Improving the Bioconversion of Lignocellulosic Feedstock to Bio-fuels and Chemicals

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A submission presented in partial fulfilment of the requirements of the University of South Wales for the degree of Doctor of Philosophy
October 2015
CONTENT

ABSTRACT................................................................................................................................. iv

ACKNOWLEDGEMENTS............................................................................................................. v

1. INTRODUCTION .................................................................................................................. 1

1.1 Aims of the research ......................................................................................................... 3

2. ANAEROBIC DIGESTION PROCESSES .......................................................................... 5

2.1 Microbiology of Anaerobic digestion .............................................................................. 5

  2.1.1 Hydrolysis ................................................................................................................... 5

  2.1.2 Acidogenic Phase ...................................................................................................... 6

  2.1.3 Acetogenic phase ...................................................................................................... 7

  2.1.4 Methanogenic phase ................................................................................................. 9

2.2 Biochemistry and Microbial Adaption in the Anaerobic Digestion process ................. 10

  2.2.1 The Hydrogenic/Acidogenic Fermentation System .................................................. 10

  2.2.2 Biomethane System .................................................................................................. 11

3. LIGNOCELLULOSE AS FEEDSTOCK FOR ANAEROBIC DIGESTION ......................... 12

3.1 Chemical Structure of Lignocellulosic Biomass ............................................................. 13

3.2 The Recalcitrance of Lignocellulosic Biomass to Enzymatic Hydrolysis and the role of Pre-treatment ........................................................................................................... 16

3.3 Pre-treatments ................................................................................................................ 17

  3.3.1 Physico-chemical Pre-treatment .............................................................................. 18

  3.3.2 Biochemical Pre-treatment ...................................................................................... 19

4 POTENTIAL PRODUCTS FROM ANAEROBIC PROCESSES ........................................ 23

4.1 Biofuels from Dark Fermentation of Lignocellulosic Biomass ........................................ 23

  4.1.1 Biohydrogen ............................................................................................................. 24

  4.1.2 Biomethane .............................................................................................................. 27

4.2 Biochemicals and Biopolymers ....................................................................................... 28
### 4.2.1 Volatile Fatty Acids .......................................................... 28

### 4.2.2 Polyhydroxyalkanoates (PHA) ................................................. 32

### 4.2.3 Lignin ........................................................................ 34

### 5 MATERIALS AND METHODS ......................................................... 37

#### 5.1 Biomass and Digestate ............................................................... 37

#### 5.2 Characterisation of the Substrates, Effluents and Digestates of the Anaerobic Processes ................................................................................. 37

#### 5.2.1 Solid Content .................................................................... 37

#### 5.2.2 Determination of the Lignocellulosic Components of Biomass ................................................................. 39

#### 5.2.3 Klasson Lignin Determination .................................................. 44

#### 5.2.4 Dry Matter and Total Ash Content ........................................... 46

#### 5.2.5 Robustness of the procedures for the determination of the lignocellulosic components of biomass ......................................................... 47

#### 5.2.6 Carbohydrates .................................................................. 47

#### 5.2.7 Protein Content .................................................................. 48

#### 5.2.8 Volatile Fatty Acids ............................................................... 48

#### 5.2.9 Soluble Chemical Oxygen Demand Determination ................................................................. 49

#### 5.2.10 Cation and Anion Analyses ................................................... 49

#### 5.2.11 Elemental Analysis ............................................................... 50

#### 5.2.12 Cellulase Enzyme Assay ....................................................... 50

#### 5.2.13 Characterization of Lignin by Fourier Transform Infrared spectroscopy (FT-IR) ................................................................. 51

#### 5.2.14 PHA Quantification ............................................................... 52

#### 5.2.15 Total suspended solids .......................................................... 53

#### 5.3 Substrates ........................................................................ 53

#### 5.3.1 Sucrose ........................................................................ 53

#### 5.3.2 Perennial Rye Grass ............................................................. 54
6.3 Effect of Structural Components of Lignocellulosic Biomass on Bio-Hydrogen and Bio-Methane Anaerobic Digestion (Results and Discussion - Experiment CA)......78
6.4 Comparative Analysis of the Degradation of Lignocellulose in Single-Stage AD and Two-Stage Anaerobic Process (Results and Discussion - Experiment CA1 and CA2) 85
6.4.1 Perennial Rye Grass Substrate.................................................................85
6.4.2 Wheat-feed Substrate.................................................................................86
6.5 The Role of Lignin in the Lignocellulosic Component Degradation in Anaerobic Digestion (Results and Discussion - Experiment CA1 and CA2). .......................89
6.6 Chapter Conclusion ........................................................................................92
7 ENHANCING THE YIELDS OF BIOHYDROGEN AND VOLATILE FATTY ACIDS FROM PERENNIAL RYE GRASS IN ACIDOGENIC FERMENTATION ...............94
7.1 Principle and Hypotheses of Study.................................................................94
7.2 Development of Effective Enzymatic Technique for Solubilisation of Recalcitrant Lignocellulosic Biomass (Results and Discussion - Experiment PT1) ......................95
7.2.1 Results of Experiment PT1.1: Cellulase Pre-treatment...............................97
7.2.2 Results of Experiment PT1.2: Xylanase Pre-Treatment...............................99
7.2.3 Results of Experiment P1.3: Ferulic Acid Esterase (FAE) .........................100
7.2.4 Results of Experiment P1.4: Enzyme Combinations ...............................101
7.2.5 Selection of Suitable Enzyme Cocktail Concentration for Effective Solubilisation of Perennial Rye Grass................................................................................103
7.2.6 The role of alkaline pre-treatment in the homogeneity of the perennial rye grass substrate........................................................................................................104
7.3 Enhancement of Acidogenic Lignocellulosic Fermentation due to Pre-treatment (Results and Discussion – Experiment HAG-U, HAG-ALK and HAG-ALKENZ) 108
7.3.1 The Role of Alkaline Pre-treatment in Biohydrogen Fermentation ............109
7.3.2 Effect of Enzymatic Simultaneous Saccharification and Fermentation on Lignocellulosic Degradation.............................................................................112
7.3.3 Biohydrogen Yield .................................................................115

7.3.4 Enhancement of Volatile Fatty Acids Production during Acidogenic
Fermentation ..............................................................................121

7.4 Chapter Conclusions ................................................................126

8 BIOFUELS AND BIOCHEMICALS FROM ACIDOGENIC ANAEROBIC
FERMENTATION OF LIGNOCELLULOSIC BIOMASS .........................127

8.1 Principle and Hypotheses of Study .............................................127

8.2 Results and Discussion – Experiment BMP: Effect of Enhanced Acidogenesis on
Bio-methane Fermentation ..........................................................128

8.2.1 Residual composition of perennial rye grass digestate ...............132

8.3 Effect of Enzymatically Enhanced Hydrolysis of Lignocellulose on
Polyhydroxyalkanoate (PHA) Production (Discussion of Results – Experiment PB)
133

8.3.1 The Pattern of Utilisation of Volatile Fatty Acids in the Perennial Rye Grass
Hydrolysate during PHA Bio-synthesis by Cupriavidus necator ............137

8.3.2 The Pattern of Utilisation of soluble carbohydrates in a culture of Cupriavidus
necator fed with the HAG-ALKENZ effluent for PHA production ............139

8.4 Lignin as a Potential Product from Anaerobic Digestion .................144

8.4.1 Lignin Determination Technique .............................................145

8.4.2 Lignin Biorefinery (Results and Discussion of Experiment L) ........149

8.4.3 Characterization of extracted lignin using FT-IR ..........................150

8.5 Economic Viability of the Secondary Processes that can be integrated with the
Acidogenic Processes .....................................................................155

8.5.1 Biofuels ...............................................................................155

8.5.1 Finding Alternative Uses for the product of the Acidogenic fermentation ...158

8.6 Chapter Conclusions ..................................................................163

9 CONCLUSIONS ............................................................................165

10 FUTURE WORK .............................................................................168
LIST OF FIGURES

Figure 3.1: Structure of lignocellulose ................................................................. 16
Figure 5.1: Refluxing unit used in NDF and ADF analyses ..................................... 40
Figure 5.2a: Photograph of mechanical press with mortar and pestle ..................... 52
Figure 5.2b: Photograph of KBr disc for FTIR measurement .................................. 52
Figure 5.3: Schematic illustration of the biohydrogen reactor. ................................. 56
Figure 5.4: Photograph of the biohydrogen reactor set-up .................................... 56
Figure 5.5: Schematic illustration of the BMP reactor ............................................ 59
Figure 5.6: Photograph of the Bioprocess control AMPTS II .................................. 59
Figure 5.7: Schematic illustration of the experimental plan ..................................... 69
Figure 5.8: Schematic illustration of the experimental plan ..................................... 70
Figure 5.9: Schematic illustration of the experimental plan ..................................... 71
Figure 5.10: Schematic illustration of the experimental plan ................................... 72
Figure 6.1a: Perennial rye grass composition before and after anaerobic digestion ...... 88
Figure 6.1b: The composition of wheat-feed before and after anaerobic digestion ...... 88
Figure 7.1: Additional soluble COD released from dried perennial rye grass due to cellulase enzyme .................................................................................... 98
Figure 7.2: Additional soluble COD released from perennial rye grass due to xylanase enzyme .................................................................................... 100
Figure 7.3: Additional soluble COD released from perennial rye grass due to Ferulic Acid Esterase (FAE) enzyme ................................................................. 101
Figure 7.4: Enzyme combinations actual yield versus expected yield (sum of individual activities) ................................................................. 102

Figure 7.5: Release of soluble substrates due to enzyme concentration of 0.20ml/gVS of perennial rye grass (0.8% Cellulase ± 0.15 FAE) ................................................................. 104

Figure 7.6: Untreated perennial rye grass in water ................................................................................. 106

Figure 7.7: Effect of pre-treatment on dried perennial rye grass ................................................................. 107

Figure 7.8: Effect of alkaline pre-treatment on fresh perennial rye grass ................................................. 107

Figure 7.9: Production of biohydrogen due to Chemical and Biochemical Pre-treatment of perennial rye grass substrate ....................................................................................... 117

Figure 7.10: Comparative yield of hydrogen of biochemically pre-treated perennial rye grass, 10g/l sucrose and the residual sugars present in the enzyme cocktail ........................................... 118

Figure 7.11: Comparative yield of Total VFAs due to pre-treatments ....................................................... 122

Figure 7.12: VFAs proportions in a. HAG-U_{effluent}, b. HAG-ALK_{effluent}, c. HAG-ALKENZ_{effluent} ..................................................................................................................... 124

Figure 8.1: Cumulative methane production during BMP test ................................................................. 131

Figure 8.2: Methane yield from BMP assay ......................................................................................... 131

Figure 8.3: Digestate composition after batch anaerobic digestion process .............................................. 133

Figure 8.4: Profile of online capacitance (pF/cm) and PHA in a culture of Cupriavidus necator fed with the HAG-ALKENZ_{effluent} ..................................................................................... 136

Figure 8.5: Profile of optical density, cell dry weight and PHA in a culture of Cupriavidus necator fed with the HAG-ALKENZ_{effluent} ..................................................................................... 136

Figure 8.6a: Pattern of utilisation of VFAs in a culture of *Cupriavidus necator* fed with the HAG-ALKENZ_{effluent} for PHA production .................................................................................. 138

Figure 8.6b: Pattern of utilisation of VFAs in a culture of *Cupriavidus necator* fed with the HAG-ALKENZ_{effluent} for PHA production .................................................................................. 139

Figure 8.7: Profile of Total COD and soluble carbohydrates in a culture of *Cupriavidus necator* fed with HAG-ALKENZ_{effluent} for PHA production ......................................................... 141
Figure 8.8: Utilisation of soluble carbohydrates in a culture of *Cupriavidus necator* fed with HAG-ALKENZeffluent for PHA production ............................................................................................................ 142

Figure 8.9: Profile of fructooligosaccharides metabolism in a culture of *Cupriavidus necator* fed with HAG-ALKENZeffluent for PHA production ............................................................................................................ 144

Figure 8.10: Lignin determination from HAG-Usubstrate comparing ADL and Klasson procedures (ASL and AIL) ...................................................................................................................................... 147

Figure 8.11: Lignin determination from HAG-ALKeffluent comparing ADL and Klasson procedures (ASL – Acid Soluble Lignin; AIL - Acid Insoluble Lignin) ............................................................................................................ 148

Figure 8.12: FTIR spectra of dried perennial rye grass. ...................................................................................................................................... 153

Figure 8.13: FT-IR spectra of extracted lignin relative to dried perennial rye grass and standard alkali lignin ...................................................................................................................................... 153

Figure 8.14: Photograph of extracted lignin tablets ...................................................................................................................................... 155

Figure 8.15: Suggested approach for lignocellulose bioconversion to biofuels and biochemicals ...................................................................................................................................... 162
LIST OF TABLES

Table 2.1: Pathways of degradation of organic monomers in the acidogenic phase ..........7
Table 2.2: Acetogenic metabolic processes .....................................................................8
Table 4.1a: Biomass Sources of VFA ..........................................................................30
Table 4.1b: Biomass Sources of VFA ..........................................................................31
Table 4.2: The current principal markets of lignosulphonate ......................................35
Table 5.1: Reagents of Neutral Detergent Fibre Solution and their Function ...............40
Table 5.2: Average values of total and volatile solid content of biomass in experimental period ........................................................................................................................................60
Table 6.1: Correlation between NDF and CHNSO in perennial rye grass ..................75
Table 6.2: Correlation between NDF and CHNSO in Wheat-feed .................................76
Table 6.3: Compositional Changes of the Perennial Rye Grass Substrate in the Anaerobic Digestion Processes ..........................................................................................................................83
Table 6.4: Compositional Changes of the Wheat-feed Substrate in the Anaerobic Digestion Processes ..........................................................................................................................84
Table 7.1: Experimental setup- enzyme proportions based on unit enzyme activity ....96
.............................................................................................................................................Error! Bookmark not defined.
Table 7.2: Typical characteristics of the seed sludge ..................................................108
Table 7.3: The Effect of Alkaline Pre-treatment on Biohydrogen Fermentation ..........110
Table 7.4: Biochemical Component of Effluents of Anaerobic Bioreactors ...............120
.............................................................................................................................................Error! Bookmark not defined.
Table 8.1: Lignin Determination Procedures: comparative yield ................................149
Table 8.2: Characteristics of lignin extracted from the effluents of acidogenic fermentation ..................................................................................................................................................150
Table 8.3: Economic evaluation of the anaerobic fermentation processes ...............157
LIST OF EQUATIONS

Equation 1 ................................................................................................................. 9
Equation 2 ................................................................................................................. 9
Equation 3 ................................................................................................................... 25
Equation 4 ................................................................................................................... 25
Equation 5 ................................................................................................................... 38
Equation 6 ................................................................................................................... 38
Equation 7 ................................................................................................................... 38
Equation 8 ................................................................................................................... 39
Equation 9 ................................................................................................................... 41
Equation 10 ............................................................................................................... 42
Equation 11 ............................................................................................................... 43
Equation 12 ............................................................................................................... 44
Equation 13 ............................................................................................................... 44
Equation 14 ............................................................................................................... 44
Equation 15 ............................................................................................................... 45
Equation 16 ............................................................................................................... 45
Equation 17 ............................................................................................................... 46
Equation 18 ............................................................................................................... 46
Equation 19 ............................................................................................................... 53
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AD</td>
<td>anaerobic digestion</td>
<td>HRT</td>
<td>hydraulic retention time</td>
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<td>ADF</td>
<td>acid detergent fibre</td>
<td>IPCC</td>
<td>Intergovernmental Panel on Climate Change</td>
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<tr>
<td>ADL</td>
<td>acid detergent lignin</td>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
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<td>AIL</td>
<td>acid insoluble lignin</td>
<td>NDF</td>
<td>neutral detergent fibre</td>
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<tr>
<td>ASL</td>
<td>acid soluble lignin</td>
<td>OD</td>
<td>Optical Density</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
<td>OLR</td>
<td>organic loading rate</td>
</tr>
<tr>
<td>BA</td>
<td>bicarbonate alkalinity</td>
<td>PHA</td>
<td>polyhydroxyalkanoates</td>
</tr>
<tr>
<td>CDW</td>
<td>Cell dry weight</td>
<td>SSF</td>
<td>Simultaneous saccharification and fermentation</td>
</tr>
<tr>
<td>COD</td>
<td>chemical oxygen demand</td>
<td>TS</td>
<td>total solids</td>
</tr>
<tr>
<td>CSTR</td>
<td>continuously stirred tank reactor</td>
<td>TSS</td>
<td>total suspended solids</td>
</tr>
<tr>
<td>FID</td>
<td>flame ionisation detector</td>
<td>VFA</td>
<td>volatile fatty acid</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatograph</td>
<td>VFAs</td>
<td>volatile fatty acids</td>
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<tr>
<td>HPLC</td>
<td>high performance liquid</td>
<td></td>
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<tr>
<td></td>
<td>chromatography</td>
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<td>VS</td>
<td>volatile solids</td>
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ABSTRACT

This study investigated the fate of lignocellulosic biomass (wheat-feed and perennial rye grass) in different anaerobic digestion systems, evaluating the role of substrate specificity on the pattern of degradation. The two-stage (biohydrogen-biomethane) anaerobic system was found to be more effective in the degradation of lignocellulose, when compared to the conventional single-stage system. The perennial rye grass substrate possessed about 21% higher holocellulose concentration when compared to the wheat-feed; its exploitation in the acidogenic digestion was however poor, resulting in a 2.9% lower biogas yield in an equivalent two-stage system. The study therefore developed a treatment technique involving the use of cellulase and ferulic acid esterase enzyme combinations for the treatment of perennial rye grass. The enzyme cocktail at 0.202 ml enzyme/g VS added resulted in efficient bioconversion of the complex polymers to soluble carbohydrates, evident in the yield increase of soluble COD, to 321.0±10.9 mg/gVS, a 393.2% yield increase, when compared to the no enzyme added control. The yield of bio-hydrogen after enzymatic addition was 48ml/gVS, 335% higher when compared to the alkaline treatment; and more than seven fold higher than the yield obtained from the fermentation with no pre-treatment. The acetate to butyrate ratio varied from 4:1, when no pre-treatment was used, to 2:1 when alkaline pre-treatment was used, then to 1:1 after the enzymatic treatment. The downstream effect of the prior hydrolysis on the subsequent processes to acidogenic fermentation like biomethane and PHA production and lignin recovery were also investigated. The hydrogenic/acidogenic fermentation resulted in methane yield improvement of 45.7%. The study shows that the more effective a hydrolysis procedure is in the depolymerisation of complex polymers, the greater the accumulation of PHA in the PHA biosynthesis operations. The enhanced hydrogenic/acidogenic fermentation having effectively degraded the holocellulose component of the perennial rye grass substrate ensured that relatively high quality lignin was obtained in an Organosolv lignin-extraction procedure. FT-IR profile show less contamination of polysaccharides and proteins in the lignin extracted from the enzymatically enhanced acidogenic fermentation. An evaluation of the economic viability of the investigated secondary processes showed that direct integrations of those processes to the biohydrogen process may not be as economically advantageous, when compared to a 2nd - stage biomethanation system.
ACKNOWLEDGEMENTS

I wish to express my deep gratitude to Prof. Richard Dinsdale, my director of study whose kind but firm direction followed the project from inception to completion. I am also sincerely grateful to Prof. Alan Guwy, my supervisor, who offered valuable insights and research guidance to the current study. I also want to express my profound gratitude to my other supervisor, Dr. James Reed, for his guidance, suggestions and editorial comments. I would like to also express my heartfelt appreciation to my line manager, Dr. Sandra Esteves without whom I could not have completed this project. My sincere thanks also go to Dr. Gregg Williams, Dr. Jaime Massanet-Nicolau, Dr. Des Devlin, and Dr. Shee Ming Thai for their technical assistance.

Special thanks go to Garry Shipley, who provided effluent and digestate from his two-stage (biohydrogen-biomethane) anaerobic process for compositional analysis, and to Pearl Passanha for the quantification of the amount of PHA after the biosynthesis procedure. I also want to thank Dr. Amandeep Kaur, Dr. Katrin Fradler and Mr. Savvas Savvas for their immense support during the study.

I also wish to express my sincere gratitude and appreciation to ERDF for funding the H₂ Wales project and A4B for funding CIRP; without which my dreams would not have materialised.
CHAPTER 1

1. INTRODUCTION

Lignocellulosic biomass stands out as one of the most important renewable carbon sources for energy in the world today. The history of the use of lignocellulosic biomass as an energy source goes beyond the pre-industrialization times; and it is currently the most important energy source in developing countries (Braun et al., 2009). Though the potential of lignocellulosic biomass as an energy source is fundamentally established, its commercial exploitation has been relatively minimal in developed countries such as the UK. Reports on the use of biomass in 1996 showed that in the UK, just a small fraction of the annual production (approximately 6 million tonnes) of recoverable cereal straw was used as feed for animals, with the rest being disposed of by burning (Hawkes et al., 2008; Lawther et al., 1996). It is important to say, however, that in recent times, scientific research and development into the adequate exploitation of the biomass resource as a renewable energy source, has received significant political backing in many countries. This has become increasingly important, largely due to the potential economic instability and consequential greenhouse gas emissions attributed to the use of fossil fuels.

The earth’s atmospheric equilibrium has notably changed over the past 15 decades, as a result of increases in the concentration of greenhouse gases; leading to about 0.74°C rise in the global surface temperature in the twentieth century (Dai, 2013; IPCC, 2007). With fossil fuels being the major source of the discharge of greenhouse gas into the atmosphere, its replacement by an alternative energy vector is of critical importance. The United Nations Intergovernmental Panel on Climate Change (IPCC), for example, is promoting the reduction of greenhouse gases emission in the G8 countries by more than 50% by the year 2050, when compared with the greenhouse gases levels at year 1990 (IPCC, 2014).

The search for renewable and sustainable alternative fuels to replace fossil fuels and their harmful emissions has intensified progressively in the last two decades. Bioenergy in particular, among other sources of renewable energies like wind, solar and hydroelectricity, has been identified as having the potential to play an important role in meeting the energy challenges, due to its availability (Menon and Rao, 2012).
Conventional approaches for the conversion of lignocellulosic biomass to energy include both thermal (combustion, pyrolysis, and gasification) and biochemical (fermentation and anaerobic digestion) processes. Among these technologies, anaerobic digestion stands out as the most promising technology necessary for effective conversion of higher moisture content lignocellulose biomass to energy. The biogas produced from the anaerobic process has the advantage of being able to be used directly or cleaned-up for distribution in either compressed cylinders or natural gas pipe networks. The digestate because of its rheology, nutrient availability to plants, and organic carbon content, can also be used as a soil improver and fertilizer (Pain and Hepherd, 1985; Tafdrup, 1995).

In the UK, 4.8-5.7 Mtoe (million tonnes of oil equivalent) of biomass resources has been identified as being available for bioenergy production (Defra, 2005). Grass, for example, has been found to be the most abundant potential anaerobic feedstock in the UK. The permanent grassland (over 5 years old) is estimated to be 1.36 million ha in Scotland (Scottish Agriculture Census, 2009); 3.22 million ha in England (Defra, 2010); 0.68 million ha in Northern Ireland (DARD, 2013), and about 1.05 million hectares in Wales (Welsh Agriculture Statistical Survey, 2012). The potential of anaerobic digestion of lignocellulosic biomass in the UK is therefore highly positive.

Hawkes et al. (2007) highlighted that that potential use of lignocellulosic biomass cannot be adequately exploited unless a viable pre-treatment stage to solubilise the lignocellulose complex is adopted. Pre-treatment is therefore seen as the potential way forward for the improvement of efficiency of lignocellulose degradation in anaerobic digestion (Kohlman et al., 1995; Lynd et al., 1996; Mosier et al., 2003). The dark fermentation of lignocellulosic biomass to produce biohydrogen, due to its reduced emissions, was identified by Martinez-Perez et al. (2006) as one of the important approaches to achieving the Europe and European Commission’s target of achieving 10% of the transport fuel from biomass by 2020 (Directive 2009/30/EC). The potential yield of biohydrogen from lignocellulosic biomass is however indirectly linked to the effectiveness of an initial pre-treatment process applied to a particular substrate.

The current study attempts to obtain a viable pre-treatment approach that has the potential of enhancing the technical and practical aspects of the yield of biohydrogen from lignocellulose
substrates and its downstream effect on the products that can be obtained from the biohydrogen and anaerobic digestion process.

1.1 Aims of the research

The anaerobic digestion of lignocellulosic biomass, using the two-stage (hydrogenic/acidogenic-methanogenic) anaerobic digestion process, with effective pre-treatment, is considered as an important technique for potentially increasing the energy recovery from the biomass resource. The overall aim of the current study was to critically review the potential of lignocellulosic biomass as a substrate for anaerobic digestion; investigate the challenges posed by its complex structure in anaerobic digestion systems, and evaluates the range of products that can potentially be obtained from the fermentation of lignocellulosic substrates.

The major aims of this project are:

1. To investigate the fate of two lignocellulose substrates (wheat-feed and dried perennial rye grass (*Lolium perenne*)) in single-stage and two-stage biohydrogen and methane/anaerobic processes.

   *Objectives:*
   
   - To evaluate how a separated acid-phase affects anaerobic degradation of wheat-feed and perennial rye grass, and the extent to which the degradation efficiency achieved in the digestion of both lignocellulosic substrates differ.
   - To examine how the pattern of degradation of the lignocellulosic complex in the two substrates (wheat-feed and perennial rye grass) correlates with their corresponding biogas yields in the anaerobic digestion processes.

2. To optimise biohydrogen production from perennial rye grass by tackling the limiting factor posed by the recalcitrance of the lignocellulosic complex through pre-treatment
Objectives

- To determine an ideal enzyme concentration that has the potential of effectively accelerating the hydrolysis of the complex polysaccharides of the perennial rye grass to soluble carbohydrates.

- To evaluate the extent to which alkaline pre-treatment and enzymatic simultaneous saccharification and fermentation (SSF) enhance the hydrogenic/acidogenic fermentation of the *Lolium perenne* for the production of biohydrogen and volatile fatty acids (VFAs).

3. To evaluate the downstream effect of enhanced hydrolysis, through pre-treatment, on processes that can be integrated to the biohydrogen process, such as methane production, PHA biosynthesis and lignin biorefinery.

Objectives:

- To evaluate the downstream effect of the enhanced enzymatic hydrolysis on a second-stage biomethane process, the production of polyhydroxyalkanoates (PHA), and the lignin biorefinery from the acidogenic effluent.

- To investigate the economic viability of integrating the biohydrogen fermentation process directly with PHA or lignin biorefinery.
CHAPTER 2

2. ANAEROBIC DIGESTION PROCESSES

2.1 Microbiology of Anaerobic digestion

The anaerobic degradation of biomass to produce biofuels (either hydrogen or methane biogas) is a complex process (particularly for methane production), needing a number of syntrophic interrelations of different groups of bacteria and archaea, to convert complex organic molecules to methane and carbon dioxide. The microbial fermentation process has been categorized into four main phases. Each of the four phases have specific environmental conditions for optimal performance and carried out by different group of bacteria in the syntrophic consortium. The phases of degradation include hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Deublein and Steinhauser, 2008; Mosey, 1983). Some of the sensitive parameters that define the ideal environmental condition for the fermentation process include pH, redox and hydrogen partial pressure.

2.1.1 Hydrolysis

The hydrolysis phase (first phase) involves the exo-enzymatic degradation of particulate organic materials, such as proteins, cellulose and fats into monomers by facultative and obligate bacteria, referred to as hydrolytic bacteria. As highlighted by Deublein and Steinhauser (2008), the mode of mechanism of the hydrolytic bacteria involves the release of hydrolases - exoenzymes (extracellular enzymes), which catalyse a reaction where covalent bonds of the complex compound are split with water. The oxygen dissolved in the water is utilised by the facultative anaerobes, resulting in a low redox potential environment suitable for the activities of the obligate anaerobes.

Some of the hydrolytic bacteria identified by researchers to be involved in the hydrolysis process include those from genera *Clostridium*, *Bacillus*, *Staphylococcus*, *Cellulomonas*, and *Mycobacterium*. During the hydrolysis process, complex polysaccharides are converted to short chain oligosaccharides and sugars by saccharolytic bacteria; proteins to amino acids by
proteolytic bacteria, and lipids to fatty acids and glycerol by lipolytic bacteria (Stronach et al., 1986).

The rate and degree of hydrolysis is substrate specific. The hydrolysis of carbohydrates has been found to occur in a few hours, whilst the hydrolysis of proteins and lipids could take a couple of days to complete. As described by Deublein and Steinhauer (2008), the recalcitrant nature of lignocellulose and lignin makes it difficult to be degraded, resulting in slow and incomplete degradation. When biologically recalcitrant substrates are considered, the rate-limiting step for the anaerobic degradation process is the hydrolysis phase.

Other factors that also affect the rate and degree of hydrolysis include pH, particle size, enzyme production, and diffusion and rate of adsorption of enzymes to the substrate (Bengtsson et al., 2008, Dabrock et al., 1992).

2.1.2 Acidogenic Phase

The acidogenic phase involves the breakdown of the monomers produced in the hydrolysis phase by acidogenic bacteria (facultative and obligate anaerobes) into hydrogen, carbon dioxide, ammonia, VFAs (C1-C5 molecules), lactate, alcohol, H₂S, and other by-products such as humic acids (Appels et al., 2008). As noted by McCarty and Mosey (1991), the particular species and proportions of the products of the acidogenic phase, are highly dependent on the concentration of the intermediates such as hydrogen and the pH. For example, as the partial pressure of hydrogen increases, the production of reduced compounds like acetate decreases. Mosey (1983) reported that the production of acetate is favoured at a partial pressure of 10⁻³ atm and lower. Deublein and Steinhauer (2008) also emphasized that a favourable partial pressure suitable for a specific microbial species is dependent on the type of substrate. As highlighted by McCarty and Mosey (1991), the production pathway by NADH is more favourable to the production of propionic acid at neutral pH; the pathway however tends to skew towards the production of butyrate as the environment becomes more acidic. A stable anaerobic environmental condition in the anaerobic fermenter is however maintained by syntrophic relationship with the hydrogen utilising methanogens (Harper and Pohland, 1986). Table 2.1 shows the degradation of organic monomers to acidogenic products.
Some of the important factors that influence the acidogenic fermentation process include interspecies hydrogen transfer, pH, hydraulic retention time and previous acclimation of the anaerobic culture.

### Table 2.1: Pathways of degradation of organic monomers in the acidogenic phase

<table>
<thead>
<tr>
<th>Organic Monomers</th>
<th>Degradation Pathway</th>
<th>Acidogenic product</th>
<th>Representative Bacteria Genus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrates</td>
<td>Succinate; acrylic pathway</td>
<td>propionic acid</td>
<td>Propionibacterium</td>
</tr>
<tr>
<td></td>
<td>butyric acid pathway</td>
<td>butyric acid</td>
<td>Clostridium</td>
</tr>
<tr>
<td>Fatty acids:</td>
<td>β - oxidation</td>
<td>acetic acid</td>
<td>Acetobacter</td>
</tr>
<tr>
<td>Amino acids</td>
<td>Stickland reaction</td>
<td>acetate, ammonia, CO₂ and H₂S</td>
<td>Clostridium botulinum</td>
</tr>
</tbody>
</table>

(Adapted from Deublein and Steinhauser, 2008)

### 2.1.3 Acetogenic phase

The Acetogenic phase involves the endogenic activities of acetogenic (obligate anaerobes) archaea, which convert the organic products from the acidogenesis stage to acetic acid. The symbiotic relation of the acidogens and the methanogens, where the methanogen through interspecies hydrogen transfer utilise the hydrogen by-product of the acidogens, is highly important in the anaerobic digestion process. This is because the methanogens can only metabolise a limited range of substrates such as hydrogen and acetate. The exergonic H₂ and CO₂ produced from the acidogenic phase are also constantly reduced to acetic acid in the acetogenic phase by the homoacetogens (Deublein and Steinhauser, 2008). The conversion of ethanol, propionic and butyric acids to acetate and hydrogen are endergonic and therefore energetically unfavourable. The energy used or released during acetogenic reactions (the Gibbs free energies) is substrate specific. The degradation of propionic acid for example occurs at Δ\( \dot{G}_0 \) = +76.1 KJ mol⁻¹; the degradation of butyric acid at Δ\( \dot{G}_0 \) = +48.1 KJ mol⁻¹; and ethanol at Δ\( \dot{G}_0 \) = +9.6 KJ mol⁻¹ (van Lier et al., 1993). The acidogenic degradation via
oxidation of long-chain fatty acids to acetic acids is only thermodynamically possible at significantly low hydrogen partial pressures. The microbial activities of the methanogenic anaerobe, on the other hand, can occur at high hydrogen concentrations. The syntrophic relationship between the acetate and methanogenic microbes ensures that the products of the acetogenic reaction are constantly removed by the methanogens. The metabolic activity of methanogens therefore provides a conducive environment for acetogenic growth by reducing local hydrogen partial pressures.

The range of hydrogen partial pressures deemed acceptable for acetogenesis has been found to be dependent on the species of bacteria involved, as well as the type of substrates been used. Nearly 95% of the methane produced by the acetoclastic methanogens (acetate utilising methanogens) is formed from direct uptake of hydrogen through the syntrophic relationship. The other 5% is attributed to the reduction of the CO₂ by dissolved hydrogen (Deublein and Steinhauser, 2008).

**Table 2.2: Acetogenic metabolic processes**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propionic acid</td>
<td>CH₃(CH₂)COOH + 2H₂O → CH₃COOH + CO₂ + 3H₂</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>CH₃(CH₂)₂COO⁻ + 2H₂O → 2CH₃COO⁻ + H⁺ + 2H₂</td>
</tr>
<tr>
<td>Valeric acid</td>
<td>CH₃(CH₂)₃COOH + 2H₂O → CH₃COO⁻ + CH₃CH₂COOH + H⁺ + 2H₂</td>
</tr>
<tr>
<td>Isovaleric acid</td>
<td>(CH₃)₂CHCH₂COO⁻ + HCO₃⁻ + H₂O → 3CH₃COO⁻ + H₂ + H⁺</td>
</tr>
<tr>
<td>Capronic acid</td>
<td>CH₃(CH₂)₄COOH + 4H₂O → 3CH₃COO⁻ + H⁺ + 5H₂</td>
</tr>
<tr>
<td>Carbon dioxide / hydrogen</td>
<td>2CO₂ + 4H₂ → CH₃COO⁻ + H⁺ + 2H₂O</td>
</tr>
<tr>
<td>Glycerine</td>
<td>C₃H₅O₃ + H₂O → CH₃COOH + 3H₂ + CO₂</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>CH₃CHOHCOO⁻ + 2H₂O → CH₃COO⁻ + HCO₃⁻ + H⁺ + 2H₂</td>
</tr>
<tr>
<td>Ethanol</td>
<td>CH₃(CH₂)OH + H₂O → CH₃COOH + 2H₂</td>
</tr>
</tbody>
</table>
2.1.4 Methanogenic phase

The methanogenic phase is the final stage of the anaerobic digestion process, requiring strict anaerobic conditions. Sahm (1984) reported that a redox potential as low as -330 mV is required for the methanogenic degradation to occur. Some of the major substrates that can be accessed by the methanogens include H₂, acetate, formate, methanol and methylamine. Since methanogens can only utilise a limited number of substrates (by-products of the acidogens), their function is directly dependent on the acidogenic metabolic activities. An optimal performance of the acidogenic phase could potentially lead to an optimal yield in the methanogenic phase. In cases where the acidogenic bacteria have a symbiotic relationship with other organisms instead of the methanogens, the H₂ is competed for, often leading to a reduction in digester performance, as the methanogens are deprived of the hydrogen source. The relationship of the acidogen with some of these competitive hydrogen utilising microorganisms, result in the reduction of sulphate to hydrogen sulphide, which has been found to be inhibitory to the metabolic activities of the methanogens. Some of the sulphate-reducing bacteria identified by researchers include those of the genera Desulfococcus, Desulfsarcina, Desulfobacter, Desulfuromonas, Desulfobulbus, Desulfovibrio, Desulfonema and Desulfotomaculum (Deublein and Steinhauser, 2008).

Two main groups of bacteria are involved in the methanogenic reaction. These are: the acetoclastic methanogens which produces methane by oxidizing acetate (eqn. 1), and the lithotrophic (hydrogen utilising) methanogens which involve the reduction of CO₂ to form methane (eqn. 2)

\[ \text{CH}_3\text{COOH} \leftrightarrow \text{CH}_4 + \text{CO}_2 \text{ at } \Delta \text{G}_0 = -31 \text{ KJ mol}^{-1} \quad \text{Equation 1} \]
\[ \text{CO}_2 + 4\text{H}_2 \leftrightarrow \text{CH}_4 + 2\text{H}_2\text{O} \text{ at } \Delta \text{G}_0 = -131 \text{ KJ mol}^{-1} \quad \text{Equation 2} \]

As shown in the Equation 2, the methanogenic reaction involving the reduction of CO₂ and H₂ is comparatively more exergonic than the oxidation of acetic acid, it is however responsible for only 27-30% to the total methane produced. Nearly 70% of the total methane production is attributed to the activities of acetoclastic methanogens (Deublein and Steinhauser, 2008). The acetoclastic methanogens including those from the genera Methanosarcina and Methanobacterium, both have low theoretical growth rate (with a long generation time of nearly 100 hours).
2.2 Biochemistry and Microbial Adaption in the Anaerobic Digestion process

2.2.1 The Hydrogenic/Acidogenic Fermentation System

The hydrogenic dark fermentation system involves both obligate and facultative bacteria that catalyse the production of VFA, hydrogen (H$_2$) and carbon dioxide (CO$_2$) using organic carbon as substrate (Venkata Mohan, 2009; Hallenbeck and Benemann, 2002; Hawkes et al., 2008). The metabolic activity of the hydrolytic and acidogenic bacteria during the dark fermentation process increases the availability of the hydrogen ions (H$^+$) and electrons, important precursors for the production of H$_2$.

The electron transport chain in the bacteria system is governed by redox mediators (NAD$^+/$/FAD$^+$) and membrane bound proteins (NADH dehydrogenase and cytochrome bc1). The H$^+$ released from NADH/FADH in the presence of the NADH dehydrogenase and electron undergoes series of inter-conversions involving the transfer of the electrons to the cytochrome bc1 complex, and the H$^+$ to the inter-membrane space. The electron from the cytochrome bc1 becomes excited and is transferred to the protein ferredoxin where hydrogenases catalyse the reduction to H$_2$. (Vardar-Schara et al., 2008)

VFAs and CO$_2$ are the major by-products of the biohydrogen production process. The anaerobic system that maintains active hydrolytic and acid-forming microbial consortium in one reactor; and acetogenic and methanogenic microbes in a second reactor, has been suggested to be a promising and practical option for scaling up the efficiency of the conversion of biomass to energy. A number of published papers show the two-stage anaerobic system as the most promising biological approach for efficient energy recovery from lignocellulosic biomass (Parawira et al., 2005; Yu et al., 2002).

