings presented in Chapter 5 show the assessment process for selecting the best inoculum pre-treatment, keeping in mind that substrate type (glucose: sole carbohydrate was used) is an influence parameter for the assessment, as the hydrogen production values presented in this study may differ if a different substrate is used, such as food waste, sewage sludge or agricultural waste. In terms of general guidelines, the proposed method and analysis in this thesis are one step toward improving knowledge in this field and helping researchers assess and select the best pre-treatment method for their own type of substrate.

8.3 Glucose recovery/production from sewage sludge

The limited hydrogen production from sewage sludge in DF is the main obstacle to using it as a feedstock for this technology. The findings in the literature review presented in this thesis show that DF is a sustainable process with the ability to utilise a wide range of substrates, such as food waste, solids waste and agricultural waste. Unfortunately, the contrary is true with HSS, as the complex structure of its contents makes it difficult for fermentative bacteria to use it, as

shown in Chapter 5 in this thesis, while its low C/N ratio is another limiting factor (Xia et al., 2016). Therefore, in this research, EH was chosen for use as a biological pre-treatment for HSS for several reasons. First, this process uses enzyme, which has the ability to disintegrate hard-to-digest macro sewage flocs into easily digestible micro flocs under specific operation conditions. The findings from the literature review show that EH has a positive impact on breaking down the complex structure (lignocellulosic biomass) to simpler and more easily digestible micro flocs (i.e., glucose) (Hsu et al., 1980). Second, the findings presented in Chapter 5 show that simple carbohydrate (glucose) is among the main fermentable substrates for hydrogen production and the most favourable and primary substrate for fermentative bacteria (e.g., *Clostridium* bacteria) (Finlay, 1995). In addition, sewage sludge has the potential to be a sustainable source for glucose production. It has been reported that an estimated 6.22 Mt/yr of sugar can be produced from municipal sludge and livestock manure generated in Canada (Champagne, 2007). Finally, EH is more favourable than mechanical, chemical and physical pre-treatments from the perspective of environmental impact and may become more economic in the future. Therefore, we decided to pre-treat HSS with EH, as the expected outcome of this experiment was to convert HSS to a suitable substrate (i.e., glucose) that has a suitable fermentable structure which can be easily utilised by fermentative bacteria for hydrogen production.

The findings presented in Chapter 6 show that the EH process as a pre-treatment for HSS enhanced its glucose content and converted some of the macro sewage flocs to more easily digestible micro flocs (glucose). The glucose content in HSS was increased/released by the aid of enzyme, as expected, but several

parameters influenced the glucose production from HSS. For example, enzyme dosage is one influence parameter; therefore, in this research, a range of enzyme (cellulase) dosages was tested for glucose production. Substrate concentration is another influence parameter for the EH process; thus, different sewage sludge concentrations (based on TCOD) were used to assess its impact on glucose production and the overall EH process. The findings in the literature review show that operation temperature and pH are also important to maximise glucose production in EH and can affect enzyme activity and hydrolysis rate. However, we decided to choose the enzyme dosage and two different substrate concentrations in this research. In addition, cellulase enzyme was used, which is a commercial enzyme that may become costly when used at commercial scale for EH. Thus, more investigation is needed to assess EH on sewage sludge and how to reach the optimum enzyme dosage for maximum glucose production and optimise the whole process so as to maximise glucose production and minimise commercial enzyme usage (which is expensive) or find an alternative enzyme source (e.g., enzyme recovery for sewage sludge is possible). This will help to improve our knowledge and direct waste industries to use enzyme for glucose production. It will also create another sustainable source of glucose and be available for food processing, industrial and/or bio-hydrogen production applications.

