Promoters:

Prof. dr. ir. Korneel Rabaey  
Center for Microbial Ecology and Technology (CMET),  
Department of Biochemical and Microbial Technology,  
Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

Dr. Han Vervaeren  
Department of Biochemical and Microbial Technology,  
Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

Members of the examination committee:

Prof. dr. ir. Peter Bossier (Chairman, UGent)  
Laboratory of Aquaculture & Artemia Reference Center,  
Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

Prof. dr. ir. Veerle Fievez (Secretary)  
Department of Animal Production,  
Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

Dr. Marta Coma  
Centre for Sustainable Chemical Technologies,  
University of Bath, Bath, United Kingdom

Prof. dr. ir. Frederik Ronsse  
Department of Biosystems Engineering,  
Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

Dr. Marta Carballa  
Department of Chemical Engineering  
University of Santiago de Compostela, A Coruña, Spain

Dean Faculty of Bioscience Engineering

Prof. dr. ir. Marc Van Meirvenne

Rector Ghent University

Prof. dr. Anne De Paepe
Production of lactic acid and derivatives from grass using mixed populations

Way Cern Khor

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences
Titel van het doctoraat in het Nederlands:
De productie van melkzuur en derivaten van gras met behulp van gemengde populatie

Please refer to his work as:

Cover illustration:

ISBN:

This work was funded by Special Research Fund (BOF, project number: DEF13/AOF/010) of the University of Ghent (Belgium).

The author and the promoters give the authorization to consult and to copy parts of this work for personal use only. Every other use is subjected to the copyright laws. Permission to reproduce any material contained in this work should be obtained from the author. Copyright © 2017
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>acetic acid</td>
</tr>
<tr>
<td>AEM</td>
<td>anion exchange membrane</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BMP</td>
<td>biomethane potential</td>
</tr>
<tr>
<td>CA</td>
<td>chronoamperometry</td>
</tr>
<tr>
<td>CCD</td>
<td>central composite design</td>
</tr>
<tr>
<td>CE</td>
<td>counter electrode</td>
</tr>
<tr>
<td>COD</td>
<td>chemical oxygen demand</td>
</tr>
<tr>
<td>CP</td>
<td>chronopotentiometry</td>
</tr>
<tr>
<td>CV</td>
<td>cyclic voltammetry</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>GM</td>
<td>genetically modified</td>
</tr>
<tr>
<td>HMF</td>
<td>hydroxymethylfurfural</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IC</td>
<td>ion chromatography</td>
</tr>
<tr>
<td>IL</td>
<td>ionic liquid</td>
</tr>
<tr>
<td>LA</td>
<td>lactic acid</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>LAB</td>
<td>lactic acid bacteria</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>OTU</td>
<td>operational taxonomy unit</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RE</td>
<td>reference electrode</td>
</tr>
<tr>
<td>rpm</td>
<td>rotation per minute</td>
</tr>
<tr>
<td>RSM</td>
<td>response surface methodology</td>
</tr>
<tr>
<td>sCOD</td>
<td>soluble chemical oxygen demand</td>
</tr>
<tr>
<td>STP</td>
<td>standard temperature and pressure</td>
</tr>
<tr>
<td>SWOT</td>
<td>strength, weakness, opportunity, threat</td>
</tr>
<tr>
<td>TCD</td>
<td>thermal conductivity detector</td>
</tr>
<tr>
<td>TKN</td>
<td>total Kjeldahl nitrogen</td>
</tr>
</tbody>
</table>
TS   total solid
VS   volatile solid
VS_{initial}  initial volatile solid
VSS  volatile suspended solid
WE   working electrodes

mg g^{-1} VS_{initial} Carbon: milligram per gram initial volatile solid of Carbon (e.g. 1 mol of acetic acid is 60 g and contains 2 mol of carbon molecules, 2 \times 12.01 = 24.02 g Carbon of acetic acid)
Table of Contents

Chapter 1 Introduction ........................................................................................................... 1
  1.1 Climate change .................................................................................................................. 2
  1.2 Grass! .................................................................................................................................. 2
  1.3 What to do with grass? ....................................................................................................... 4
  1.4 Production from grass ...................................................................................................... 6
    1.4.1 Challenges specific for grass ....................................................................................... 6
    1.4.2 Current production from grass ................................................................................... 13
    1.4.3 New products from grass .......................................................................................... 13
    1.4.4 Finding the right conditions ..................................................................................... 17
    1.4.5 Getting the product: The extraction challenge ......................................................... 18
  1.5 Objective of thesis .......................................................................................................... 20