The hydrogen-producing bacteria are able to form protective spores under extreme environmental conditions, such as high temperature, high acidity and alkalinity. The inability of the methanogens to form spores ensures that only the acid formers are selected in the inoculums by thermal pretreatment. The application of this pre-treatment technique helps to prevent competitive growth and co-existence of other H$_2$ utilising bacteria (Venkata Mohan and Goud, 2012).
2.2.2 Biomethane System

The methanogenic anaerobes are methane producers encompassing two groups of microorganisms belonging to the Archaea domain, exhibiting synergistic relationship with other groups of microorganisms (Shah et al., 2014). The methanogens based on the carbon source used as substrates are capable of functioning as heterotrophs or autotrophs, producing CH₄ as the main end product of the biomethanisation process (anaerobic digestion) (Prescott et al., 2008). The methanogenesis process involves the reduction of CO₂ using the energy obtained from the inter-conversion of the acidogenic products (H₂, formate, methanol, acetate, CO₂). The metabolic activity involves the coenzyme methanofuran (MFR) and the co-factors tetrahydromethanopterin (H₄MPT) and F430 (nickel tetrahydromethanopterin).

During the methanogenesis process, inorganic molecules such as H₂ and formate are oxidised to release electrons and protons. The electrons released from the electron donors are carried through a membrane bound transport chain, which catalyses a redox reaction; being coupled with H⁺ translocation across the cytoplasmic membrane (Goldman et al., 2009). The transfer of the electrons across the membrane creates a chemical gradient by which ATP is synthesized through chemiosmosis by the membrane-bound ATPase (Angenent et al., 2004; Jablonski and Ferry, 1992). The release of ATP is very important, as the methanation process requires a lot of energy to reduce CO₂ to CH₄.

Methanogens, being archae, differ extensively from the acidogens both biologically and ecologically. The difference is evident in their physiology, growth rate, nutritional needs and versatility to environmental changes (Damirel and Yenigün, 2002; Gujer and Zehnder, 1983). Though anaerobic digestion allows the co-existence of methanogens and acidogens, research has confirmed that the physical separation of the two anaerobic systems in a two-stage (hydrogenic/acidogenic-methanogenic) system improves the efficiency of the bioconversion of biomass to energy; increasing the net energy gained from the anaerobic process. The two-stage anaerobic system maintains an active hydrolytic and acid-forming microbial consortium in one reactor, and acetogenic and methanogenic anaerobes in a second reactor (Parawira et al., 2005).
3. LIGNOCELLULOSE AS FEEDSTOCK FOR ANAEROBIC DIGESTION

The selection of feedstock is highly important in the performance of an anaerobic digester (Hawkes et al., 2007). To obtain sustainable biohydrogen and/or biomethane production in the anaerobic process, a suitable feedstock must be highly biodegradable, abundant, readily available and available at relatively low cost (Guo et al., 2010). Different feedstocks have different methane and/or hydrogen potentials (Amon et al., 2007). The type and concentration of the feedstock have also been found to have a direct relationship to operational parameters such as the selection of hydraulic retention time (HRT) in the anaerobic digestion process. The success of anaerobic digestion process in the UK, for example, can directly be linked to the availability of feedstocks like: food and farm waste (e.g. animal manure), and sewage sludge (DECC, 2010).

Lignocellulosic biomass is a promising feedstock for anaerobic digestion, due to its high incorporated polysaccharide content and the potential high ultimate methane yield from that substrate (Ward et al., 2008), but often this energy potential is not adequately utilised in typical anaerobic digestion process implementation (Deublein and Steinhauser, 2008). The biochemical and microbiological processes of anaerobic digestion might seem straightforward, however its application to lignocellulosic substrates is however a very complex one. Lignocellulosic biomass is composed of a heterogeneous complex of carbohydrates, interlinked with lignin (made up of units of a complex polymer of phenylpropanoid) (Fengel and Wegener, 1984; Wright et al., 1987). The holocellulose components of biomass have been found an important carbon source, suitable for efficient biogas production; nevertheless, adequate exploitation is not easily realized. The recalcitrance of the lignocellulosic biomass is due to the complex nature of the chemical structure, influenced by chemical factors including the crystallinity of cellulose, salicification and lignification (Tomlin et al., 1965); this typically mean that it makes a poor quality substrate in current anaerobic digestion systems. For example the enzymatic digestibility of untreated lignocellulosic biomass has been found to be 20% below the theoretical potential yield (Sun, 2010). Though the potential of bioconversion lignocellulosic biomass to energy vectors cannot be overemphasized, the difficulty of converting complex lignocellulose substrates to
fermentable sugars poses a huge challenge for the anaerobic digestion of lignocellulose, vis-à-vis its commercialization potential. Martínez-Pérez et al. (2007) emphasized that, biomass substrates required for effective anaerobic degradation must be of low lignin concentration.

3.1 Chemical Structure of Lignocellulosic Biomass

The carbohydrate proportion of lignocellulose is estimated to be around 75% (Jørgensen et al., 2007). The potential release of fermentable sugars from lignocellulose during the anaerobic fermentation processes is not proportional to that fraction, due to the compact and rigid structure of the lignocellulose matrix. Lignocellulosic biomass has been found to be recalcitrant to enzymatic hydrolysis due to the following factors including: cellulose crystallinity, quantity and the spatial inter-linkage of lignin, hemicellulose and pectin, the quantity of acetyl groups, the degree of polymerization, particle size and specific surface area (Chang and Holtzapple, 2000; Sun and Cheng, 2002; Zhu et al., 2008). The degree of recalcitrance of lignocellulose can be assessed by the composition of the plant cell wall i.e. the differences in the types of hemicellulose and the lignin-polysaccharide ratios (Pauly and Keegstra, 2010).

Cellulose consists of β (1-4) linkages that link a chain of D-glucose. The D-glucose chains are linked by strong hydrogen bonds, forming microfibrils in the cell wall (Van Dyk and Pletschke, 2002). The microfibrils are insoluble and their complex framework can directly be linked to the crystalline nature of cellulose and its resistance to enzymatic hydrolysis. Apart from the crystalline cellulose polymorph, there exist amorphous portions in the cellulose structure that are otherwise readily available to hydrolases, and are easily degraded in fermentation process. The cellulose polysaccharides represent 15-30% of the primary cell wall dry mass, and approximately 40% of the secondary cell wall (Sticklen, 2008). The cellulose microfibrils are implanted in a matrix of hemicellulose, lignin and pectin, in the middle lamellae and (in the primary and secondary cell walls) (Eriksson and Bermek, 2009; Raven et al., 1999) lignin-hemicellulose arrays are found in the spaces between the cellulose microfibrils in the cell walls.

Hemicellulose is usually classified as heterogeneous polysaccharides with diverse structure and composition; consisting of a short chain branched polymer of sugars. Hemicellulose polymers include: xylan, galactan, mannan and arabinan. The hemicellulose structure is
composed of 1, 4-linked β-D-hexosyl residues, forming the backbone of the structure. Some of the factors that influence the diversity of hemicellulose include: the type of cell tissue, the type glycosidic linkages, side-chain composition and the degree of polymerisation (Chundawat et al., 2011). The chemical configuration of hemicellulose, for example, is responsible for some of the major difference in softwood and hardwood plants. Whereas softwood contains non-acetylated xylans consisting of α-L-arabinofuranose linked by α-1, 3-glycosidic bonds, the hardwood xylans consist of O-acetyl-4-O-methylglucuronoxylan with significant degree of acetylation (Sunna and Antranikian, 1997).

Pectin is a hydrophilic polysaccharide that is highly varied in structure, with galacturonic acid as its major component; forming part of the intracellular framework of the plant. The presence of the galacturonic acid contributes to the high level of acetylation or methyl-esterification, usually associated with pectin (Willats et al., 2006). The hydrophilic nature of pectin contributes to the flexibility of the plants interaction with water. Other components of pectin include fructose, galactose, arabinose, rhamnose, and apiose (Van Dyk and Pletschke, 2002).

Lignin polymers play an important role of strengthening and protecting the plant against insects and parasites (Sticklen, 2008). The biosynthesis of lignin and deposition occurs in the plant cell wall during xylem formation stage, and the process involves the oxidation of phenolic monomers by enzymes (including, peroxidases and laccases) to form phenoxy radicals, which are then able to spontaneously polymerize (Eriksson and Bermek, 2009). The phenolic monomers that undergo free-radical polymerisation to form basic lignin structure are p-hydroxyphenyls (H), guaiacyls (G), and syringyls (S). The chemical orientation of these three phenylpropanoid units is very diverse depending on the type cell tissue and the specific plant species. Herbaceous plants (e.g. grass) are made up of fair distribution of the all the three phenylpropanoid units. The p-hydroxyphenyl units that make up 5-10% of the grass lignin have been found to be esterified at the γ-position of its propyl group. Softwood (e.g. spruce and pine), consist almost entirely of guaiacyls (coniferyl alcohol) units, whereas hardwood (e.g. willow, beechwood and poplar) are composed mainly of monolignols of guaiacyls and syringyls. As emphasized by Eriksson and Bermek (2009), the specific three-dimensional structure of lignin is unknown; understanding the structural orientation of the phenolic monomers is however very important, as it highlights the degree of recalcitrance of a potential biomass feedstock.
The complexity of the chemical structure of lignocellulose makes it difficult to degrade in a fermentation process. The real promise of using lignocellulosic biomass as a viable substrate for anaerobic digestion therefore lies in the effective disruption of the crystalline structure of cellulose, breaking also the lignin seal (Mosier, 2005). Figure 3.1 shows a schematic illustration of the chemical structure of the lignocellulose complex.
3.2 The Recalcitrance of Lignocellulosic Biomass to Enzymatic Hydrolysis and the role of Pre-treatment

Studies on lignocellulosic biomass as a bioprocess feedstock include: wheat straw (Kongjan and Angelidaki, 2010), wheat bran (Noike, 2002), rice straw (Lo et al, 2010), rice bran (Noike, 2002) and corn stover (Cao et al., 2009; Datar et al., 2007). These studies have demonstrated that attaining commercial quantities of biofuels and biochemical products from lignocellulosic feedstock is low without pre-treatment processes. The readily solubilized cellulose proportion of lignocellulosic untreated biomass that is hydrolysed to fermentable sugars has a conversion efficiency estimated to be less than 20% (Zhang and Lynd, 2004). It
is often argued that pre-treatment is expensive and may not be economically viable; Eggeman and Elander (2005) however emphasized that the net cost of not pre-treating lignocellulose may even be higher due to the low efficiency bestowed by the recalcitrant nature of the lignocellulose complex.

Hydrolysis of lignocellulose to fermentable sugars can be enhanced by several pre-treatment techniques, with different specific targets and mode of actions. Van Dyk and Pletschke (2002) outlined the modes of action of the pre-treatment agents, these included:

i. partial or total removal of lignin from the substrate resulting in increased porosity, thus making it more available to hydrolases (Mansfield et al., 1999);
ii. Lignin redistribution (Zhang and Lynd, 2004);
iii. Disruption of the lignin-polysaccharide linkage;
iv. Disruption and/or removal of the hemicellulose; v. diminution of the degree of cellulose polymerisation; v. breakdown of the particle size (Chundawat et al., 2007)

The concentration of lignin in lignocellulose has been identified as important factor limiting the rate of hydrolysis of polysaccharides to simple sugars (Dijkerman et al., 1997; Jung et al., 2000; Varnai et al., 2010). The disruption of lignin is therefore the fundamental goal of many pre-treatment techniques; including physical disruption (mechanical, thermal), chemical (acid, alkaline, ionic liquids, steam) and biological (enzyme, fungal) (Alvira et al., 2010; Chandra et al., 2007; Lucas et al., 2011; Mora-Pale et al., 2011)

### 3.3 Pre-treatments

The pre-treatment of lignocellulosic biomass is vital for the enhancement of the enzymatic digestibility of the lignocellulosic complex, by removing the hemicellulose/lignin, thereby weakening the crystalline structure of the cellulose and hence increasing the surface area of the substrate for biochemical activities (Xu et al., 2011). A pre-treatment technique can be said to be of great potential, if along with its effective enhancement of the accessibility for hydrolysis, it is capable of minimizing carbohydrate loss during the process (Xu et al., 2011). The pre-treatment should also be cost effective (Sun and Cheng, 2002)
3.3.1 Physico-chemical Pre-treatment

Chemical pre-treatments have widely been used to improve the yields of biofuels and biochemicals from lignocellulosic biomass. The applications of alkaline and acid pre-treatment in anaerobic digestion have been well studied, bringing improved performance in the degradation process and biogas yield.

Zhong et al. (2011), compared biological pre-treatments (using fungus *Pleurotus florida*) and chemical pre-treatments (using urea, NaOH and ammonia), and observed a higher biogas yield in the chemically pre-treated samples, when compared to the biologically treated samples. The NaOH pre-treatment for example resulted in a yield that was 16.58% higher than the biologically pre-treated corn straw, and 207.07% higher than the untreated corn straw used as control. Though the experiment contributed to the knowledge on the performance of biological/chemical pre-treatment on a lignocellulosic substrate, it did not clearly select the best available technique for hydrolysing lignocellulose, with the potential of passing the test of being both cost effective and sustainable (eco-friendly).

Thermo-chemical pre-treatment of lignocellulose (chemical pre-treatment at higher temperature) has been identified as being capable of achieving high solubilisation yields (Fernandes et al., 2009), which might not be achievable in the current studies of enzyme hydrolysis. However, the release of inhibitory compounds at such conditions, especially in higher lignin containing biomass, makes the technique less sustainable in anaerobic digestion (Kaar and Holtzapple, 2000).

The resulting inhibitory compounds from the pre-treatment based on their mode of action, have been categorised as follows (Mills et al., 2009; Palmqvist and Hahn-Hagerdal, 2000):

i. Un-dissociated weak organic acids, which inhibit the microbial growth by penetrating microbial cells and decreasing the intracellular pH;

ii. furan derivatives, that interfere with fermentative and/or glycolytic enzymes, and

iii. phenolic compounds and macromolecules that cause damage to microbial cellular membranes.

Barakat et al. (2012) investigating the effect of furans and phenolic compounds on the methane production from a xylose rich hydrolysate, observed no inhibitory effect of the
lignin polymers or oligomers on the anaerobic digestion of the xylose. They however explained that higher biogas yield could only be achieved in substrates with higher syringyl/guaïacyl units (S/G) ratio and lower molecular weight of lignin polymers. Quéméneur et al. (2012) investigating the effect of furan derivatives (furfurals and 5-hydroxymethylfurfural; HMF), phenolic monomers (vanillin, phenol and syringaldehyde) and lignin (Organosolv and Kraft lignin) on the biohydrogen production from xylose observed a significant inhibitory effect on the biohydrogen process, by these compounds. The furan derivatives, when added to the culture, led to a significant decrease in H₂ yield to 0.45 mol-H₂/mol xylose compared to the yield of the control, with a yield of 1.67 mol-H₂/mol xylose. The outcome of both studies, and many others, indicate that acidogenesis is more susceptible to the lignin derived inhibitory compounds, when compared with methanogenesis. Further work is however needed to clearly justify these conclusions and identify a mechanism.

3.3.2 Biochemical Pre-treatment

Duff and Murray (1996) highlighted a key advantage of enzymatic hydrolysis over conventional acid or alkaline hydrolysis; that, enzymatic hydrolysis is carried out normally under mild conditions (about 50°C; pH 4.8) thereby requiring comparatively less operational cost of e.g. thermal energy.

The pre-hydrolysis of lignocellulose with enzymes is a key area of scientific research today, principally targeted at releasing soluble carbohydrates for the anaerobic degradation process. The enzymatic studies have focused on the use of cellulases to hydrolyse cellulose; hemicellulases (including xylanases) to hydrolyse hemicellulose and other accessory enzymes including, ferulic acid esterase, acetylxylan esterase, α-L-arabinofuranosidase, p-coumaric acid esterase and aglucuronidase (Saha, 2003).

The combination of chemical and biological pre-treatments for effective hydrolysis has been identified as very promising. This kind of pre-treatment, which can be referred to as biochemical pre-treatment has led to a significant improvement in the hydrolysis of lignocellulose. The process adopts the tested and approved physico-chemical pre-treatment methods, combined with the reliable fungal or enzyme pre-treatments. Redding et al. (2011) used dilute sulphuric acid for the pre-treatment of Cynodon dactylon (Bermuda grass), with the addition of 40 FPU g⁻¹ cellulase to enhance the yield of sugar, and 70 CBU g⁻¹ cellobiase to prevent the feedback inhibition caused by the accumulation of cellobiose. Operating at
55°C, they observed a 95% theoretical yield of sugar. Wang et al. (2010), using sodium hydroxide pre-treatment, under the same conditions, observed a 90.4% glucan conversion and 65.1% xylan conversion. The use of lime under similar conditions also resulted in an 87.4% glucan conversion and 67.5% xylan conversion (Wang, 2009).

Ma et al. (2010) observed that a combination of 0.25% H₂SO₄ with E. taxodii (incubated over a 10-day period) improved the hydrolysis compared to the pre-treatment with the acid alone. Bruni et al. (2010), on the other hand observed a 66% increase in yield of biogas from a chemical pre-treated substrate, compared to the 34% achieved in a biochemical pre-treatment (NaOH and subsequent enzymatic treatment) of the same substrate. The difference in the results gives an indication that further research is needed to identify the best achievable technique of pre-treating lignocellulose. The performance of a biochemical pre-treatment relative to anaerobic biohydrogen production; along with its high yielding solubilisation effect, would also need to be justified with regard to the yield of biogas. Xu et al. (2011) explained that a pre-treatment process (depending on the concentrations of the pre-treatment agent used) could potentially cause carbohydrate loss, as well as, the increase of by-products such as VFAs that have potential to inhibit the biohydrogen yield. Therefore, the determination of the effect of the pre-treatment process on the yield of biohydrogen is imperative.

The ratio of the polymers and polysaccharides in the lignocellulosic biomass has a direct influence on the type of pre-treatment selected for a specific substrate. Research has found that single enzyme preparations give the best indication of the degree of enhancement attained due to improved biochemical activities achieved by the addition of the biochemical catalyst. Banerjee et al. (2010c) emphasized the importance of having a better understanding of the types of enzyme, specific activities, and their proportion in order to design cost effective enzyme mixtures. Kumar and Wyman (2009) have also explained synergism (a process by which two or more enzyme combinations produce an improved hydrolysis yield, which would not be independently attainable) as the most promising approach of biological pre-treatment. Studies on different enzyme combinations have produced many interesting results in recent studies. Lin et al. (2011), for example, using ball-milled pre-treatment and enzyme combinations (including β-glucosidase and xylanase), on corn stover could obtain a yield in sugars, as high as 95%.
The degree of synergy is however difficult to interpret due to the use of commercial or crude enzyme cocktails instead of single enzyme mixtures; leading to complexities resulting from the influence of additional activities of enzymes (Bernergee et al., 2010a; van Dyk and Pletschke, 2012). The degree of synergy between different enzymes can therefore be said to be unpredictable and only actual biochemical assays with enzyme and substrate specificity can determine the optimal synergy between different enzymes (Bernergee et al., 2010b). Chundawat et al. (2008) explained that the hydrolytic potential of different lignocellulose substrates does not show a perfect correlation with their enzymatic standard assays. van Dyk and Pletschke (2012) also emphasized that the current studies of pre-treating cellulose does not show a clear distinction between the contribution of the pre-treatment of the substrates (structural changes) and that of the enzyme synergy. Whilst some authors have found no enzymatic synergy in acid-pre-treatment of lignocellulosic substrates (Samejima et al., 1998), others have found synergy to exist in acid pre-treated substrates (Ramirez-Ramirez et al., 2008), making the relationship between different pre-treatments and enhanced hydrolysis a complicated one (van Dyk and Pletschke, 2012).

Tabka et al. (2006) used a combination of enzymes (cellulase, xylanase and ferulic acid esterase (FAE)), simultaneously with dilute sulphuric acid and steam explosion to pre-treat wheat straw, and achieved a soluble sugar yield of 51.4% glucose for ethanol production. The use of both acid pre-treatment and steam explosion, alongside the enzyme mixture however, leave a fundamental economic doubt on the technique. The challenge of cost effectiveness of pre-treatment techniques remains as the major hurdle for commercial lignocellulosic fermentation processes.

Banerjee et al. (2010c) utilized 16 individual purified enzymes to design an enzyme mixture that resulted in a glucose yield of 60-70% from corn stover. Their pre-treatment technique eliminated the challenge of the enzymatic synergy interference often observed in the use of commercial enzymes. van Dyk and Pletschke (2012) however argued that, though the approach of Banaerjee et al. (2010c) is very promising, their evaluation of glucose or xylose released, rather than the total monomer sugars, meant that “completely different enzymes in different ratios were required for glucose or xylose release”, making the approach not viable commercially. They therefore suggested measurement of the total monomer sugars as an imperative tool for the evaluation of the degree of optimisation of synergic ratios and enzyme prediction. Many researchers have designed multicomponent enzyme mixtures that surpass
commercial enzymes in efficiencies (Banerjee et al., 2010a, b, c; Gao et al., 2010; Selig et al., 2008). It must be noted that, the aim of the current study was not to achieve an ideal enzymatic study using pure enzymes, but to utilise commercially available enzymes to obtain mixtures that could enhance the hydrolysis of perennial rye grass. As suggested by Banerjee et al. (2010c) the addition of accessory enzymes has the potential of enhancing the synergy of commercial enzymes. The current research used the release of soluble COD for the evaluation of the solubilizing effect of the enzymes, as recommended by van Dyk and Pletschke (2012).
CHAPTER 4

4 POTENTIAL PRODUCTS FROM ANAEROBIC PROCESSES

4.1 Biofuels from Dark Fermentation of Lignocellulosic Biomass

The drive for renewable electricity in the UK has increased in recent times. Recent DECC reports indicate that the UK in general has made significant progress towards the achievement of the country’s 15% renewable energy usage target, introduced in 2009, under the EU Renewable Directive. The contribution of renewable resources to the UK electricity demand was reported to have increased by 3.6% between 2012 and 2013 to 14.9% (DECC 2014). Significant increases were observed in use of biofuels for transport and heat from renewable sources, with a 14% rise in biofuels (biodiesel and bioethanol) for transport usage and a 19% rise in usage of renewable sources for heat (DECC, 2014). The assessment of the commodity balances for renewables and waste, during years 2011-2013, revealed that nearly 70% of the contribution of renewable resource came from biomass, wind and hydroelectricity (DECC, 2014). The contribution of anaerobic digestion was however low (2.2% of total bioenergy, excluding sewage gas - 2.8% of bioenergy), with an estimated 150 MW installed capacity. Though the net contribution to the energy demand was relatively low, the anaerobic digestion resource observed a capacity increase of nearly 42% in 2012-2013 - an increase that could be attributed to the corresponding increase in Feed-in Tariffs (FiT) levels within the period. The FiT is the financial support given by energy suppliers to renewable energy generators based on the energy generated in kilowatt-hours. The FiT rate for anaerobic digesters of size less than 250kW is 14.7p/kWh. A digester of a size between 250 and 500 kW is given a FiT rate of 13.7 p/kWh; and a digester size between 500 kW and 5 MW will attract FiT at a rate of 9.9 p/kWh. (DECC, 2014)

A proposal adopted by the European Parliament in 2008, instructed a binding target of 10% of renewable energy in the transport system of each member state by 2020 (Kreuger et al., 2011). Significant progress is being made to achieve the target in the UK through the Renewable Transport Fuel Obligation (RTFO), which placed legal requirement on the suppliers of fuel in the transportation industry to ensure that a minimum of 10% (v/v) of fuel sales are from renewable sources by 2020. The contribution of biogas from anaerobic process as renewable energy resource is only towards the generation of heat and electricity. Biogas from anaerobic digestion processes has a great potential, and therefore if upgraded to
biomethane for transportation use, significant reduction in the carbon footprint could be attained. The use of agricultural land for energy crop production for use in anaerobic digestion has been a subject of contentious political and scientific debate. In spite of the many scenarios adopted in several models for global agriculture and land use (Clarke et al., 2009; Luderer et al., 2011), the complexity of food demand, population growth and the elasticity of the energy demand pose inevitable uncertainties on the future of AD industry. In order to ensure sustainable use of cultivated land and pastures, Fischer et al. (2009) adopted the “food first” paradigm in their modelling of scenarios of land use for the production of biofuels and the potential risk to food supply. It has been suggested that the use of agro-industrial waste, the municipal solid waste and wastewater sludge as feedstocks for biogas production is the way forward, favoured not only politically but also socially with the emphasis that waste could be converted to clean energy (Molino et al., 2013). Lignocellulosic biomass due to its availability, low cost and abundant residual carbohydrates stands out as important renewable organic carbon source for the production of biogas (biohydrogen and biomethane) in anaerobic processes.

4.1.1 Biohydrogen

The production of hydrogen from untreated lignocellulosic biomass is achievable (Hawkes et al., 2008). The yield is however very low due to the recalcitrant nature of the lignocellulose complex. The maximum theoretical yield of hydrogen that could be produced from carbohydrates in the anaerobic fermentation process has been estimated to be 4 moles H₂ per mole hexose sugar, considering acetic acid as the end product (Hawkes et al., 2007) (Equation 3). If butyric acid is the end-product, then a theoretical yield of 2-moles H₂ per mole hexose sugar converted is achievable (Hussy et al., 2003; Roger et al., 1993) (Equation 4). The actual hydrogen yield obtained from the lignocellulosic feedstock is considerably lower, this in spite of the many pre-treatment techniques that have been used to attempt to optimize the process. Hawkes et al. (2008) highlighted that if the 1.2 million ton/year of the flour mill co-product (wheat-feed) produced in the UK alone, were to be utilised as a substrate for biohydrogen production, the resulting energy would be more than enough to power the entire transportation fleet of the UK flour industry. Hydrogen from the dark fermentation process, once purified, could be used in fuel cells to produce electricity to power vehicles and machinery, having been earmarked as the most efficient energy carrier system.
A mixture of hydrogen and methane has been identified as a more realistic approach to utilise the fuel in the already established internal combustion engine industry (Bauer and Forest, 2001; Karim et al., 1996).

\[
\text{C}_6\text{H}_{12}\text{O}_6 + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COOH} + 2\text{CO}_2 + 4\text{H}_2 \quad \text{Equation 3}
\]

\[
\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow \text{CH}_3\text{CH}_2\text{CH}_2\text{COOH} + 2\text{CO}_2 + 2\text{H}_2 \quad \text{Equation 4}
\]

As emphasized by Cortright et al. (2002), the true benefits of hydrogen can only be realised if it is produced from renewable sources such as from the dark fermentation of biomass. Martínez-Pérez et al. (2007) emphasized that a biomass substrate ideal for biohydrogen production must be low in lignin but high in carbohydrates, have a low demand/input of energy for its treatment and fermentation, and moderate or zero requirement of nutrients (McKendry, 2002).

Lignocellulosic biomass has significant amounts of lignin inter-linked with the polysaccharides. The recalcitrant nature of the complex arising from the inter-linkage between the aromatic polymer and the polysaccharides, pose a serious challenge to the bio-fermentation process. That notwithstanding, tremendous energy gains have been attained when pre-treatment processes are adopted to enhance the hydrolysis process, even though such yields are below the expected theoretical yields. Cao et al. (2012) investigated the role of lime pre-treatment in the mechanism hydrogen production from corn stover, observed a hydrogen yield of 155.3 ml/g-TVS, an approximately 38% improvement when compared to the yield obtained from the untreated corn stover. The procedure outlined by Cao et al. (2012) demonstrated that calcium hydroxide has a high potential of disrupting the recalcitrant lignocellulosic structure, thereby making the cellulose component readily available to hydrolytic enzymes. Their procedure however involved the pre-treatment of the substrate for lime at 0.10 g/g biomass, at ambient temperature for 96 hours. The technique also involved the washing of the pre-treated slurry with water before being digested. It can be suggested that though the yield enhancement was impressive, the actual application of the technique is far from ready for commercial scale implementation, as several questions including; time, energy, and the inhibitory effect of residual calcium ions after the pre-treatment were not answered.
Many pre-treatment techniques which have been found to be very effective in the solubilisation of lignocellulosic biomass, and have been applied with great success in the bio-ethanol industry, have failed to attain similar achievement in dark fermentation biohydrogen production due to factors including inhibition posed by the pre-treatment agent. Hawkes et al. (2007) compared biohydrogen production from wheat-feed after treatment with 2.0% H$_2$O$_2$ with NaOH to wheat-feed substrate in a semi-continuous anaerobic fermentation, and observed a hydrogen production of 56 m$^3$/ton from the raw wheat-feed, and 31 m$^3$/ton from the NaOH-H$_2$O$_2$ hydrolysate. Hawkes et al. (2007) explained that the relatively low conversion of hydrolysed sugar to hydrogen (58%) was essentially due to the limitations of solubilisation of complex carbohydrates by the inhibition in the hydrolysis processes.

Han et al. (2012) digested soybean straw with H$_2$O$_2$ as pre-treatment agent and observed that as the concentration of the H$_2$O$_2$ was decreased, the conversion efficiency of the sugars to hydrogen increased. At a concentration of 30% H$_2$O$_2$, the sugar conversion efficiency was found to be 45.66% resulting in a hydrogen yield of 18.04 ml/g-substrate. When 16% H$_2$O$_2$ was used to pre-treat the substrate, the yield efficiency improved to 47.15%, resulting in a H$_2$ yield of 23.0 ml/g-substrate. At 2.0% and 0.5% H$_2$O$_2$, degradation efficiency increased to 60.32% and 65.83% respectively. The yield of hydrogen on the contrary decreased, with 4.48 ml/g-substrate produced at 2% H$_2$O$_2$ pre-treatment and 1.76 ml/g-substrate produced because of the 0.5% H$_2$O$_2$ pre-treatment. They explained that though H$_2$O$_2$ was effective in the solubilisation of the substrate for higher energy yield, further increase of concentration created a less favourable environmental condition for the microbial activities, hence the low sugar yield.

Han et al. (2012) also evaluated the effect of the concentration of NaOH (8%, 4%, 2%, 1%, and 0.5%) as a pre-treatment agent on the sugar conversion efficiency, as well as the H$_2$ yield. The highest substrate solubilisation efficiency was found at 4% NaOH, resulting in a H$_2$ yield of 6.60 ml/g-substrate. The highest H$_2$ yield was however obtained at NaOH concentration of 0.5%, indicating that high NaOH concentrations resulted to increased Na ion toxicity, which inhibited the fermentation process. Han et al. (2012) selected 4% HCl as the ideal pre-treatment method for the soybean straw, having compared its solubilisation/degradation efficient to HCl concentrations including: 8%, 4%, 2%, 1% and 0.5% HCl. Though they observed very similar sugar degradation efficiencies in all the studied HCl concentrations, the H$_2$ yield was found to be 40.77, 47.65, 33.77, 25.01 and 17.26 ml/g-substrate, respectively.
Their work demonstrated that the concentration of pre-treatment agent and the yield of H₂ do not follow a linear pattern. Han et al. (2012) further explained that cationic/anionic toxicity could occur at a certain threshold specific to the microbial population involved in the fermentation process, thereby affecting the metabolic efficiency. It must also be noted that the type of substrate also plays a significant role in the selection of a pre-treatment agent.

The utilisation of mild acid or alkaline with enzymatic pre-treatment has been viewed as very promising for the enhancement of the hydrolysis of lignocellulosic biomass (Cheng et al., 2011), an important step for enhancing biohydrogen production. Rowena et al. (2009) have worked with the addition of enzymes in three different digester configurations, including pre-treatment of the lignocellulosic substrate before a single-stage digestion; direct addition of enzyme to a single-stage reactor; and the addition of enzymes to the first stage (hydrogenic/acidogenic) of a two-stage process. However, they observed no significant difference in the yield of biogas in the three configurations that were studied. Enzymatic treatment in an acidogenic SSF has the potential of being an ideal process for fermentation of lignocellulosic biomass because of the fact that the optimum pH (5.2 – 5.5 pH) maintained in the acidogenic reactor provides ideal environmental conditions for the activity of the enzyme, and the process requires less operational time and energy.

4.1.2 Biomethane

The integration of lignocellulosic biohydrogen fermentation systems with methanogenesis has been well studied. VFAs, which are co-products of the biohydrogen fermentation process, are important precursors for the production of methane (Guwy et al., 2011). The two-stage anaerobic digestion process has been found to have enormous advantage over the single-stage system. The system has also been proven to give improved COD reduction, allow a relatively higher organic loading rate, improved process stability, and enhanced biogas yield (Cohen et al., 1980). DiStefano and Palomar (2010), comparing a two-stage biohydrogen and biomethane process to single-stage anaerobic digestion systems, showed that a two-stage process resulted in an improved specific energy yield, nearly 32% higher when compared to the single-stage AD process.

Many researchers, as highlighted by Guwy et al. (2011) have investigated the application of the two-stage AD system on different substrates including sewage sludge, dairy waste, and food waste, but very few have applied the system to recalcitrant lignocellulosic biomass.
Massanet-Nicolau et al. (2013) made a more detailed comparison of the single-stage and the two-stage AD process, using real time gas production data from the digestion of a lignocellulosic feedstock (wheat-feed pellets). With the same overall retention time, the methane yield obtained from the two-stage system was found to be 359 L/kg VS, which was an approximately 37% yield increase when compared to the single-stage system. They also showed that even at a shorter retention time (12 day HRT), the two-stage system performed better than a single-stage system operated at the longer HRT of 20 days. The major difference in the performance of the two anaerobic digestion systems could essentially be attributed to the readily available VFA (particularly, acetate and butyrate) which were made available by the hydrolytic-acidogenic first stage of the two-stage system. The energy yield improvement obtained by Massanet-Nicolau et al. (2013) was higher than many other anaerobic digestion systems designed to enhance biomethane production (Lafitte-Trouqué and Forster, 2002; Ferrer et al., 2008). The results emphasized that significant energy yield could be obtained from recalcitrant substrates such as lignocellulosic biomass, if the acid phase of the anaerobic digestion process is improved.

4.2 Biochemicals and Biopolymers

4.2.1 Volatile Fatty Acids

The yields of VFAs during acidogenic fermentation, as well as its composition, have been identified to be substrate specific (Jiang et al., 2007; Rajagopal and Beline, 2011). Rajagopal and Beline (2011), described the fermentation of carbohydrates, lipids and proteins as being directly associated with the production of the lower molecular weight VFAs (acetic, propionic, iso-butyric and n-butyric). The higher molecular weight VFAs (iso- and n-valeric acids) are mainly associated with the reductive deamination of amino acids during acidogenic fermentation of proteins (Chen et al., 2007) or through the Stickland reaction by reductive oxidation between amino acids pairs (McInerney, 1988) in (Chen et al., 2007). Research has shown that the type of substrate plays an inevitable role in determining the yield of VFA as well as the proportions of the individual VFA products. Ucisik and Henze (2008) stressed that even the origin of the substrate to some extent determines the VFA yield and composition.
Significantly, higher yields of biologically produced VFAs - a co-product from anaerobic fermentation, has been achieved from a wide range of substrates, including: sucrose, food wastes, wastewater and wheat-feed, as illustrated in Table 4.1. The main focus of scientific research with regards to VFA production has fundamentally been placed on the environmental conditions/operational parameters that affect the acidogenic fermentation process (Bengtsson et al., 2008); and that has laid an important platform for the optimisation of acidogenic fermentation. The effects of process conditions including pH, temperature, organic loading rate and retention time have been demonstrated to be essential for the optimisation the acidogenesis process (Bengtsson et al., 2008, Dabrock et al., 1992). Daddario et al. (1993) also identified the type of reactor as an important factor in the enhancement of VFA production. Table 4.1 shows some of the work on VFA production, highlighting some of the operational conditions that affect the yield of VFAs. Lignocellulosic biomass, because of its recalcitrant nature does not stand out as an ideal substrate for the sole production of VFA. Finding a value for it as a co-product of the biohydrogen process is viewed as the most viable approach.
Table 4.1a: Biomass Sources of VFA

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Focus of study</th>
<th>Reactor Type</th>
<th>Parameters</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food waste</td>
<td>Varying Temperature</td>
<td>Batch AD</td>
<td>Temp. (°C)</td>
<td>Komemoto et al. (2009)</td>
</tr>
<tr>
<td>Molasses based synthetic wastewater</td>
<td>two-stage AD</td>
<td>AnMBR</td>
<td>pH</td>
<td>Wijekoon et al. (2011)</td>
</tr>
<tr>
<td>Molasses based synthetic wastewater</td>
<td></td>
<td>H₂-R</td>
<td>HRT (hours)</td>
<td>Sreethawong et al. (2010)</td>
</tr>
<tr>
<td>Molasses based synthetic wastewater</td>
<td></td>
<td>CH₄-R</td>
<td>OLR (g COD/L/d)</td>
<td>Kyazze et al. (2006)</td>
</tr>
<tr>
<td>Cassava Wastewater</td>
<td></td>
<td></td>
<td>H₂ (L H₂/L/d)</td>
<td>Banks et al. (2011)</td>
</tr>
<tr>
<td>Sucrose</td>
<td></td>
<td></td>
<td>CH₄ (L CH₄/L/d)</td>
<td></td>
</tr>
<tr>
<td>Source segregated domestic food waste</td>
<td>Varying Substrate concentrations CSTR</td>
<td></td>
<td>2.5 kg VS m⁻³ day⁻¹</td>
<td></td>
</tr>
</tbody>
</table>

| VFAs                             |                        |                      |                    |                          |
| Acetic acid                      | 1.0                    | 1.0                  | 1.0                | 2.01                     |
| n-Butyric acid                   | 3.0                    | 4.0                  | 5.8                | 6.34                     |
| i-Butyric acid                   | -                      | -                    | -                  | -                        |
| Propionic acid                   | <1.0                   | 5.0                  | 5.0                | -                        |
| n-Valeric acid                   | -                      | -                    | -                  | -                        |
| Total VFA                        | 7.0                    | 7.0                  | 12.0               | 15.0                     |

Table 4.1b: Biomass Sources of VFA

<table>
<thead>
<tr>
<th>Substrate</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>Swine wastewater</th>
</tr>
</thead>
<tbody>
<tr>
<td>Focus of study</td>
<td>Varying HRT/OLR</td>
<td>two-stage AD</td>
<td>Ammonia Stripping</td>
<td>Thermophilic</td>
</tr>
<tr>
<td>Reactor Type</td>
<td>ASBR</td>
<td>CSTR</td>
<td>Batch AD</td>
<td>CSTR</td>
</tr>
<tr>
<td>Parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temp. (°C)</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>51</td>
</tr>
<tr>
<td>pH</td>
<td>5.3</td>
<td>5.5, 7.0</td>
<td>7.0</td>
<td></td>
</tr>
<tr>
<td>HRT (Hrs)</td>
<td>4, 6, 9, 18</td>
<td>18, 11.3</td>
<td>20 days</td>
<td>48</td>
</tr>
<tr>
<td>OLR (g COD/L/d)</td>
<td>120, 80</td>
<td>4.17, 24.2</td>
<td>4.71, 70</td>
<td>70</td>
</tr>
<tr>
<td>H₂ (L H₂/L/d)</td>
<td>0.88, 4.12</td>
<td>2.57, 1.78</td>
<td>4.04</td>
<td></td>
</tr>
<tr>
<td>CH₄ (L CH₄/L/d)</td>
<td></td>
<td>1.32</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>Acetic acid</td>
<td>2.0, 3.9</td>
<td>4.35, 0.21</td>
<td>14.2, 13.0</td>
<td>10.3</td>
</tr>
<tr>
<td>n-Butyric acid</td>
<td>1.4, 5.2</td>
<td>2.98, 0.04</td>
<td>1.53, 1.52</td>
<td>3.5</td>
</tr>
<tr>
<td>l-Butyric acid</td>
<td>-</td>
<td>0.03</td>
<td>4.88, 4.55</td>
<td>-</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>1.8, 1.1</td>
<td>1.65, 0.05</td>
<td>4.35, 4.28</td>
<td>3.3</td>
</tr>
<tr>
<td>n-Valeric acid</td>
<td>0.53, 0.93</td>
<td>1.12, 0.02</td>
<td>1.70, 1.64</td>
<td>2.0</td>
</tr>
<tr>
<td>Total VFA</td>
<td>8.73, 13.1</td>
<td>10.2, 0.34</td>
<td>27.4, 25.7</td>
<td>20.3</td>
</tr>
</tbody>
</table>

4.2.2 Polyhydroxyalkanoates (PHA)

The high yields of VFA produced as a co-product of the biohydrogen process is finding new potential markets in the bio-plastic industry. The integration of the biohydrogen process and PHA biosynthesis provides the possibility of adding value to the biohydrogen fermentation process. The mechanism involves the utilisation of the effluent from the acidogenic fermentation as a nutrient and carbon source for some gram positive and gram negative bacteria (including bacteria from the genera: *Pseudomonas, Bacillus* and *Methylobacterium*), which produce polymers of hydroxyl fatty acids as intracellular carbon and an energy reserve (*Keshavarz and Roy, 2010; Passanha et al., 2013*). The polymer produced from the process has the advantage of being utilised as high value plastic in the packaging, biomedical/medical, textile and photographic industries (*Chen, 2009; Keshavarz and Roy, 2010*), and have been classified as being renewable, biodegradable, and non-toxic (*Akaraonye et al., 2010; Albuquerque et al., 2010a,b*). The use of low cost substrates such as the effluent of biohydrogen reactors after the fermentation of lignocellulosic substrates has the potential to improve the sustainability of the process and the reduction of the cost of the plastic produced, hence improving the economic viability of the technique.