8.4 The role of enzymes in enhancing the hydrogen potential of sewage sludge

The increment in glucose content in HSS due to EH pre-treatment shows the potential of this process in converting HSS to suitable substrate for enhancing bio-hydrogen production in DF reactor. However, these trial EH experiments

were operated as a separate test from DF and without monitoring the hydrogen production, as the objective was to assess glucose production by EH. Therefore, in Chapter 7, a mixed substrate, which included HSS (collected from Esholt WWTP, Bradford, UK) and cellulase, enzyme blend (purchased from Sigma-Aldrich) was tested in a DF reactor for biogas production. The bacteria source (inoculum) was digestate, also collected from the AD reactor in Esholt WWTP, Bradford, UK, and inoculated into the DF reactor in a BHP test. The primary results from the first trials were unexpected as the hydrogen production was limited, showing the need to modify the BHP to overcome this limitation of hydrogen production. Therefore, some adjustments were made for the following BHP trials, including the following: (i) increasing the operation time of BHP from five to eight days as there was a lag phase in hydrogen production during the first trials because of the lag in bacteria activity which may occur during a BHP test; (ii) increasing the bacteria population by increasing the ISR from 1:1 to 2:1 (double the amount of inoculated bacteria), as this may enhance the hydrogen production, according to the literature review; (iii) an alternative HST method (furnace: 105°C for 60 min) was used to prepare the inoculum for BHP, as the results showed that some hydrogen-producing bacteria were affected by HST (autoclave: 115°C for 20 min).

All these modifications were applied to the BHP, and the findings in Chapter 7 show that using a mixed substrate (enzyme + HSS) in BHP and applying the modifications enhanced the overall DF process and that the hydrogen was enhanced along with VFA production, accumulation and consumption. These findings show the potential of integrating the two processes (DF and EH) in one system (reactor). Doing so will make the method more efficient than other

methods, which use separate reactors, because the footprint of the reactor at commercial scale will be reduced.

More conclusions can be drawn from comparing the results presented in Chapter 5 (testing the BHP of HSS) and Chapter 7 (using mixed substrate: HSS + enzyme). The comparison shows that both hydrogen and carbon dioxide yield were enhanced when enzyme was used in BHP. This finding related to the results presented in Chapter 6 (treating HSS with EH), which show how EH enhanced the glucose content in HSS by breaking down the complex structure of HSS. Therefore, HSS became more favourable and more easily digestible for hydrogen-producing bacteria. Beside biogas production, VFA production/ accumulation was massively enhanced when enzyme was used in the BHP, as acetate and butyrate were the dominant acids. This indicates the increase in glucose content due to using EH, as these VFAs are the main products of glucose conversion in the DF process. Overall, therefore, BHP processing of mixed substrate (enzyme and HSS) enhanced both glucose concentration and VFA production and, thus, hydrogen yield was enhanced.

More investigations are needed to optimise the influence parameters of this process, as changing pH, operation temperature, ISR and enzyme dosage may positively or negatively affect bio-hydrogen production due to the sensitivity of the process. Moreover, finding an alternative source of enzyme to be used instead of the expensive commercial enzymes will improve the process efficiency and make it more attractive for industrial application.

Chapter 9
Conclusions and recommendations

This chapter gives conclusions and recommendations which can guide future research work related to bioenergy (methane and hydrogen) production from sewage sludge.

9.1 Overall conclusions

The outcomes and findings from this thesis can be concluded and summarised in the following points.

- Managing sewage sludge still remains a challenging task for the water industry because of continuous increment in urban population and more tightening environmental restrictions affecting current routes for the final disposal and expectations on developing more sustainable (net-zero) treatment processes.
- Sewage sludge has been processed by AD for biogas production for many years in the UK, but still methane yields are limited and biogas quality cannot go beyond 70% CH_4, without the use of biogas upgrading/cleaning processes that produces biogas suitable for grid injection or transport use. That limits the use of biogas to in-situ energy production with great inefficiencies and energy losses.
- The changes in the characteristics of the collected samples (HSS and digestate from Esholt a WWTP in Bradford) in this research were assessed and observed over one year of collection and testing, which helped to assess the performance of HTP and AD reactors at the Esholt WWTP. Moreover, understand the fluctuation that can occur in HTP

performance and how this can affect AD performance in terms of process stability and high biogas production. Also, the BMP results help to create a baseline for comparison with any works related to upgrading methane yield in future and show the methane potential of the current biogas production applications (THP+AD). Therefore, alternative biogas upgrading methods can be assessed to enhance methane yield and improve AD processes.