Chapter 2 Pretreatment of Biomass ....................................................................................... 22
  2.0 Abstract ........................................................................................................................... 23
  2.1 Introduction ..................................................................................................................... 23
  2.2 Materials and Methods .................................................................................................. 25
    2.2.1 Materials ................................................................................................................... 25
    2.2.2 Pretreatment of grass with Ca(OH)\(_2\) ..................................................................... 25
    2.2.3 Extrusion and Ca(OH)\(_2\) post-treatment of grass, maize straw and sprout stem ....... 26
    2.2.4 Biochemical methane potential (BMP) test .............................................................. 26
    2.2.5 Kinetic modelling ....................................................................................................... 27
    2.2.6 Analytical methods .................................................................................................. 27
    2.2.7 Power measurement .................................................................................................. 28
  2.3 Result and Discussion ...................................................................................................... 28
    2.3.1 Low temperature 10 °C Ca(OH)\(_2\) pretreatment ...................................................... 28
    2.3.2 Pretreatment temperature and time effect ............................................................... 29
    2.3.3 Ca(OH)\(_2\) loadings effect ........................................................................................ 30
    2.3.4 Statistical analysis and result validation ................................................................... 30
    2.3.5 Extrusion and subsequent Ca(OH)\(_2\) post-treatment on different feedstock ............ 33
Chapter 3 Storage of Biomass

3.0 Abstract .................................................................................................................................39
3.1 Introduction .............................................................................................................................39
3.2 Materials and Methods ..........................................................................................................41
  3.2.1 Materials ............................................................................................................................41
  3.2.2 Process overview ................................................................................................................41
  3.2.3 Pretreatment .......................................................................................................................42
  3.2.4 Storage ...............................................................................................................................42
  3.2.5 Biochemical methane potential (BMP) test .....................................................................42
  3.2.6 Fermentation test ...............................................................................................................43
  3.2.7 Analytical methods ............................................................................................................43
  3.2.8 Statistical analysis .............................................................................................................44
3.3 Result and Discussion ..............................................................................................................44
  3.3.1 Effect of pretreatment on the biomass storability in terms of biomass properties ..........44
  3.3.2 Effect of pretreatment and storage on methane production during anaerobic digestion .48
  3.3.3 Effect of pretreatment and storage on carboxylate production during fermentation ......50

Chapter 4 Lactic acid fermentation ..............................................................................................53

4.0 Abstract .................................................................................................................................54
4.1 Introduction .............................................................................................................................54
4.2 Materials and Methods ..........................................................................................................56
  4.2.1 Substrate ............................................................................................................................56
  4.2.2 Microorganisms and cultivation .......................................................................................56
  4.2.3 Batch fermentation ............................................................................................................57
  4.2.4 Semi-continuous fermentation .........................................................................................57
  4.2.5 DNA extraction ..................................................................................................................58
  4.2.6 DNA sequencing and bioinformatics processing ...............................................................58
  4.2.7 Statistical analysis .............................................................................................................59
  4.2.8 Analytical methods ..........................................................................................................60
4.3 Result .......................................................................................................................................60
  4.3.1 Impact of acetate addition on lactic acid concentration – batch mode .........................60
Chapter 6

6.0 Abstract ..................................................................................87
6.1 Introduction ..............................................................................87
6.2 Materials and Methods ............................................................89
   6.2.1 Substrate, microorganisms and cultivation ..........................89

Chapter 5 Extraction of carboxylic acids ........................................72

5.0 Abstract ..................................................................................73
5.1 Introduction ..............................................................................74
5.2 Materials and Methods ............................................................75
   5.2.1 Materials ...........................................................................75
   5.2.2 Membrane electrolysis .......................................................75
   5.2.3 Nanofiltration .....................................................................76
   5.2.4 Pertraction ..........................................................................76
   5.2.5 Ion exchange ......................................................................77
   5.2.6 Ionic liquid extraction .........................................................77
   5.2.7 Analytical methods .............................................................77
5.3 Result and Discussion ...............................................................77
   5.3.1 To achieve selectivity during extraction ..............................77
   5.3.2 To achieve a high extraction efficiency ..............................79
   5.3.3 To achieve a high concentration extract ..............................80
   5.3.4 Product utilization ...............................................................81
   5.3.5 Comparison of extraction methods ....................................82

Chapter 6 Alkane production from grass ........................................86