As highlighted by Akaraonye et al. (2010), *Cupriavidus necator*, unlike bacterial strains like *Alcaligenes latus*, lack a PHA biosynthetic gene and therefore do not accumulate PHA alongside growth, but require the limitation of essential nutrients for PHA accumulation. *Cupriavidus necator* has therefore been widely used for the production of PHA from mainly simple sugars and fatty acid substrates (Akaraonye et al., 2010). *Cupriavidus necator* because of its high production potential from low molecular weight carbon sources is a preferred inoculum for the production of PHA from the effluents of acidogenic fermentation (mainly VFAs and soluble carbohydrates). Aravind et al. (2013) utilised hydrolysed grass and *Syzygium cumini* seed as sole carbon sources for PHA production and *Ralstonia eutropha* as inoculum, and obtained a PHA accumulation of 0.097 g/l (28.97% of cell dry weight) and 0.10 g/l (42.2% of cell dry weight), respectively. Passanha et al. (2013) using *Cupriavidus necator* as inoculum, investigated the use the effluent and digestate of anaerobic digestions systems as substrates for PHA biosynthesis. They observed that a mixture of the effluent and digestate of biohydrogen and biomethane digesters treating wheat-feed and effluent from a biohydrogen reactor digesting food waste, in 1:1:1 ratio, provided a media that was rich in nutrients and trace elements. The nutrients provided in the media were important for the high
growth rate of *Cupriavidus necator*, and its depletion resulted in high accumulation of PHA. The maximum PHA yield of 12.29 g/l (which was 90% of cell dry weight) is the highest reported in the utilisation of effluents/digestate of anaerobic digestion process with complex primary substrates (Passanha et al., 2013). The use of soluble organic matter (VFAs and soluble carbohydrates) obtained from the acidogenic fermentation of lignocellulosic biomass as substrate for PHA production, will provide a cheaper source of nutrients and organic carbon source for the bioplastic biosynthesis.

Aravind et al. (2013) observed that the presence of residual mass apart from the carbohydrates from the hydrolysis process, led to a reduction of the production of PHA, as the microorganisms could not adapt to such complex substrates (Yang et al., 2010). It can be deduced from their work that enhancing the hydrolytic-acidogenic fermentation of recalcitrant lignocellulosic substrates in the biohydrogen fermentation process, has a direct influence on their utilisation in PHA biosynthesis. Though many attempts have been made to enhance the acidogenic fermentation of lignocellulosic biomass, no work has been done to investigate the downstream effect of the enhanced hydrolysis on the PHA production.
4.2.3 Lignin

The production of lignin worldwide is estimated be around 50 million tons per annum (NNFCC, 2011). The annual global production of isolated lignin for lignosulphonate alone is estimated to be 1.1 million tonnes and an estimated growth rate of 1.4% is predicted per annum, with a market price of around £250 - £2,000 per tonne (depending on the form) (NNFCC, 2011). Lignin is a naturally occurring heterogeneous biopolymer with highly reactive groups, making it an important source for the production of a wide range of renewable material and chemicals (Varanasi et al., 2013). Table 4.2 summarises some of the many applications of lignin and its principal markets according to Ek (2005). Lignin has also been used as a raw material in the production of value-added chemicals, including: phenol, benzene, vanillic acid, guaiacol, methanol, dimethyl sulfoxide (DMSO) and acetic acid (Gandini, 2011; Goheen, 1971). As emphasized by Varanasi et al. (2013), the application of lignin for the production of these low-molecular weight chemicals has the potential of substantially increasing the market value of lignin. Vanillic acid for example has a market value of about $13.0 per kilogram, with a global demand of about 30,000-50,000 tonnes per annum. Currently, the highest global demand of lignin derived products is the lignin polyblend; with an annual worldwide demand of nearly 90 million tonnes, at a market price of around $3.2/kg. (Holladay et al., 2007)

The extraction of lignin from lignocellulosic biomass has been achieved through many different techniques including acid sulphite pulping using salts of sulphurous acid to produce lignosulphonate (Pratt, 2012). Unsulphonated lignin can be produced through the Kraft pulping process, which involves treatment of the biomass with sodium carbonate and sodium sulphate, breaking down the lignin component and some hemicellulose. The lignin is then isolated via acid precipitation. Lignosulphonate has the advantage of being water soluble, and this led to the process of sulphomethylation of the otherwise insoluble Kraft lignin to improve its water solubility (Ek, 2005). The production route of lignin also includes steam explosion pre-treatment and pyrolysis, which normally results in a lignin product classified as being lower molecular weight than Kraft lignin (Holladay et al., 2007).
Table 4.2: The current principal markets of lignosulphonate

<table>
<thead>
<tr>
<th>Scale</th>
<th>Uses</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large scale</td>
<td>Dust control, Animal feed, Concrete admixtures</td>
<td>Dimrel et al. (2002); Varanasi et al. (2013); Ek (2005); Holladay et al. (2007)</td>
</tr>
<tr>
<td>Medium Scale</td>
<td>Dye dispersants, Gypsum wallboard dispersant, vanillin</td>
<td>Ek (2005), Lauten et al. (2010); Hoyt and Goheen (1971); Northey (2002); Berry and Viswanathan,</td>
</tr>
<tr>
<td>Small-scale</td>
<td>Oil well drilling mud additive, resins, binders</td>
<td>Dimrel et al. (2002); Varanasi et al. (2013); Ek (2005); Holladay et al. (2007)</td>
</tr>
<tr>
<td>Small medium</td>
<td>Carbon black, emulsifier, water treatment, cleaning chemicals, micronutrients, leather tanning, battery expander, pesticides dispersant, rubber additive, electrolytic refining agents, protein precipitants, grinding agents</td>
<td>Kadla et al. (2002); Nagel et al. (2002); Feldman (2002); Montanari et al. (2002); Dimrel et al. (2002)</td>
</tr>
</tbody>
</table>

The Organosolv pulping process which involves the use of a mixture of formic acid, acetic acid and water (Lam et al., 2001), has been found as having very high lignin recovery efficiency, resulting in less contaminated lignin (Vanderghem et al., 2012). Another extensively developed Organosolv process is the alcohol-Organosolv process, which involves a series of extractions using 50% ethanol for the delignification of hardwood to produce lignin referred to as Alcell lignin (Pye and Lora, 1991). The application of the Alcell process in softwood has been reported to be weak, because of the low yields of lignin obtained from that substrate. Ionic liquids, such as 1-ethyl-3-methylimidazolium acetate, have also been reported to have great potential for depolymerizing extracted lignin or technical lignin to produce renewable chemicals and materials (Varanasi et al., 2013).
The quality of lignin is highly dependent on the type of extraction technique adopted. The contaminants such as carbohydrates, particulates, volatiles and salts have been found to hinder the production of chemicals from lignin. The production of high quality lignin is therefore the major challenge of scientific research in this area of study. The current study aims to examine the quality of lignin after Organosolv extraction of lignin from an acidogenically fermented perennial rye grass biomass.
CHAPTER 5

5 MATERIALS AND METHODS

5.1 Biomass and Digestate

The lignocellulosic substrates utilised in the current study were wheat-feed pellets (obtained from Rank Hovis Ltd., UK) and perennial rye grass pellets (obtained from Perennial rye feed, Hampshire, UK). The wheat-feed and perennial rye grass stocks were kept in a refrigerator at 4-8°C to ensure physiochemical consistency and then used when needed. The substrates were treated with sodium hydroxide, before being fed to a biohydrogen reactor at 49.1 g/l TS equivalent and the effluent from the hydrogen reactor was fed to methanogenic reactors, as described in Massanet-Nicolau et al., (2013). Samples were taken from feedstocks; the effluents from the hydrogen; and digestates from the methane reactors operated by Massanet-Nicolau et al. (2013) for compositional analysis.

5.2 Characterisation of the Substrates, Effluents and Digestates of the Anaerobic Processes

5.2.1 Solid Content

The total solids (TS) and volatile solids (VS) were determined according to the standard method outlined by the American Public Health Association (APHA) as mentioned in Clesceri et al. (1999). An empty Pyrex beaker of 50 ml working volume was placed in a furnace (Carbolite, Sheffield, UK) maintained at 550±25°C for about 1 hour until a stable weight was attained. The beaker was then taken to cool in the atmosphere for 3 minutes, before being placed in a desiccator (Fisher Scientific, Loughborough, UK) to cool further to ambient temperature. The empty beaker was weighed ($W_a$), using a 4 decimal place balance (Fisherbrand). An amount of biomass /slurry (5 g to 15 g) was then added to the beaker and the weight ($W_b$) recorded. The beaker and its content (sample) was then dried at 105±2°C in a Forced-air oven (Gallenkamp, Leicester, UK), until a constant weight was achieved. The sample was then placed in the desiccator to cool to ambient temperature and the weight then recorded ($W_c$). The sample after being weighed was placed in the furnace at 550±25°C until a constant weight was achieved. The beaker was removed and placed in the desiccator to cool to room temperature, allowing a 3-minute drop in temperature in atmosphere before being placed.
in the desiccator, after which the weight was recorded ($W_d$). Each biomass sample was analysed in triplicate.

The TS was calculated using Equation 5, and VS was expressed in terms of dry weight (Equation 6), or in terms of wet weight (Equation 7).

\[
TS = \frac{W_c - W_a}{W_b - W_a} \times 100 \quad \text{Equation 5}
\]

\[
VS_{(DW)} = \frac{W_c - W_d}{W_c - W_a} \times 100 \quad \text{Equation 6}
\]

\[
VS_{(WW)} = \frac{W_c - W_d}{W_b - W_a} \times 100 \quad \text{Equation 7}
\]

Where: TS – Total solids in the sample (%)

\[VS_{(DW)}\] – Volatile solids in sample according to the dry weight (%)

\[VS_{(WW)}\] – Volatile solids in the sample according to wet weight (g)

$W_a$ – Weight of empty beaker (g)

$W_b$ - Weight of beaker and sample prior to drying (g)

$W_c$ – Weight of beaker and sample after drying (g)

$W_d$ – Weight of beaker and sample after incineration (g)

$V$ – Sample volume

The TS were also reported in concentration as gram of solids per litre of slurry added. Equation 8 indicates the calculation of TS in grams per litre (g/l).
5.2.2 Determination of the Lignocellulosic Components of Biomass

5.2.2.1 Sample Preparation:
Samples taken from the substrates and effluents of the hydrogen reactors and digestates of the methane reactors were dried in a forced-air drying oven (Gallenkamp Hotbox; Grant Instruments Ltd., Cambridge, UK) at 55°C for 72 hours for the compositional analyses. Each of the dried samples was ground with a coffee grinder to pass through a 2 mm screen. The prepared samples were used for the determination of the neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) and for the dry matter (DM) determination.

5.2.2.2 Neutral Detergent Fibre: Amylase Procedure
The quantity of residual lignocellulose component was determined according to the NDF-refluxing procedure, outlined in Undersander et al. (1993), a modification of methods mentioned in Goering and Van Soest (1970), Van Soest et al. (1991), and Metertens (1992).

The procedure involves the use of the neutral detergent solution to dissolve the easily degradable content of the biomass, including sugars, protein, pectin and lipids, leaving a fibrous residue, which is essentially composed of cellulose, hemicellulose and lignin. Table 5.1 shows the reagents used in the preparation of the NDF solution and their role in the solubilisation of the easily digested plant content.

\[
TS \ (g/l) = (Wc - Wa) \times \frac{1000}{V}
\]

Equation 8
Table 5.1: Reagents of Neutral Detergent Fibre Solution and their Function

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium dodecyl sulphate</td>
<td>The detergent is used in the Solubilisation of proteins</td>
</tr>
<tr>
<td>Sodium borate, decahydrate (Na₂B₄O₇.10H₂O)</td>
<td></td>
</tr>
<tr>
<td>Sodium phosphate, dibasic (Na₂HPO₄),</td>
<td></td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid (EDTA)</td>
<td>Chelating of calcium and removal of pectin at boiling temperatures</td>
</tr>
<tr>
<td>Triethylene glycol</td>
<td>Removal of non-fibrous matter from concentrate feeds</td>
</tr>
<tr>
<td>Heat-stable alpha amylase</td>
<td>Removal of starch</td>
</tr>
<tr>
<td>Sodium sulphite</td>
<td>Removal of some nitrogenous matter</td>
</tr>
</tbody>
</table>

Filter bags (F57, ANKOM Technology, UK) were marked with solvent resistant marker and weighed (W1), using an analytical balance (A & D Instruments Ltd., GR-202-EC, Japan). Each bag after weighing was zeroed and 0.55-0.95 g of the prepared sample was weighed into each bag (W2), and the bag sealed with a heat sealer (RS Instruments, UK). An empty bag was also weighed (C1) and sealed to be used for the determination of the blank bag correction.

A refluxing unit was filled with the NDF solution, at 50 ml for each sample. The refluxing unit and its content were then pre-heated and an amount of 0.55 g sodium sulphite per each sample was weighed and added to the solution. After 4 to 5 minutes of boiling, a volume of 50 µl of the heat stable amylase (dietary fibre kit; A3306; Sigma Chemical Co, St. Louis, MO) per bag was added to the solution, ensuring thorough mixing. The refluxing was then carried out under heat (150˚C) for 60 minutes, allowing gentle stirring of the solution. After removing the samples from the extraction-unit, they were washed five times with boiling water. A 1litre beaker was immediately filled with 250 ml hot water and a volume of 50 µl of the heat stable amylase per each sample added. The samples were then added and allowed to react for about 3 minutes. The samples were washed thrice with warm water, allowing 2 minutes of soaking between the washes. The samples were washed twice with acetone (enough to cover all the
filter bags), and placed on a screen to air-dry. The samples were then dried in an oven at 105±2°C for 24 hours. The dried samples were removed from the oven, cooled in a desiccator and weighed (W3). All experiments were carried out in triplicate. Equation 9 shows the calculation of NDF. Figure 5.1 shows the refluxing set-up used for the NDF extraction

$$\%\text{NDF (DM basis)} = \frac{W3 - (W1 \times C1)}{W2 \times DM} \times 100$$  \hspace{1cm} \text{Equation 9}$$

Where: 
- W1 = Bag tare weight
- W2 = Sample weight
- W3 = Weight after extraction process
- C1 = Blank bag correction (final oven-dried weight/original blank bag weight)
- DM = laboratory dry matter

5.2.2.3 Acid Detergent Fibre Determination

The ADF was determined adapting the procedure outlined in Undersander et al. (1993) and AOAC (1990) (Association of Official Agricultural Chemists). The procedure involves the use of acid detergent solution; an acidified quaternary detergent to dissolve the soluble content of the plant cell, soluble minerals and hemicellulose, leaving a fibrous residue which is composed of cellulose, lignin, and a trace amount of cell wall proteins and minerals (ash). The samples under investigation were prepared as in 5.12. The filter bags (F57, ANKOM) were weighed empty (W1), recording the weight to the nearest 0.1 mg. An amount of 0.55-0.95 g of the prepared sample was then weighed directly into each bag (W2), and sealed with the heat sealer. An empty filter bag was weighed (C1) to be used for the determination of the blank bag correction factor. The samples were then placed in a holder and the holder placed in the refluxing unit. A volume of 100 ml acid-detergent solution per sample was added, and a few granules of anti-bumping granules were added in order to smoothen the boiling. Refluxing was carried out for 120 minutes, adjusting the heat to ensuring slow boiling, allowing gentle stirring of the solution. After the refluxing process, the samples were taken out and washed five times with boiling water. The samples were then washed twice in acetone. The samples
were removed and air-dried on a wire screen. The samples were then completely dried in an oven at 105±2°C. The samples were then removed from the oven, cooled in a desiccator to ambient temperature and then weighed (W3) to the nearest 0.1 mg. Equation 10 shows the calculation of ADF, and Figure 5.1 shows the refluxing set-up used for the ADF extraction.

\[
\% \text{ADF (DM basis)} = \frac{W_3 - (W_1 \times C_1)}{W_2 \times \text{DM}} \times 100 \quad \text{Equation 10}
\]

Where: 
- \(W_1\) = Bag tare weight
- \(W_2\) = Sample weight
- \(W_3\) = Weight after extraction process
- \(C_1\) = Blank bag correction (final oven-dried weight/original blank bag weight)
- DM = laboratory dry matter

**Figure 5.1: Refluxing unit used in NDF and ADF analyses**
5.2.2.4 Acid Detergent Lignin Determination

Acid detergent lignin was determined according to the 2-beaker system by Ankom Technology (2005). The dried residue of the ADF after extraction process is used in the determination of the ADL. The ADL extraction process involves the use of 72% sulphuric acid. Filter bags were weighed (W1), after which the balance was zeroed. The filter bags with samples treated in the ADF procedure were cut open and the content weighed to about 0.5 g in to weighed filter bags (W2), and sealed using a heat sealer as in the NDF/ADF procedure. Again, one blank bag was weighed and sealed (C1), for the determination of the blank bag correction determination. The filter bags (W2) were then placed in a 3-litre beaker, adding a sufficient quantity of the 72% H₂SO₄. A 2-litre beaker was placed in the 3-litre beaker, in order to keep the filter bags submerged in the acid. The set-up was agitated at the start and then every 15 minutes, by gentle up and down movement of the 2-litre beaker. After 3 hours of treatment, the samples were removed and washed with tap water to remove acid (repeating washing until pH was neutral). The water was then removed from the samples by rinsing with sufficient acetone. The samples were then air-dried for 30 minutes, before being placed in the oven for complete drying at 105°C. The samples after being removed from the oven were dried directly in a desiccator, and weighed (W3) after cooling to ambient temperature. Equation 11 shows the calculation ADL component.

\[
\text{% ADL (DM basis)} = \frac{W_3 - (W_1 \times C_1) \times 100}{W_2 \times DM} \quad \text{Equation 11}
\]

Where: W1 = Bag tare weight

W2 = Sample weight

W3 = Weight after extraction process

C1 = Blank bag correction (final oven-dried weight/original blank bag weight)

DM = laboratory dry matter

Note: %NDF, %ADF and %ADL were multiplied with the TS (g/l) and presented as NDF (g/l), ADF (g/l) and ADL (g/l)
5.2.2.5 *Hemicellulose and Cellulose*

The NDF is composed of mainly cellulose, hemicellulose and lignin; whilst the ADF is composed of cellulose and lignin. As mentioned by Van Soest et al. (1991), the subtraction of the ADF value from the NDF gives a fair estimation of the quantity of the hemicellulose. The quantity of the hemicellulose was therefore estimated by subtracting the ADF from the NDF, whilst the cellulose quantity was estimated by subtracting the ADL from the ADF. The NDF, ADF and ADL were determined as described in Section 5.2.2.

\[
\text{Hemicellulose} = \text{NDF} - \text{ADF} \quad \text{Equation 12}
\]

\[
\text{Cellulose} = \text{ADF} - \text{ADL} \quad \text{Equation 13}
\]

\[
\text{Holocellulose} = \text{NDF} - \text{ADF} \quad \text{Equation 14}
\]

5.2.3 *Klasson Lignin Determination*

5.2.3.1 *Acid Insoluble Lignin*

The sample for the Klasson lignin determination was prepared as described in Section 5.2.2.1. The lignin determination adapted in the study, as highlighted by Sluiter et al. (2011) is suitable for extractive free biomass. The dried sample was subjected to the extraction process using the NDF refluxing procedure. It must be noted however, that sodium sulphite was not added as a reagent since it has the capability of breaking down some amount of the lignin (Van Soest et al., 1991).

Glass tubes were placed in an oven for one hour to remove any moisture and ensure consistent weight. The tubes were then cooled in the desiccator, and weighed to the nearest 0.1 mg \((W_{\text{crucible}})\). A 0.3 ± 0.01 g of prepared sample was weighed to the nearest 0.1 mg and placed in the glass tube (10 ml volume). About 0.55 g was weighed at the same time for the determination of the dry matter content, as described in Section 5.2. A volume of 3.00 ± 0.01 ml (4.92 ± 0.01 g) of 72% \(\text{H}_2\text{SO}_4\) was then added to the sample, mixing thoroughly with a glass-stirring rod until the sample was thoroughly wetted. The glass tubes were then incubated using a water bath at 30 ± 1°C for 2 hours, stirring every 15 minutes to ensure complete mixing and wetting. The hydrolysate was then transferred to a 100 ml Duran laboratory bottle (Duran Group, Germany). The hydrolysate was then diluted to a 4% acid concentration by adding deionised water until the combined weight of the hydrolysed sample
and water was 89.22 ± 0.04 g. The bottle was then sealed and the sample autoclaved at 121 ± 3°C for 1 hour. After the autoclaving, the sample was allowed to cool to room temperature, followed by vacuum filtration using a Whatman three piece filter funnel (mounted to a Buchner flask) equipped with a vacuum pump (Charles Austen Pumps, Lancs., UK). The filtrate was collected for the determination of the quantity of acid soluble lignin (Klasson-ASL) in the biomass and the residue was washed twice with deionised water. The residue on a filter paper was dried at 105 ± 3°C for 2 hours, in a pre-weighed beaker. The dried sample was then cooled in the desiccator, and weighed to the nearest 0.1 mg (W_{hydrolysed}). The quantity of acid insoluble lignin (AIL) was calculated as shown in Equation 15. The sample was then incinerated at 550 ± 25°C in the furnace for 4 hours. The samples were removed from the furnace and allowed to cool for 10, and placed in a desiccator to cool to room temperature, after which it was weighed (W_{ash}).

5.2.3.2 Acid Soluble Lignin

The filtrate sample obtained from filtration of the acid hydrolysed biomass (Section 5.6.1.1) was diluted with deionised water to a require concentration based on an absorbance range of 0.7 – 1.0. The absorbance of the diluted sample and blank (deionised water) was measured using the spectrophotometer at a wavelength of 320 nm.

\[
\% \text{ Acid insoluble lignin} = \frac{W_{\text{hydrolysed}} - W_{\text{crucible}} - W_{\text{ash}}}{\text{DM}} \times 100 \quad \text{Equation 15}
\]

\[
\% \text{ Acid soluble lignin} = \frac{\text{UV} \times \text{Volume} \times \text{Dil}}{\varepsilon \times \text{DM}} \times 100 \quad \text{Equation 16}
\]

Where: \( \text{UV} \) = average UV-Vis absorbance for the sample at 320 nm

Volume = volume of filtrate, 87 ml

Dil = dilution factor

\( \varepsilon \) = Absorptivity of biomass at specific wavelength
The total amount of Klasson lignin on an extractive free basis was determined as follows:

\[
\% \text{ Klasson lignin} = \% \text{AIL} + \% \text{ASL}
\]  

Equation 17

5.2.4 Dry Matter and Total Ash Content

The dry matter of the biomass (prepared as in Section 5.2.2.1) was carried out as described by Undersander et al. (1993). The dry matter determination process involved the use of all the equipment used in the determination of the solid content (described in Section 5.2.1). Similar to the solid process, an empty beaker of 50 ml working volume was placed in the furnace for 1 hour, then placed in a desiccator to cool to ambient temperature. The weight of the empty beaker was then recorded \(W_4\) using the balance. Approximately 2 g of the sample was then weighed to the nearest 0.1 mg into the beaker \(W_5\). The sample was then placed in the oven to dry for 24 hours at 105°C. After drying, the sample was removed from the oven and placed in a desiccator to cool to ambient temperature. The beaker and its contents were then weighed to the nearest 0.1 mg \(W_6\).

To determine the total ash content, the dried sample \(W_6\) was placed in the furnace to be incinerated at 550°C. The sample was then removed from the oven, allowed to cool for 3 minutes in the atmosphere before being placed in the desiccator to cool to ambient temperature. The sample (beaker plus burnt biomass) was then weighed to the nearest 0.1 mg \(W_7\). The percentage dry matter and ash content were calculated using Equation 18.

\[
\% \text{Dry Matter (DM)} = \frac{W_6 - W_4}{W_5 - W_4} \times 100
\]  

Equation 18

Where \(W_4\) = Weight of empty beaker (g)

\(W_5\) = Weight of beaker and sample (g)

\(W_6\) = Weight of beaker and sample after drying at 105°C (g)
5.2.5 Robustness of the procedures for the determination of the lignocellulosic components of biomass

The procedure designed for the lignocellulosic components determination was applied in a Round Robin test organized by the Anaerobic Digestion Research Inter-laboratory Project (ADRIP). The purpose was to justify the validity of the experimental procedure/ concept for the biomass compositional analyses. The research materials provided for analyses included forage substrates, including: RM-05c (legume wrapping) and RM-06 (dried dactyl silage). The compositional analyses procedures adopted were the NDF, ADF and ADL. As illustrated in Appendix I, the experimental values of NDF, ADF and ADL were within the range of the assigned values with the only deviation occurring in ADL of the RM06 (dried dactyl silage sample). The results proved the validity of the lignocellulose compositional Analysis procedure adopted in the current study.

5.2.6 Carbohydrates

The total carbohydrate content of the dried perennial rye grass and wheat-feed substrates (as described in 5.2.2) were determine by grinding the samples with a ball mill (Retsch, MM1900). The ground samples were then dissolved in water and further diluted with deionised water until the concentration was between 200 – 50 mg/l glucose equivalent.

The samples for soluble carbohydrates were prepared by centrifuging the slurry at 15,000 rpm for 10 minutes (Accuspin Micro R, Fisher Scientific), followed by dilution to the appropriate concentration.

The carbohydrate content was analysed by the phenol sulphuric acid method in accordance with the procedure in outlined in Dubois et al (1956). Glucose was used as a standard; diluted within the ranges of 25 -200 mg /l. An amount of 400 µL of both standard, samples were placed in a test tube, and 400 µL of 5% phenol was added and mixed, followed by the addition of 2 ml of concentrated sulphuric acid. The mixture was capped, vortexed thoroughly mix, and then incubated at ambient temperature for 30 minutes. After the incubation period, the samples were vortexed again, allowed to stand for 3 minutes before being measure using a UV-Vis spectrophotometer (Perkin Elmer Lamda 25), at 490 nm. The carbohydrate content of the sample was determined by comparing it to a calibration curve ($R^2 \geq 0.98$) of the standard
at glucose concentrations within the range of 25 mg to 200 mg/l. All the carbohydrate measurements were reported in glucose equivalent.

5.2.7 Protein Content

The protein content of the samples was determined according to Hartree (1972), a modification of the Lowry method. The procedure involved the initial treatment of the sample with alkaline in the presence of tartrate (2 g KNa Tartrate, 100 g Na₂CO₃, 500 ml 1M NaOH), followed by a 10 minutes incubation at 50°C with alkaline copper sulphate in the presence of tartrate (2 g KNa Tartrate, 1 g CuSO₄, 10 ml 1M NaOH, 90 ml H₂O). After the incubation, Folin-phenol reagent was added to the mixture. After another 10-minute incubation period at 50°C, the samples and standard were allowed to cool to ambient temperature. During the reaction, a reduction of the Folin-phenol complex occurs, resulting in a blue colour (Sengupta, S. and Chattopadhyay, 1993). The quantity of protein, which correlates with intensity of the absorbance, was then measured at 650 nm using a UV-Vis spectrophotometer (Perkin Elmer Lamda 25). The protein content of the sample was determined by comparing the absorbance to a linear curve of known concentrations (20 – 160 mg/l) of the bovine serum albumin (BSA) standard.

5.2.8 Volatile Fatty Acids

VFAs in the samples were determined using the static headspace gas chromatographic method according to the method outlined by Cruwy et al. (2002). The VFA analyser consisted of a headspace-sampling unit (Perkin Elmer, HS40XL, Beaconsfield, UK) connected to a gas chromatographic unit (Perkin Elmer, AutosystemXL), equipped with flame ionisation detector (FID) and a free fatty acid phase (FFAP) column (Supelco Ltd, Poole, UK). The VFA analyser functioned by using the headspace unit to automatically pick a vial containing a prepared sample, heating with an internally built oven at 190°C and pressurizing it at 14 psi, using nitrogen as a carrier gas. Using an injector port maintained at temperature of 200°C and a split flow of 5.0 ml/min, the volatilized sample was then carried by enhanced capillarity through the column (maintained at 200°C for 1 minute, increasing from an initial 60°C at 10°C/minute) and flame ionisation detector (optimized at 250°C). The quantity of VFA having been detected by the flame ionisation detector was evaluated based on the calibration of the machine using a standard made from known concentrations (0 - 1000 mg/l) of acetic, propionic, iso-butyric, n-butyric, iso-valeric and n-valeric acids.
The samples and standards were prepared according to the procedure described in Cruwys et al. (2002) (1 ml of 62% (w/v) NaHSO$_4$; 0.1 ml of 2 – ethylbutyric acid prepared at a stock concentration of 1.80 g/l).

5.2.9 Soluble Chemical Oxygen Demand Determination

The COD quantity of the sample was analysed using the Hach Lanch COD kit which was developed based on the Standard Methods (HMSO, 1986). The method involved the addition of 2.5 ml of diluted filtrate of a centrifuged sample, and the blank (deionised water) to a vial containing the COD reagents including potassium dichromate (K$_2$Cr$_2$O$_7$), followed by thorough mixing before incubating the sample at 150°C for 2 hours on a heating block (DriBlock - Techne Ltd, Cambridge, UK). K$_2$Cr$_2$O$_7$ with its strong oxidising properties under acid conditions was used to oxidize the organic carbons (C) of the sample. During the reaction, the hexavalent dichromate ion (Cr$_2$O$_7^{2-}$) gives up oxygen (O$_2$) to bond with the C atom to form CO$_2$, resulting in the reduction of the Cr$_2$O$_7^{2-}$ ion to Cr$^{3+}$. The dichromate ion resulted in a colour change, which was measured at 600 nm using a UV-VIS spectrophotometer.

5.2.10 Cation and Anion Analyses

The quantity of cations and anions in the sample were determined using a Dionex ion chromatograph equipped with a separation column; the Ionpac ICE-AS6 for anions analysis and IonPac CS12A column for cations analysis (Dionex, Camberley, UK). The analyser was also fitted with a self-regenerating suppressor (ASRS- ultra II for anion analysis or the CSRS-ultra II for cations analysis) and a conductivity detector. Methanosulphonic acid (CH$_3$SO$_3$H) at a concentration of 20 mM was used as the eluent for the cations analysis whilst eluent of the anions analysis was a reagent contained 1.8mM sodium carbonate (Na$_2$CO$_3$) and 1.7mM sodium bicarbonate (NaHCO$_3$).

The ion analyser worked by automatically taking the sample from the vial, using inbuilt syringe equipped with a 1.0 µm syringe filter (Gelman Sciences, USA), injecting it into the sampling port. The ions were extracted along the chromatograph column using the eluent, which was carried by means of a gradient pump at a flow rate of 1 ml/min. The type of ion was then detected using the conductivity detector.
The quantity of individual ions were evaluated by comparing the samples to a 10 point calibration curve of anion standards (chloride, fluoride, nitrate, nitrite, sulphate and phosphate) or cations standards (sodium, potassium, calcium and ammonium) prepared at a concentration of 0 to 100 ppm.

5.2.11 Elemental Analysis

Elemental compositional analysis that measures the proportions of carbon, hydrogen, nitrogen, sulphur and oxygen (C, H, N, S and O) in the biomass was determined using the Vario EL III elemental analyser (Elementar Analysensysteme GmbH, Germany). The thermal analysis process involved a controllable combustion of the samples in catalytic tubes in an oxygenated atmosphere and high temperature. Helium is used as a carrier gas, helium/hydrogen mixture as pyrolysis gas and oxygen as combustion gas. After combustion of the sample, the adsorption columns of the elements are detected by a thermal conductivity detector (TCD). Dry samples were ball milled to fine powder and weighed into an aluminium foil. Sulfanilic acid was used as standard for the determination of CHNS and benzoic acid was used as standard for the determination of O.

5.2.11.1 Determination of Total Nitrogen (TN)

Nitrogen in the biomass samples are present in the reduced form such as ammonia or ammonium, as well as in oxidized form such as nitrite and nitrate. During the determination of the nitrogen in the elemental analysis, nitrogen is lost through decomposition of ammonia/ammonium. The total nitrogen was then determined using the sum of the N determined by the elemental analysis and the quantity of N in the ammonia determined by the cation analysis.

5.2.12 Cellulase Enzyme Assay

The effluent of the acidogenic reactor was analysed for residual cellulase enzyme activity in accordance with the EnzChek fluorescence-based cellulase substrate protocol (Invitrogen, 2005). The principle of the technique is based on the amount of enzyme that is able to cause a change in fluorescence in the substrate. The samples were diluted to match the detection range of 4-3000 mU/ml. Reagents used in the assay included: 100 mM sodium acetate buffer (pH
5.0), Cellulase Substrate (E33953 EnzChek® blue fluorescent, Invitrogen, UK) and 50% DMSO, used in the preparation and storage of aliquots of the cellulase substrate. A 50 µL of control (digestion buffer), standard (Cellulase 13L, Biocatalysts Ltd, UK) were added to wells in a 96-well microplate. An amount of 50-µL enzyme substrate was added to each test and control well, and the mixture incubated at room temperature for 30 minutes. The fluorescence was then measured at excitation maxima of 360 nm and emission maxima at 460 nm, using the fluorimeter (Fluostar Optima Microplate Reader, BMG Labtech - Germany). Cellulase activity was determined by plotting a linear curve of amount of standard vs. fluorescence, and comparing the fluorescence of the sample to the data points that correspond to the amount of enzyme on the standard curve.

5.2.13 Characterization of Lignin by Fourier Transform Infrared spectroscopy (FT-IR)

The FT-IR analysis was carried out according to the method of Manaraa et al. (2013) using a Perkin Elmer Spectrum RX1. The freeze-dried extracted lignin (about 5 mg) was mixed with potassium bromide in 1:20 ratio (about 10 mg dried-sample to 200 mg KBr). The mixture was ground and then mechanically pressed at 2 bar to form a disc, before being scanned (Figure 5.2a shows the mechanical press). The sample was scanned in the 2000–400 cm⁻¹ range, with 25 scans, at a resolution of 4 cm⁻¹. Alkali lignin (Sigma, UK) was used as the reference substrate. The spectra of the extracted lignin were then compared with that of the alkali lignin.
5.2.14 **PHA Quantification**

Optical density (OD) was measured with a spectrophotometer (Hach DR 2800, US) at a wavelength of 650 nm. A 5 ml sample was measured, using deionised water as a blank.

The cell dry weight of the sample was determined by centrifuging an aliquot of known weight at 13,000 rpm for 10 minutes (Accuspin Micro R centrifuge), followed by washing of the residue with deionised water before freeze-drying (Lyotrap freeze dryer, LTE Scientific Ltd.). The final weight after freeze-drying was measured, and used in the determination of the cell dry weight.
PHA quantification was carried out according to the procedure described by Law and Slepecky (1961). The principle of quantification is based on the conversion of the residual polymer to crotonic acid by heat treating the substrate with concentrated sulphuric acid at 150˚C, and measuring the quantity of crotonic acid using Spectrophotometry (235 nm).

5.2.15 Total suspended solids

Total suspended solid (TSS) was determined using the standard method outlined in the APHA (1989). The procedure involved filtration of the sample though a pre-weighed fibreglass filter paper and measuring the dried weight of the residue. The filtration process was carried out using a fibreglass filter paper mounted on a Whatman three-piece filter funnel and Buchner flask, connected to a vacuum pump. The residue of the mixed samples after the vacuum filtration process was dried to a constant weight at 105±0.5˚C. TSS (mg/l) was determined by subtracting the weight of the filter paper from the total weight of the dried residue and the filter paper.

\[
\text{mg total suspended solids/l} = \frac{(A-B) \times 1000}{\text{sample volume, ml}}
\]

where:

\[A = \text{weight of filter + dried residue, mg, and}\]

\[B = \text{weight of filter, mg.}\]

5.3 Substrates

5.3.1 Sucrose

Sucrose was used as a model substrate in a control experiment. A commercial food grade granulated sugar was used as the sucrose source (Tate & Lyle, London, UK). The sugar was dissolved and diluted to a 20 g/L concentration. An amount of 0.1% antifoam (Dow Corning, Coventry, UK) and trace elements from CR Teplex were then added to the mixture at a concentration of 0.28 g/kg VS, and the mixture stored at 4˚C, and continuously stirred (Heidolph stirring unit, Cole Parmer, UK). The mixture was fed in a semi-continuous mode directly from the refrigerator to the bioreactor using a peristaltic pump (Watson Marlow, Falmouth, UK).
5.3.2 Perennial Rye Grass

perennial rye grass pellets (Perennial rye feed, Hampshire, UK) was used as substrate for the anaerobic digestion process. The perennial rye grass pellets were soaked in water and kept in a refrigerator overnight. The substrate was then diluted with water, containing suspended antifoam (polydimethylsiloxane, Dow Corning, Coventry, UK). Trace element from CR Teplex was then added to the mixture at a concentration of 0.28 g/kg VS. The perennial rye grass substrate was prepared to obtain a total solid equivalent of 49.1 g/l. The treated feed was then transferred to a feed storage tank, which was kept a 4-6°C in a refrigerator to limit microbial growth. At pH 11.5, microbial activity in the feed storage tank was limited as observed by Massanet-Nicolau et al. (2013).