- There is potential in upgrading the quality (>80% CH_4) and yields of produced biogas from sewage sludge by developing hydrogenotrophic methanogenesis in existing AD reactors. That can help to achieve higher energy outputs, maximise sewage sludge utilization and minimise digestate volumes to be disposed.
- DF is one of several methods used for bio-hydrogen production, whereby fermentative bacteria are used to hydrolyse organic substrates to produce hydrogen gas. And It is considered more sustainable to produce hydrogen via biological processes than conventional processes such as natural gas decomposition, petroleum oxidation and coal gasification. Therefore, The BHP of HSS was assessed, and the results will help to create a baseline for comparison any works related to enhancing hydrogen yield from DF in future.
- Very limited hydrogen was achieved for HSS, while a high amount of hydrogen was produced by the same inoculum but with a different substrate (glucose). This finding indicates that it is necessary to apply one of the substrate pre-treatments to HSS to enhance hydrogen production. Overall, the main target is to find an efficient and economical biological

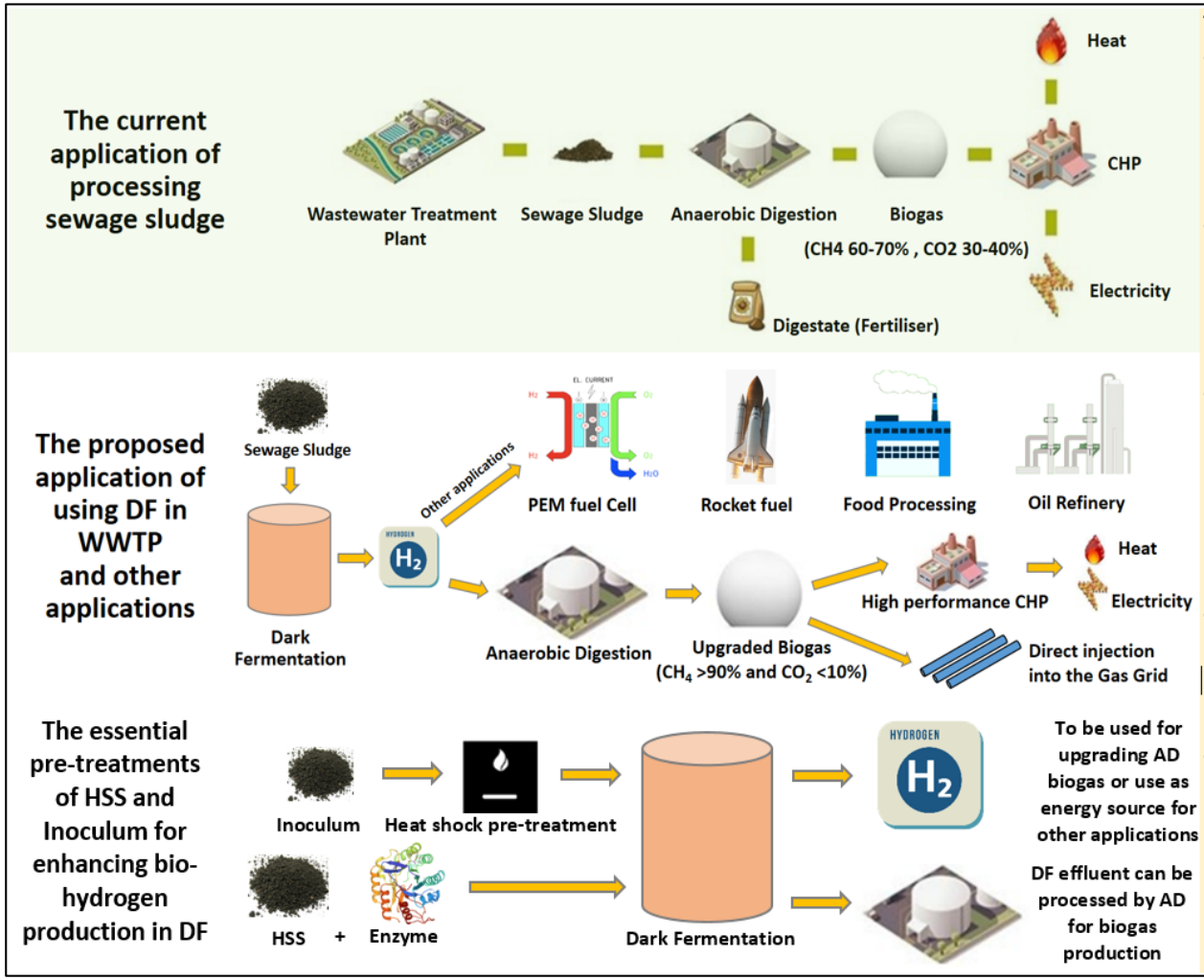
hydrogen production method, conditional on using available renewable resources, such as sewage sludge.