6.0 Abstract ..................................................................................87
6.1 Introduction ..............................................................................87
6.2 Materials and Methods ............................................................89
   6.2.1 Substrate, microorganisms and cultivation ..........................89
6.2.2 Semi-continuous fermentation ................................................................. 89
6.2.3 Semi-continuous microbial elongation ..................................................... 90
6.2.4 Maximum rate of caproic acid production test ........................................ 90
6.2.5 Maximum concentration of caproic acid test .......................................... 90
6.2.6 Electrochemical extraction ..................................................................... 91
6.2.7 Kolbe electrolysis .................................................................................... 91
6.2.8 Analytical methods ................................................................................ 92
6.3 Result and Discussion ................................................................................ 92
  6.3.1 Semi-continuous fermentation of lactic acid from grass ....................... 92
  6.3.2 Semi-continuous elongation of caproic acid through lactic acid ............ 93
  6.3.3 Maximum rate of caproic acid production and maximum concentration of caproic acid test 94
  6.3.4 Electrochemical extraction of caproic acid and fuel production through Kolbe electrolysis . 97
  6.3.5 Feasibility of turning grass into aviation fuel ........................................... 98

Chapter 7 General discussion and perspectives ................................................. 100
  7.1 Main findings ............................................................................................ 101
  7.2 Discussion .................................................................................................. 102
    7.2.1 Biomass preparation .......................................................................... 102
    7.2.2 Product conversion ........................................................................... 104
    7.2.3 Product extraction and synthesis ....................................................... 106
  7.3 Grass biorefinery ....................................................................................... 108
  7.4 Moving grass biorefinery forward ............................................................. 111

Abstract ............................................................................................................. 114
Biobliography ........................................................................................................ 117
Appendix A .......................................................................................................... 130
Appendix B .......................................................................................................... 138
Curriculum Vitae ................................................................................................. 144
Acknowledgments .............................................................................................. 148
Introduction
Chapter 1 Introduction

1.1 Climate change

Climate change has been one of the major concerns emerging in the past decades. Worldwide development requires an ever-growing need for energy and fuels, in industrial, transportation and residential sectors, and these demands are mainly satisfied by fossil fuels. The use of fossil fuels has been linked to the increase in concentration of atmospheric greenhouse gases, which leads to environmental issues such as global warming, ocean acidification, Artic sea ice loss, etc.

On the quest of searching for new sources of chemicals and fuels, crops and plants are often the primary target due to their availability and human’s capability to cultivate them. Along with the urban development, various types and streams of wastes are also generated. Among others are paper pulp, fermented molasses waste, poultry waste, food waste, and more diluted streams such as dairy wastewater, paper mill waste water, and palm oil mill effluent. Thus far, despite its vast abundance and ubiquitous nature, grass has not been considered extensively.

1.2 Grass!

The family Poaceae or Gramineae, known with its common name – grass, is the 5th largest plant family on Earth with over 700 genera and 10 000 species (Clayton et al., 2016; Smole & Hribenik, 2011) at present time. They are monocotyledonous flowering plants where their seeds typically contain only one embryonic leaf. They can be found in almost every environmental (inhabited) niche on the Earth, except the coldest region of the Artic and Antarctica. Some of the most common grass are portrayed in Figure 1.1. Properties of grass can vary widely depending on the surrounding conditions and harvest time. For example, winter grass will normally have a higher lignin content than summer grass. The moisture content of grass can also fluctuate depending on the weather. Depending on the intended application, these factors are taken into consideration when determining the harvest time in order to obtain grass with desirable properties. Properties which are ubiquitous among grass species are they typically contain structural carbohydrate in the form of lignin (100 – 300 g kg$^{-1}$ total solid), hemicellulose (150 – 500 g kg$^{-1}$ total solid) and cellulose (250 – 400 g kg$^{-1}$ total solid) content and non-structural carbohydrate such as sugars and starch (40 – 250 g kg$^{-1}$ total solid).
solid) (Cherney et al., 1988), which are the targeted compound for chemical conversion. Main sources of grass which can be utilized are found from nature grassland, agriculture and clippings.

Major grasslands of the world can be divided into 2 main types, namely savannah (or tropical), and temperate grassland (e.g. prairie and steppe). Area of grassland has been declining through the past decades, mostly due to intensive agriculture activity. The grassland coverage is dynamic and although exact quantification of grass is nearly impossible, approximate figures are summarized in Figure 1.2. In Flanders alone (north part of Belgium), grasslands represent an estimated $42 \times 10^6$ kg of available biomass annually. This can be related to the area of grassland, which is available in Belgium ($0.005 \times 10^6$ km$^2$), which in turns gives a rough idea on the scale of grass availability.