5.3.3 Fructooligosaccharides

Fructo-oligosaccharide used as the substrate in the PHA production studies was obtained from Biocare Limited, UK. The concentration of the fructo-oligosaccharide used was 1.8 g/l equivalent to the residual soluble carbohydrate found in the perennial rye grass hydrolysate after the acidogenic fermentation. The substrate was prepared by the addition of peptone, meat and yeast extract as nutrient source for Cupriavidus necator, the pure culture PHA bacteria strain used in the Experiment PB2 (Section 5.8.5).

5.4 Pre-treatments

5.4.1 Alkaline and Enzymatic Pre-treatment

The perennial rye grass pellets were alkaline treated with 0.46% NaOH by adding 4.6 ml of concentrated NaOH in 1 litre of perennial rye grass slurry.

The perennial rye grass substrate having been found to be more recalcitrant to hydrolytic-acidogenic fermentation when compared to the wheat-feed substrate was selected for further pre-treatment to improve the biohydrogen process. The enzymatic pre-treatment was carried out in for major experiment, involving the use of commercial enzymes including cellulase (Accellerase ® 1500, Genencor, USA), xylanase (Accelerase ®XY, Genencor, USA) and ferulic acid esterase (Depol™ 740L, Biocatalyst, UK). The cellulase enzyme stock had an enzyme activity of 25,500 kU/ml. The xylanase and FAE enzymes had specific activity of 20,290 kU/ml and 11, 590 kU/ml, respectively.
Single enzyme and enzyme combinations treatment were carried out on perennial rye grass, using increasing concentrations of enzymes based on unit enzyme activity. In each experiment 49 g of dried perennial rye grass pellets was weighed into a 1 litre plastic container and the container was half filled with water. A concentration of 0.01% (w/v) sodium azide was added in order to inhibit microbial growth, followed by addition of the require concentrations of enzymes (based on enzymatic activities). After mixing the content of the container, it was topped up with water to the 1 L mark. The samples were then placed in an incubator (Gallenkamp, UK), mixing at 150 rpm. The incubation was carried out at a temperature of 40˚C, for 3 days, taking samples every 24 hours. All experiments were carried out in triplicate. Samples were taken at start of the experiment and then after every 24 hours for 3 days. The analysis carried out on the samples included soluble chemical oxygen demand (sCOD) and soluble carbohydrates.

5.5 Continuous Biohydrogen/ Acidogenic Fermentation Reactor

5.5.1 Inoculum

The sewage sludge used to inoculate the biohydrogen reactors (Section 5.8.3.2) was obtained from the Cog Moors Sewage Treatment Works, Cardiff, UK; an operational full scale anaerobic digester. The sludge was coarsely sieved with a 2 mm mesh, in order to remove particulates such as grit and rags. Prior to seeding the digester, the seed sludge was heat treated at 110˚C for 15 minutes in a domestic pressure cooker. The quantity of heat treated sludge added at start of the biohydrogen reactor was equivalent to 3 g/l of the TS (HMSO, 1988).

5.5.2 Reactor Design

The reactor used in the biohydrogen fermentation process was a continuously stirred tank reactor (CSTR) with a working volume of 5 L. The stainless steel reactor was built with ports for feeding, nutrients addition, pH control, sampling, gas-outlet and pH probe. The feedstock in a 30 L storage tank was pumped using Watson Marlow 620S pumps fitted with marprene tubing (Watson and Marlow, Falmouth, UK). Same pumping set-up was used to pump the effluent. The mixing of the reactor content was carried out using an adjustable electric overhead stirrer (Heidolph Instruments, Schwabach, Germany) at 150 rpm. Figure 5.3 shows a schematic illustration of the hydrogen reactor and Figure 5.4 shows a photograph of the reactor set-up.
Figure 5.3: Schematic illustration of the biohydrogen reactor.

Figure 5.4: Photograph of the biohydrogen reactor set-up.
5.5.2.1 Temperature control
Warm water, which had been heated using Grant flow heaters (Grant Instruments, Cambridge, UK), was re-circulated through silicone tubing coiled around the vertical peripheral of the reactor, in order to maintain the conditions of the reactor at a mesophilic temperature of 35±0.5°C. The temperature data was continuously logged online, being measured using a Temperature-pH probe (Mettler Toledo).

5.5.2.2 Control of pH
The pH of the reactor was maintained between 5.4 and 6.0 pH, using a pH electrode (HA405-DXK-S8/120, Mettler Toledo) connected to a pH meter (Mettler Toledo, pH 20250e, Leicester, UK). The pH meter was calibrated before each experiment using standard buffer solutions of pH 4.01±0.01 and pH 7.01±0.01 (Thermo Scientific, the Netherlands). The pH readings were processed on a computer via a data acquisition card (National Instrument, Hungary). The pH information was then used to communicate to the pH control pump via data acquisition software (Labview, Newbury, UK). The pH control pump upon receiving instructions from the computer delivered 2M NaOH to maintain the pH when it dropped to pH 5.4. The activity of the hydrolytic-acidogenic bacteria ensured that the pH in the reactor was always below pH 6, therefore it was not necessary to have acidic buffer to reduce the pH.

5.5.3 Online monitoring

5.5.3.1 Biogas Volume
The volume of the biogas produced in the digestion process was determined using a wet tip gas meter (Wet Tip Gas Meter, USA). The gas meter utilises the liquid displacement principle. As the volume of gas increases, it displaces the water, which causes a plastic counter incorporated with internal magnet to trigger a reed switch, sending information via the data acquisition card to the computer equipped with Labview data acquisition program. The tip metre was calibrated to a known volume of gas, and total volume of gas was then determined by multiplying the discrete volume increase per tip by the total tip count.

5.5.3.2 Hydrogen Content
The hydrogen content of the biogas was determined using a Robust Hydrogen Sensor (HY-OPTIMA 740, Valencia - USA). The hydrogen analyser used a thin film palladium-nickel as solid-state sensor that absorbed hydrogen molecules, thereby increasing the resistance of the
sensor. The Pd/Ni sensor then interpreted the resistance, converting it to a voltage output. The Hy-Optima 740 hydrogen analyser has a voltage output of 0 to 5 volts, corresponding to a hydrogen percentage range of 0.5% to 100% (v/v) at 1atm. According to the manufacturer, the sensor has an accuracy of ± 0.15% absolute for 0.5 to 10% H2, and ± 0.50% absolute for 10 to 100% H2. The hydrogen sensor was calibrated with gas with hydrogen concentrations ranging from 0.5 to 100%, achieving linear correlation with an R² value of 0.98 or greater.

5.5.3.3 Data Acquisition

The monitoring of the bioreactor was carried on a custom interface Labview programme developed with the Labview data acquisition system, consisting of data acquisition card (PCI-036E) and NI-DAQ driver software installed on a windows desktop computer. The Labview programme logged measurements data every second. At every 3-minute interval, the compile data were averaged and saved on a text file. The averaged data was displayed as graphs on the computer screen; giving an indication of the performance of the reactor.

The pumping (influent/sustrate, effluent, nutrient and pH) were all controlled via the data acquisition card (DAQ). All the reactor controlling sensors and actuators were connected directly to the DAQ, then interpreted the control signals, except the feed and effluent pumps, which were connected to the DAQ via a solid state relay (RS Components, UK), which allowed on and off switching of the pump at defined intervals.

5.6 Batch Reactors

The seed sludge used for the inoculation of the BMP reactors was obtained from the Cog Moors Sewage Treatment Works, Cardiff, UK. The seed sludge was sieved through a 2.0 mm sieve, in order to remove relatively large size particulate matter in the sludge. The inoculum to substrate ratio used in the batch biomethane potential experiment was 2:1.

The BMP procedure was used as a simple and inexpensive technique to evaluate the process kinetics and the quality of the the pre-treated perennial rye grass substrates for anaerobic digestion; leading to a fairly accurate estimation of the yield of biomethane from the substrates (Angelidaki et al., 2009; Owen et al., 1979; Abu-Dahrieh et al., 2011). Batch methane digestion involved the use of the Bioprocess Control AMPTS II (shown in Figure 5.5), which is based on the protocol described by Angelidaki et al. (2009). The anaerobic digestion process involved the use of a 500 ml reaction vessel, mounted with a stirrer and
sampling and gas outlets. The gas outlet was connected to 3M sodium hydroxide scrubber, which was then connected to a wet-tip meter.

Figure 5.5: Schematic illustration of the BMP reactor.

Figure 5.6: Photograph of the Bioprocess control AMPTS II.
5.7 Experimental Regimes

5.7.1 Experiment CA: The Fate of Lignocellulosic Biomass in Anaerobic Processes

The compositional analyses involving wheat-feed and perennial rye grass as substrates for anaerobic digestion systems were based on the quantity of NDF, ADF and ADL in the residual mass obtained from a single-stage anaerobic digester and a two-stage (biohydrogen-biomethane) anaerobic digester, operating at the same overall hydraulic retention times (HRT) (Massanet-Nicolau et al., 2013; 2014). The single-stage methanogenic reactor (A20) and the two-stage reactor (M19) were both maintained at a 20-day HRT. The two-stage AD system consisted of an acidogenic reactor, operating at 0.75 day HRT (HA), and a methanogenic reactor (M19) operating at 19.25 days.

Aliquots of the substrates and effluents and digestates from the three reactors were taken and stored for compositional analysis weekly, for 3 weeks, for both the wheat-feed and the perennial rye grass, after the reactor was observed to have achieved stability after being operated for about 100 days. The average TS (g/l) and VS$_{\text{DW}}$ (%) recorded from day 101 to day 122 was found to be consistent or stable (as shown in Table 5.2), with the standard deviations (SD) in both substrates, in all the reactors, below 2%, giving an indication of the stability of the environmental conditions in the reactors.

Table 5.2: Average values of total and volatile solid content of biomass in experimental period

<table>
<thead>
<tr>
<th></th>
<th>Wheat-feed</th>
<th>Perennial rye grass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TS (g/l)</td>
<td>SD</td>
</tr>
<tr>
<td>Feed</td>
<td>51.98</td>
<td>0.97</td>
</tr>
<tr>
<td>HA</td>
<td>43.07</td>
<td>0.99</td>
</tr>
<tr>
<td>A20</td>
<td>18.45</td>
<td>0.86</td>
</tr>
<tr>
<td>M19</td>
<td>16.20</td>
<td>0.17</td>
</tr>
</tbody>
</table>

The compositional analyses experiments were undertaken; with the first batch of samples taken on the 107th day of the anaerobic digestion process for analysis; the second batch of samples were taken on the 115th day of continuous digestion, and the last batch of samples were taken on the 122nd day. Having consistent results in the VS as an indication of the study...
state meant that the results of the residual biomass compositional analysis on the effluent the HA reactor and digestates of the M19 and A20 reactors, was a good representation of the degradation process within the period under study. The analyses carried out on the samples from the bioreactors included: determination of the TS, VS, NDF, ADF and ADL, VFAs, carbohydrates, proteins, ion analysis and pH. All analyses were undertaken in triplicate. Experiment CA involved the compositional analyses of the feedstock and effluent and digestate of the reactors involving wheat-feed and perennial rye grass as substrates. A schematic illustration of the experimental equipment is illustrated in Figure 5.7.

5.7.2 Enzymatic pre-treatment for the depolymerisation of the lignocellulosic complex in Perennial rye grass

5.7.2.1 Experiment PT1: Enzymatic Pre-treatment

The determination of suitable concentrations of commercial enzymes with effective hydrolysis potential, involved four main modes of enzymatic treatment experiments. The experiments were carried out using dried perennial rye grass in water at a concentration of 49.1 g/l TS as substrate, and maintained at a temperature of 40°C. The experiment was run for 3-days, and samples were taken at time 0, 24, 48 and 72 hours, to evaluate the yield of soluble COD. The experiments sought to determine the ideal concentration of individual enzymes or the combination of enzymes with high-yielding enzymatic hydrolysing potential on perennial rye grass. The experiments included.

i. Experiment PT1.1: the use of different concentrations of cellulase enzyme,
ii. Experiment PT1.2: the use of different concentrations of xylanase enzyme,
iii. Experiment PT1.3: the use of different concentrations of ferulic acid esterase (FAE), and
iv. Experiment PT1.4: the use of different combinations of cellulase and xylanase, cellulase and FAE, xylanase and FAE.

The additional soluble COD released due to the enzyme and the percentage yield increase of soluble COD due to the enzyme pre-treatment was calculated as shown in Section 5.7.2.2
5.7.2.2 Calculation of soluble chemical oxygen demand (COD) yield

<table>
<thead>
<tr>
<th>Concentration COD released (g/l)</th>
<th>COD of sample – COD of enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>COD released (g/l) X volume (l)</td>
<td>Amount COD released (g)</td>
</tr>
<tr>
<td>Yield COD (mg/g VS) =</td>
<td>(\frac{\text{Amount of COD released (mg COD)}}{\text{g VS of grass added}})</td>
</tr>
<tr>
<td>[g VS added = amount of perennial rye grass pellets added x TS% x VS%]</td>
<td></td>
</tr>
</tbody>
</table>

**Additional yield released by enzyme** = Yield (Enzyme)- Yield (control)

\[
\% \text{ increase due to enzyme} = \frac{\text{Yield (Enzyme)} - \text{Yield (control)}}{\text{Yield (control)}}
\]

5.7.2.3 Experiment PT2: The role of alkaline pre-treatment in the homogeneity of dried perennial rye grass substrate to prevent clogging of pumps

A 50-gram amount of dried perennial rye grass was weighed into 1-litre beakers and half filled with water. The dried pellets were allowed to dissolve and the mixture was thoroughly stirred using a plastic stirring rod. To one of the beakers (PT1), 0.46% of concentrated NaOH (10M) was added bringing the concentration to pH 11.0±0.5. To another beaker (PT2), cellulase enzyme concentration of 1% (w/w) was added. The last setup involved perennial rye grass in with water with no addition of alkaline or enzyme (PT0). The total volume of the mixture was brought to 1 litre by adding water. The mixture was stirred vigorously for a minute, and then allowed to settle for 30 minutes. The top layer (comprising of the liquid fraction and the suspended solids) was was poured into a separated beaker for TSS measurements. A schematic illustration of the experimental is illustrated in Figure 5.8.
5.7.3 Biohydrogen Experiments

5.7.3.1 Control/Reference Experiment

The control experiment was carried out using sucrose as a model substrate and was operated for 17 days in a 5 litre CSTR. By knowing the quantity of COD in the enzyme combinations, the experiment helped to ascertain what quantity of the overall biogas yield could be attributed to the residual carbohydrates in the enzyme combinations. The experiment was fed continuously at a 24 hour HRT, at a feed concentration of 10 g/l. The digester was operated at an organic loading rate of 10 g/l/day. The temperature was maintained at 35±0.5°C, and pH was maintained around 5.5 using 3M NaOH solution.

5.7.3.2 Experiment HAG-U and HAG-ALK: Dried perennial rye grass as substrate for biohydrogen fermentation

Continuous biohydrogen production was carried out using dried perennial rye grass as substrate. Experiment HAG-U involved the use of untreated dried perennial rye grass pellets as substrate, whilst Experiment HAG-ALK involved the use of alkaline pre-treated perennial rye grass pellets as substrate.

In Experiment HAG-U, the HAG-U\textsubscript{substrate} was prepared by soaking the dried perennial rye grass pellets in water and adding trace elements and antifoam. In Experiment HAG-ALK, 0.46% NaOH (w/w) was also added to the perennial rye grass, increasing the pH in the HAG-ALK\textsubscript{substrate} to about 11. In both HAG-U and HAG-ALK experiments, the feedstock was maintained around a final total solid concentration of about 49.1 g/l. The 5 litre reactor (as described in Section 5.5) was operated at an organic loading rate of 42 kgVS/m\textsuperscript{3}/day and an HRT of 24 hours; and the environmental conditions was maintained at a temperature of 35±0.5°C, and pH of 5.4 to 5.6. Aliquots of the feedstock and the effluent from the bioreactor were taken every day for compositional analyses. The CSTR reactor was initially fed with the untreated perennial rye grass substrate (HAG-U\textsubscript{substrate}, after 10 days of continuous running; the substrate was then changed to the alkaline pre-treated substrate (HAG-ALK\textsubscript{substrate}).
5.7.3.3 Experiment HAG-ALKENZ: Enzymatic Simultaneous Saccharification and Fermentation

The enzymatic SSF was carried out using alkaline pre-treated perennial rye grass pellets as feedstock (HAG-ALK_{substrate}) for biohydrogen generation. The reactor (as described in Section 5.5) was operated at an organic loading rate of 0.42 g/g VS, and an HRT of 24 hours. An enzyme cocktail consisting of a mixture of cellulase enzyme and ferulic acid esterase (selected from the enzymatic hydrolysis experiment) was fed daily to the reactor at 0.202 ml/g. The effect of the enzyme addition on hydrogen yield and VFA production was then monitored. The reactor was maintained at a temperature of 35.0±0.5°C and pH of 5.5, for 21 days. Schematic illustration of the experimental is illustrated in Figure 5.9. Samples were taken daily from the effluent tank for compositional analysis.

5.7.4 Bio-methane Potential Experiment

The bio-methane potential test was carried out in four reactor setups, and each setup was made of three replicates. Reactor 1 (B-blank) was the blank, which involved the treatment of the seed sludge alone. Reactor 2 (B-REF) was the reference substrate (cellulose); used for evaluating the quality of the inoculum by comparing the results to previous studies. Reactor 3 (B-ALK) was fed with effluent of the acidogenic reactor treating perennial rye grass with 0.46% NaOH pre-treatment. The effluent of the acidogenic fermentation involving the enzymatic SSF treating perennial rye grass with 0.46% NaOH pre-treatment, was used as substrate for reactor 4 (B-ALKENZ). The reactors had a working volume of 0.5 litres. All the substrates (blank, cellulose, and perennial rye grass) were loaded into the bioreactors based on the inoculum to substrate ratio of 2:1. 400 ml of inoculum was maintained in all reactors, whilst the substrate was loaded to achieve the same substrate to inoculum ratio based on the VS. After seeding, the reactors were sealed and the headspace was flushed with nitrogen to ensure anaerobic conditions for the fermentation process. The temperature of the reactor was maintained at 35°C. The content of the reactors were mixed automatically at 3 minute intervals. The gas outlet of each reactor was connected to a wet tip meter via a 3M NaOH - CO_{2} scrubber (Mahmoudkhani and Keith, 2009). The scrubber removed the CO_{2} from the biogas so that the gas volume recorded via the wet tip was entirely methane. The reactors were operated for 30 days, after which samples were taken for analysis. The experimental equipment is illustrated in Figure 5.10.
5.7.5 PHA Biosynthesis Experiment

PHA production was carried out using effluent from the acidogenic reactor after fermentation of the enzymatic SSF as substrate (as illustrated in Figure 5.9). The behaviour of soluble carbohydrates degraded in the fermentation process was compared with the utilisation of a commercial fructo-oligosaccharide as substrate. *Cupriavidus necator* (DSMZ 54, DSMZ, Germany), a pure culture gram-negative bacteria strain was used as inoculum.

i. Experiment PB1: involved the use of the acidogenic effluent as a nutrient source

ii. Experiment PB2: involved the use of commercial fructo-oligosaccharide (Section 5.3.3). Oligofructan media was prepared by mixing 1.8 g of the oligofructan (as substrate), 5g/l peptone and 3 g/l meat extract (as nutrients).

The initial pH of both media were adjusted to pH 7.00 by adding 5M NaOH; and acetic acid was supplied as pH control during experiments. Antifoam (Antifoam 204, Sigma Aldrich) was then added reduce forming during the fermentation. The 5-litre CSTR fermenter and its content was the autoclaved at 121°C. The fermentation process was monitored online using a dielectric spectroscopy probe (Aber Instruments, UK) which measured the capacitance, giving an indication of the rate of bioplastic accumulation by the microbes, as described by Kedia et al. (2013). The fermentation was controlled at neutral pH, 30°C temperature and 40% DO.

5.7.6 Experiment L: Lignin Extraction

Experiment L involved the extraction of lignin from the HAG-ALK<sub>effluent</sub>, and HAG-ALKENZ<sub>effluent</sub>, as illustrated in Figure 5.9. The slurries from both experiments were dried at 55°C for 48 hours. The dried samples were ground with a ball mill to pass through a 1mm sieve. The dried perennial rye grass residue was then placed in a 500 ml Duran bottle. The sample was then pre-treated using the Organosolv (formic/acetic acid method), described by Manaraa et al. (2013), which involves the use of formic acid/acetic acid/water mixture in a volume ratio of 30/50/20. The sample was initially soaked with the reagent at a liquid:dry matter ratio of 25:1 for 30 minutes at 50°C. The mixture was then stirred to initiate the reaction, and the temperature was increased to 107°C (Stuart hotplate stirrer, RS Components, UK). The pre-treatment was carried out for 3 hours maintaining stirring at 450 rpm. The mixture was then removed from the heating and stirring plate, and allowed to cool to ambient
temperature. The hydrolysate was then filtered with a gravity filter funnel fitted with a fritted disk (40–100 µm pore size; Robu Glasfilter-Geräte GmbH). The residue was washed twice with the Organosolv solution. After filtration, acidified water (HCl at pH 2) was added in 1 in 5 dilutions to all the washes while stirring in order to precipitate the lignin. The precipitated lignin was then recovered by centrifuging and washing with acidified water, followed by freeze-drying. The extraction process was carried out in duplicates. The quality of the extracted lignin from the HAG-ALK\textsubscript{effluent} (LA) and the extracted lignin from the HAG-ALKENZ\textsubscript{effluent} (LE) were the compared with a standard alkali lignin (LR). Schematic illustration of the experimental is illustrated in Figure 5.9.

5.7.7  Statistical Methods

5.7.7.1  Pearson’s Correlation

The IBM SPSS Statistics software was used in the evaluation of the Pearson’s correlation. The Pearson’s correlation was used to assess the linear correlation (dependence) between the NDF of all the dried samples from the substrates and effluent of the HA reactor and digestates of the A20 and M19 reactors, and the concentrations of chemical elements, CHNSO, that form their corresponding structure. The correlation utilised a sample size of 48 for each of the variable (NDF and chemical elements). The sample size (N=48) included a sum of 12 samples from each stage of the fermentation process (HA, A20 and M19).

5.7.7.2  Independent Sample T-Test

The IBM SPSS Statistics program was used to analyse the independent samples t-test. The independent t-test statistical analysis measures the level of difference between the mean of the quantity of the undigested components of the substrates and effluent and digestates (NDF, soluble carbohydrate, cellulose, hemicellulose, ADL, protein) of:

i. the HA reactor and M19 reactor.
ii. A20 reactor and M19 reactor

The independent samples t-test helped ascertain whether there was a statistical evidence that the means of the two variables are significantly different. The sample size was 12 for each of the variables.
5.7.7.3 One-way ANOVA

The one-way analysis of variance (ANOVA), was used to determine whether there was any significant difference in the means of ADL of the samples from the substrates, the $\text{HA}_{\text{effluent}}$ and the $\text{M19}_{\text{digestate}}$. The one-way ANOVA helped to evaluate the homogeneity variances of the ADL means in the two-stage (biohydrogen-biomethane) AD system. The sample size was 36, comprising of 12 samples from each of the stages.

5.7.8 Economic Calculations for biogas

Assumptions: A centralized fermenter of size about 173 m³, treating 10,000 tonnes of perennial rye grass per year.

Substrate (perennial rye grass) = 10,000 tonnes/year

Biogas yield = BY (m³/kg VS)

VS content (tonnes/year) = St x TS (%) x (VS)

Expected biogas (m³ biogas/year) = VS content (kg/year) x BY (m³/kg VS)

Amount of biogas produced per year is then converted to amount of biogas per hour, BY (m³/hour)

Calorific value (High heating value) - methane = 39 MJ/m³; hydrogen 12.75 MJ/m³

Power available to CHP, $P_w$ (Watts) = BY (m³/hour) x calorific value (MJ/m³) x $10^6/3600$

Assumptions: CHP unit has 30% conversion efficiency for electricity and 55% conversion efficiency for heat.

Electricity production = $P_e$ (kW) x 0.3

Electricity production per year = $P_e$ (kW) x 24 x 365 = $P_e$ (kWh/y)

Heat production = $P_h$ (kW) x 0.55

Heat production per year = $P_h$ (kW) x 24 x 365 = $P_h$ (kWh/y)

Generation tariff = 10.37 pence/kWh

Export tariff = 4.77 pence/kWh (FiT, 2014)
The assumptions for the calculation of the heat and electricity production from the biohydrogen produced in the process were as follows:

Calorific value of hydrogen 121 MJ/kg = 10.043 MJ/m³

Electricity production via fuel cell has a conversion efficiency of 50%, and heat production via CHP unit has a conversion efficiency of 60%
Part 1: Experiment CA – Compositional analysis on ample from the reactors of Massanet-Nicolau et al. (2013, 2014)

Figure 5.7: Schematic illustration of the experimental plan
Part 2: Experiment PT - Perennial rye grass pre-treatment

Experiment PT1 – Enzymatic Pre-treatment

Perennial rye grass pellets

+ Water and 0.01% sodium azide

perennial rye grass

Control + Cellulase (C) + Xylanase (X) + FAE (F) + CX + CF + XF

PT₀

PT₂

Analysis: soluble COD, soluble Carbohydrates

Experiment PT2 - The role of alkaline pre-treatment in the feedstock homogeneity

Perennial rye grass pellets

+ 0.46% (v/v) of conc. NaOH

+ 1% cellulase

PT₀

PT₁

PT₂

TSS analysis

Definitions:

PT₀: control or untreated perennial rye grass

PT₁: alkaline treated perennial rye grass

PT₂: enzyme treated perennial rye grass

Figure 5.8: Schematic illustration of the experimental plan
Part 3: Experiment G – Biofuel and bio-products production

Definition:
HAG-U: Biohydrogen reactor with untreated PGR feed
HAG-ALK: Biohydrogen reactor with alkaline treated PGR feed
HAG-ALKENZ: Biohydrogen reactor with alkaline treated PGR feed in enzymatic SSF
HAG-U_{effluent}: slurry effluent from HAG-U reactor
HAG-ALK_{effluent}: slurry effluent from HAG-ALK reactor
HAG-ALKENZ_{effluent}: slurry effluent from HAG-ALKENZ reactor
LR: KBr disc of standard alkali lignin as reference lignin for FTIR analysis
LA: KBr disc of lignin extracted from dried effluent of HAG-ALK reactor
LE: KBr disc of lignin extracted from dried effluent of HAG-ALKENZ reactor
BMP: Biomethane potential (go to Figure: 5.7)
PHA: Bioplastic production

Figure 5.9: Schematic illustration of the experimental plan
Part 3b: Biomethane potential (BMP) batch experiments

Definition:

B-BLANK: BMP reactor treating sewage sludge feed as blank
B-REF: BMP reactor treating cellulose as reference substrate
B-ALK: BMP reactor treating effluent of HAG-ALK reactor as feedstock
B-ALKENZ: BMP reactor treating effluent of HAG-ALKENZ reactor as feedstock
B-BLANK_{digestate}: digestate effluent from B-BLANK reactor
B-REF_{digestate}: digestate effluent from B-REF reactor
B-ALK_{digestate}: digestate effluent from B-ALK reactor
B-ALKENZ_{digestate}: digestate effluent from B-ALKENZ reactor

Figure 5.10: Schematic illustration of the experimental plan
CHAPTER 6

6 COMPARATIVE STUDY OF THE FATE OF TWO LIGNOCELLULOSE SUBSTRATES (WHEAT-FEED AND DRIED PERENNIAL RYE GRASS) IN ANAEROBIC DIGESTION PROCESSES

6.1 Principle and Hypothesis of Study

Massanet-Nicolau et al. (2012), using dried wheat-feed pellets as a model lignocellulosic feedstock in a two-stage biohydrogen and biomethane anaerobic process, reported a 38.5% increase in energy yield (based on the VS wheat-feed added), compared to a conventional single-stage. Massanet-Nicolau et al. (2014) also used real time gas production data to compare continuous single-stage and two-stage anaerobic digestion of dried perennial rye grass pellets in parallel experiments, using the same conditions as in the wheat-feed. The energy yield of biogas estimated from the perennial rye grass was 13.4% higher in the two-stage system when compared to the single-stage anaerobic digestion system. The energy yields they obtained were relatively high, when compared to previously published reports on biogas yields from lignocellulosic biomass (Hills and Roberts, 1981; Jin et al., 2012).

In their work, the hydrogen yield from the acidogenic reactor (HA) treating the wheat-feed was found to be 7.48 L H₂/kg VS. When the effluent of the hydrogen reactor running at an HRT of 0.75 days, was fed to a second-stage methanogenic reactor at 19.25 day HRT (M19), making a two-stage AD process of overall HRT of 20 days, the methane yield was found to be 359.65 L CH₄/kg VS. The digestion of the wheat-feed directly in a single-stage anaerobic digester resulted in a methane yield of 261 CH₄/kg VS. The perennial rye grass substrate was also treated in a conventional single-stage AD process (A20) at 20 day HRT resulting in a biogas yield of approximately 310 L CH₄/kg VS. In the two-stage system, the hydrogen yield from the first-stage acidogenic fermentation was found to be 6.7 L H₂/kg VS. When the acidogenic effluent was fed to a second-stage methanogenic reactor (M19) at an overall HRT of 20 days, the methane yield was found to be 349.4 L CH₄/kg VS. (Massanet-Nicolau et al., 2012; 2013)

The average yield of biogas produced in the acidogenic reactor in both lignocellulosic substrates was nearly 40 times lower than the biogas yield in the single-stage AD reactors.
The current study sought to find answers to the differences and similarities in the degradation of the lignocellulose substrates that resulted in the performance of the two substrates in the AD systems. The focus of this work was to compare the trend of degradation of the basic components of the two lignocellulosic substrates in the AD systems. In summary, the major questions this chapter was set out to investigate were:

- To what extent does a separate acid-phase affect the anaerobic degradation of wheat-feed and perennial rye grass?
- How does the pattern of degradation of the lignocellulose components in the two substrates (wheat-feed and perennial rye grass) correlate with their corresponding biogas yield in anaerobic digestion processes?

6.2 Effect of the Chemical Structure of Lignocellulose on Anaerobic Digestion (Results and Discussion)

The chemical structure of lignocellulosic substrates plays a significant role in its degradation in anaerobic processes, vis-à-vis, biofuel and biochemical yields. Table 6.1 and 6.2 show the correlation between the NDF of the dried samples from the substrate and effluents/digestate of the bioreactors (HA, A20 and M19) and the concentration of chemical elements, C, H, N, S, and O, that form their corresponding structure. As emphasized by Jeffries (1994), the pattern of degradation of lignocellulosic biomass is defined by the diverse and complex configuration of the polysaccharide-lignin linkages. As shown in Table 6.1, there was a strong significant positive relationship between the NDF, the carbon, the hydrogen, and the oxygen contents in the degradation of the perennial rye grass in the anaerobic processes (A20, HA, MA). The relationship between NDF and carbon ($r (98) = 0.954, p< 0.01$), and hydrogen ($r (98) = 0.984, p<0.01$), and oxygen ($r (98) = 0.982, p<0.01$) indicated that, as the lignocellulose complex was degraded, the carbon, hydrogen and oxygen composition showed a similar pattern of degradation. The carbon, hydrogen and oxygen contents therefore accounted for approximately 90% of the variance in the NDF content of the perennial rye grass. The nitrogen content showed a significant positive correlation with the NDF ($r (98) = 0.876, p<0.01$), indicating that as the NDF decreased, the nitrogen level also decreased. The sulphur content on the other hand showed no significant correlation at the 0.01 significance level (2-tailed) with the NDF ($r (98) = 0.207, p< 0.172$).
Table 6.1: Correlation between NDF and CHNSO in perennial rye grass

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**. Correlation is significant at the 0.01 level (2-tailed); PCC – Pearsons Correlation Coefficient
Table 6.2: Correlation between NDF and CHNSO in Wheat-feed

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**. Correlation is significant at the 0.01 level (2-tailed).

As shown in Table 6.2, there was a strong significant positive correlation between the NDF and the carbon, the hydrogen, and the oxygen contents in the degradation of the wheat-feed. The relationship between NDF and carbon (r (98) = 0.988, p< 0.01), and hydrogen (r (98) = 0.985, p<0.01), and oxygen (r (98) = 0.997, p<0.01) indicated that the fate of the lignocellulose components in the biomass had a direct link with the basic elements of the biomass. The carbon, hydrogen and oxygen content of the wheat-feed, similar to the
perennial rye grass, also accounted for approximately 90% of the variance in the NDF content. The nitrogen content, on the other hand, show a negative correlation with the NDF ($r(98) = -0.811$, $p<0.01$), indicating that as the NDF value decreased the nitrogen level increased. Contrary to the observation made from perennial rye grass, the sulphur content in the wheat-feed showed a significant positive correlation with the NDF ($r(98) = 0.746$, $p<0.01$).

The results indicate that the distribution of the C, H, N, S and O in the chemical structure was responsible for the variation in the pattern of degradation of the NDF in the perennial rye grass and the wheat-feed substrate. It can be seen that the fate of C, H and O were very similar in both substrates. The variation in the chemical matrix was therefore attributed to the distribution and utilisation of sulphur and nitrogen. The difference in the NDF-nitrogen relationship in the perennial rye grass and wheat-feed substrate was attributed to the varying amount of the incorporated protein and the influence of the microbial population growth patterns, which influence the release of ammonia in the system. The correlation of the NDF with the chemical elements gave an indication that the quantity of undigested components of the lignocellulosic biomass (cellulose, hemicellulose and ADL), had a direct link with the basic constituent of the chemical structure. The quantity of the residual lignocellulose components before and after the anaerobic digestion was therefore sufficient to indicate behaviour of the chemical structure of the biomass.

### 6.2.1 Nutrients (C:N:P:S - ratio)

The C/N ratio in the wheat-feed and perennial rye grass substrates were both found to be about 14:1. Deublein and Steinhauser (2006) recommended a C/N-ratio of range of 16: 1 - 25: 1 for most biomass substrates. The C/N-ratio was around the recommended range, but as explained by Deublein and Steinhauser (2006) some of the nitrogen in the lignocellulosic substrates is bound to the lignin structure limiting their actual availability in metabolism. The addition of nutrients in the AD process ensured that the right balance of carbon to nitrogen was obtained. The ammonium ($\text{NH}_4^+$) concentration in the HA$_{\text{effluent}}$ with perennial rye grass as substrate was found to be $0.56\pm0.03$ g/l. After the digestion in the M19 reactor the NH$_4^+$ concentration was found to be $1.11\pm0.01$ g/l, compared to a concentration in the A20$_{\text{digestate}}$ which was found to be $0.84\pm0.04$ g/l.
In the wheat-feed substrate digestion, the NH$_4^+$ concentrations in the HA$_{\text{effluent}}$ and M19$_{\text{digestate}}$ were found to be 0.24±0.04 g/l and 1.37±0.02 g/l, respectively. The NH$_4^+$ concentration in A20$_{\text{digestate}}$ was found to be 0.96±0.03 g/l, nearly 30% less that the concentration in the M19$_{\text{digestate}}$.

The concentrations of the NH$_4^+$ levels in all the reactors were all below the inhibitory levels of NH$_4^+$-N > 1500 mg/l (Deublein and Steinhauser, 2006). The concentrations of the NH$_4^+$ ions in the effluents and digestates of the AD systems gave an indication that the C/N ratios were not so low as to cause any inhibitory effect by increasing ammonium or ammonia content.

The C:N:P:S nutrient ratio in dried perennial rye grass was found to be 303:21:4:3. After the acidogenic fermentation, the HA$_{\text{effluent}}$ which is the substrate for the M19 reactor had a nutrient ratio of C:N:P:S of 250:18:5:3. The C:N:P:S ratio in dried wheat-feed substrate was found to be 346:25:3:3. After the acidogenic fermentation of the wheat-feed substrate, the HA$_{\text{effluent}}$ was found to contain a nutrient ratio of C:N:P:S of 394:31:9:3. Deublein and Steinhauser (2006) highlighted that a nutrient ratio of C:N:P:S of 500–1000:15–20:5:3 was sufficient for a stable anaerobic process. The concentration of micronutrients (N, P, and S) in the substrate for each reactor or stage of the AD process was therefore considered adequate for robust and sustainable anaerobic microbial activities. The sulphur concentration as a micronutrient was ideal, and therefore posed no threat of excess H$_2$S formation to inhibit the methanogenic process.

6.3 Effect of Structural Components of Lignocellulosic Biomass on Bio-Hydrogen and Bio-Methane Anaerobic Digestion (Results and Discussion - Experiment CA)

The study evaluated the effect of the structural components of the lignocellulosic biomass (NDF, cellulose and hemicellulose, and ADL) on the anaerobic digestion of wheat-feed and perennial rye grass substrates in the HA reactor and M19 reactor, as described in Experiment CA in Method-Section 5.7.1. Both substrates were subjected to anaerobic digestion in a two-stage (biohydrogen-biomethane) AD system (operating at an overall HRT of 20 days) and a conventional single-stage anaerobic digester. The lignocellulosic
constituents of the substrates and effluents/digestate of the AD systems were then critically evaluated.

As shown in Table 6.3, the NDF component of the perennial rye grass substrate was found to be 33.9± 0.38 g/l, representing 62.2% of the TS. The substrate was then degraded in an acidogenic reactor (HA) at an HRT of 0.75 days. The NDF component of the HA effluent was found to be 32.1± 0.69 g/l (94.6% of the initial NDF in the substrate), indicating only a 5.4% reduction. When a portion of the HA effluent was fed to M19 reactor, the NDF at a concentration of 6.4± 0.23 g/l was observed to have been reduced by 81.2% from the levels originally in the substrate.

As shown in Table 6.4, the NDF concentration in the wheat-feed substrate was found to be 27.50 ± 0.48 g/l, representing 54.96% of the TS. The quantity of the NDF after the substrate was degraded in HA reactor, was found to be 25.3± 0.17 g/l (92.5% of NDF in in the substrate), indicating about an 8.0% NDF reduction. When the HA effluent was fed to the M19 reactor, the NDF component in the M19 digestate was found to be 7.75± 0.09 g/l (29.2% of NDF in the substrate). The results indicated that approximately 71% of the lignocellulose component was potentially degraded in the two-stage process.