- Inoculum pre-treatment is an essential step toward enhancing hydrogen production in the DF process. HST was the best pre-treatment for hydrogen production and the stability of DF, while AST and BST made no difference to BHP performance. The highest cumulative hydrogen production was achieved with HST, which could be attributed to the longer inhibition of hydrogen consumers.
- Fermentation operation conditions, such as temperature, alkalinity, initial pH and ISR, have a crucial impact. Hence, without selecting the proper conditions for the inoculum and substrate types, a high re-activation of hydrogen-consuming bacteria (acetobacteria or methanogens) will occur.
- Initial pH and ISR have impact on hydrogen and VFA production in DF, as the conversion pathway for glucose or other substrates can be changed by changing these operation parameters. Therefore, it is very important to control the operating conditions to ensure the best VFA route to gain maximum hydrogen production from DF.
- The hydrogen production values in this study may differ when a different substrate, such as food waste, sewage sludge or agricultural waste, is used. To create a guideline procedure for all DF experiments in future, therefore, the first step is to understand the capability of using inoculum (sole or complex) for hydrogen production and how it may react with different pre-treatment methods (HST, BST and AST). Moreover, it is necessary to understand which pre-treatments lead to maximum

hydrogen production and ensure maximum time for inhibiting the activity of hydrogen consuming bacteria.

- Benedict's quantitative method was assessed to detect and quantify glucose content in a solution that contains HSS. With some modifications (finding the optimum wavelength for a mixed sample and modified glucose curve), Benedict's quantitative method can be more reliable and more accurate than the original Benedict's method, for measuring glucose concentration in HSS samples.
- Finding the optimum wavelength should be the first step in any future works that use Benedict's method, as this will give more accurate results. Moreover, creating a modified glucose curve is another approach to ensure accurate glucose concentration measurements in an EH test.
- The limited hydrogen production from HSS in DF can be resolved by the aid of enzyme. This research has proved the positive impact of EH in enhancing the biodegradability and digestibility of HSS, as both hydrogen and VFA production improved in DF. Using the EH process as pre-treatment for HSS enhanced its glucose content and converted some macro sewage flocs to easily digestible micro flocs (glucose). Therefore, the substrate will be better and more easily digested by bacteria in a DF reactor, which will lead to enhanced production of hydrogen and VFAs.
- The limited research related to hydrogen production from mixed substrate (enzyme and sewage sludge) and the positive results presented in this research show that more investigation and assessment is required to find the optimum enzyme dosage and operation condition to optimise hydrogen production via DF.

- More investigation is needed to enhance bio-hydrogen production in DF to increase scale-up feasibility. The results presented in this research show that bio-hydrogen production from DF is still considered lower than the other conventional technologies. But the idea of using a biological method for hydrogen production, conditional on using available renewable resources, such as sewage sludge, remain attractive for investors and could meet environmental and sustainability targets.
- In summary, Figure (9.1) shows the current application of processing sewage sludge in WWTP and the research proposal of using sewage sludge as feedstock for DF for bio-hydrogen production. Also, Figure (9.1) shows the potential application of using bio-hydrogen for enhancing AD/biogas (increase methane content) and other applications (as an energy source). Also, this figure summarises the research findings and recommendations for future work.