It is worth noting that these figures do not include agricultural grass and clippings, which are also substantial source of grass. Agricultural grass is normally well managed, which facilitates utilization. Moreover, grass clippings from roadside, garden or parks often contain impurities such as plastic, metals, as such. Removal of impurities is often necessary before the grass can be utilized.
1.3 What to do with grass?

Grass may have been around for around 55 to 70 million years (Kellogg, 2001). Discovery and study of silicified plant tissues (phytoliths) preserved in Late Cretaceous coprolites indicates that dinosaurs might have already dined on grass some 65 million years ago (Piperno & Sues 2005). Nowadays, it is still mainly used as grazing and feed for animals, either in its fresh state or stored form (silage, Figure 1.3).

Grass and grassland have a grave importance for Europe as well as rest of the world. Not only it can act as carbon source or sink and graze for animals, it also helps to prevent soil erosion, provide water storage and watershed protection for river system. Furthermore, it is home to massive biodiversity within and surrounding its coverage (Huyghe et al., 2014), and not to mention that grass also gives the landscape an aesthetic appearance.

In the interest of industrial processes, grass can serve as a renewable carbon source for chemical production. There are many sources of grass which are clipped for convenience with minimal or no further processing, for example at the airport, roadside, sport facilities, etc. Even more grass sources are only extensively managed, if they are done at all. Considering the sheer amount of grass available worldwide, the potential of this substrate is humongous. In the past, structural and chemical properties of grass have been investigated for biofuel production (Anderson & Akin, 2008), however, the potential of grass is still largely untapped.
As an example of its variety of uses, grass can also be used for constructions such as bridges. Q’eswachaka is the name of a suspension bridge made entirely of grass over the Apurimac River in Quehue, Peru (Figure 1.4). Single cords of grass twisted together can create cables that can support more than 2268 kg. In order to expand the use of grass, it has also been tested and utilized to produce cardboard, carton and paper in industry. At a more advanced level, grass is considered a high potential feedstock for biorefinery (Shekhar Sharma et al., 2011).
A biorefinery is a facility that integrates conversion processes to produce fuels, power, chemicals and materials from biomass, with the ultimate aim of closing the world carbon loop and achieving a zero or even positive carbon economy (Kamm et al., 2000). It is an analogue of the conventional petroleum refining process, with its distinctive difference of biomass utilization and incorporation of biological processes. While other renewable energy sources such as solar, hydropower, wind, tidal, waves and geothermal show very promising potential for energy generation, biomass is the only form which provides a direct supply of carbon and hydrogen source. In that sense, there is a great opportunity to utilize this renewable resource of carbon and hydrogen molecules for materials and fuels production (Parajuli et al., 2015).

A biorefinery can get its feed from dedicated crops, either from agriculture or from forestry, and it has been differentiated into several generation depending on its feedstock (Parajuli et al., 2015). 1st generation biorefineries deal with readily digestible substrate such as sugar and corn. A well-known example of commercial biorefinery is the production of ethanol from corn. Getting dedicated crops from agriculture to feed biorefinery, however, is a contentious issue because it is seemingly in conflict with food availability. Thus, biomass waste can be used as an alternative feedstock. This gives birth to the 2nd generation biorefinery, which takes a step further and focuses on the use of non-food, waste or lignocellulosic biomass. While 3rd generation biorefinery aims to utilize algae biomass, a concept of 4th generation which focusses on photobiological solar fuels and electrofuels has also been proposed (Aro, 2016). In a 2nd generation biorefinery, the pipeline can be visualized as process steps presented in Figure 1.5. In this context, grass serves as the feedstock for the process pipeline.

![Figure 1.5 Typical 2nd generation biorefinery process pipeline](image)

### 1.4 Production from grass

#### 1.4.1 Challenges specific for grass

Why is grass, despite its potential, not utilized more than just as food for animals? One of the main challenges is the scale of biomass. While there may be plenty of grass around, it is spread over a large surface area. Moreover, most grass species have a very high growth rate but the availability of grass may not be consistent over the year. At tropical zones where the temperature is warm (30 °C and
higher) and constant throughout the year, grass can grow very rapidly and it is available throughout the year. In the temperate zones such as Mediterranean and subtropics, grass is only available during certain seasons. Hence a storage system is often necessary to ensure steady supply of grass. One of the most effective storage methods is ensiling, where grass is compressed to a density between 140 to 260 kg m$^{-3}$ (Honig, 1991). This process induces lactic acid production by lactic acid bacteria (mainly *Lactobacillus* spp.), which lowers the pH of silage to below 4. The low pH inhibits the growth of other microorganisms and results in preservation of grass silage. Other storage methods involve dosing of chemicals such as sulphuric acid and calcium hydroxide to achieve a pH either too high (> 11) or too low (< 3) for bacterial growth. Extensive research has been performed on the storage of grass to ensure a steady supply (Digman et al., 2010; Jatkauskas & Vrotniakiene, 2011). To utilize grass, it needs to be harvested, transported to processing facility, and stored, with all the process steps being highly efficient to make the process feasible.