The soluble carbohydrates component of the perennial rye grass substrate was found to be 8.8± 0.39 g/l. The HA reactor resulted in the reduction of approximately 79% of the total mass of the soluble carbohydrates in the substrate, resulting in an undigested soluble sugar concentration of 1.8± 0.39 g/l. When the HA effluent was fed to the M19 reactor, the undigested soluble carbohydrate was found to be 0.52± 0.02 g/l, with an observed percentage reduction of 94%. The soluble carbohydrate component in the wheat-feed was also found to be 17.6± 0.37 g/l. After the fermentation in the HA reactor, the soluble carbohydrate was found to be 2.79± 0.25 g/l (15.9% of soluble carbohydrates in the substrate), indicating an approximately 84% reduction of the initial soluble carbohydrate concentration in the fermentation process. Similarly, digestion in the M19 reactor resulted in 97% reduction of the soluble carbohydrates, with an undigested soluble carbohydrates concentration of 0.56± 0.15 g/l.

The results indicate that the gas yield and VFAs obtained by the HA reactor could largely be attributed to the utilisation of the soluble carbohydrate (79-84% soluble carbohydrates reduction in both substrates). This was because only 5.4% of the structural carbohydrates (hemicellulose and cellulose) in the perennial rye grass and 8.0% in the wheat-feed substrate
were utilised in the acid phase, whilst a perceived significant reduction of the protein and ADL contents were observed. The results further highlighted that, soluble carbohydrate is a bioavailable substrate for AD process, with 94-97% degraded after the two-stage process.

The wheat-feed substrate had more originally available readily soluble carbohydrates compared to the perennial rye grass substrate. The wheat-feed substrate had a soluble carbohydrate content of 17.6 g/l, making it a preferred substrate for acidogenic fermentation when compared to the perennial rye grass, which had a soluble carbohydrate content of 8.8 g/l. The yields of biohydrogen and VFAs from the two lignocellulosic substrates were also consistent with the compositional analyses. The VFAs produced from wheat-feed substrate (9.21±0.15 g/l) were 46.2% higher than that achieved in the perennial rye grass (6.3 ± 0.3 g/l). The yield of biohydrogen from the wheat-feed substrate was found to be 7.48 l H₂/kg VS, whereas the yield of biohydrogen from the perennial rye grass was found to be 6.7 l H₂/kg VS. The approximately 10% greater yield in the wheat-feed substrate was attributed to the higher availability of soluble carbohydrates, as compared to the perennial rye grass.

The perennial rye grass at an initial holocellulose content of 28.79 g/l (61.1% of the VS), was reduced to a holocellulose content of 26.3 g/l, indicating an 8.4% reduction. The overall reduction of the residual holocellulose in the two-stage process was found to be 95.1% (a residual holocellulose content of only 1.4 g/l). The wheat-feed substrate, on the other hand, had an initial holocellulose content of 22.96 g/l. After the HA fermentation, the holocellulose content was found to be 21.0 g/l, indicating a reduction of 8.7%. The overall holocellulose reduction after the two-stage process was found to be 86.8% (a residual holocellulose content of 3.03 g/l. Both the perennial rye grass and the wheat-feed substrates show similar trend of degradation of holocellulose in the HA reactor (8.4% and 8.7% reduction respectively). The holocellulose content of the perennial rye grass was relatively more readily solubilized in the M19 reactor when compared to that of the wheat-feed substrate. Even though the perennial rye grass substrate possessed relatively higher incorporated holocellulose, and therefore higher biogas potential in the two-stage process, than the wheat-feed substrate, it resulted in yields lower than expected. To extrapolate more clearly, it was envisaged that, if the wheat-feed substrate at an initial holocellulose content of 22.96 g/l and a conversion efficiency of 86.8%, could result in a yield of 181.12 L CH₄/kg VS, then the perennial rye grass substrate at an initial holocellulose content of 28.79 g/l and a conversion efficiency of 95.1%, should result in a higher yield of 240.81 L CH₄/kg VS. The relatively low yield of methane could be
linked to the higher ADL content in the perennial rye grass substrate compared to the wheat-feed substrate, which limited the cellulosic degradation in the HA reactor. Furthermore, the relatively higher content of the readily soluble carbohydrate of the wheat-feed substrate resulted in a higher yield of VFAs in the HA fermentation process (Table 6.4). The perennial rye grass substrate, on the other hand, resulted in a relatively low yield of VFA after the first-stage of the two-stage AD process. An increased yield of VFAs is in direct correlation with an increased methane yield, since VFAs are an major precursor for second-stage methanogenic process (Table 6.3). The lignocellulosic substrates with higher incorporated soluble carbohydrates, such as the wheat-feed, can be suggested as the preferred choice of substrate for a two-stage AD process.

Comparing the yield of methane from the wheat-feed and perennial rye grass substrates in the A20 digestion process however showed that the degradation of the perennial rye grass resulted in a relatively higher yield of methane (310.0 L CH₄/kg VS), than the wheat-feed substrates (261.14 L CH₄/kg VS). The results indicated a yield difference of nearly 19%, in favour of the perennial rye grass. The only component that was effectively degraded in the HA fermentation was the soluble carbohydrate, making it therefore the most important component based on which comparative evaluation of the acidogenic fermentation of the two substrates can be carried out. In the M19 digestion however, the holocellulose were equally effectively degraded alongside the soluble carbohydrates, making the holocellulose content the major subject of comparative evaluation of the two substrates in the single-stage AD process.

The complex polysaccharides require slow hydrolysis to fermentable sugars before being utilized for VFA production, then to methane, and that was essentially allowed in the A20 and M19 processes, ensuring effective utilisation of the holocellulose. Though lignocellulosic biomass with structural composition similar to the dried perennial rye grass (having low initial available soluble carbohydrates) may not stand out as a substrate of choice for biohydrogen production, an application of an effective pre-treatment process to solubilize the recalcitrant lignocellulosic complex will dramatically improve the energy value of such substrates.

Few species of bacteria are capable of releasing certain xylanases and lignin-degrading enzymes that have been identified to be effective in the degradation of the lignocellulosic
components of biomass. The current study suggested that the acidogenic bacteria (largely clostridia species) involved in the acidogenic fermentation process do not possess adequate enzymatic potential to degrade the lignocellulosic component of the wheat-feed and perennial rye grass substrate. The complex polymers in the substrate require a longer time to decompose to soluble carbohydrates and oligomers, before being converted to VFAs, hydrogen and CO₂ (Jeong et al., 2008). The slow decomposition of organic polymers meant that only the originally available and readily solubilized carbohydrates were utilised for hydrogen and VFAs production.
Table 6.3: Compositional changes of the perennial rye grass substrate in the anaerobic digestion processes

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<td>5.4±0.42 -71.7</td>
<td>6.8±0.32 -64.6</td>
</tr>
<tr>
<td>ADL</td>
<td>5.2±0.21</td>
<td>5.8±0.32 13.6</td>
<td>5.0±0.27 -3.3</td>
<td>5.7±0.50 13.8</td>
</tr>
<tr>
<td>Ash</td>
<td>5.4±0.13</td>
<td>6.8±0.66 26.4</td>
<td>5.85±0.07 8.2</td>
<td>6.3±0.12 16.7</td>
</tr>
<tr>
<td>Sol. Carbs</td>
<td>8.8±0.39</td>
<td>1.8±0.39 -79.2</td>
<td>0.52±0.02 -94.0</td>
<td>1.0±0.087 -88.5</td>
</tr>
<tr>
<td>Protein</td>
<td>4.3±0.2</td>
<td>5.0±0.20 17.1</td>
<td>1.55±0.04 -63.8</td>
<td>1.9±0.04 -55.6</td>
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<tr>
<td>Total VFAs</td>
<td>0.5±0.02</td>
<td>6.3±0.3 1133.7</td>
<td>0.1±.005 -81.1</td>
<td>0.1±.002 -79.8</td>
</tr>
<tr>
<td>Na</td>
<td>0.9±0.04</td>
<td>1.04±0.02 10.6</td>
<td>1.3±0.03 38.8</td>
<td>1.1±0.015 22.0</td>
</tr>
<tr>
<td>NH₄</td>
<td>0.11±0.03</td>
<td>0.56±0.03 408.6</td>
<td>1.11±0.01 906.1</td>
<td>0.84±0.04 666.7</td>
</tr>
<tr>
<td>K</td>
<td>0.57±0.04</td>
<td>0.72±0.008 27.1</td>
<td>0.74±0.00 29.3</td>
<td>0.7±0.005 27.6</td>
</tr>
<tr>
<td>Mg</td>
<td>0.02±0.000</td>
<td>0.14±0.001 498.1</td>
<td>0.04±0.0 94.6</td>
<td>0.06±0.00 146.3</td>
</tr>
<tr>
<td>Ca</td>
<td>0.04±0.003</td>
<td>0.15±0.002 315.1</td>
<td>0.092±0.0 160.4</td>
<td>0.1±0.001 216.5</td>
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<td>Cl</td>
<td>0.36±0.000</td>
<td>0.4±0.003 12.3</td>
<td>0.3±0.006 -10.0</td>
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<tr>
<td>NO₃</td>
<td>0.009±0.001</td>
<td>0.0018±0.0 -80.2</td>
<td>0.002±0.0 -77.7</td>
<td>0.001±.0 -86.5</td>
</tr>
<tr>
<td>Phosphate</td>
<td>0.23±0.097</td>
<td>0.49±0.01 115.4</td>
<td>0.2±0.015 -11.1</td>
<td>0.2±0.008 -10.0</td>
</tr>
<tr>
<td>sulphate</td>
<td>0.16±0.002</td>
<td>na na</td>
<td>na Na</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>2.2±0.12</td>
<td>1.8±0.06 -24.1</td>
<td>0.91±0.02 -58.2</td>
<td>1.18±.01 -46.0</td>
</tr>
<tr>
<td>C</td>
<td>27.6±1.7</td>
<td>22.6±0.4 -18.2</td>
<td>8.2±0.15 -70.2</td>
<td>9.41±7 -66.0</td>
</tr>
<tr>
<td>S</td>
<td>0.41±0.04</td>
<td>0.2766±0.02 -32.2</td>
<td>0.16±.003 -61.8</td>
<td>0.18±.01 -56.0</td>
</tr>
<tr>
<td>H</td>
<td>4.36±0.09</td>
<td>4.14±0.17 -4.98</td>
<td>1.3±.05 -70.4</td>
<td>1.50±.02 -65.7</td>
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</table>
## Table 6.4: Compositional Changes of the Wheat-feed Substrate in the Anaerobic Digestion Processes

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Substrate</th>
<th>Hydrogen Reactor (HA)</th>
<th>Methane Reactor (M19)</th>
<th>Single-stage AD (A20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Quantity</td>
<td>Residual Quantity</td>
<td>Increase/Decrease (%)</td>
<td>Residual Quantity</td>
</tr>
<tr>
<td></td>
<td>(g/l)</td>
<td>(g/l)</td>
<td>(%)</td>
<td>(g/l)</td>
</tr>
<tr>
<td>NDF</td>
<td>27.5±0.48</td>
<td>25.3±0.17</td>
<td>-8.0</td>
<td>7.75±0.09</td>
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<tr>
<td>ADF</td>
<td>11.3±0.30</td>
<td>11.1±0.55</td>
<td>-2.1</td>
<td>6.79±0.14</td>
</tr>
<tr>
<td>ADL</td>
<td>4.5±0.06</td>
<td>4.3±0.84</td>
<td>22.5</td>
<td>4.72±0.08</td>
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<tr>
<td>Ash</td>
<td>2.44±0.07</td>
<td>2.05±0.07</td>
<td>-15.8</td>
<td>2.41±0.07</td>
</tr>
<tr>
<td>Sol. Carbs</td>
<td>17.6±0.37</td>
<td>2.79±0.5</td>
<td>-84.1</td>
<td>0.56±0.015</td>
</tr>
<tr>
<td>Protein</td>
<td>5.55±0.12</td>
<td>6.81±0.21</td>
<td>22.7</td>
<td>2.38±0.014</td>
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<tr>
<td>Total VFAs</td>
<td>0.37±0.06</td>
<td>9.21±0.15</td>
<td>2363.0</td>
<td>0.24±0.03</td>
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<td>Na</td>
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<td>1.29±0.07</td>
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<td>1.20±0.02</td>
</tr>
<tr>
<td>NH₄</td>
<td>0.13±0.003</td>
<td>0.24±0.04</td>
<td>94.2</td>
<td>1.37±0.02</td>
</tr>
<tr>
<td>K</td>
<td>0.39±0.005</td>
<td>0.52±0.08</td>
<td>34.9</td>
<td>0.55±0.02</td>
</tr>
<tr>
<td>Mg</td>
<td>0.004±0.0</td>
<td>0.12±0.01</td>
<td>3152.0</td>
<td>Na</td>
</tr>
<tr>
<td>Ca</td>
<td>na</td>
<td>0.01±0.000</td>
<td>na</td>
<td>0.01±0.00</td>
</tr>
<tr>
<td>Cl</td>
<td>0.05±0.006</td>
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<td>0.05±0.001</td>
</tr>
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<td>NO₃</td>
<td>0.001±0.0</td>
<td>na</td>
<td>na</td>
<td>Na</td>
</tr>
<tr>
<td>Phosphate</td>
<td>0.31±0.013</td>
<td>0.82±0.029</td>
<td>162.7</td>
<td>0.52±0.02</td>
</tr>
<tr>
<td>sulphate</td>
<td>0.06±0.006</td>
<td>0.03±0.002</td>
<td>-56.8</td>
<td>0.01±0.00</td>
</tr>
<tr>
<td>N</td>
<td>1.12±0.15</td>
<td>1.45±0.01</td>
<td>29.6</td>
<td>1.57±0.01</td>
</tr>
<tr>
<td>C</td>
<td>21.8±0.26</td>
<td>18.3±0.15</td>
<td>-15.9</td>
<td>5.73±0.12</td>
</tr>
<tr>
<td>S</td>
<td>0.22±0.018</td>
<td>0.14±0.005</td>
<td>-36.0</td>
<td>0.10±0.003</td>
</tr>
<tr>
<td>H</td>
<td>3.43±0.056</td>
<td>2.87±0.042</td>
<td>-16.5</td>
<td>0.90±0.027</td>
</tr>
</tbody>
</table>
6.4 Comparative Analysis of the Degradation of Lignocellulose in Single-Stage AD and Two-Stage Anaerobic Process (Results and Discussion - Experiment CA1 and CA2)

6.4.1 Perennial Rye Grass Substrate

The digestion of the perennial rye grass substrate (33.9±0.38 g/l) in the A20 reactor resulted in NDF of 8.6 ± 0.16 g/l, representing a 74.8% reduction. The M19_{digestate} on the other hand had NDF component of 7.75±0.09 g/l, representing an 81.2% reduction. The results illustrated in Figure 6.1 show that the two-stage process was more effective in degrading lignocellulose than the single-stage AD process.

By treating the perennial rye grass substrate in a single-stage AD process, the hemicellulose component was found to have degraded by 86.97%, with an undigested amount of 1.92 ± 0.23 g/l. The M19_{digestate} similarly had 0.97 ± 0.14 g/l hemicellulose, representing a reduction of 93.5%. (Table 6.3)

The cellulose component was also found to have been more degraded in the M19_{digestate} when compared to the A20_{digestate}. After the methanation process, the undigested cellulose in the A20_{digestate} was found to be 0.89±0.12 g/l, representing a 93.7% reduction, whereas the undigested cellulose in the M19_{digestate} was found to be 0.42 ± 0.14 g/l, representing a 97.0% reduction. (Table 6.3)

The soluble carbohydrate component in the M19_{digestate} and in the A20_{digestate}, also show that the two-stage AD process was more effective than the single-stage AD process. The undigested soluble carbohydrate in the A20_{digestate} was found to be 1.01 ± 0.09 g/l, representing an 88.5% reduction, and that of the M19_{digestate} was found to be 0.52 ± 0.02 g/l, representing a 94% soluble carbohydrates reduction. (Table 6.3)

The residual protein content in the A20_{digestate} was found to be 1.89±0.04 g/l, compared to that in the M19_{digestate}, which was found to be 1.55±0.04 g/l, representing a 55.6% and 63.8% respectively. (Table 6.3)

By comparing the proximate compositional values of the of the HA_{effluent}, M19_{digestate}, and A20_{digestate}, it was observed that the two-stage biohydrogen-biomethane AD process was more effective in the degradation of the lignocellulose component in the perennial rye grass than the conventional single-stage AD process. It was observed that the hemicellulose, cellulose,
the soluble carbohydrates and proteins were degraded more in the M19\textsubscript{digestate} than in the A20\textsubscript{digestate}. An independent t-test conducted to compare the quantities of the following gave a strong indication that there was a statistically significant reduction of the lignocellulose components in M19 process, when compared to the A20 process:

- Undigested hemicellulose for M19 (M=0.705 g/l, SD=0.26) and A20 (M= 1.47 g/l, SD= 0.524), p= 0.001;
- Undigested cellulose for M19 (M=0.318 g/l, SD= 0.308) and A20 (M=0.659 g/l, SD=0.269), p = 0.026;
- Soluble carbohydrates for M19 (M=0.082 g/l, SD=0.011) and A20 (M=0.158 g/l, SD=0.0389), p = 0.001.

6.4.2 Wheat-feed Substrate

Using wheat-feed as the substrate, the NDF in the A20\textsubscript{digestate} was found to be 7.66±0.23 g/l, indicating a 71.1% reduction. The NDF in the M19\textsubscript{digestate} was also found to be 7.8 ±0.23 g/l, indicating a NDF reduction of 70.8% (Table 6.4). The results show that the performances of the A20 and M19 reactors in the breakdown of the lignocellulose components in both substrates were similar, resulting in similar quantities of NDF. It was therefore deemed important to critically evaluate the fate of the hemicellulose, cellulose, soluble carbohydrates and the protein utilisation in the A20 and M19 reactors in order to ascertain the reasons behind the difference in biogas yield.

The undigested hemicellulose in the A20\textsubscript{digestate} when the wheat-feed was used as substrate, was found to be 1.91±0.09 g/l, indicating 87.4% reduction. The M19 process, on the other hand, resulted in undigested hemicellulose of 0.95± 0.15 g/l, indicating a hemicellulose reduction of 93.7% (Table 6.4). It can be seen from Figure 6.1 that a perceived significant variation was observed the performance of the single-stage AD and the two-stage process per virtue of the composition of the digestate. The reduction of the hemicellulose in the M19\textsubscript{digestate} was 6.3% greater than that observed in the A20\textsubscript{digestate}. The level of difference, using independent t-test statistical analysis, comparing the mean (M) of the quantity of the undigested hemicellulose in the M19\textsubscript{digestate} (M=1.18 g/l, SD=046) and A20\textsubscript{digestate} (M= 1.02
g/l, SD= 0.69), p= 0.59, however show that the difference in performance was not statistically significant.

The undigested cellulose in the A20\textsubscript{digestate} was found to be 2.33±0.13 g/l, representing a 70.1% cellulose reduction. In M19 digestate, the undigested cellulose was found to be 2.08±0.21 g/l, representing a reduction of 73.3% (Table 6.4). The reduction of cellulose in the M19\textsubscript{digestate} was found to be 3.3% greater than that observed in the A20\textsubscript{digestate}. Statistical analysis however show that, there was no significant difference between the mean values of the cellulose in M19 (M=1.28 g/l, SD=0.91) and A20 (M=1.46 g/l, SD=0.87), p=0.69.

The unutilised soluble carbohydrates concentration in the A20\textsubscript{digestate} was found to be 0.54±0.10 g/l; indicating that by the end of the single-stage AD process, 96.9% of the soluble carbohydrates in the substrate had been degraded. In the M19\textsubscript{digestate}, the undigested soluble carbohydrate was found to be 0.56± 0.15 g/l sugars, with an observed reduction of 96.8% (Table 6.4). The statistical analysis comparing the mean values of the undigested soluble carbohydrates in M19 (M=0.44 g/l, SD= 0.13) and A20 (M= 0.49 g/l, SD= 0.23) confirmed that there was no significant difference in the performance of the two systems regarding the reduction of soluble carbohydrates.

The protein component was also found to have been more degraded in the M19\textsubscript{digestate} than into the A20\textsubscript{digestate}. The undigested protein in the A20\textsubscript{digestate} was found to be 2.92±0.12 g/l, representing a 47.4% reduction, whereas the undigested protein in the M19\textsubscript{digestate} was found to be 2.38±0.14 g/l, representing a 57.2% reduction (Table 6.4). An independent t-test conducted to compare the quantity of the undigested proteins in the M19\textsubscript{digestate} (M=2.05 g/l, SD=0.33) and A20\textsubscript{digestate} (M= 2.50 g/l, SD= 0.41), p= 0.020, show a statistically significant difference in the performance between the M19 and A20 processes, with the M19 having as superior degradation efficiency.
Figure 6.1a: perennial rye grass composition before and after anaerobic digestion

Figure 6.1b: The composition of wheat-feed before and after anaerobic digestion
The Role of Lignin in the Lignocellulosic Component Degradation in Anaerobic Digestion (Results and Discussion - Experiment CA1 and CA2).

The ADL in the perennial rye grass substrate was found to be 5.2 ± 0.61 g/l, and after the A20 process, the ADL was found to be 5.7 ± 0.50 g/l. After the M19 process, the ADL was found to be 5.0 ± 0.27 g/l (Table 6.3). The ADL component appeared to have increased by 9.6% in the A20_dig state and 3.8% in the M19_dig state. A one-way ANOVA testing the homogeneity variances of the ADL mean values using a sample size of 36 however show that there was no significant difference in the mean values of the ADL in the A20_dig state and M19_dig state (p=0.73). Similarly, the ADL component in the wheat-feed substrate was also found to be 4.5 ± 0.06 g/l. The ADL component was found to be 4.72 ± 0.08 g/l in the M19_dig state and 4.29 ± 0.10 g/l in the A20_dig state (Table 6.4). The ADL component was found to have increased by 4.89% in the M19_dig state and reduced by 4.6% in the A20_dig state. Again, a one-way ANOVA testing the homogeneity variances of the ADL means in the A20_dig state, using a sample size of 45, indicated that there was no significant difference in the means obtained in the A20_dig state (p=0.62). The observed differences in the ADL values can therefore be attributed to experimental error.

According to Triolo et al. (2012), the quantity of lignin can significantly reduce the biomethane potential when it exceeds a critical point of 100g/kg of the VS. The ADL content in the perennial rye grass substrate was 108 g/kg VS, when compared to ADL content of 95.3 g/kg VS, observed in the wheat-feed substrate. The ADL contents in both substrates were around the critical threshold, but that had no perceived significant effect on the biomethane potential of the substrates. Even though the ADL content of the perennial rye grass substrate was only 13% higher than in the wheat-feed substrate, its effect on the cellulosic degradation, though marginal, was higher than that observed in the wheat-feed substrate.

The HA_effluent, using perennial rye grass as substrate, had a ADL content of 131.7 g/kg, whilst HA_effluent of the wheat-feed substrate was found to contain ADL of 113.3 g/kg VS. In line with the lignin-CH₄ principle, defined by Triolo et al. (2012), the quantity of lignin in the perennial rye grass posed greater resistance to the biomethanation of the substrate, when compared to the wheat-feed.

The recalcitrant behaviour of both substrates in the current study can also be attributed to the complex carbohydrate-lignin linkages. As described by Jeffries (1994), the carbohydrate
chain linkage with lignin is attacked by exo-enzymes during biodegradation from the reducing end of the polysaccharide, with a progressive removal of the substituents towards the reducing end of the molecule. He further explained that the mode of action of the exo-splitting enzymes makes it nearly impossible for a complete degradation of the polysaccharide complex to occur.

The hydrolysis of polysaccharides occurs through the release of exoenzymes by saccharolytic bacteria in the hydrolysis phase of the anaerobic digestion process, which occurs in a few hours during the AD process and because the lignocellulose component of biomass requires slow degradation, a complete degradation is impossible (Deublein and Steinhauser, 2008). The limited degradation of the lignocellulose component (NDF) (5.4% reduction in the perennial rye grass and 8.0% reduction in the wheat-feed) in the HA reactor can therefore be explained by the principle highlighted by Deublein and Steinhauser (2008). As described by Triolo et al. (2011), the hemicellulose and lignin components in lignocellulosic biomass are intermeshed, with chemical bonds identified to be covalent cross linkages. Jeffries (1994) highlighted that the complex barrier to biodegradation of lignocellulose can largely be attributed to the nature of the lignin-carbohydrate interaction. Wallace et al. (1991) also emphasized that the covalent linkage of lignin to carbohydrate could be direct or indirect, depending on the specific plant species, making the trend of degradation of lignocellulose complex and substrate specific. Kirk and Chang (1981) and Milstein et al. (1984), all attributed the recalcitrant nature of the lignocellulosic component of biomass, as limiting the rate of biodegradation to the complex lignin-structural carbohydrate complexes.

The evaluations of the results show that the recalcitrance of lignocellulose component degradation was more prominent in the HA reactor compared to the M19 reactor. In the perennial rye grass, 5.4% NDF reduction was observed in the HA \textit{effluent} compared to 81.2% NDF reduction in the M19 \textit{digestate} (Table 6.3). Similarly, in the wheat-feed, the NDF reduction observed after the HA fermentation (8.0% reduction) was lower than that in the M19 digestion (70.8% reduction) (Table 6.3). As described by Cornu et al., (1994), the effect that the structural configuration of the lignocellulose complex has on the degradation of the cell wall polymers is dependent on the specific conditions of the microbial attack, such as the rate of production of exoenzymes. It can therefore be suggested that the limited period of metabolic activity (0.75-day HRT), optimum for the acidogenic bacteria, was not enough for the effective degradation of the lignocellulose complex, when compared to the methanogenic.
process, which had a relatively long exposure of the lignocellulosic complex to metabolic activity.

Mok and Antal (1992) showed that the quantity and structure of lignin has a strong correlation with the efficiency of the yield of fermentable sugars, whereas hemicellulose was found to have a weak correlation. As determined in the current study, the lignin component in the perennial rye substrate (ADL) was found to be 9.3% of the dry matter, whilst the ADL in the wheat-feed after the acidogenic fermentation was found to be 6.4% of the dry matter. Pronyk et al. (2011) highlighted that higher lignin concentration in a biomass substrate increases the recalcitrance of the substrate to acidogenic fermentation. They showed the effect of lignin in anaerobic digestion in a hydrothermal treatment of lignocellulosic biomass. They also observed that the higher lignin containing substrates resulted in the lowest yield of dissolved mass, when compared to the lower lignin containing substrates. Tamaki and Mazza (2010) also reported that the lower content of lignin in triticale resulted in higher yields of fermentable sugars, as compared with other high lignin containing substrates, explaining that hydrolytic enzymes have easy access to the hemicellulose at lower lignin concentrations. Besle et al. (1995) also emphasized that the chemical nature of lignin linkages to the polysaccharides and, most importantly, the total amount of lignin in a substrate all have significant effects on the biodegradation of the particular substrate. They also emphasized that specific plant species exhibit different behaviour during the biodegradation based on the nature of the lignin component.

Lignin, apart from its structural effect on formation of biomass, also act by adsorbing and inactivating hydrolytic enzymes thereby restricting enzymatic hydrolysis (Wyman et al., 2005). Nakagame et al. (2011) emphasized that the spontaneous absorption of the cellulase enzyme by lignin is caused by hydrophobic and electrostatic interactions between the enzyme and the lignin polymer.

Wyman et al. (2005) observed that high lignin content in canola and mustard feedstock preparation caused significant reduction in the yield of fermentable sugars. It must be noted that the recalcitrance caused by chemical nature of lignin and its linkage with the polysaccharides is different from the impediments of lignin derivatives on biodegradation (Cornu et al., 1994; Op den Camp et al., 1988). The inhibition of lignocellulose degradation due to phenolic compounds can directly or indirectly be attributed to the interactions of
phenolics and the carbohydrates, in contrast to the structural features of the lignocellulose complex that can directly prevent polysaccharide degradation (Cornu et al., 1994).

The percentage reduction of the cellulose in the wheat-feed substrate was found to be 13.2%, when compared to the 10.8% cellulose reduction that occurred in the perennial rye grass substrate. In perennial rye grass, a higher quantity of ADL meant that it was more recalcitrant to acidogenic fermentation when compared to the wheat-feed substrate. The results from HA fermentation showed consistency with the biogas yield reported by Massanet-Nicolau et al. (2012), in that the wheat-feed gave higher yield of biohydrogen, compared to the perennial rye grass. Therefore, by virtue of accessibility of the hydrolytic enzymes to cellulose, it can be said that the chemical structure of the substrate based on the quantity of lignin correlates well with the extent of biodegradation.

The ADL component in both the perennial rye grass and wheat-feed substrates remained largely unchanged during the AD process. At an initial percentage of VS at approximately 11% in the perennial rye grass substrate, the ADL proportion in both the $\text{A20}_{\text{digestate}}$ and $\text{M19}_{\text{digestate}}$ was found to be about 32%. The ADL proportion was also approximately 9% in the wheat feed substrate, but was found to have increased to about 34% for both the $\text{A20}_{\text{digestate}}$ and $\text{M19}_{\text{digestate}}$. Even though the actual ADL proportion was largely unchanged during the digestion processes, the percentage of ADL relative to the VS content increased. The percentage rise in the ADL in both substrates was attributed to the reduction of the holocellulose content which was effectively degraded unlike the ADL component. The increase of ADL content after the digestion process (in both A20 and M19 processes) gave an indication of its potential as a value added product from lignocellulosic substrates. The AD process therefore boosted the economic value of lignocellulosic biomass by making the recovery of lignin easier after AD processing.

### 6.6 Chapter Conclusion

Many researchers have highlighted the effect of the recalcitrance of the lignocellulose component of biomass in anaerobic digestion. However, this is the first time that two lignocellulosic biomasses (perennial rye grass and wheat-feed) have been degraded in anaerobic digestion systems alongside a critical evaluation of the behaviour of the residual lignocellulose constituents in the anaerobic processes; this led to an understanding of how the
structural polysaccharides and lignin polymers affect the digestion process and the yields of biofuel obtained from the process.

- The separate acid phase in the two-stage anaerobic digestion system potentially enhanced the hydrolysis and acidogenic process, leading to the release of more VFA and hence an improvement of the yield of biogas when compared to the single-stage anaerobic digestion. The acid phase also improved the dissociation of the fatty acids produced in the anaerobic digestion process, limiting the inhibitory effect of undissociated fatty acids on the fermentation process. This led to an effective degradation of the lignocellulosic component of the biomass substrate in the M19 digestion when compared to the conventional A20 system.

- The study showed that the quantity of ADL and recalcitrance of the cellulose to degradation affected the yield of biogas in the two substrates investigated. The quantity of originally available soluble carbohydrates was also found to have direct effect on the quantity of biogas produced in the anaerobic digestion process.

- It was observed that, having an initial ADL concentration of approximately 10% in both substrates, the effluents and digestates (HA_{effluent}, M19_{digestate} and M19_{digestate}) were found to be rich in lignin (approximately 32-34% of the VS), as the ADL proportion remained largely undegraded in the AD process when compared other organic components in the substrate. The study therefore proposed that lignin could be extracted as a valuable product from AD process.
CHAPTER 7

7 ENHANCING THE YIELDS OF BIOHYDROGEN AND VOLATILE FATTY ACIDS FROM PERENNIAL RYE GRASS IN ACIDOGENIC FERMENTATION

7.1 Principle and Hypotheses of Study

Extensive review of literature indicates that lignocellulosic biomass has a considerable potential as substrate for biohydrogen and VFA production. The economic sustainability of the process is however in great doubt due to the recalcitrant nature of lignocellulosic substrates needing complex pre-treatment techniques. After the evaluation of the fate of lignocellulosic biomass in three anaerobic fermentation systems in Chapter 6, it was concluded that the enzymatic hydrolysis in the acidogenic fermenters for digestion of the perennial rye grass was not enough to solubilize the complex polysaccharides to provide readily available soluble carbohydrates for production of biogas and VFAs. Soluble carbohydrates were found to be the only major component of the biomass that was effectively utilized in the hydrolytic-acidogenic process for biohydrogen and VFA production. Though perennial rye grass was found to be rich in structural carbohydrates, the relatively low amount of readily solubilized sugars resulted in low yield of biohydrogen and VFAs. The current study also seeks to improve the yields of biohydrogen and VFA, through enhanced hydrolysis by pre-treatments. In summary, the current study was set out to answer the following questions:

- What concentration of enzyme(s) will potentially be effective in accelerating the hydrolysis of the complex polysaccharides of the perennial rye grass to soluble carbohydrates?

- To what extent to does alkaline pre-treatment and enzymatic SSF enhance the acidogenic fermentation of the lignocellulose content in the perennial rye grass, the yields of biohydrogen and VFA?
7.2 Development of Effective Enzymatic Technique for Solubilisation of Recalcitrant Lignocellulosic Biomass (Results and Discussion - Experiment PT1)

The enzymatic pre-treatment experiments were designed with the aim of obtaining an enzyme cocktail that has the potential of improving the degradation of the lignocellulosic complex in the acidogenic fermentation process, and the yields of bio-hydrogen and VFAs (as described in Experiment PT1 in Method Section 5.7.2). The combinations of enzymes used in the development of optimal enzymatic pre-treatment technique for perennial rye grass are illustrated in Table 7.1.

The enhancement of hydrolysis was evaluated by the measurement of soluble COD release (a recommended analytical technique by van Dyk and Pletschke (2012)), as a reasonable representation of the total carbohydrate that are made available for anaerobic digestion,
Table 7.1: Experimental setup - enzyme proportions based on unit enzyme activity

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cellulase</th>
<th>Xylanase</th>
<th>FAE</th>
<th>Amount added (ml enzyme/ gVS)</th>
<th>Enzyme activity</th>
</tr>
</thead>
<tbody>
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<td>PT1.1</td>
<td>(%) (v/v)</td>
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<td>(%) (v/v)</td>
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<td>34.77 kU/ml esterase</td>
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<tr>
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<td>0.3</td>
<td>Nil</td>
<td>0.15</td>
<td>0.096</td>
<td>76.5 kU/ml cellulase; 17.4 kU/ml esterase</td>
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<tr>
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<td>0.5</td>
<td>Nil</td>
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<td>0.15</td>
<td>0.202</td>
<td>204.0 kU/ml cellulase; 17.4 kU/ml esterase</td>
</tr>
</tbody>
</table>
7.2.1 Results of Experiment PT1.1: Cellulase Pre-treatment

The additional soluble COD released due to the cellulase enzyme treatment at 0.05% cellulase concentration was found to be 54.9±9.7 mg COD/gVS at 24 hours of treatment; representing a percentage yield improvement of 35.3%. The additional soluble COD yield further improved to 96.6±22.9 mg COD/gVS at 48 hours, then to 143.1±5.0 mg COD/gVS after 72 hours of treatment; representing increased yields of 57.02% and 64.2% respectively. (Figure 7.1)

At cellulase concentration of 0.15%, the yield of soluble COD was found to be 82.79±4.1 mg COD/gVS, 98.3±13.1 mg COD/gVS, and 109.9±10.8 mg COD/gVS, at 24, 48, 72 hours respectively. Yield of soluble COD was found to have improved by 63.2% after 24 hours of incubation, and that was observed to have the highest yield rate when compared to the rate of release of soluble COD 48 and 72-hour sampling times gave no further percentage yield increase, as shown in Figure 7.1.

The soluble COD yield further increased as the cellulase concentration was increased to 0.3% cellulase. After 24 hours of incubation, the yield was found to be 116.2±4.7 mg COD/gVS. The yield further improved to 132.2±4.0 mg COD/gVS and 151.5±4.7 mg COD/gVS, at 48 and 72 hours respectively; representing increased yields of 80.5%, 86.9% and 68.0% (Figure 7.1).

At 0.5% cellulase, the additional yield of soluble COD due to the enzyme was found to be 125.2±14 mg COD/gVS at 24 hours; 147.3±2.0 mg COD/gVS at 48 hours and 252.5±3.4 mg COD/gVS at 78 hours. The yields obtained due to enzyme addition corresponded to a percentage increase in yield of 74.7%, 78.0% and 113.3%; after incubation with enzyme of 24, 48 and 72 hours respectively. (Figure 7.1)

The 0.8% cellulase treatment yielded additional soluble COD of 245.6±1.8 mg COD/gVS of, after 24-hour incubation; 299.4±14.0 mg COD/gVS, after 48-hour incubation; and 355.3±8.6 mg COD/gVS after 72-hour incubation. The yield obtained corresponded to percentage yield increase of 182.7%, 144.9% and 159.4%, at 24, 48 and 72 hours respectively, when compared to the no enzyme added control. (Figure 7.1)

The highest cellulase concentration used in the biomass treatment process was 1.2%, having an activity of 426.0 kU/ml cellulase. The additional soluble COD released, due to the
enzyme, was found to be 417.3±10.9 mg COD/gVS (192.5% yield increase) after 24 hours; 491.0±9.3 mg COD/gVS (215.2% additional yield increase); and 505.0±14.3 mg COD/gVS (197.4% additional yield increase). (Figure 7.1)

There was a 50.8% increase in the release of soluble COD after the cellulase concentration was increased from 0.05% to 0.15%. When the enzyme concentration was further increased to 0.3% cellulase, the increase in release of soluble COD was found to be 51.2%, indicating a progressive increase. When the cellulase concentration was further increased to 0.5% cellulase however, there was no perceived significant increase in the yield of soluble COD, when compared to the 0.3%. The 0.8% cellulase concentration on the other hand resulted in a yield increase of 111.3%, when compared to the value at 0.5% cellulase, and 96.2%, when compared to the value at 0.3% cellulase concentration. The yield further increased by 69.9% as the concentration was increased from 0.8% to 1.2%. It can be seen that the 0.8% cellulase resulted in more effective solubilisation effect on the perennial rye grass. The 0.8% was therefore selected as the concentration of choice for the treatment (Figure 7.1).

![Figure 7.1: Additional soluble COD released from dried perennial rye grass due to cellulase enzyme](image)

Note: Percentage (%) cellulase was based on the unit enzyme activity shown in Table 7.1
7.2.2 Results of Experiment P1.2: Xylanase Pre-Treatment

The treatment of perennial rye grass with 0.05% xylanase resulted in soluble COD of 48.4±5.3 mg COD/gVS, after 24 hours of incubation. After 48 hours of incubation, the yield improved to 55.6±2.7 mg COD/gVS, indicating a soluble COD yield increase of 14.9%. After 72 hours of incubation, the yield was found to be 61.24±2.0 mg COD/gVS, indicating a 10.1% further yield increase. (Figure 7.2)

The yield of soluble COD due to treatment with 0.15% xylanase, was found to be 81.5±2.7 mg COD/gVS at 24 hours; and by 48 hours of treatment, the yield was found to be 79.8±1.8 mg COD/gVS, indicating no perceived significant increase in the soluble COD yield. By the 72nd hour, the yield had increase to 91.0±2.4 mg COD/gVS, indicating a 14.1% increase when compared to the value obtained at 48 hours (Figure 7.2).