The research findings and recommendations:

- There is potential in upgrading the quality (>80% CH4) and yields of produced biogas from sewage sludge by developing hydrogenotrophic methanogenesis in existing AD reactors. That can help to achieve higher energy outputs, maximise sewage sludge utilization and minimise digestate volumes to be disposed. Energy source for other applications.
- Inoculum pre-treatment is an essential step toward enhancing hydrogen production in the DF process. HST was the best pre-treatment for hydrogen production and the stability of DF. The highest cumulative hydrogen production was achieved with HST, which could be attributed to the longer inhibition of hydrogen consumers.
- This research has proved the positive impact of EH in enhancing the biodegradability and digestibility of HSS, as both hydrogen and VFA production improved in DF. Using the EH process as pre-treatment for HSS enhanced its glucose content and converted some macro sewage flocs to easily digestible micro flocs (glucose). Therefore, the substrate will be better and more easily digested by bacteria in a DF reactor, which will lead to enhanced production of hydrogen and VFAs.
- The limited research related to hydrogen production from mixed substrate (enzyme and sewage sludge) and the positive results presented in this research show that more investigation and assessment is required to optimise hydrogen production via DF.
- Further works in this field should be carried out by other researchers and can investigate different configurations, for example reactor type (batch, semi-continuous and continuous reactors), reactor volume, operation temperature (thermophilic), range of ISR (3:1 and 4:1), different types of commercial enzymes, range of enzyme dosage, mixture of enzymes or/and fungi, reduce the energy consumption of HST and HSS concentration (larger than 10 g TCOD/L).

Figure 9.1 A summary of the research findings on managing/using sewage sludge in WWTP.

9.2 Recommendations and future work

Certain recommendations can be considered for further research activities related to hydrogen and methane production from AD and DF, as listed below.

- The BMP and BHP of HSS in this study were carried out by a small-volume reactor and in batch mode. A larger-volume reactor and continuous mode should be considered in further works to improve our understanding of the AD and DF processing of sewage sludge and the effect of influence parameters on the overall process. Also, this will help to better understand the methane and hydrogen potential of HSS and the potential for scale-up.
- More inoculum pre-treatments, such as physical pre-treatment (freezing-thawing and aeration), chemical pre-treatment (sodium 2-bromoethanesulfonate or 2-bromoethanesulfonic acid and iodopropane) and combined pre-treatment (e.g., combined HST and BST) should be considered to enable a better comparison and assessment of the enrichment process of hydrogen-producing bacteria and the inhibition process of hydrogen-consuming bacteria.
- Beside the inoculum pre-treatment methods, testing different operation conditions, such as thermophilic instead of mesophilic, range of initial pH and alkalinity adjustment and automated mixing instead of hand shaking, will improve our knowledge and generate more results for use in optimising the BHP through modelling software.
- In this study, Benedict's quantitative method was used to measure glucose concentration in a solution. Using specific analytical methods

High performance liquid chromatography (HPLC) can improve data accuracy and provide a better insight on the production of sugars.

- In EH trials, cellulase, blend enzyme was used, so further works should consider different types of commercial enzymes; moreover, a mixture of enzymes or/and fungi may enhance the glucose production from sewage sludge.
- In addition, further works need to optimise the enzyme dosage in the EH process, as very limited studies have investigated the impact of enzyme on HSS.
- BHP trials for mixed substrate had promising results toward enhancing hydrogen production from HSS; however, due to the time limitation on this study, further works in this field should be carried out by other researchers and can investigate different configurations, for example reactor type (batch, semi-continuous and continuous reactors), reactor volume, operation temperature (thermophilic), range of ISR (3:1 and 4:1), enzyme dosage and HSS concentration (larger than 10 g TCOD/L).
- Further work needs to assess the use of DF by-products (Solids and liquid), as this effluent is reached with VFAs (especially butyrate and acetate). One possible solution is to process DF effluent by AD for methane production. Ruggeri et al. (2010) reported that the combined process (DF+AD) resulted in a positive net energy over the whole range of tested reactor dimension with 45–90% of available energy.

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