At xylanase concentration of 0.3%, the yield of soluble COD was found to be 73.2±2.8 mg COD/gVS, 81.67±3.5 mg COD/gVS, and 121.3.0±3.8 mg COD/gVS, at 24, 48, 72 hours respectively (Figure 7.2).

The 0.5% xylanase treatment yielded additional soluble COD of 91.4±1.6 mg COD/gVS, after 24-hour incubation; 85.9±5.3 mg COD/gVS, after 48-hour incubation; and 98.6±3.8 mg COD/gVS (Figure 7.2).

It can be seen from Figure 7.2 that most of the solubilisation from the use of the xylanase enzyme occurred within 24 hours of treatment. The linear curve through the yields of all the concentrations of xylanase at the 24-hour treatment period show that the 0.15% xylanase treatment resulted in soluble COD yields comparable to the 0.3% and 0.5% concentrations. The 0.15% xylanase was therefore selected as the most effective concentration for the solubilisation of the perennial rye grass substrate.
7.2.3 Results of Experiment P1.3: Ferulic Acid Esterase (FAE)

As shown in Figure 7.3, the yield of soluble COD after treatment with 0.05% FAE was found to be 41.3±5.6 mg COD/gVS after 24 hours of incubation. The yield appeared to have reduced to 34.5±3.1 mg COD/gVS, after 48 hours of incubation; but then increased to 57.0±3.0 mg COD/gVS yield after 72 hours of incubation. The results show that most of the solubilisation activity occurred within the first 24 hours of treatment.

The treatment of the perennial rye grass with 0.15% FAE resulted in a soluble COD yield of 68.1±3.0 mg COD/gVS after 24 hours of incubation, indicating nearly 64.7% increase when compared to the yield with 0.05% FAE concentration. The yield further improved to 79.1±3.3 mg COD/gVS, then to 84.5±2.0 mg COD/gVS; after 48 hours and 72 hours respectively. The results indicated a 16.2% increase in yield from the value at 24 hours by the 48 hours and additional 6.8% by the 72 hour of treatment. (Figure 7.3)

The 0.3% FAE resulted in a soluble COD release of 102.6±1.2 mg COD/gVS at 24 hours; 102.5±7.9 mg COD/gVS at 48 hours; and 109.3±5.6 mg COD/gVS by 72 hours. The yield obtained from the 0.3% FAE concentration at 24 hours, was about 50.7% greater than the yield obtained at half that concentration (0.15% FAE). No perceived significant increase was however observed at the 48 hours and only an increase of 6.5% was observed by the 72 hours of treatment. The results as can be seen from the Figure 7.3 gave an indication that the
solubilisation effect of the FAE enzyme largely occur within 24 hours of treatment, with the 0.15% FAE, standing out as the concentration of choice for solubilising perennial rye grass.

![Graph showing solubilisation effect of FAE enzyme over time](image)

**Figure 7.3**: Additional soluble COD released from perennial rye grass due to *Ferulic Acid Esterase* (FAE) enzyme.  
Note: Percentage FAE was based on the unit enzyme activity shown in Table 7.1

### 7.2.4 Results of Experiment P1.4: Enzyme Combinations

The enzyme combination experiment was designed by using cellulase and xylanase as core enzyme and FAE and xylanase as accessory enzymes to test the synergistic relationship in the combinations. Based on the analyses of different concentrations of the individual enzymes, the 0.15% concentration was selected as a suitable concentration of the individual enzymes to evaluate their combined effect. The 24-hour treatment period based on the analyses from the individual enzymes was also selected as a suitable point for the evaluation of the solubilisation effect of the enzyme combinations.

The yield obtained from the treatment with 0.15% xylanase + 0.15% FAE was found to be 154.6±6.8 mg COD/g VS after 24 hours of incubation; about 3.3% higher than the expected yield of 149.6 mg/g VS computed from the combination of the individual xylanase and FAE at same concentrations. (Figure 7.4)

The 0.15% cellulase + 0.15% xylanase combination resulted in a soluble COD yield of 87.7 ± 4.5 mg COD/g VS after 24 hours of incubation, nearly 46.7% less than the expected yield.
from the combination of the values from the yield from the individual cellulase and xylanase at same concentrations. (Figure 7.4)

The 0.15% cellulase + 0.15% FAE resulted in a COD yield of 167.0±2.6 mg COD/g VS. The yield obtained from the cocktail was about 10.7% higher than the expected results from the addition of the values of the individual enzymes. The results suggested a poor or no synergistic relationship between the xylanase and the cellulase. The cellulase as core enzyme showed a more positive synergy with the FAE, when compared to the xylanase and the FAE. (Figure 7.4)

The cellulase-FAE mixture was therefore selected as the suitable enzyme combination for effective solubilisation of perennial rye grass to soluble carbohydrates.

Figure 7.4: Enzyme combinations actual yield versus expected yield (sum of individual activities)
7.2.5 Selection of Suitable Enzyme Cocktail Concentration for Effective Solubilisation of Perennial Rye Grass

Having selected the cellulase-FAE mixture as having positive synergistic relation, different ratios of cellulase and FAE were tested in order to select a high yielding enzyme combination, capable of effectively solubilizing perennial rye grass. The selection of an ideal pre-treatment technique was based on high yielding/hydrolysing potential of the enzyme, as well as, the ability to minimize carbohydrate loss (Xu et al., 2011). Although sodium azide was added in the studies described here to limit microbial activity, the enzyme of choice was selected based on its ability release soluble carbohydrates in the shortest period of time in order to avoid product loss due to consumption by the microbes. It can be seen from Figure 7.5 that the optimal enzymatic activities during the treatment process in all the enzyme combinations seem to have occurred within 24 hours of incubation. The most suitable enzyme for hydrolysis was therefore selected based on their performance at 24 hours of treatment. Having established that the FAE showed a positive synergistic relationship as an accessory enzyme to the cellulase core enzyme, the 0.15% FAE concentration was combined with cellulase concentrations of 0.15%, 0.3%, 0.5% and 0.8%. The most suitable cellulase concentration for the solubilisation of the perennial rye grass substrate was therefore selected based on how positive the synergistic relationships between the FAE and the cellulase enzyme concentrations. The highest yield of soluble COD was obtained from the 0.8% cellulase ± 0.15 FAE enzyme-cocktail. The soluble COD yield was found to be 365.5±3.0 mg COD/g VS, about 119% higher than the yield obtained with the 0.15% cellulase ± 0.15 % FAE enzyme mixture, 65% higher than the yield obtained with the 0.3% cellulase ± 0.15 % FAE enzyme, and 41% higher than the yield obtained with the 0.5% cellulase ± 0.15% FAE enzyme mixture. The actual yield of soluble COD in all the enzyme combinations was higher than the expected yields. Even though the 0.5% cellulase + 0.15% FAE gave a greater difference between the actual and expected yields, the actual yield compared to the 0.8% cellulase ± 0.15% FAE was low due to the low yield of COD from the individual 0.5% cellulase used in the expected yield estimation. The actual yield of soluble COD from the 0.8% cellulase ± 0.15% FAE mixture was found to be nearly 17%, higher than the expected yield. The 0.8% cellulase ± 0.15% FAE cocktail was therefore selected as being the best available technique for the solubilisation of recalcitrant components of perennial rye grass, capable of releasing soluble carbohydrates for biohydrogen production. (Figure 7.5)
After 24 hours of incubation, the yield of soluble COD was found to be 365.5±10.9 mg /gVS; representing a 393.2% yield increase, when compared to the yield obtained with no enzyme control (Figure 7.5). The soluble carbohydrate gained in the process represented 56.1% of soluble COD.

**Figure 7.5: Release of soluble substrates due to enzyme concentration of 0.20ml/ gVS of perennial rye grass (0.8% Cellulase ± 0.15 FAE)**

Note: Enzyme percentages expressed in the figure was based on unit enzyme activities shown in Table 7.1

7.2.6 *The role of alkaline pre-treatment in the homogeneity of the perennial rye grass substrate.*

The use of alkali pre-treatment to alter the chemical and structural composition of the lignocellulosic biomass for enhanced hydrolysis, in order to obtain soluble carbohydrates for anaerobic processes (*Chang and Holtzapple, 2000*), was found to play an important role in the improvement of the homogeneity of the lignocellulosic substrate. The role of the alkaline in
the enhancement of the homogeneity and rheology of the perennial rye grass substrate was evaluated as described in Experiment PT2 in Method Section 5.7.2.3.

The primary mode of action of NaOH in the pre-treatment of lignocellulosic biomass, as already mentioned, has been defined as the delignification of the lignocellulosic complex and the solubilisation of the xylan by removing acetyl and uronic acid substitutions of the hemicellulose (Chang and Holtzapple, 2000). The alkaline (NaOH) hydrolysis attack the ester bond cross-linkages between the lignin and xylan, resulting in increased porosity of the biomass (Tarkov and Feist, 1969). As described by Silverstein et al. (2007), significant removal of lignin is dependent on several factors, including the concentration of the alkali reagent, the time of exposure, the temperature and the pressure. Whilst pre-treating a lignocellulosic biomass at NaOH concentration of 0.5%, they observed 7.6% further lignin reduction when the pre-treatment conditions were changed from 90°C/15 psi to 121°C/15 psi. They also observed significant lignin reduction at increasing concentrations of the NaOH. In the current study, the NaOH pre-treatment was carried out at 0.46% (v/v) concentration, at atmospheric temperature and pressure. An ADL reduction of 0.47%, as observed in the current study, could be considered low and within the margin of error. Though only a small fraction of the ADL was removed, physical observation of the perennial rye grass slurry indicated that, the NaOH addition improved the homogeneity the substrate, eradicating the frequent clogging of the pumps, which occurred when the continuous fermentation was carried on the perennial rye grass without pre-treatment. Figure 7.6 shows the state of the perennial rye grass substrate before pre-treatment. The TSS in the untreated perennial rye grass was found to between 5 to 9% of the TS (Figure 7.6). After the perennial rye grass was treated with 0.46% NaOH, the TSS was found to be <0.5% (Figure 7.7). The treatment of perennial rye grass with 0.202 ml enzyme/g (as shown in Section 7.3) resulted in TSS of 4-7% of TS, after 24 hours of treatment (Figure 7.7).

A reactor failure was observed during the HAG-U fermentation after about 10 days of running because of clogging of the feeding pump. The clogging was attributed to the high quantity of floating mass (caused by the waxy nature of the plant cuticle) even under continuous stirring. With continuous stirring at 120 rpm in the Feed-tank, the alkali addition notably improved the homogeneity of the substrate. It can be suggested that as the lignin seal was disrupted by the action of the NaOH, the porosity of the biomass increased. Moreover, as
highlighted by Cogger et al. (2002), the bulk density of the biomass increased with increasing moisture content, allowing the biomass to be absorbed into the alkaline solution, thereby improving the homogeneity of the biomass. When the experimental principle was applied to fresh perennial rye grass, as observed in Figure 7.8, similar observations were made.

Smyth et al. (2009) carried out a critical reviewed the use of grass as a viable substrate for anaerobic digestion. They emphasized that untreated grass presents significant difficulty in mixing and pumping, and therefore suggested conversion of grass to silage for better exploitation of the energy inherent in grass.

Figure 7.6: Untreated perennial rye grass in water
Figure 7.7: Effect of pre-treatment on dried perennial rye grass

Figure 7.8: Effect of alkaline pre-treatment on fresh perennial rye grass
7.3 **Enhancement of Acidogenic Lignocellulosic Fermentation due to Pre-treatment**

*(Results and Discussion – Experiment HAG-U, HAG-ALK and HAG-ALKENZ)*

The untreated perennial rye grass was used as substrate (HAG-U\textsubscript{substrate}) for acidogenic fermentation in the HAG-U reactor. Alkaline pre-treated perennial rye grass was used as substrate (HAG-ALK\textsubscript{substrate}) for acidogenic fermentation in reactor HAG-ALK. In the HAG-ALKENZ reactor, HAG-ALK\textsubscript{substrate} used as substrate in an enzymatic SSF using enzyme cocktail (0.8% Cellulase \(\pm\) 0.15 FAE) developed in Chapter 7.2. The yields of biohydrogen and VFAs, and the degradation of the lignocellulosic components were then comparatively evaluated. The 5-litre CSTR reactor was inoculated with heat-treated seed sewage sludge. The reactor was maintained at environmental conditions as described in Section 5.6. Typical characteristics of the seed sludge are shown in Table 7.2. The sewage sludge seed at a high biochemical alkalinity (BA) of 3.77 g/l, gave an indication that the source fermenter had greater capacity for resisting sudden changes in pH, hence the provision of healthy strains of bacteria for start-up. Effluents from the three-biohydrogen reactors (HAG-U, HAG-ALK and HAG-ALKENZ) were taken for compositional analysis including: NDF, ADF, ADL (Method Section 5.7.3.2).

**Table 7.2: Typical characteristics of the seed sludge**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values</th>
</tr>
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<tbody>
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<td>TS (%)</td>
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<td>VSww (%)</td>
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<tr>
<td>pH</td>
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</tr>
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<td>Bicarbonate alkalinity (as CaCO(_3)) (g/l)</td>
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<tr>
<td>VFA (g/l)</td>
<td>1.354</td>
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<td>VFA/BA ratio</td>
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</table>
7.3.1 The Role of Alkaline Pre-treatment in Biohydrogen Fermentation

The quantity of NDF in the HAG-U\textsubscript{substrate} was found to be 25.6±0.21 g/l. After the substrate was degraded in the HAG-U reactor, the quantity of NDF in the HAG-U\textsubscript{effluent} was found to be 22.38±0.92 g/l; indicating 12.5% reduction. (Table 7.3)

The NDF in the HAG-ALK\textsubscript{substrate} was found to be 24.96±0.59 g/l. It can be seen from the results (Table 7.3) that the addition of the alkaline (0.46% NaOH, pH 10.9) resulted 2.5% NDF reduction. The NDF component in the HAG-ALK\textsubscript{effluent} was found to be 18.31±0.1.08 g/l, indicating 26.6% degradation. When compare to the NDF in the HAG-U\textsubscript{effluent}, an 18.2% reduction was observed to have occurred due the the alkaline pre-treatment.

The hemicellulose component in the HAG-U\textsubscript{substrate} was found to be 12.37±0.1.8 g/l. After the acidogenic fermentation, a hemicellulose quantity of 10.87±1.86 g/l was determined. The results indicated the anaerobic process achieved a 12.1% reduction of hemicellulose. The hemicellulose component of the HAG-ALK\textsubscript{substrate} was found to be 12.21±0.23 g/l. The quantity of the hemicellulose in the HAG-ALK\textsubscript{substrate} was found to be similar to the quantity detected in the HAG-U\textsubscript{substrate}, which was also found to be 12.37±0.1.8 g/l; indicating that the alkaline addition resulted in hemicellulose reduction of 1.29% even before the fermentation process. After the HAG-ALK fermentation, however, the quantity of the residual hemicellulose in the HAG-ALK\textsubscript{effluent} was found to be 9.73±0.20 g/l, indicating a 20.3% reduction, compared to the 12.12% reduction in the HAG-U\textsubscript{effluent}. (Table 7.3)

The undigested cellulose component after the acidogenic fermentation of the HAG-U\textsubscript{substrate} was found to be 7.34±1.23 g/l, approximately 19%, from an initial value of 9.05±0.94 g/l. The cellulose component of the HAG-ALK\textsubscript{substrate}, on the other hand, was found to be 8.59±0.17 g/l, indicating that the alkaline pre-treatment, even before the fermentation, led to a cellulose reduction of 5.08%. After the HAG-ALK fermentation, the cellulose was found to have reduced to 6.18±0.49 g/l indicating a 28.1% reduction of the cellulose component. It was observed from the results that the alkaline addition resulted in relatively greater degradation of the cellulose after the HAG-ALK fermentation. (Table 7.3)
Table 7.3: The Effect of Alkaline Pre-treatment on Biohydrogen Fermentation

<table>
<thead>
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<th>Parameters</th>
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<th>Alkaline Pre-treatment (HAG-ALK)</th>
</tr>
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<td>Effluent</td>
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<tr>
<td>HRT (days)</td>
<td>NA</td>
<td>1.000</td>
</tr>
<tr>
<td>Total VFA (g/kg VS/day)</td>
<td>0.10±0.013</td>
<td>1.74±0.19</td>
</tr>
<tr>
<td>Soluble Carbohydrates (mg/gVS)</td>
<td>126.03±1.7</td>
<td>4.97±0.85</td>
</tr>
<tr>
<td>Total COD (g/gVS)</td>
<td>19.57±2.63</td>
<td>21.31±1.45</td>
</tr>
<tr>
<td>TS (g/l)</td>
<td>48.16±1.11</td>
<td>44.76±0.68</td>
</tr>
<tr>
<td>VSNw (%)</td>
<td>87.92±0.24</td>
<td>75.78±0.65</td>
</tr>
<tr>
<td>NDF (g/l)</td>
<td>25.60±0.21</td>
<td>22.38±0.92</td>
</tr>
<tr>
<td>Hemicellulose (g/l)</td>
<td>12.37±1.8</td>
<td>10.87±1.86</td>
</tr>
<tr>
<td>Cellulose (g/l)</td>
<td>9.05±0.94</td>
<td>6.34±1.23</td>
</tr>
<tr>
<td>ADL (g/l)</td>
<td>4.20±0.36</td>
<td>4.18±0.19</td>
</tr>
<tr>
<td>Cellulase activity (kU/ml)</td>
<td>0.00</td>
<td>0.0061</td>
</tr>
</tbody>
</table>
As shown in Table 7.3, the quantity of ADL in the HAG-U\\text{substrate} was found to be 4.20±0.86 g/l. By comparing the HAG-ALK\\text{substrate} to the HAG-U\\text{substrate}, it was observed that the ADL component had been reduced by 0.95% after the alkaline treatment. After the HAG-U fermentation, the ADL component was found to be 4.18±0.79 g/l, indicating a reduction of 0.48%. The ADL component of the HAG-ALK\\text{substrate} was found to be 4.16±0.27 g/l, and after the acidogenic fermentation, the residual ADL was found to be 3.6±0.96 g/l, indicating a reduction of 13.5%.

By comparing the composition of the HAG-ALK\\text{effluent} to HAG-U\\text{effluent}, it was observed that the alkaline pre-treatment fermentation compared to the no pre-treatment fermentation resulted in further NDF reduction of 18.19%.

The results show that alkaline pre-treatment improved the enzymatic hydrolysis of the holocellulose in the acidogenic fermentation. The sodium hydroxide used in the pre-treatment is known to be a strong agent that is specific for the extraction of hemicellulose and lignin. Xu et al. (2006) defined the mode of action of the NaOH as targeting the α-ether bonds between hemicellulose and lignin of the cell wall to cleave it. Sun et al. (2003) reported that the efficiency of degradation of the lignocellulosic component of biomass significantly decreases with increasing concentrations of NaOH. They however observed a maximum yield of hydrogen and VFAs, at 0.5% NaOH concentration. The concentration of NaOH used in the pre-treatment process in the current study was 0.46%, which was in accordance with the work of Sun et al. (2003), as that concentration compared to lower or higher NaOH concentrations was found to be most suitable for the pre-treatment of lignocellulosic biomass for biohydrogen production.

The alkaline pre-treatment was found to have a relatively greater effect on the hemicellulose component when compared to the cellulose component. The removal of the hemicellulose has the potential of increasing the pore size of the lignocellulose substrate, making the cellulose more accessible for hydrolysis (Chandra et al., 2007). It can be seen in the results that the concentration of alkaline used in the pre-treatment did not affect the solubilisation of the lignin component of the substrate in the anaerobic process. The results, however, show that there was an improved yield of biohydrogen and VFAs from the acidogenic reactor when alkaline pre-treatment was adapted. It can therefore be concluded that, though a complete delignification was not achieved, the concentration of the alkaline used in the experiment was
enough to change the structure of the lignin, thereby improving the yields of products obtained from the process. As described by Chandra et al. (2007), the change of the structural location of lignin is also an important mode of action, which results in improved access of the hydrolytic enzymes to the cellulose and hemicellulose, without the removal of the lignin component. It can be concluded from the results that the alkaline pre-treatment adopted was effective; capable of causing increased release of soluble carbohydrates without severe delignification, which has the potential of releasing derivatives of lignin such as phenolics, furfural, 5-hydroxymethyl furfural, ρ-coumaric acid, etc., that have been found to be inhibitory to acidogenic fermentation (Gossett et al., 1982; Palmqvist and Hahn-Hagerdal, 2000). Ishizawa et al. (2009) emphasized that cellulose digestibility can significantly be affected by feedback inhibition due to severe delignification. They observed that delignification resulted in aggregation of the cellulose microfibrils, which was found to be responsible for the reduction of the accessibility of enzymes.

7.3.2 Effect of Enzymatic Simultaneous Saccharification and Fermentation on Lignocellulosic Degradation

The enzymatic-SSF also improved the reduction of the lignocellulose component of the perennial rye grass substrate in the acidogenic reactor effluents. The NDF in the HAG-ALKsubstrate was found to be 23.96±0.59 g/l. After the enzymatic-SSF in the HAG-ALKENZ reactor, the NDF in the HAG-ALKENZeffluent was found to be 11.45±0.75 g/l, indicating 54.1% reduction. By comparing the residual NDF in the HAG-ALKeffluent to the residual NDF in the HAG-ALKENZeffluent, it can be observed that 28.6% further reduction could be attributed to the addition of the enzyme cocktail. The combined effect of alkaline and the enzyme was therefore found to have caused NDF reduction of 55.3%, which is 42.7% higher than the reduction of the lignocellulosic complex observed after the acidogenic fermentation of the HAG-Usubstrate. (Table 7.4)

The residual hemicellulose component in the HAG-ALKENZeffluent was also determined to be 7.22 ± 0.38 g/l; 12.4% further reduction when compared to the quantity obtained in the HAG-ALKeffluent. The combined effect of the alkaline and enzyme was therefore found to have resulted in a hemicellulose reduction of 40.9%; 29.5% higher than the reduction of hemicellulose observed in the HAG-Ueffluent. (Table 7.4)
The residual cellulose concentration in the HAG-ALKENZ\_effluent was found to be 0.42 ± 0.20 g/l, 67.1% further reduction, when compared to the content in the HAG-ALK\_effluent. As discussed in Section 7.2, no further reduction was observed upon addition of the weak alkaline, as both the HAG-U\_substrate and HAG-ALK\_substrate were found to have degraded by 28.0%. The addition of the enzyme cocktail however resulted in a cellulose reduction of 95.1% when compared to the initial quantity in the substrate. (Table 7.4)

The ADL component in the HAG-ALKENZ\_effluent was determined to be 3.80 ± 0.17 g/l, compared to the 4.16 ± 0.27 g/l quantity observed in the substrate. It can be deduced that the combine effect of the weak alkaline and enzyme led to ADL reduction of 8.7%. A comparison of the ADL content in the HAG-ALK\_effluent (3.7 ± 0.96 g/l) to the quantity in the HAG-ALKENZ\_effluent, however, showed that the lignin reduction that could be attributed to the addition of the enzyme was relatively low. (Table 7.4)

The use of enzyme to enhance the effective degradation of the holocellulose complex in lignocellulosic biomass to soluble carbohydrates has been reported by Van Dyk and Pletschke (2012), as being pivotal in making anaerobic processes economically competitive. The multiple enzymes at defined ratios, adopted in the current study to pre-treat perennial rye grass, resulted in high-level bioconversion of the complex polymers to soluble carbohydrates, consistent with the mode of mechanism defined in Van Dyk and Pletschke (2012). The extracellular hydrolases involved in the depolymerisation of complex carbohydrates, provide the individual bacteria with a highly active feeding mechanism (Juillard et al., 1995), releasing soluble sugars for the anaerobic digestion process. The efficacy of the extracellular hydrolases have however been found to be highly prominent in substrates of wastewater conditions (Vetter et al., 1998). The recalcitrant nature of untreated lignocellulosic biomass makes it poor substrate for enzymatic hydrolysis (Sun, 2010). Sun and Cheng (2002) observed a degradation efficiency of less than 20%, when untreated lignocellulosic biomass degraded in anaerobic fermentation. Adelsberger et al. (2004) described that different lignocellulosic biomass require different mode of action of the microbial hydrolytic enzymes, making the microbial enzymatic functionality highly substrate specific.

The primary function of the 0.8% cellulase (in the enzyme cocktail) used in the experiment was to hydrolyse the β-1, 4-glycosidic linkages, which is a prominent bond in lignocellulosic biomass; and is thus a major contribution to solubilising the recalcitrant biomass. Cellulase
was selected ahead of xylanases based on availability and economic viability, since both enzymes share similar function of hydrolysing β-1,4-glycosidic linkages (Gilbert and Hazlewood, 1993). Kumar and Wyman (2009a) highlighted that commercial cellulases and xylanases have similar functionality in cellulosic digestions.

The degradation efficiency of soluble carbohydrates was determined to be 79.2% in the HAG-U reactor, indicating that, if the biochemical pre-treatment technique developed were utilised, a dramatic improvement of hydrogen yield could be realized; given that, the technique with its effective solubilisation potency could result in approximately 393% release of additional soluble carbohydrates when compared to no enzyme control.

The ‘0.8% Cellulase ± 0.15% FAE’ enzyme cocktail, stood out as the most effective enzyme mixture for the solubilisation of the perennial rye grass substrate. Banerjee et al. (2010a) however, emphasized that the accuracy and consistency of the results obtained when pure enzymes are not used could be compromised, since there are usually additional enzyme activities in the commercial or crude enzyme mixtures which are not accounted for in the commercial specification (Selig et al., 2009). The results of the current study however highlight the effectiveness of using a selected combination of commercial enzymes to enhance solubilisation yield, making it a more practical approach for deployment in industry.

The optimal enzymatic synergy predicted based on the relative composition of substrate, rather than the actual biochemical assays using pure enzymes mixtures, may be less reproducible as emphasized by Banerjee et al. (2010b). That notwithstanding, as efficiency of enzymatic activity on complex polymers has a direct relationship with the release of readily solubilized monomers, a comparative assessment of different commercial enzyme combinations can be said to be scientifically justifiable.

The cellulase (0.8% cellulase) used in the treatment was considered as a core enzyme, according to principle defined by Banerjee et al. (2010c). The commercial Accellerase core enzyme used in the current study consisted of endo-β-1, 4-D-glucanase, exo-β-1, 4-D-glucanase and β-1, 4-D-glucosidase. The FAE therefore acted as an additional “accessory” enzyme enhancing enzymatic activities specific to the substrate (perennial rye grass). Similar to the work of Banerjee et al. (2010c), the addition of the right quantity of the accessory enzyme to a known concentration of the core enzyme was the major basis for the enzyme cocktail development, and this was specific to the substrate being studied. As proposed by
Banerjee et al. (2010c), the ideal approach for identifying a specific enzyme cocktail for solubilising a selected lignocellulosic biomass is to identify a core set of enzymes, with the subsequent evaluation of additional enzymes known as accessory enzymes. They observed that accessory enzymes were specific to different substrates. For example, β-mannanase and amylloglucosidase were essential for the hydrolysis of dried distilled grains (DDG), but was not necessary for the solubilisation of corn stover. Xiros et al. (2009), on the other hand, identified protease as important accessory enzyme for DDG. Meyer et al. (2009) described the principle underlining the contributions individual enzymes in a cocktail, and their role in the cumulative enzymatic effect as the ‘minimal enzyme cocktail principle’.

In the current study, after various concentrations of the FAE were tested (Experiment PT1.3), the 0.15% FAE concentration was selected to combine with 0.8% Cellulase (core enzyme) to form a cocktail found to be highly effective for the solubilisation of perennial rye grass. The quantity of the enzyme cocktail used in the pre-treatment was 0.202 mg enzyme/g VS, which is competitive with those, mentioned in literature. Boussaid and Saddler (1999) for example, highlighted that an enzyme load of 40 mg protein/g cellulose (40 FPU/g cellulose) was ideal for effective solubilisation of standard microcrystalline cellulosic substrate Avicel PH-101(Fluka, Switzerland). They also observed that de-lignified Kraft pulp, on the other hand, required 60 mg protein/g cellulose (60 FPU/g cellulose) enzyme concentration as a minimum enzyme for adequate solubilisation. The current study therefore uses low dose of enzyme to avoid the occurrence of delignification.

Massanet-Nicolau et al. (2007) using sewage sludge as a substrate, observed a 13.5% increase in soluble carbohydrates because of enzymatic pre-treatment. Cui et al. (2010) using poplar leaves as substrate, obtained a hydrogen yield of 44.92 ml H$_2$/g TS, after pre-treatment with an enzyme cocktail consisting of cellulase, β-glucanase, arabinase, xylanase and hemicellulase. The current study obtained a hydrogen yield of 48.44 ml H$_2$/gVS after treatment with 0.202 mg enzyme/gVS (5 Cellulase: 1 FAE).

**7.3.3 Biohydrogen Yield**

Comparative evaluation biogas production from the HAG-U reactor, the HAG-ALK reactor and HAG-ALKENZ reactor, as described in Method Section 5.7.3, were carried out.
As shown in Figure 7.9, the yield of hydrogen in the no pre-treatment control was found to be less than 1.0 cm³ per minute over a period of 14 days; releasing an average of 6.67 l H₂/kgVS. The yield of bio-hydrogen from HAG-ALK_substrate was however found to have improved to approximately 2.0 ml/minute over 14 days, resulting in an average of 10.95 l H₂/kgVS. The results indicated that the alkaline pre-treatment led to approximately a 64% improvement in hydrogen yield. The average hydrogen yield over 15 days from the HAG-ALKENZ reactor was approximately 8 ml/ minute, corresponding to 48.44 l H₂/kgVS; indicating a substantial 335% further yield increase. (Figure 7.9)

The experiments show that the enzyme cocktail added had an incorporated COD concentration of 291.1 mg/l. Figure 7.10 shows the yield of hydrogen obtained from a 10 g/l sucrose substrate to be 47.65 l/kgVS. The yield of hydrogen from the available COD in the enzyme cocktail (0.291 g/l COD) was therefore calculated based on the yield obtained from the 10g/l sucrose to be 1.387 l/kgVS. Approximately 3% of the hydrogen yield could therefore be attributed to the soluble carbohydrates in the enzyme added to the mixture. The actual yield due the biochemical activity (total yield minus yield due to enzyme sugars) was therefore estimated to be 47.1 l/kgVS. (Figure 7.10)

The yield of hydrogen from the HAG-ALK reactor was found to be 10.95 l/kgVS; the yield due to the enzymatic activity only, was therefore observed to be 36.15 l/kgVS indicating yield improvement of 74.6% due to the enzymatic activity (Table 7.10).
Figure 7.9: Production of biohydrogen due to Chemical and Biochemical Pre-treatment of perennial rye grass substrate
Figure 7.10: Comparative yield of hydrogen of biochemically pre-treated perennial rye grass, 10g/l sucrose and the residual sugars present in the enzyme cocktail

Improving the yield of the hydrogen producing fermentation, has a positive effect on any secondary bioprocess it might be coupled with; including methane production (Ghosh, 1991); PHA production (Yu, 2001); photo-fermentation to produce additional hydrogen (Tao et al., 2007). Enhancing the efficiency of the biochemical activities in the acidogenesis stage results not only in higher yields of bio-hydrogen, but also important by-products such as VFA; an important precursor for the secondary processes (Atonopoulou, 2008). Ghosh (1991) observed that, adopting a hydrogen first stage in a 2-phase anaerobic process, for sewage sludge resulted in an enhanced production of methane in the second stage. Lovely and Klug
(1986) identified acetate, a major product from the hydrogen producing reactor, as an ideal substrate for bio-methanation. Several researchers also emphasize that a two-phase anaerobic process has overwhelming advantage over a classical one-phase process, in the production of biofuels (Adney et al., 1989; Ghosh, 1987; Shin et al., 2001).

Banerjee et al. (2010b) investigating the effect of pre-treatment of lignocellulosic biomass for bio-hydrogen production, emphasized that the target of most of the research into enzymatic pre-treatment (using commercial enzyme mixtures) of lignocellulosic biomass, have been on the development of optimal hydrolysis of acid treated corn stover, therefore not optimal for other pre-treatment techniques and other lignocellulosic substrates.

Zhang et al. (2007) obtained a biohydrogen yield of 3.16 ml H$_2$/g VS when a raw corn stover was digested. When they used 0.5% NaOH to pre-treat the corn stover, they obtained a maximum hydrogen yield of 56.70 ml H$_2$/ gVS. In the current study, the HAG-U$_{substrate}$ resulted in a yield of 6.67 ml/gVS. By using 0.46% NaOH to pre-treat the substrate, the hydrogen yield only improved to 10.95 ml H$_2$/ gVS. The variation in results further emphasizes that substrate specificity is a critical factor for effective pre-treatment, for maximized yields (Van Dyk, 2012). There is a great deal of variation in the structural composition of lignocellulose, a major contribution to varying accessibility of hydrolytic enzymes to the complex recalcitrant polymers.

The HAG-ALKENZ fermentation resulted in an improved hydrogen yield of 48.44 ml/gVS, indicating an approximately 335% further yield increase, when compared to the HAG-U fermentation; and that is one of the highest reported in literature. Yang et al. (2010) using thermo-alkaline pre-treatment at 100°C on lipid-extracted micro-algal biomass residue, observed a hydrogen yield of 45.54 ml/gVS. Cui and Shen (2010), after pre-treatment of dry grass with 4% HCl in a batch bio-hydrogen fermentation process, observed a cumulative bio-hydrogen yield of 72.21 ml/g.

The result confirmed reported research into lignocellulose degradation that substrate specificity is critical for the selection of a particular pre-treatment. Sills and Gossett (2011), however questions the commercial viability of customizing enzymatic hydrolysis for every single lignocellulosic biomass.
# Table 7.4: Biochemical Component of Effluents of Anaerobic Bioreactors

<table>
<thead>
<tr>
<th>Parameters</th>
<th>No Pre-treatment</th>
<th></th>
<th></th>
<th>Alkaline/ Enzymatic Pre-treatment</th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Substrate</td>
<td>HAG-</td>
<td>% Increase/decrease</td>
<td>Substrate</td>
<td>HAG-</td>
<td>% Increase/decrease</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ueffluent</td>
<td></td>
<td></td>
<td>ALKeffluent</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrogen Yield (l/kg VS)</td>
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<td>6.667</td>
<td>NA</td>
<td>NA</td>
<td>10.95</td>
<td>64.29*</td>
</tr>
<tr>
<td>OLR (kg VS/m³/day)</td>
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<td>NA</td>
<td>NA</td>
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</tr>
<tr>
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<td>NA</td>
<td>1</td>
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</tr>
<tr>
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<td>NA</td>
<td>0.0023</td>
<td>0.170</td>
<td>316.67*</td>
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<td>184.01</td>
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<td>NDF (g/l)</td>
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<td>-12.58</td>
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<td>18.31</td>
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</tr>
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<td>Hemicellulose (g/l)</td>
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</tr>
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<td>Cellulose (g/l)</td>
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<td>ADL (g/l)</td>
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<td>Cellulase activity (kU/ml)</td>
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<td>0.0061</td>
<td>NA</td>
<td>0</td>
<td>0.065</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Relative to the yield obtained from the HAG-Usustrate; **The enzyme cocktail at 0.202ml enzyme/gVS had Total carbohydrate concentration of 0.153g/l which correspond to hydrogen yield of 0.729 l/kg VS therefore actual hydrogen yield due to biochemical pre-treatment was: 47.707 l/kg VS/

Thesis – Philemon Kumi
7.3.4 Enhancement of Volatile Fatty Acids Production during Acidogenic Fermentation

As shown in Figure 7.11, the yield of VFA in the HAG-U fermentation was found to be an average of 1.92±0.09 g/l VFAs (0.045 g VFA/ g VS) taken after the first 14 days of continuous anaerobic digestion, the results indicate a VS to VFA conversion efficiency approximately 5%.

The yield of VFAs the average yield of VFAs from the HAG-ALKsubstrate was found to be 7.02±0.14 g/l VFAs (0.170 g VFA/ g VS), indicating VS to VFAs conversion efficiency of 16.6%. The results indicate a VFA yield increase of approximately 266% to due to alkaline pre-treatment.

The total VFAs in the HAG-ALKENZeffluent was found to be 9.77±0.42 g/l VFAs (0.232 g VFA/ g VS). The results indicate VFA conversion of 23.2%. The enzyme activity therefore improved the VFA yield observed in the alkaline pre-treatment experiment by 39.3%, giving an overall VFA yield improvement of 409.3%, when compared to the VFA yield from the HAG-Usubstrate.
The predominant VFA produced in the HAG-U fermentation was acetic acid, and was found to be 1345.6±39.2 mg/l VFA (31.2±0.9 mg VFA/ gVS), representing 70.83% of the total VFAs produced. The average n-butyric acid production was found to be 409.14 mg/l VFA (9.5±1.3 mgVFA/ gVS), representing approximately 21% of the total VFAs. Acetate to n-Butyrate ratio was identified to be 4:1. The average quantity of propionic acid produced was found to be 140.9±49.4 mg/l VFAs (3.3±1.1 mg VFA/gVS), representing approximately 7% of the total VFAs. The yields of i-butyric, i-valeric and n-valeric were found to be less than 1% of the total VFA.

The average acetic acid produced in the HAG-ALK fermentation was found to be 2901.0±76.4 mg/l VFA (68.8±1.8 mgVFA/ gVS), representing 41.3% of the total VFAs produced. The average yield of n-butyric was found to be 1815.8±48.8 mg/l VFA (48.1±1.2 mgVFA/ gVS, representing 25.9% of the total VFAs. The acetate to butyrate ratio was determined to be 2:1. The average propionic acid production was found to be 1590.5±51.1 mg/l VFA (37.7±1.2 mgVFA/ gVS, representing 22.6% of the total yield of VFAs. The average n-valeric acid was found to be 631.8±16.7 mg/l VFA (15.0±0.4 mgVFA/ gVS),
representing 9.0% of the total VFAs. The total yields of i-butyric and i-valeric was less than 1% of the total VFAs.

The HAG-ALKENZ fermentation resulted in an acetic acid yield of 3556±179.3 mg/l VFAs (84.3±4.2 mgVFA/ gVS), representing 36.4% of the total VFAs. The average yield of butyric acid was found to be 3920.9±168.0 mg/l VFA (93.0±4.2 mgVFA/ gVS), representing approximately 40% of the total VFA. The acetate to butyrate ratio was 1:1. The average propionic acid was found to be 825.8±60.6 mg/l VFA (19.6±1.4 mgVFA/ gVS), representing 8.4%. The average n-valeric acid was found to be 1257.8±93.4 mg/l VFA (29.8±2.2 mgVFA/ gVS), representing approximately 13% of the total VFAs. Again, total i-butyric acid (2.16±0.12 mg VFA/gVS) and i-valeric acid (2.9±0.15) was less than 2%. 

123

Thesis – Philemon Kumi
Figure 7.12: VFAs proportions in a. HAG-U effluent, b. HAG-ALK effluent, c. HAG-ALKENZ effluent
The results show that the pre-treatment procedures enhanced the yield of VFAs. The total yield of VFAs obtained after the HAG-U fermentation was 1.74 g/l (47.9 g/kg VS). The total VFA production from the HAG-ALK fermentation was 7.25 g/l (216.9 g/kg VS, indicating 21.7 % VS to VFA conversion. The yield of VFA from the HAG-ALK fermentation was found be approximately 317% enhancement of the yield observed in the HAG-U fermentation. The yield of VFAs from the HAG-ALKENZ fermentation was 10.45 g/l (325.9 g/kg VS, indicating 32.6% VS to VFA conversion), approximately 38% enhancement of the yield from the HAG-ALK fermentation. The pre-treatment improved the accessibility of the holocellulose (cellulose and hemicellulose) to hydrolytic enzymes to release soluble carbohydrates; allowing effective bioconversion to VFA.

The results also show that the type of pre-treatment used affected not only the total quantity of VFA produced, but also the proportions of individual VFAs. The yields of VFA in the HAG-U substrate were predominantly acetic acid, which represent approximately 70% of the total VFAs. The ratio of acetic to butyric acid was 4:1. The total acetic acid produced in the HAG-ALK fermentation was about 41.3% of the total VFAs, with the acetate to butyrate ratio being 2:1. After HAG-ALKENZ fermentation, the acetic acid percentage of the total VFAs was 36.4%, with the acetate to butyrate ratio being, 1:1. It can be concluded from the results that the type of pre-treatment can alter the proportions or quality of VFAs. The yields of VFAs during acidogenic fermentation, as well as its composition, have been identified to be substrate specific (Jiang et al., 2007; Rajagopal and Beline, 2011). According to Rajagopal and Beline (2011) the fermentation of carbohydrates, proteins and lipids, are directly associated with the production of lower molecular weight VFAs (acetic, propionic, iso- and n-butyric). The production of higher molecular weight VFAs (iso- and n-valeric acids) are, on the other hand associated with reductive deamination of amino acids during acidogenic fermentation of proteins (Rajagopal and Beline, 2011; Chen et al., 2007) or through the Stickland reaction by reductive oxidation between amino acids pairs (McInerney, 1988). It can be concluded therefore that the type of VFAs produced in acidogenic fermentation is directly linked with the composition of the substrate and its bioavailability to the hydrolytic enzymes. Ucisik and Henze (2008) also established that origin of the substrate (sewage sludge) has a direct relationship with the quantity, as well as, the composition of VFAs.
7.4 Chapter Conclusions

As discussed in chapter 6, the soluble carbohydrate was identified as the major component of the perennial rye grass substrate responsible for the yields of biohydrogen and VFAs obtained in the HA reactor. The hydrolysis enhancement study in this chapter was therefore set to improve the yield of biohydrogen and VFAs through biochemical pre-treatment.

- The enzyme cocktail (0.8% Cellulase ± 0.15% FAE) at 0.202 ml enzyme/g VS resulted in high-level bioconversion of the complex polymers to soluble carbohydrates after 24 hours of incubation. The yield of soluble carbohydrates was determined to be 321.0 ± 10.9 mg/gVS; representing a 393.2% yield increase, when compared to the yield obtained with no enzyme control.
- The yield of bio-hydrogen HAG-ALKENZ was 48 ml/gVS, approximately 335% higher when compared to the HAG-ALK fermentation; and more than 7 fold increase in the yield obtained from the HAG-U substrate. The enzymatic-SSF resulted in improved degradation of the lignocellulose component inherent in the substrate by approximately 43%. The degradation of the initial cellulose after enzymatic SSF was approximately 95%, compared to 5% cellulosic degradation in the HAG-U fermentation.
CHAPTER 8

8 BIOFUELS AND BIOCHEMICALS FROM ACIDOGENIC ANAEROBIC FERMENTATION OF LIGNOCELLULOSIC BIOMASS

8.1 Principle and Hypotheses of Study

The enzyme addition improved the yield of bio-hydrogen and VFAs (as described in Chapter 7). As noted by Bengtsson (2008), the acidogenic stage is the rate-limiting step of the anaerobic digestion process. The biochemical methane potentials of the effluents of the acidogenic reactors (HAG-ALK and HAG-ALKENZ) were evaluated, in order to access the effect of the enhancement of the acidogenic phase on methanogenic digestion process. As discussed in Chapter 7, the enhanced hydrolysis via enzymatic pre-treatment resulted in more readily solubilized monomers for biohydrogen and VFA production. As emphasized by Sannigrahi et al. (2010), the final utilisation of the treated substrate has the potential of giving a fair indication of the effectiveness of a pre-treatment technique. The compositional analyses showed that, the HAG-ALKENZ effluent was rich in nutrients and VFA, which are essential precursors for the production of biogas. The current study was set out to investigate the downstream effect of the enhanced hydrolysis on processes with the potential of secondary integration with the hydrolytic-acidogenic fermentation process, such biomethane production, PHA biosynthesis, and lignin biorefinery. The design of the study was to find answers the outlined questions:

- What will downstream effect of the enhanced enzymatic hydrolysis have on a second-stage biomethane process, the production of PHA, and the lignin biorefinery from the acidogenic effluent?
- Is the integration of biohydrogen fermentation process directly with PHA or lignin biorefinery economically viable?
8.2 Results and Discussion – Experiment BMP: Effect of Enhanced Acidogenesis on Bio-methane Fermentation

The two-stage anaerobic digestion system involving the physical separation of the acidogenic bacteria from the methanogenic microbial consortium has been identified as one of the most promising techniques capable of ensuring effective degradation complex substrates (Demirel and Yenigün, 2002; Kaseng et al., 1992). The two-stage anaerobic digestion of grass may also increase the operational stability of the digestion process (Rubio-Loz et al., 2010). It is therefore increasingly important to access the biochemical methane potential of the effluent of the acidogenic reactor.

As discussed in Chapter 7, the improvement of enzymatic hydrolysis using enzymatic SSF resulted in the formation more soluble and bioavailable substitutes, when compared to the control with no enzyme addition. The cellulase enzyme used in the acidogenic SSF, supplemented the saccharolytic activities of the acidogenic microbes, resulting in a more efficient degradation of the cellulose component of the substrate to readily solubilized monosaccharides. In effect, the enzymatic SSF increased the soluble organic matter concentration of the hydrolysate; producing essentially readily bioavailable short chain organic acids, which are important precursors for downstream processes, such as the production of biomethane.

The improvement of the yield of biomethane from grass was identified by Abu-Dahrieh et al. (2011), as involving many factors, including: reactor design (Singh et al., 2001), the use of adsorbents (Weiß et al., 2011) and substrate pre-conditioning (Richter et al., 2011). As emphasized by Bengtsson (2008) acidogenesis is the rate-limiting step in anaerobic digestion; improving the environmental conditions/ operational conditions that affect the acidogenic, leads to direct enhancement of biogas yield / energy balance. The current study investigated the downstream effect on biogas yield after a prior acidogenic enhancement. Biomethane potential (BMP) assay was adopted to assess the improvement of biogas yield after an enhanced acidogenic fermentation using enzymatic SSF. As described in the Method-Section 5.7.4, the HAG-ALK\textsubscript{effluent} HAG-ALKENZ\textsubscript{effluent} were used as substrate for BMP reactors B-ALK and B-ALKENZ respectively; using sewage sludge as blank substrate in B-BLANK reactor, and cellulose as reference substrate in B-REF reactor.
Figure 8.1 shows the cumulative biomethane gas production, whilst Figure 8.2 shows a normalised illustration of the biodegradability/biomethane potential of the three major substrates used in this study; thus, the mean biogas obtained from the sludge alone (B-BLANK) was subtracted from the quantity of biogas obtained in the other BMP units.

As shown in Figure 8.2, all the three BMP units (B-REF, B-ALK and B-ALKENZ) resulted in an exponential increase in biogas in the first 5 days, and that was similar to the observation made by Abu-Dahrieh et al. (2011). The sharp yield increase can be attributed to the rapid growth of different bacteria in the microbial consortium, with increased rate of hydrolytic activities, resulting in the high rate of conversion of the available substrate (Abu-Dahrieh et al., 2011). As the readily solubilized components of the substrates (including VFAs) were utilised, the remaining available substrate for the methanogenic digestion was largely the complex lignocellulosic proportion of the substrate, this needing a longer time period for hydrolysis and fermentation. The results gave a strong indication that the rate of biogas production in the first 5 days of the BMP digestion process has the potential of being used an indicator for the efficiency of the hydrolysis of an acidogenic reactor.

The yield of methane from the B-REF reactor was 385.5±35.7 ml CH₄/ gVS added, after 21 days of digestion. At the end of the digestion process (30 days), the yield of the methane was 385.8±29.0 ml CH₄/ gVS added. The results indicated that the last 9 days of digestion resulted in a yield increase of less than 0.1%. The results show that a 21-day HRT could be enough for the effective degradation of the cellulose substrate. The yield obtained from cellulose was in agreement with other peer reviewed studies, including Adu-Dahrieh et al. (2011) (approximately 350 ml CH₄/ gVS added); Nallathambi Gunaseelan (2009) (approximately 404 ml CH₄/ gVS cellulose) and Hansen et al. (2004) (approximately 379 ml CH₄/ gVS cellulose). The consistency of the reference substrate compared to other studies indicated that the operational conditions of the BMP assay were analytically robust.

The B-ALKsubstrate also showed similar trend of methane yield based on the HRT, resulting in a yield of 223.17.58±24.9 ml CH₄/ gVS added, after 21 days of digestion. A cumulative yield of 223.10±28.2 ml CH₄/ gVS was obtained after 30 days of digestion, indicating a yield difference of less than 0.1% in the last 9 days, similar to the yield observed in the B-REF reactor. Shiralipour and Smith (1984) studying the conversion of untreated Napier grass to methane in a BMP assay, observed a methane yield of 240 – 310 ml CH₄/ gVS added,
depending on the age of the grass. The yield of biomethane from the HAG-ALK_{effluent} was consistent with reported research in other similar substrates such as fresh Napier grass (260±0.019 ml CH\(_4\)/gVS added), and Aleman grass (293±0.003 ml CH\(_4\)/gVS added) (Chynoweth et al., 1993).

The HAG-ALKENZ_{effluent}, treated in the B-ALKENZ reactor, resulted in a methane yield of 317.67 ± 14.9 ml CH\(_4\)/g VS added after 21 days of digestion. At the end of the AD process (30 days) however, the yield of methane was found to be 325.58±18.2 ml CH\(_4\)/gVS added; indicating a yield increase of 1.84%. The data show that the enzymatic effect in the B-ALKENZ run resulted in the release of more readily available carbon and energy source for biogas production, thereby requiring less time to degrade.

As shown in Figure 8.2, the B-ALKENZ digestion resulted in higher yield of biogas when compared to the B-ALK process. By the 30\(^{th}\) day of the digestion, the enzymatic activity in the B-ALKENZ had resulted in 45.7.8% yield increase, compared to the biogas yield in the B-ALK reactor at the same time. (Figure 8.2)

The quantity of total carbohydrate found in the commercial enzyme used in the HAG-ALKENZ process was determined to be 0.153 g/l. With the yield of cellulose as model carbohydrate resulting in a methane yield of 385 ± 35.7 ml CH\(_4\)/gVS added, the yield of methane in the B-ALKENZ digestion attributed to the added COD of the residual enzyme in the HAG-ALKENZ_{effluent} was determined to be 7.751 ± 1.48 ml CH\(_4\)/gVS added. Consequently, the actual yield of methane from the B-ALKENZ reactor, 30 days of digestion was determined to be 317.58 ml CH\(_4\)/gVS added, which is 42.3% higher than the yield obtained after the digestion of the HAG-ALK_{effluent}. (Figure 8.2)
Figure 8.1: Cumulative methane production during BMP test

Figure 8.2: Methane yield from BMP assay

B-BLANK: Blank; B-REF – Cellulose as reference substrate; B-ALK – effluent of HAG-ALK reactor as substrate; B-ALKENZ – effluent from HAG-ALKENZ reactor as substrate; Biogas yield has not been normalized to standard temperature and pressure (STP).
8.2.1 Residual composition of perennial rye grass digestate

A study of the residual mass in the digestate after the anaerobic digestion process gave an indication of which constituents of the substrate were affected by differences in the enzymatic activities, and consequently, the differences in the biogas yield in the two lignocellulosic substrates under investigation. The NDF in the B-ALK\textsubscript{digestate} was 8.13±0.27 g/l, approximately 37% of the TS; compared to the 6.11±0.03 g/l determined in the B-ALK\textsubscript{ENZ\textsubscript{digestate}}, approximately 27% of the TS. The results indicated approximately 25% higher NDF degradation due the supplemented enzymatic hydrolysis in the B-ALK\textsubscript{ENZ}, compared to the NDF reduction in the B-ALK process. (Figure 8.3)

The ADL component in the B-ALK\textsubscript{digestate} and B-ALK\textsubscript{ENZ\textsubscript{digestate}} were found to be 4.88±0.07 g/l and 4.05±0.13 g/l respectively. The ADL component was degraded by approximately 17% due to the enhanced enzymatic activity in the B-ALK\textsubscript{ENZ} reactor. The residual cellulose component in the B-ALK\textsubscript{digestate} was 0.53±0.2 g/l, compared to that obtained in the B-ALK\textsubscript{ENZ\textsubscript{digestate}}, which was determined to be 0.25±0.02 g/l. The results indicated approximately 51% cellulose degradation efficiency in the B-ALK\textsubscript{ENZ} reactor, when compared to the B-ALK reactor, because of the improved enzymatic activity. The residual hemicellulose in the B-ALK\textsubscript{digestate}, was found to be 2.73±0.03 g/l, whilst the residual hemicellulose in the B-ALK\textsubscript{ENZ\textsubscript{digestate}} was found to be 1.87±0.05 g/l; representing 31.3% hemicellulose reduction due to the enhance enzyme activity in the B-ALK\textsubscript{ENZ} reactor. (Figure 8.3)

The soluble carbohydrates component was 0.16±0.01 g/l in the B-ALK\textsubscript{digestate}. Similarly, the soluble carbohydrate in the B-ALK\textsubscript{ENZ\textsubscript{digestate}} was determined to be 0.16±0.01. The results indicated similar pattern of utilisation of the soluble carbohydrate was in both the B-ALK and B-ALK\textsubscript{ENZ} reactors. The results therefore indicated that the hydrolysis of the structural carbohydrate to soluble carbohydrates (the rate limiting step of the AD of lignocellulosic substrates) was largely responsible for the difference in the yields of biogas in the substrates under-study. (Figure 8.13)
8.3 Effect of Enzymatically Enhanced Hydrolysis of Lignocellulose on Polyhydroxyalkanoate (PHA) Production (Discussion of Results – Experiment PB)

The PHA production was carried out using the HAG-ALKENZ\textsubscript{effluent} (as described in Experiment PB in Method-Section 5.7.5). The supplemented enzymatic activity improved the availability of soluble carbohydrates and VFAs; the study therefore assessed the extent to which the available products from the enhanced acid phase were utilised in the PHA biosynthesis.

The current study investigated the use of the effluent from an acidogenic fermentation process as a source of cheap carbon and nutrient for PHA production. It is important to note that the hydrolysis of complex lignocellulose to the glucose and other monosaccharide monomers is not fully complete. The commercial cellulase enzyme used in the enzymatic SSF to obtain the HAG-ALKENZ\textsubscript{effluent} the substrate for the PHA biosynthesis contained
endo-β-(1→4)-d-glucanases (EGs), which act by cleaving cellulose in its internal non-terminal regions to produce oligosaccharides. β-Glucosidase, an exohydrolase, also present in the cellulase enzyme preparations, is responsible for the conversion of the oligosaccharides (e.g. cellobiose) to glucose. It is important to note however that, the conversion of the oligosaccharides to glucose is not fully complete in the hydrolysis process, leading to significant amount of residual oligosaccharides after the enzymatic hydrolysis of the lignocellulosic biomass. The current study evaluated the pattern of degradation of the total soluble carbohydrates available in the HAG-ALKENZ effluent, focusing on the potential influence of the complex carbohydrates (oligosaccharides) on the PHA biosynthesis process.

The HAG-ALKENZ effluent used as the substrate, providing nutrients, as well as, VFAs, as the carbon source for the PHA biosynthesis. Pure acetic acid was used as pH control, allowing additional and continuous supply of carbon source. This was in accordance with Albuquerque et al. (2011) and Chen al. (2013) that continuous feeding of VFA ensures maximum PHA synthesis, when compared to other feeding regimes. As shown in Figure 8.4 and 8.5, the PHA yield increased progressively from the third hour of fermentation, until it reached a peak at the 24th hour of fermentation. The yield of PHA at 24 hours of fermentation was determined to be 1.42 g/l, representing 28.0% (w/w) of the cell dry weight after extraction. The highest yield was determined between 24 and 28 hours, as shown in Figure 8.4. Another notable yield peak was determined around 52 hours of fermentation. The yield of PHA at 52 hours of fermentation was to be 1.34 g/l, 30.45% of the cell dry weight after the PHA extraction process. A PHA yield content as high as 70-90% has been reported in literature (Albuquerque et al., 2007; Kedia et al., 2013), when VFAs (particularly acetic acids) were supplied as major carbon source, with standard nutrient media supplied. The current study however used effluent from a hydrogen reactor as the nutrient source. The purpose of the current study was to investigate the pattern of utilisation of the residual soluble carbohydrates in hydrolysed perennial rye grass after acidogenic fermentation process and not the identification of optimum conditions to obtain maximum PHA yield. Albuquerque et al. (2010a,b) emphasized that substrate concentration and composition, feeding regimes, and type of nutrient media, are important key factors necessary for the achievement of optimum/maximum PHA yield.

Kim (2000) showed the use of pure culture strains for the biosynthesis of PHA using soluble carbohydrates, as having great potential. The production cost of PHA production could however be further decreased if far more cheaper but complex substrates such as
lignocellulosic biomass were to be used. As emphasized by Albuquerque et al. (2010a), ideal culture selection for specific substrates is highly important for achieving optimum accumulation of PHA. The robustness of microbes in the mixed culture consortium, allows a more efficient response to transient conditions in the PHA biosynthesis pathway, compared to pure culture strains. The enriched mixed culture has the potential of allowing a more effective utilisation of complex feedstock, when compared to pure culture inoculum, as often observed in the degradation of complex substrate by mixed microbial consortium. The current study used a pure culture facultative *Cupriavidus necator* as the inoculum. Figure 8.5 shows the results of the optical density (OD), the cell dry weight (CDW) and yield of PHA analyses, undertaken on samples periodically taken from the bioreactor during the fermentation process. As highlighted by Kedia et al. (2013), OD is used to correlate with CDW, with a linear calibration curve, to determine the yield of PHA. As they explained, the optical properties and densities of certain bacteria strains, including *C. necator* are altered and unstable when the biopolymer accumulates, leading to poor correlation with total PHA yield (Jiang et al., 2008; Łabuśek and Radecka, 2001).

The OD measured in the current study, did not show perfect correlation with the PHA yield (as shown in Figure 8.5). Kedia et al. (2013) explained that the imperfect relationship of the OD to PHA yield could largely be attributed to fact that the OD could potentially increase even when no increase is occurring in the viable cells as PHA accumulation takes place more readily in nutrient limiting conditions. Similar lack of correlation also occurred in the later stages of the fermentation process when autolysis of the cells resulted in a potential released of PHA into the medium. The measurement of capacitance using dielectric spectroscopy (shown in Figure 8.4) show good correlation with the PHA yield, helping to trace the pattern of degradation of the substrates understudy. The highest PHA yield was determined between 20 to 30 hours of fermentation, verified by the profile expressed in the capacitance measurement. Again, another peak of increasing PHA accumulation was determined around 52 hours, which also correlated with the capacitance measurements. The study was consistent with the work of Pedrós-Alió et al. (1985) and Kedia et al. (2013), who emphasized the correlation between PHA accumulation and the cell bacteria density.
Figure 8.4: Profile of online capacitance (pF/cm) and PHA in a culture of Cupriavidus necator fed with the HAG-ALKENZ effluent

Figure 8.5: Profile of optical density, cell dry weight and PHA in a culture of Cupriavidus necator fed with the HAG-ALKENZ effluent
8.3.1 The pattern of utilisation of volatile fatty acids in the perennial rye grass hydrolysate during PHA bio-synthesis by Cupriavidus necator

Figure 8.6 shows the metabolism of VFAs in the PHA microbial fermentation process. The concentration of the total VFAs in the substrate for the PHA production (HAG-ALKENZ_effluent) was found to be 12.61 g/l, with the acetic, propionic and n-butyric acid contents being found to be 4.47 g/l, 0.956 g/l and 4.74 g/l, respectively. The n-valeric concentration was found to be 2.14 g/l; iso-butyric, and iso-valeric acid concentrations were determined to be 0.136 g/l and 0.176 g/l, respectively. As shown in Figure 8.6b, by the 3rd hour of fermentation, 8.5% of the acetic acid component in the media had been utilised. At the same time, the propionic, i-butyric, n-butyric, i-valeric and n-valeric acids concentration appeared to have increased by 10.5%, 8.8%, 0.25%, 15.9% and 11.2%, respectively. As illustrated in Figure 8.6b, acetate, propionate, butyrate and valerate are all major by-products of PHA production. The biosynthesis of PHA involves the glycolysis of the residual glucose in the media (carbon source) to two molecules of pyruvates. The pyruvate is converted in the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, during which VFAs are produced as essential precursors for PHA production (Mohan et al., 2013). At the 24th hour of fermentation, it was presumed that nearly all the initial acetic acid in the media had been consumed, in that 0.82 mg/ml acetic acid was taken into the bioreactor to control the pH of the media. The entire consumption of propionic acid occurred by the 24th hour of fermentation. The iso-butyric acid was found to have degraded by approximately 64%, the valeric acid by approximately 9%; whilst no notable degradation was observed in the n-butyric, during the same fermentation time (24 hours of fermentation).

An interesting observation from Figure 8.6 was that, the concentrations of n-butyric acid, i-valeric acid and n-valeric acid were all notably utilized between the 30th to 32nd hours of fermentation. By the 46th hour of fermentation however, n-butyric acid was observed to have increased by 16.7%, i-valeric by 27.9%, and n-valeric by 21.3%, when compared to the concentrations obtained at the 30th hour of fermentation. The results indicated that the increase in VFA concentrations between the 32nd to 50th hours of fermentation corresponded to the loss of PHA (Figure 8.4 and 8.5). Kedia et al. (2013) emphasized that relationship between VFA increase and PHA loss could be attributed to release of non-utilised VFAs from...
the intracellular regions into the medium due to lysing of the cell. However, as they suggested, further verification need to be carried out to justify that concept.

At the end of the fermentation process (72th hour), the original acetic acid, propionic acid and i-butyric acid residual in the media were all entirely consumed. The n-butyric acid content was degraded by 31.3%, whilst i-valeric and n-valeric were both degraded by 16.0%.

As illustrated in Figure 8.6a and 8.6b, the lower molecular weight short-chain fatty acids were easily available to the microbes and utilized quickly when compared to the relatively higher molecular weight short-chain fatty acids. It was observed that the 2-carbon (C) acetic acid was readily available to the microbes, compared to the 3C – propionic acid and so on.

The valeric acids were found to be less degradable, having been degraded by approximately 16% after the fermentation process.

![Figure 8.6a: Pattern of utilisation of VFAs in a culture of Cupriavidus necator fed with the HAG-ALKENZeffluent for PHA production](image)

Thesis – Philemon Kumi
8.3.2 The Pattern of Utilisation of soluble carbohydrates in a culture of Cupriavidus necator fed with the HAG-ALKENZ effluent for PHA production.

Glucose and sucrose have been the main type of sugar used as substrates in PHA production studies (Choi et al., 1998; Tsuge, 2002). Choi et al. (1998) studied the productivity of PHA from glucose, and achieved a PHA yield as high as 4.63 g/l h, “using recombinant E. coli that carried Alcaligenes lactus PHA biosynthesis genes (phaCAB)”. The utilisation of pure glucose and sucrose as substrates for PHA biosynthesis have been well optimized (Tsuge, 2002); however, the search for cheaper and easily solubulised sources of carbohydrates has been a key goal of recent research. Albuquerque et al. (2010a, b) have investigated the use of sugars in sugar cane molasses as a carbon source, using mixed microbial culture as inoculum for PHA biosynthesis. Aravind et al. (2013) also investigated the use glucose from hydrolysed grass using the zinc chloride method adopted from Cao et al. (1995) as cheap source of carbon, and a soil microbial isolate for the PHA biosynthesis. Enriched mixed cultures have been found be more effective in the degradation of complex substrates during PHA biosynthesis compared to pure cultures of single strains. Nevertheless, as emphasized by Dircks et al. (2001), mixed cultures store carbohydrates as glycogen, as opposed to pure cultures that typically store carbohydrates directly as PHA. It is therefore essential to
investigate the path of metabolism of carbohydrates in PHA biosynthesis, focusing on the substrate-product relationship.

The residual fermentation effluent apart from providing essential carbon source needed for PHA production, the nutrient and trace elements present in the media were essential for the growth of the bacteria. The initial chemical oxygen demand (COD) was found to be 31.89±0.81 g COD/L. By the sixth hour of fermentation, the COD of the culture was found to be 27.63±0.55 g COD/L, representing 13.3% reduction (Figure 8.7). The results gave an indication that the exponential growth phase of the *Cupriavidus necator* occurred around the 6th hour of fermentation. By the 24th hour of fermentation, the residual COD in the bacterial culture was found to be 27.42 ± 0.42 g COD/L, indicating no notable reduction when compared to the values obtained by the 6th hour of fermentation. The results suggested that the stationary phase of the bacteria occurred around the 24th hour of fermentation, by which time the readily available lower molecular weight short chain fatty acids had entirely been utilized, initiating a period of ‘partial starvation’, thereby allowing the PHA accumulation to commence. The higher molecular short chain acids and glucose (6 Carbon) began their process of slow cycle of PHA biosynthesis as the lower molecular weight short chain acids are been utilized and depleted; this then formed the basis of the principle/strategy of ‘partial starvation’. At this point extra acetic acid was added to buffer the loss and the percentage conversion to PHA began to fall significantly. As shown in Figure 8.7, approximately 7% of the soluble carbohydrate had been utilized by the 6th hour of fermentation, compared to nearly 100% degradation observed in the acetic and propionic acids at the same time. It was observed that after the readily available carbon sources were utilized, a notable degradation of approximately 31% of soluble carbohydrates was observed. It must be noted that the soluble carbohydrate present in the HAG-ALK_effluent was not entirely composed of glucose, but contained complex carbohydrates such as fructo-oligosaccharides which are not easily utilized by the PHA producing microbes. It can be observed from Figure 8.8 that a major utilisation of the soluble carbohydrates also occurred at around 54 hours of fermentation, and that corresponds to the pattern of PHA production. As shown in Figure 8.4 and Figure 8.5, two major peaks were observed in the graphs illustrating PHA concentration, the cell dry weight, as well as the capacitance. Figure 8.8 shows the degradation pattern of soluble carbohydrates and its relationship with PHA production. The study emphasizes the fact that
the accumulation PHA in the biosynthesis process is highly dependent on the composition, as well as the chemical structure of the substrate.

Figure 8.7: Profile of Total COD and soluble carbohydrates in a culture of *Cupriavidus necator* fed with HAG-ALKENZ effluent for PHA production
Figure 8.8: Utilisation of soluble carbohydrates in a culture of *Cupriavidus necator* fed with HAG-ALKENZ effluent for PHA production

Again, it was observed that at the 72nd hour of fermentation, the soluble sugar content was found to have increased by nearly 16% from the 1.13±0.05 g/L to 1.34±0.04 g/L (Figure 8.8). The rise in the residual soluble carbohydrates by 72 hours was attributed to the effect of the relationship between the complex carbohydrates, such the fructo-oligosaccharides found in the HAG-ALKENZ and the readily available VFAs. It can is likely that the complex carbohydrates require a longer time for hydrolysis to be converted to monosaccharides before going through glycolysis to be converted to VFAs, the essential precursors for the completion of the PHA biosynthesis cycle. Accumulation of VFAs at that stage of the biosynthesis processes where the *Cupriavidus necator* is undergoing starvation of readily available short chain sugar, have a great potential of triggering feedback inhibition, leading to gluconeogenesis; a process whereby the metabolic pathway is skewed towards the production of glucose. It has been suggested that odd-chain fatty acids have greater contribution to glucose formation through gluconeogenesis, as compared to even-chain fatty acids (Garrett et al., 2002). The fatty acids undergo oxidation resulting in the production of propionyl CoA, which are then converted to succinyl CoA; an essential precursor for pyruvate, which takes up 4 molecules of ATP and 2 molecules of GTP, resulting in the production of glucose-6-phosphate.

To further elucidate on the observations made in this study, 1.9 g/l commercial oligofructans was used as the main substrate with the addition of standard meat extract – peptone nutrient media (as described in Experiment PB2 in Method-Section 5.7.5). The initial soluble carbohydrates concentration was found to be 2.61±0.04 g/L. By the 24th hour of fermentation, the residual soluble carbohydrate was found to be 3.08±0.15 g/L, indicating 15.3% increase. The highest rise in soluble carbohydrates was observed around 28 hours of fermentation, where 26.0% increase was observed when compared to initial values. As shown in the graph, a notable decrease in soluble carbohydrates was observed around 52 hours of fermentation (2.51±0.23 g/L), compared to the soluble carbohydrate concentration observed at 28 hours (3.53±0.30 g/L) (Figure 8.9). A notable increase in PHA accumulation, similar to the pattern observed in the fermentation of the HAG-ALKENZ effluent was also observed at this time (Figure 8.9), and that was in agreement with the work of Majone et al. (1996).
(1998) and Albuquerque et al. (2010a), who observed that excess substrate availability occurring simultaneously with internal growth limitation of the bacteria, favoured the accumulation of PHA.

The concentration of soluble carbohydrates at the 52nd hour of fermentation was also observed to be similar to the observed soluble carbohydrate concentration at the start of the experiment, found to be 2.61±0.04 g/L. At the end of the fermentation process (72 hours), the residual soluble carbohydrates was found to be 2.86±0.30 g/L. It can be seen from the results that the residual soluble carbohydrate concentration in the effluent from the fermentation process was nearly at the same concentration as that observed at the start of the experiment. It was concluded that the oligosaccharides present in at the substrate was essentially not degraded in the fermentation process. Since acetic acid was supplied as pH control, it became a preferred carbon source for the microbes. Fructooligosaccharides, due to the configuration of their osidic bonds, have been discovered to be highly resistant to the hydrolytic enzymes released to aerobic microbes. The presence of the higher concentration complex soluble carbohydrates in the fermentation process, potentially contributed a shift in the microbial pathway from PHA synthesis to gluconeogenesis, releasing glucose detected as rise in soluble sugar levels during the fermentation process. This readily available carbohydrate was however utilized by the PHA producing microbes, until excess glucose is accumulated towards the microbial regeneration equilibrium phase. The perennial rye grass hydrolysate showed similar pattern of metabolism, and that contributed to the notable peak that was observed at the later stage of the PHA biosynthesis process as shown by the online capacitance measurement in Figure 8.4.
Figure 8.9: Profile of fructooligosaccharides metabolism in a culture of *Cupriavidus necator* fed with HAG-ALKENZ effluent for PHA production

8.4 Lignin as a Potential Product from Anaerobic Digestion

The current study sought to contribute to the knowledge of obtaining high quality products from the anaerobic process residues. The study accessed the extraction of high quality lignin from the effluent of acidogenic reactor. As emphasized by Zhang (2011), most technical lignin (Kraft lignin and lignosulfonate) contain sulphur, which devalues the lignin quality for the conversion to value-added products. As discussed in Chapter 6 and 7, the lignin component in lignocellulosic biomass remained largely not degraded during the anaerobic fermentation process. The residual ADF proportion of the biomass in the effluent was about 80-95% after the fermentation process, when compared to the initial concentrations in the substrate. The study compared the Klasson lignin protocol which include the acid soluble lignin (Klasson-ASL) and the acid insoluble lignin (Klasson-AIL) procedures, to the acid detergent lignin (ADL) procedure, in order to determine if there is any bias in these two widely used techniques for the determination of the residual lignin in anaerobically treated biomass. The lignin purification process was then carried out, having a prior knowledge of the quantity of the residual lignin.
8.4.1 Lignin Determination Technique

It was also observed from the study that the recalcitrant lignocellulosic component of the HAG-U\textsubscript{substrate} was essentially not degraded after that acidogenic fermentation. The enzymatic HAG-ALKENZ fermentation (in Chapter 7) however, resulted in a high degradation of the holocellulose component of the perennial rye grass, with the lignin component only marginally degraded. The HAG-ALK\textsubscript{effluent} and HAG-ALKENZ\textsubscript{effluent} were therefore compared ascertaining the value and quality of the lignin component. The study therefore sought to extract the residual lignin in the effluent of the acidogenic fermentation, as a valuable product from the anaerobic process.

The HAG-U\textsubscript{substrate} and the HAG-ALK\textsubscript{effluent} were used as biomass materials for a comparative total Klason lignin and acid detergent lignin (ADL) analyses. The lignin technique found to be robust and accurate for the determination of the residual lignin quantity, in the comparative study analysis was then used in evaluation of expected lignin quantity in the lignin purification process.

The samples were dried at 55°C for 36 hours. The dried biomass was then analysed for the NDF, ADF and ash content. The NDF component of the dried matter was found to be 6.11±0.03 g/l; representing 27.0% of the TS (22.6±0.06 g/l), and 37.44% of the dry matter. The ash content was found to be 8.22 g/l, representing 36.3% of the TS, and the holocellulose content were found to be 2.13±0.08 g/l, representing approximately 9% of the TS. The current study carried out a comparison of the total Klason lignin and the acid detergent lignin procedures, in order to have accurate measurement of the expected lignin before the extraction process.

The mean value of lignin in the HAG-ALK\textsubscript{substrate} determined using the acid detergent lignin (ADL) procedure was found to be 3.59 ± 0.09 g/l. The Klason lignin procedure, on the other hand resulted in a total lignin value of 5.83 ± 0.06 g/l, comprising Klason-AIL component of 3.70 ± 0.05 g/l, and Klason-ASL component of 2.14 ± 0.06 g/l. (Figure 8.10)

In the HAG-ALK\textsubscript{effluent}, the residual ADL value was found to be 4.12 ± 0.16 g/l, and the total Klason lignin was found to be 5.48 ± 0.14 g/l, comprising of an Klason-AIL value of 3.73 ± 0.17 g/l, and an Klason-ASL value of 1.74 ± 0.09 g/l. (Figure 8.11)
As shown in Figure 8.10 and 8.11, the residual lignin component can be estimated more correctly with the total Klasson lignin procedure. Though the ADL showed close correlation with the Klasson-AIL, this technique could not account for soluble lignin proportions in the samples.

A null hypothesis was therefore established stating that there is no significant difference in the values of residual linin obtained using the ADL determination procedure and the Klasson–AIL procedures.

An independent t-test was conducted to determine if a difference existed between the mean lignin values obtained by the ADL procedure and the Klasson-AIL procedure in the HAG-$U_{\text{substrate}}$. No statistically significant difference was observed between the mean quantities of ADL (M = 3.59 g/l, SD = 0.28) and Klasson-AIL (M = 3.70 g/l, SD = 0.15), $t (18) = -1.174$, $p = 0.26$. The 95% confidence interval was -0.33 to 0.093. The null hypothesis was therefore accepted.

An independent t-test was also conducted to determine if a difference existed between the mean values of lignin obtained by the ADL procedure and the Klasson-AIL procedure in the HAG-$U_{\text{effluent}}$ and the HAG-$\text{ALK}_{\text{effluent}}$. There was no statistically significant difference between the mean lignin values obtained from the ADL procedure (M = 4.11 g/l, SD = 0.47) and Klasson-AIL procedure (M = 3.73 g/l, SD = 0.52), $t (16) = 1.64$, $p = 0.12$. The 95% confidence interval was -0.113 to 0.89. The null hypothesis was therefore accepted.

A null hypothesis was also proposed that there is no significant difference in the various lignin fractions of ADL, Klasson-AIL and Klasson-ASL values obtained in the HAG-$U_{\text{substrate}}$ compared to the HAG-$\text{ALK}_{\text{effluent}}$.

An independent t-test was conducted to determine if a difference existed between the lignin as ADL values obtained from the HAG-$U_{\text{substrate}}$ and the HAG-$\text{ALK}_{\text{effluent}}$. There was a statistically significant difference between the mean ADL values of HAG-$U_{\text{substrate}}$ (M = 3.59 g/l, SD = 0.28) and the HAG-$\text{ALK}_{\text{effluent}}$ (M = 4.12 g/l, SD = 0.47), $t (17) = -3.009$, $p = 0.008$. The 95% confidence interval was -0.916 to -0.159. The null hypothesis was therefore rejected.

An independent t-test was conducted to determine if a difference existed between the Klasson-AIL values obtained from the HAG-$U_{\text{substrate}}$ and the HAG-$\text{ALK}_{\text{effluent}}$. There was no
statistically significant difference between the mean Klasson-AIL values of the HAG-U (M= 3.70 g/l, SD= 0.15) and HAG-ALK_{effluent} (M= 3.73 g/l, SD= 0.52), \( t (17) = -0.16, p= 0.88 \). The 95% confidence interval was -0.39 to 0.34. The null hypothesis was accepted.

An independent t-test was also conducted to determine if a difference existed between the Klasson-ASL values obtained from the HAG-U_{substrate} and the HAG-ALK_{effluent}. There was statistically significant difference between the mean Klasson-ASL of the HAG-U_{substrate} (M= 2.13 g/l, SD= 0.19) and HAG-ALK_{effluent} (M= 1.75 g/l), SD= 0.27, \( t (17) = 3.56, p = 0.002 \). The effect size, \( d > 0.25 \), gave an indication that the magnitude of the difference in the means is large. The difference in the Klasson-ASL in HAG-U_{substrate} and HAG-ALK_{effluent} was because soluble lignin was potentially utilized in dark fermentation as a carbon source.

![Figure 8.10: Lignin determination from HAG-U_{substrate} comparing ADL and Klasson procedures (ASL and AIL)](image)
8.4.1.1 Reproducibility of test procedure

As illustrated in Table 8.1, the standard deviations of the lignin values, using the ADL and Klasson-AIL procedures on the HAG-U\textsubscript{substrate} samples, were generally lower than that determined in the HAG-ALK\textsubscript{effluent}. The results show that the data points in the HAG-U\textsubscript{substrate} tend to be very close to the mean, when compared to the HAG-ALK\textsubscript{effluent}. The HAG-ALK\textsubscript{effluent} data-points were relatively spread out over a range of values. The relative inconsistency in the lignin values in the HAG-ALK\textsubscript{effluent}, compared to the HAG-U\textsubscript{substrate} could largely be attributed to the extent of variation in the sample particle size after the acidogenic fermentation. Considering 2 standard deviations from the mean, however show that all the data points (100%) were found to have followed normal distribution based on the empirical rule, Pr (μ-2δ ≤X≤ μ+2δ). It can therefore be suggested that the procedures adopted in the current study were all consistent and reproducible.
Table 8.1: Lignin Determination Procedures: comparative yield

<table>
<thead>
<tr>
<th></th>
<th>Mean (µ)</th>
<th>SD (δ)</th>
<th>SEM</th>
<th>%1δ</th>
<th>% 2δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expected Value</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>68.27</td>
<td>95.45</td>
</tr>
<tr>
<td><strong>HAG-U_{substrate}</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADL</td>
<td>3.586</td>
<td>0.283</td>
<td>0.089</td>
<td>50.00</td>
<td>100.0</td>
</tr>
<tr>
<td>Klasson-AIL</td>
<td>3.704</td>
<td>0.146</td>
<td>0.046</td>
<td>70.00</td>
<td>100.0</td>
</tr>
<tr>
<td>Klasson-ASL</td>
<td>2.130</td>
<td>0.191</td>
<td>0.060</td>
<td>90.00</td>
<td>100.0</td>
</tr>
<tr>
<td><strong>HAG-ALK_{effluent}</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADL</td>
<td>4.118</td>
<td>0.475</td>
<td>0.158</td>
<td>90.00</td>
<td>100.0</td>
</tr>
<tr>
<td>Klasson-AIL</td>
<td>3.731</td>
<td>0.524</td>
<td>0.175</td>
<td>80.00</td>
<td>100.0</td>
</tr>
<tr>
<td>Klasson-ASL</td>
<td>1.747</td>
<td>0.274</td>
<td>0.091</td>
<td>80.00</td>
<td>100.0</td>
</tr>
</tbody>
</table>

8.4.2 Lignin Biorefinery (Results and Discussion of Experiment L)

The current study had the aim of extracting and characterizing the lignin residual in the HAG-ALK_{effluent} and the HAG-ALKENZ_{effluent}, evaluating the downstream effect of the enhance hydrolysis, by the enzymatic SSF, on the properties and structure of the extracted lignin. The dried sample of the HAG-ALK_{effluent} and HAG-ALKENZ_{effluent} were used, as described in Experiment L in Method-Section 5.7.6. The dried samples were ground with a ball mill, at a dry matter content of about 95.1%. The dried biomass were then subjected to Organosolv extraction process, using the protocol outlined by Lam et al. (2001) in Vanderghem et al. (2011), using formic acid/acetic acid/water (30/50/20 volume ratio). After the Organosolv extraction process, the total weight of the extracted biomass from the weak alkaline pre-treatment fermentation (LA_{lignin}) was 38.7%. The extracted residue from the HAG-ALKENZ_{effluent} (LE_{lignin}) was on the other hand determined to be 42.63% (w/w) of the initial weight. The quantity of lignin per gram of the initial biomass of the LA_{lignin} was 0.183 g (0.094 g/g), whilst the quantity in the LA_{lignin} was 0.267 g (0.081 g/g). The results show that more lignin was recovered from the alkaline treatment fermentation than the enzymatic SSF. However, as shown in Table 8.2, the extracted residue in the weak alkaline treatment only was found to be comparatively more contaminated with carbohydrates and proteins. The residual carbohydrates in the extract were determined to be 25.8% and 10.9%, by mass in the residue from alkaline treatment and the enzymatic SSF, respectively.

149

Thesis – Philemon Kumi
Table 8.2: Characteristics of lignin extracted from the effluents of acidogenic fermentation

<table>
<thead>
<tr>
<th>Parameters after extraction</th>
<th>Alkaline treatment (LA_{lignin})</th>
<th>Enzymatic SSF (LE_{lignin})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Weight of biomass (dried biomass)</td>
<td>6.0g</td>
<td>6.0g</td>
</tr>
<tr>
<td>Extracted Residue ( % (w/w))</td>
<td>38.7%</td>
<td>42.6%</td>
</tr>
<tr>
<td>Protein content of residue (%)</td>
<td>3.9%</td>
<td>1.04%</td>
</tr>
<tr>
<td>Total carbohydrate of residue (%)</td>
<td>25.8%</td>
<td>10.9%</td>
</tr>
<tr>
<td>Ash content of residue (%)</td>
<td>6.7%</td>
<td>6.3%</td>
</tr>
<tr>
<td>Lignin per gram of acidogenic biomass</td>
<td>0.183g</td>
<td>0.267g</td>
</tr>
<tr>
<td>Lignin (g/g TS)</td>
<td>0.094g</td>
<td>0.081g</td>
</tr>
</tbody>
</table>

8.4.3 Characterization of extracted lignin using FT-IR

After the Organosolv extraction process, the obtained lignin residue after freeze-drying was analysed with Fourier transformed infrared spectroscopy (FT-IR), according to the method of Manara et al. (2013), as described in Method-Section 5.2.13. The freeze-dried extracted lignin (5 mg) was ground with potassium bromide (KBr) at a ratio of 1:20. The mixture was then pressed mechanically before being analysed. The FT-IR analysis was carried out recording the spectra at range of 2000-600 cm⁻¹ at a resolution of 4 cm⁻¹.

The FT-IR spectra of the extracted perennial rye grass lignin were compared to spectra obtained from commercial alkali lignin relative to the spectra obtained from untreated perennial rye grass (Figure 8.12), to extrapolate the distinct types of bonds in the chemical structure of lignin (shown in Figure 8.13). The FT-IR spectra gave an indication of which derivatives of lignin was present in the biomass after the Organosolv lignin extraction process, comparing the results to previously published data (Gupta and Lee, 2010; Hage et al., 2010; Manara et al., 2013; Serrano et al., 2010; Sun et al., 1999; Villaverde et al., 2009;). Figure 8.12 shows the FTIR spectra of dried perennial rye grass; whilst Figure 8.13 shows how distinct the peaks of the extracted lignin, (LA_{lignin} and LE_{lignin}) are different from the peaks of the untreated perennial rye grass.
Each absorption band in the spectra reflects the characteristics of a particular kind of phenylpropane units present in the sample. The absorbance bands around 848 cm\(^{-1}\) was attributed to the presence of the C-H vibrations of \(p\)-hydroxyphenylpropane (H) units (Quoc Lam et al., 2001). As shown in Figure 8.13, the peaks of the absorbance at 848 cm\(^{-1}\) was visible in the extracted lignins (LA\(_{\text{lignin}}\) and LE\(_{\text{lignin}}\)), as well as alkali lignin standard (LR\(_{\text{lignin}}\)). As can be seen in the absorbance graph, \(p\)-hydroxyphenylpropane was found to be prominent in the chemical structure of the LE\(_{\text{lignin}}\) and LA\(_{\text{lignin}}\), but less distinct in the LA\(_{\text{lignin}}\).

The absorbance bands at 992 cm\(^{-1}\) and 1040 cm\(^{-1}\) were consistent with results of Baeza et al. (1990), who identified a peak around 1033.8 cm\(^{-1}\) to be associated with non-condensed guaiacyl nuclei. According to Baeza et al. (1990), the characteristic of non-condensed guaiacyl nuclei is usually observed at an absorbance band of 1033.8 cm\(^{-1}\). The band between 1068 cm\(^{-1}\) recorded in all the three samples was also linked to guaiacyl and syringyl group (example: guaiacylpropane (G) and syringylpropane (S)). (Figure 8.13)

Seisto and Poppius-Levlin (1997) highlighted that a band around 1164 cm\(^{-1}\) is a characteristic of all the three types of phenylpropane units: \(p\)-hydroxyphenylpropane (H), guaiacylpropane (G) and syringylpropane (S). In the current study, the phenylpropane units were identified around 1168 cm\(^{-1}\). As shown in the graph, the peak was less distinct in the LA\(_{\text{lignin}}\), when compared to the LE\(_{\text{lignin}}\) and the alkali lignin standard (LR\(_{\text{lignin}}\)); and that could be attributed to the contamination due the presence of higher residual carbohydrates. (Figure 8.13)

The absorption band around 1230-1210 cm\(^{-1}\) were identified by Marara et al. (2013) to be the characteristic of aromatic phenyl C-O. The LE\(_{\text{lignin}}\) and the LR\(_{\text{lignin}}\) standards show notable band peaks around 1232 cm\(^{-1}\); but again the band peak at the same band position was less distinct. (Figure 8.13)

The band around 1446 cm\(^{-1}\) and 1456 cm\(^{-1}\) in the extracted lignins and alkali lignin, are also characteristic of C-C bounds and aromatic structure vibrations of the phenylpropane groups, in accordance with the observations of Manara et al. (2013). (Figure 8.13)

The absorption bands around 1488 cm\(^{-1}\) are characteristic of C-H vibration of CH\(_3\) and CH\(_2\) groups, aromatic ring vibrations, and C-H deformations (Manara et al., 2013). As shown in
the graph, the of the C-H deformations, as illustrated by the distinctness of the absorption bands, was minimal in all the substrates under study. (Figure 8.13)

The bands found between 1603 cm\(^{-1}\) and 1513 cm\(^{-1}\) have been suggested to correspond to C-C vibrations of aromatic nuclei of the phenylpropane groups (Hortling et al., 1997). The extracted grass lignin obtained in the current study show absorbance bands around 1546 cm\(^{-1}\) and 1542 cm\(^{-1}\), consistent with the findings of Hortling et al. (1997). (Figure 8.13)

Hortling et al. (1997), studying the lignin composition in triticale straw with FT-IR, also observed an absorbance band at 1653 cm\(^{-1}\), reporting that a band around such position could be attributed to carbonyl grouping in the amide groups \{–NH(CO)}\}; which they explained could essentially be due to the presence of proteins in sample. The LA\textsubscript{lignin} in the current study, show weak bands at 1620 cm\(^{-1}\), indicating some level of protein contaminations, but there was no absorbance band around the same area in the standard LR\textsubscript{lignin} and the LE\textsubscript{lignin}. (Figure 8.13)

The absorption band between 1746 cm\(^{-1}\) and 1738 cm\(^{-1}\) gave an indication of the existence of carbonyl functional groups. The carbonyl groups (acetate carbonyl group and p-coumaryl ester group) are typically expected at absorption bands between 1790 and 1680 cm\(^{-1}\) (Manara et al., 2013). The observed presence of a strong band, characteristic of the carbonyl functional group, is inconsistent with the general nature of lignin, and of aromatic rings, which are expected to have very little of carbonyl functional groups (Gupta and Lee, 2010; Liu et al., 2008). Quoc Lam et al. (2001) however explained that, the unexpected carbonyl functional groups could largely be linked to the esterification of certain alcohol and phenol functions as a result of the Organosolv reagent (formic and acetic acids) used in the lignin extraction process. Hortling et al. (1991) emphasized that, the partially esterification of the hydroxyl groups which occurs due to the extraction process, also have the potential of altering the depolymerisation. (Figure 8.13)
Figure 8.12: FTIR spectra of dried perennial rye grass.

Figure 8.13: FT-IR spectra of extracted lignin relative to dried perennial rye grass and standard alkali lignin
Note: The scale of the vertical axis was removed because the evaluation of the FTIR scans was based on the position of the absorption band and the percentage transmittance (PGR - perennial rye grass; LA lignin – lignin extracted from HAG-ALKeffluent; LE lignin – lignin extracted from HAG-ALKENZeffluent; LR lignin – standard alkali lignin)
As observed in Figure 8.13, the FT-IR spectra profiles of the extracted grass lignins (LA\textsubscript{lignin} and LE\textsubscript{lignin}) and the LR\textsubscript{lignin} standard show peaks similar positions, indicating similarity in the fundamental chemical structure. Certain wavelengths are specific to the chemical structure of lignin, but as noted by Xiao et al. (2001), some wavelengths on the other hand, can be attributed to lignin as well as to polysaccharides, hence the need for the comparison of the extracted lignin to commercial alkali lignin. Though the FT-IR spectra of the alkali lignin ‘standard’ and the extracted grass lignins were essentially parallel, some major variations were however observed in the intensity of the peaks of the absorption bands in the three samples under-study. The LA\textsubscript{lignin} was generally determined to have relatively less distinct peaks when compared to the LE\textsubscript{lignin}, which other hand show close similarity with the peaks observed in the alkali lignin standard. It can be concluded that as the level of contamination of carbohydrates increases, the distinctness of the wavelength/spectra bands, on the other hand, decreased.

The presence of the carbohydrates in the extracted residue is highly linked to the presence of hemicellulose. Yang et al. (2007) and Dominguez et al. (2008) identified hemicellulose as common contaminants in the Organosolv lignin-extraction process. The Organosolv lignin extraction process involved the use of formic/acetic acid/water (30/50/20; v/v/v), according Manara et al. (2013), have the potential of achieving lignin purity of 80-90%; defining purity as the total amount of lignin (%) present in the after freeze drying the extracted lignin from the pre-treatment process. Vanderghem et al. (2011) also noted that lignin extracted using the Organosolv protocol is of good quality, free of sulphur. The results emphasized the fact the enhanced fermentation due to the enzyme treatment resulted in greater removal of the structural carbohydrate contaminants leading a relatively pure lignin. This agrees with other data, such as biogas yield and the compositional changes in the lignocellulosic proportions of the biomass (Section 7.3) that show that high removal of cellulose and hemicellulose increases the yield of biohydrogen.

The presence of peaks characteristic of proteins (though not high) in the LA\textsubscript{lignin} indicated that, there was some degree of contamination of the extracted sample, but that did not have great influence on the structure of the isolated lignin.

The Organosolv extraction technique using formic acid/acetic acid/water combination in a ratio of 30/50/20 (v/v/v) was proven effective in lignin extraction (Vanderghem et al., 2011).
Manara et al. (2010) studying the extraction of lignin from agro-industrial wastes reported lignin purity as high as 82% after the extraction process. A proximate evaluation of the quantity of carbohydrates, proteins and ash content in the residue after the lignin extraction process (Table 8.2), gave the purity of lignin extracted from the HAG-ALK\textsubscript{effluent} to be 63.9%; and that obtained from the HAG-ALK\textsubscript{ENZ\_effluent} to be 81.7%, consistent with the work of Vanderghem et al. (2011). The absorption bands of L\textsubscript{g} lignin show similar peaks as the standard L\textsubscript{r} lignin, when compared to the L\textsubscript{a} lignin. It can be concluded from the results that the enzymatic enhancement led to relatively higher reduction of hemicellulose, leading to a less contaminated lignin after the extraction process, hence a purer lignin. Figure 8.14 shows a photograph of the extracted lignin tablets.

![Figure 8.14: Photograph of extracted lignin tablets](image)

8.5 Economic Viability of the Secondary Processes that can be integrated with the Acidogenic Processes

8.5.1 Biofuels

The economic evaluation of the products of the anaerobic fermentation processes was based on the assumption that the perennial rye grass pellet will be fermented in a medium scale onsite anaerobic digester of tank size of approximately 175 m\textsuperscript{3}, capable of digesting about 30 tonnes perennial rye grass slurry feedstock per day. A total 10,000 tonne of perennial rye
grass pellets could be digested in the centralized plant per annum, and that will be produced from 0.056 tonne dry perennial rye grass pellets per tonne of water. The 10,000 tonnes/year of perennial rye grass pellets will therefore amount to about 178,571 tonnes of perennial rye grass slurry per year.

As discussed in Chapter 7, the yield of biohydrogen from the HAG-ALK fermentation was 10.95 l H_2/kg VS and yield from the HAG-ALKENZ fermentation was 47.71 l H_2/kg VS, equivalent to 0.530 m^3 H_2 per hour and 2.308 m^3 H_2 per hour, respectively. The biomethane yield of 223.1 l CH_4/kg VS from the B-ALK digestion and 325.58 l CH_4/kg VS from the B-ALKENZ digestion was also found to be equivalent to 7.945 m^3 CH_4 per hour and 10.253 m^3 CH_4 per hour, respectively, based on the VS consumed per year. The electricity generated from biohydrogen produced from the fermentation of the HAG-ALK substrate and the HAG-ALKENZ substrate was found to be 146.43 MWh/y and 382.91 MWh/y, respectively; resulting in gross income of £22,169.8/y and £57,972.2/y, respectively. From the biomethane production, the quantity of electricity produced from the B-ALK substrate was 4039.08 MWh/y and that produced from the B-ALKENZ substrate was 5,212.73 MWh/y, resulting in a gross income of £611,517/y and £789,208/y, respectively.

The overall electricity yield in the two-stage biohydrogen–biomethane fermentation from the HAG-ALKENZ substrate (5,595.64 MWh/y) was found to be approximately 27% higher than the overall electricity yield from the HAG-ALK substrate in the two-stage anaerobic digestion process (4,185.51 MWh/y). Though enzyme addition has great potential in the enhancement of the economic value of the lignocellulosic fermentation process, the uncertainties in the pricing of commercial enzymes pose as serious threat to technical achievement gained from them. The cellulase enzyme used in the current study was quoted at the lab scale unit-price of £2.00/kg. According to the manufacturer, the price at industrial scale could be £1.00/kg or less, depending on the quantity ordered. The ferulic acid esterase, on the other hand was quote at a lab scale price of £22.50/kg. The FAE used was a trial laboratory product, and that could be reduced £2.23 at the industrial scale depending on the quantity of the order, according to the manufacturer. The use of enzyme pre-treatment can therefore be said to be currently not economically viable. The alkaline- enzymatic treatment technique due to its high solubilisation effect on lignocellulose can however be upheld, being strengthened by the fact that, enzyme prices have been on a trend of progressive cost reduction in recent times.
The amount of heat that can be produced from the biohydrogen and biomethane using CHP, as shown in Table 8.3, could be used for digester heating or thermal hydrolysis if necessary.

**Table 8.3: Economic evaluation of the anaerobic fermentation processes**

<table>
<thead>
<tr>
<th>Type of AD</th>
<th>Bio-hydrogen</th>
<th>Bio-methane</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAG-ALK</td>
<td>HAG-ALKENZ</td>
<td>B-ALK</td>
</tr>
<tr>
<td><strong>Tonne of substrate per year</strong></td>
<td>10,000</td>
<td>10,000</td>
</tr>
<tr>
<td>Tonne of perennial rye grass slurry per year</td>
<td>178,571</td>
<td>178,571</td>
</tr>
<tr>
<td>L/kg VS</td>
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<td>47.71</td>
</tr>
<tr>
<td>m^3/kg VS</td>
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</tr>
<tr>
<td>TS %</td>
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<td>4.82</td>
</tr>
<tr>
<td>VS%</td>
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<td>87.76</td>
</tr>
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<td>VS (tpa)</td>
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</tr>
<tr>
<td>VS (kg/y)</td>
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<td>7553627</td>
</tr>
<tr>
<td>biogas (m^3/y)</td>
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</tr>
<tr>
<td>biogas (m^3/hr)</td>
<td>9.439623</td>
<td>41.13968</td>
</tr>
<tr>
<td>Calorific value of biogas (MJ/m^3)*</td>
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<td>12.75</td>
</tr>
<tr>
<td>Power (kW)</td>
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<td>145.703</td>
</tr>
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<td>Electricity (kW)</td>
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<td>electricity per year (kWh/y)</td>
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<tr>
<td>Electricity (MWh/y)</td>
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<td><strong>382.908</strong></td>
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<tr>
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<td>Export tariff @ 4.77p/kWH (£)</td>
<td>6984.814</td>
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<tr>
<td><strong>Gross income (£/year)</strong></td>
<td><strong>22169.8</strong></td>
<td><strong>57972.2</strong></td>
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</tr>
<tr>
<td>Heat (kWh/y)</td>
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<td>765815.1</td>
</tr>
<tr>
<td><strong>Heat (MWh/y)</strong></td>
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<td><strong>765.815</strong></td>
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*Note: * Citizendium (2014); Assumption: i. Biohydrogen – Fuel cell unit has a conversion efficiency of 50%, CHP for heat generation has conversion efficient of 60%. ii. Biomethane – CHP unit has a conversion efficiency of 30% for electricity and 55% for heat.
8.5.1 Finding Alternative Uses for the product of the Acidogenic fermentation

8.5.1.1 Volatile Fatty Acids

The VFAs produced after anaerobic fermentation process can be purified for other potential markets like the pharmaceutical industry. The current market for VFA as a product from renewable sources is highly unpredictable. There is currently very little information on the market value for renewable VFAs. The HAG-ALK fermentation resulted in a total VFA yield of 170 kg/ton, whilst the HAG-ALKENZ fermentation resulted in a total VFA yield of 232 kg/ton. Assuming a total of 10,000 tonnes of perennial rye grass substrate was treated with alkaline and fed every year to a centralized acidogenic reactor for VFA production (HAG-ALK), the quantity of VFA that could potentially be produced in the process (as described in chapter 7) will be approximately 1,286 tonnes/year (41% acetic acid; 26% butyric acid). The HAG-ALKENZ fermentation of 10,000 tonne of perennial rye grass/year will yield approximately 1,755 tonnes of VFA per year (36% acetic acid; 40% butyric acid).

Tecnon Orbichem (2013) quotes the European market value of acetic acid as £387-410/ton. Therefore, at acetic acid price of £410/ton, the income from the acetic acid was calculated to be about £174,268/year and £209,609/year, for the HAG-ALK fermentation and HAG-ALKENZ fermentation processes, respectively (assuming VFA extraction efficiency of approximately 80% via microfiltration).

Huang (2002) estimated the market value for butyric acid (petrochemical source) to be £0.80/kg. At £800/tonne of butyric acid unit price (at VFA extraction efficiency of approximately 90% via microfiltration), the approximately 266 tonne butyric acid produced year from the HAG-ALK fermentation of the perennial rye grass will generate a gross income of about £213,243 per year. The butyric acid yield after the HAG-ALKENZ fermentation was approximately 561 tonnes per year, generating a gross income of about £449,442 per year. The gross income from extraction of the total acetic and butyric acid yields from the HAG-ALKENZ effluent would be about £659,052 per year. The gross income from a second-stage methanogenic digestion was relatively higher (£789,208/y), making it economically feasible to convert biomass to biomethane than to convert to VFAs.
8.5.1.2 Bioplastics (Polyhydroxyalkanoates)

The integration of bioplastic production with the hydrolytic-acidogenic fermentation has been viewed as being of great potential. The yield of bioplastic obtained using the liquor from the acidogenic fermentation resulted in a PHA yield of 1.42 g/l. The yield of PHA was determined to be 29.5 kg/ton.

There are many uncertainties in the pricing/the costing of PHA product. As noticed by Barker and Safford (2009) the Environment Agency classify bioplastic containing starch under the ‘paper’ category costing £4-7/tonne; petroplastic containing bioplastics, on the other hand are classified as ‘plastic’, costing “£24-28/tonne. Barker and Safford (2009) emphasized the uncertainties that surround the classification of PHA and its costing. Serafim (2006) quoted the cost of PHA to be about £4/kg. The cost of PHA in the current study was however taken as £1.56/kg, according to the price estimation of Garcia et al. (2012).

The gross income from the PHA from the HAG-ALKENZ effluent was therefore determined to be about £393,970, if 10,000 tonnes of perennial rye grass substrate was fermented in an enzymatic-SSF system per year. A comparison of the PHA biosynthesis with the second stage biomethane process, which resulted in a gross income of £789,208/year, gave a strong indication that a direct biohydrogen-PHA process will not be as economically viable when compared to the conventional two-stage (biohydrogen-biomethane) system.

8.5.1.3 Lignin

The extraction of lignin as a co-product of the bio-fermentation of lignocellulosic biomass is considered as having a great potential of boosting the overall profitability of the bioconversion of the recalcitrant lignocellulosic feedstock. The biorefinery of lignin will allow high value-added chemicals/material co-products to be obtained from the anaerobic digestion process, including syngas products, phenols, hydrocarbons, macromolecules and oxidised products (Ek, 2005). The costing of lignin is entirely based on the chemicals and co-products obtained from the process. According to the NNFCC factsheet on renewable chemicals, published in May 2011, the market price of lignin ranges from £250 to £2000 depending on the form. The current study adopted three main scenarios of lignin biorefinery from Holladay et al. (2007) for the evaluation of the cost and the potential income from the lignin extracted after the acidogenic fermentation.
Scenario 1: Syngas alcohol

The lignin obtained from the anaerobic fermentation of lignocellulosic biomass can be converted to mixed alcohols after a gasification stage. The evaluation of the fuel value, in the current study was based on the assumption that 55 gallons of ethanol and 19 gallons of mixed alcohol can be generated from a tonne of lignin (Holladay et al., 2007). The assumed unit price of the syngas alcohol in the scenario was £1.57 per gallon. Approximately 45,280 gallons of ethanol can be produced from the lignin extracted from the HAG-ALK effluent of 10,000 tonnes of perennial rye grass substrate per year, generating a gross income of £71,181/year. The same quantity of lignin will otherwise yield approximately 15,643 gallon of mixed alcohols per year, generating a gross income of £24,591/year.

The lignin extracted from the HAG-ALKENZ effluent has the potential approximately for 38,835 gallons ethanol per year or 13,411 gallons mixed alcohol per year; generating gross income of £61,049/year or £21,083/year respectively. The gross income due the second-stage methane fermentation, when compare to a second-stage lignin biorefinery can be deduced to be comparatively highly viable, economically.

Scenario 2: Aromatic Chemicals

Lignin can be converted to aromatic chemicals, including phenol, terephthalic acid and; benzene, toluene xylene (BTX). The assumptions in the scenario were based on the conversion of lignin to BTX. According to Holladay et al. (2007), a tonne of lignin could be converted to BTX at a conversion efficiency of 20% (w/w). It was also assumed that a tonne of BTX was equivalent to 277 tonnes. At the unit price of £1.23 per gallon BTX, the extracted grass lignin from HAG-ALK effluent will produce about 25,592 tonnes of BTX per year, which will generate a gross income of £31,555/year. The lignin obtained from the HAG-ALKENZ effluent will produce 23,705 tonnes of BTX per year, which will generate a gross income of £29,228/year.

Scenario 3: Carbon Fibre

According to Holladay et al. (2007), the utilisation of lignin for the low cost production of carbon fibre could potentially displace synthetic polymers such as polyacrylonitrile (PAN). The revenue evaluation in the current study was based on the underlying assumption that lignin could be converted to carbon fibre at a conversion efficiency of 45% (w/w), as
suggested by Holladay et al. (2007). The assumed selling price of carbon fibre in the current study was £4.75 per kilogram. The lignin extracted from the HAG-ALK_efluent will yield approximately 93 tonnes of carbon fibre per year if 10,000 tonnes of perennial rye grass substrate go through the fermentation process, and that will generate a gross income of about £401,754/year. Likewise, the HAG-ALKENZ_efluent will generate approximately 86 tonnes of carbon fibre per year, which will generate an income of £373,058/year. The data show that the use of lignin residue from hydrolytic-acidogenic fermentation process for the production of carbon fibre has a great potential of improving the economic value of that fermentation process. The use of the residual lignin proportion from the digestate of a two-stage (biohydrogen-biomethane) process for the production of carbon fibre would however be more beneficial, given that the nearly the same quantity of lignin can be produced from the digestate of the second stage methane when compare with the that obtained from the acid phase system.

The economic evaluation show that, the integration of lignin biorefinery or PHA biosynthesis directly to the biohydrogen fermentation system could in no way displace biomethane process as a secondary system to the acidogenic fermentation of lignocellulosic biomass. A recommended approach capable of improving the bioconversion of lignocellulosic feedstock to bio-fuels and chemicals is illustrated in Figure 8.15.
Figure 8.15: Suggested approach for lignocellulose bioconversion to biofuels and biochemicals
8.6 Chapter Conclusions

This study contributes to the knowledge that improved enzymatic hydrolysis in the first stage (acidogenic phase), has important benefits for secondary processes such as biomethane production, PHA production and the quality of lignin extraction.

- The enhancement of enzymatic activities in the anaerobic digestion process, using the HAG-ALKENZ\textsubscript{effluent}, resulted in a sizable improvement of methane yield (approximately 59% higher than the yield obtained from the HAG-ALK\textsubscript{effluent}). The study shows that the rate of production of methane gas within the first 5 days of the digestion is essentially based on the availability of readily solubilized monomers because of enhanced hydrolysis. It was concluded therefore that the rate of methane production within the first 5 days have potential of being used to measure effectiveness of a prior hydrolytic activity in the first-stage of a two-stage AD process.

- The study suggested that the availability of the higher molecular weight short chain fatty acids at the time where the readily available VFAs have been depleted, potentially let to a shift in the path of metabolism toward gluconeogenesis, resulting in the increase of the residual soluble carbohydrates during that period. The study concluded that enhanced hydrolysis resulting in the release of higher readily solubilized carbohydrates is important for the downstream PHA production, as oligosaccharides, a product of incomplete hydrolysis was found to be largely inaccessible to the pure culture strain \textit{(Cupriavidus necator)} used in the current study.

- Extracting lignin from the effluent of an acidogenic fermentation of lignocellulosic biomass (perennial rye grass) has the potential of dramatically improving the dark fermentation process. The enhanced acidogenic fermentation having effectively degraded the holocellulose component of the perennial rye grass substrate ensured that high quality lignin was obtained. The FT-IR profile confirmed the lignin purity, showing less contamination of polysaccharides and proteins in the lignin extracted from the enzymatically enhanced hydrolytic-acidogenic fermentation process.

- Secondary process that could potentially be integrated to the acidogenic fermentation process such: PHA or lignin biorefinery may not be economic viable. Economic evaluations however show that the integration of lignin extraction and refinery, and bioplastic production with a two-stage (biohydrogen-biomethane) fermentation
system would dramatically improve the economic value for the lignocellulosic feedstock.
CHAPTER 9

9 CONCLUSIONS

- The compositional analyses of the lignocellulose proportions of the effluents and digestates of conventional single-stage AD and a two stage AD systems gave a good understanding of the substrate specificity associated with efficiency of biomass degradation in anaerobic fermentation processes. The separate acid phase in the two-stage anaerobic fermentation system potentially enhanced the hydrolysis and acidogenic process, leading the release of more VFAs and hence the improvement of the yield of biogas when compared to the single-stage anaerobic digestion. It was deduced that the acid phase also improved the dissociation of the fatty acids produced in the anaerobic digestion process, limiting the inhibitory effect of undissociated fatty acids on fermentation process, leading to effective degradation of the lignocellulosic proportions of the biomass in the two-stage system, when compared to the conventional single-stage anaerobic digestion system.

- The study found that the performance of different anaerobic fermentation systems (conventional single-stage AD and two-stage AD) are dependent on the specific feedstock, and recalcitrance of its components to degradation. The study shows that the quantity of lignin and recalcitrance of the cellulose to degradation affected the yield of biogas in the two substrates investigated. The quantity of originally inherent readily solubilized monomers was also found to directly affect the quantity of biogas produced in the anaerobic digestion process. The wheat-feed, due to the higher bioavailable monomers, resulted in higher yields of VFAs, an essential precursor for the second-phase methanogenic process, making it a preferred choice for the two-stage biohydrogen-biomethane fermentation process. It was envisaged that, lignocellulosic biomass with structural configuration similar to the dried perennial rye grass, having low initial available monosaccharides, but relatively high holocellulose content may not be ideal substrate for bio-hydrogen production when compared to the wheat-feed. An application of an effective pre-treatment process could however result in a dramatic improvement of the yields of biofuels and biochemicals from such substrates.
• It was shown that, having an initial lignin concentration of approximately 10% in both substrates. The effluent after the anaerobic processes were found to be rich in lignin, approximately 32-34% of the VS due to the recalcitrant nature of the lignin compared to the holocellulose. The study therefore envisaged that lignin could be extracted as a value added product from AD process.

• The enzyme cocktail (0.8% Cellulase ± 0.15% FAE) at 0.202 ml enzyme/g VS resulted in high-level bioconversion of the complex polymers to soluble carbohydrates after 24 hours of incubation. The yield of soluble carbohydrates was determined to be 321.0±10.9 mg/gVS; representing a 393.2% yield increase, when compared to the yield obtained with no enzyme control.

• The yield of bio-hydrogen after the enzymatic SSF was 48ml/gVS, approximately 335% higher when compared to the weak alkaline (only) treatment; and more than a 7 fold increase in the yield obtained from the untreated perennial rye grass. The enzymatic-SSF resulted in improved lignocellulose degradation of the perennial rye grass substrate by approximately 43%. The degradation of the initial cellulose after enzymatic SSF was approximately 95%, compared to 5% cellulosic degradation in the fermentation of the untreated perennial rye grass.

• The enhancement of enzymatic activities in the anaerobic digestion process, using effluent from enzymatic SSF of perennial rye grass, resulted in a dramatic improvement of methane yield (approximately 59%). The study shows that the rate of production of methane gas within the first 5 days of fermentation is essentially based on the availability of readily solubilized monomers because of enhanced hydrolysis. It was concluded therefore that the rate of methane production within the first. It was concluded therefore that the rate of methane production within the first 5 days has potential of being used to measure effectiveness of a prior hydrolytic activity in the first-stage of a two-stage AD process.

• The study suggested that the availability of the higher molecular weight short chain fatty acids at the time where the readily available VFAs had been depleted, shifted the
path of metabolism toward gluconeogenesis, resulting in the increase of the residual soluble carbohydrates during that period. The study concluded that enhanced hydrolysis resulting in the release of more bioavailable glucose is important for the downstream PHA production, as oligosaccharides, a product of incomplete hydrolysis were found to be largely inaccessible to the pure culture strain (Cupriavidus necator) used in the current study.

- Extracting lignin from the effluent of an acidogenic fermentation of lignocellulosic biomass (perennial rye grass) has the potential of improving the already well-publicised benefits of the dark fermentation process. The enhanced acidogenic fermentation having effectively degraded the holocellulose component of the perennial rye grass substrate ensured that high quality lignin was obtained. The FT-IR profile confirmed the lignin purity, showing less contamination of polysaccharides and proteins in the lignin extracted from the enzymatically enhanced hydrolytic-acidogenic fermentation process.

- In spite of the relatively higher biohydrogen yields obtained from the enzymatic-SSF of lignocellulosic biomass, the biohydrogen process was found not to be economically feasible based on the energy input required for the cultivation of the perennial rye grass and the cost of pre-treatment. Economic evaluations however show that the integration of lignin extraction and refinery, and bioplastic production with two-stage (biohydrogen-biomethane) fermentation systems would dramatically improve the economic value for the lignocellulosic feedstock.
CHAPTER 10

10 FUTURE WORK

- More research is needed on the life cycle (LCA) and economic assessments of the suggested biorefinery concepts. To facilitate this larger, perhaps pilot stage studies need to be conducted so that a more complete data set on energy and chemical inputs can be generated. Potential systems that could be compared include the production of biomethane and PHA and the production of lignin from the effluent of the hydrolytic-acidogenic fermentation. (Patterson et al., 2013)

- The elevated % of lignin in the digestate obtained from the anaerobic fermentation process could selectively be depolymerised using ionic liquids such as 1-ethyl-3-methylimidazolium acetate ([C₂ mim] [OAc]) to obtained biochemicals such as phenols, guaiacols, syringols, eugenol, catechols. These chemicals could further be oxidized to produce chemicals such as vanillin, vanillic acid, syringaldehyde. These chemicals could have a potentially significant market value and therefore could improve the value of lignin from the anaerobic digestate of lignocellulosic biomass. (Varanasi et al., 2013)

- The lignin-derived chemicals from the digestate of the AD process could be incorporated in to bioplastics derived from PHA biosynthesis using the hydolysate from lignocellulosic biomass. This incorporation could improve the properties of the PHA bioplastics, including properties such as increased elongation and water resistance, important parameters for materials used for packaging products (Northey, 2002).

- The acidogenic fermentation of perennial rye grass can be integrated with bioelectricity systems to help improve the economic viability of the perennial rye grass, as substrate for bioenergy processes. The process will involve the utilisation of the VFAs and nutrients from the biohydrogen process in microbial fuel cells to produce electricity. (Fradler et al., 2014)
• Investigating the effect of the enzyme addition on the metabolic and population profiles of bacteria and methanogens in anaerobic processes could lead to important insights into understanding anaerobic digestion performance. This would include techniques such as next generation sequencing to identify the changes in population in order to access the dominant bacteria population which were selected due to enhanced enzyme hydrolytic activity, and the use of proteomics to indicate the shift of metabolic pathways because of changes in hydrolytic performance.

• The quantification of purified lignin by subtracting the quantity of contaminants from the extracted lignin which is the standard method used in this current study has a potential significant weakness in that is not a direct measure of the desired analyte. More robust techniques which are direct measures of lignin such as thermogravimetric analyses (TGA) and gas chromatography mass spectrometry (GC/MS) will be needed for a more accurate quantification and characterisation of lignin. (Manara et al., 2013). More research is needed on lignin structure from anaerobic digestion processes using more these more advanced analytical techniques so that this chemical structural information could be used to identify end uses that are more profitable.

• Research is needed into the removal of VFAs from the hydrolytic-acidogenic reactor liquors; this could include techniques using microfiltration and ion exchange membranes. These techniques could enhance the production of biohydrogen and VFAs from lignocellulosic biomass, and therefore improve their yield and the economic value of the process. (Barnes et al., 2003)

• Methods for the detailed quantification and identification of the monosaccharides, including the monosaccharides in glycoproteins could be used, e.g. using reverse-phase high-performance liquid chromatography, for detailed characterisation and function of carbohydrates in PHA biosynthesis. (Kwon and Kim, 1993)
APPENDICES

APPENDIX I

LABORATORY REPORT-2012
SOLID ORGANIC SUBSTRATE (SOS-PTS)
SAMPLE: RM-05

SAMPLE: RM-06

170
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### Scenario 1 (Lignin as fuel)

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<th>Alkaline Treated</th>
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<tbody>
<tr>
<td>Unit price ($/pound)</td>
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</tr>
<tr>
<td>Unit price (£/pound)</td>
<td>0.025</td>
<td>0.025</td>
</tr>
<tr>
<td>Unit price (£/kg)</td>
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<tr>
<td>kg lignin/kg</td>
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<td>0.087</td>
</tr>
<tr>
<td>price of lignin (fuel) (£/kg)</td>
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<td>0.005</td>
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<tr>
<td>£/ton</td>
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### Scenario 2 (syngas alcohol: lignin gasified and converted to mixed alcohols)

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<tr>
<td>ethanol (gallons/ ton lignin)</td>
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<tr>
<td>Mixed alcohols (gallons/ ton lignin)</td>
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<td>ethanol (gallons/ ton)</td>
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<td>Mixed alcohols (gallons/ton)</td>
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<td>£ mixed alcohols/ton</td>
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### Scenario 3 (Lignin converted to simple aromatic chemicals)

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<td><strong>Efficiency of conversion</strong></td>
<td>20%</td>
<td>20%</td>
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<tr>
<td>tons lignin to BTX (BTX/ton lignin)</td>
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<td>ton lignin/ton perennial rye grass</td>
<td>0.094</td>
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<td>£ CF/ton</td>
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11 REFERENCES


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