Before the grass can be used, pretreatment is needed due to the recalcitrant nature of lignocellulosic biomass. Pretreatment of biomass is a process which breaks down complex lignocellulosic structure to improve the efficiencies of subsequent processes such as hydrolysis and biological conversion. The biodegradability of plant biomass is often limited due to the recalcitrant nature of lignin and its structural carbohydrates. Lignin, along with phenolic compounds and ferulic acid are part of the aromatic constituents of biomass, which make up the strong polymeric organic structure protecting the plants. Within the plant, hemicellulose and cellulose are strong natural polymers conferring structural support of plant, they are also the target compounds for conversion processes. Therefore, pretreatment technologies are often necessary to break down lignin and hydrolyse the cellulose and hemicellulose into simple monomer sugars such as glucose (C6 sugar) and xylose (C5 sugar) (Figure 1.6).
Early pretreatment method generally revolves around the comminution of biomass to improve biodegradability. Figure 1.7 depicts a cutter machine for size reduction of biomass during 1896. Biomass is often cut before it is stored to improve the biological activity by bacteria.
Until now, many pretreatment methods have been explored, a non-exhaustive summary can be found in Table 1.1. Pretreatment cost is the main criteria when considering pretreatment technology. While there can be many pretreatment methods available, the cost can swiftly filter out most of the options when considering large scale operation. Biocompatibility and environmental sustainability are also often taken into consideration when choosing pretreatment methods. This is often necessary to ensure a smooth coupling with biological processes. Selection of pretreatment technology is also heavily dependent on the type feedstock. For instance, methods such as steam explosion are effective against hardwood, but not suitable for softwood. Hence, an ideal pretreatment method has to be cost effective and able to enhance biodegradability significantly for subsequent conversion process. Apart from that, proactive approaches can include alteration of grass structure at the stage of plant breeding and genetic modification to reduce lignin content (Akin, 2007; Fu et al., 2011).
### Table 1.1 Overview of pretreatment technology (based on Agbor et al., 2011)

<table>
<thead>
<tr>
<th>Category</th>
<th>Method</th>
<th>Types</th>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical</td>
<td>Grinding/ Milling</td>
<td>Hammer</td>
<td>- Reduces particle size and cellulose crystallinity</td>
<td>- Power consumption usually higher than inherent biomass energy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ball</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Two-roll</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Colloid</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vibro (electroporation)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irradiation</td>
<td>Gamma ray</td>
<td></td>
<td>- Reduces the crystallinity and molecular weight of cell wall polymers,</td>
<td>- High energy input</td>
</tr>
<tr>
<td></td>
<td>Electron beam</td>
<td></td>
<td></td>
<td>- Difficult to scale up</td>
</tr>
<tr>
<td></td>
<td>Microwave</td>
<td></td>
<td></td>
<td>- Possible environmental and safety issues</td>
</tr>
<tr>
<td>Others</td>
<td>Hydrothermal / Hot liquid water/ Autohydrolysis</td>
<td></td>
<td>- Low solvent cost (water)</td>
<td>- High energy demand on downstream processing due to large water volume</td>
</tr>
<tr>
<td></td>
<td>High pressure steaming</td>
<td></td>
<td>- No chemicals required</td>
<td>- High energy input</td>
</tr>
<tr>
<td></td>
<td>Extrusion</td>
<td></td>
<td>- Scale up is possible</td>
<td>- High maintenance cost of equipment</td>
</tr>
<tr>
<td></td>
<td>Pyrolysis</td>
<td></td>
<td>- Rapid conversion to gas and liquid products</td>
<td>- High temperature</td>
</tr>
<tr>
<td></td>
<td>Torrefaction</td>
<td></td>
<td></td>
<td>- Ash production</td>
</tr>
<tr>
<td></td>
<td>Thaw/ Freezing</td>
<td></td>
<td>- No chemicals required</td>
<td>- High operating cost</td>
</tr>
<tr>
<td></td>
<td>Ionic liquid</td>
<td></td>
<td>- Effective solubilisation of lignin and hemicellulose</td>
<td>- High cost (at present)</td>
</tr>
<tr>
<td></td>
<td>Pulsed electrical field</td>
<td></td>
<td>- Can be performed at ambient conditions</td>
<td>- More research needed</td>
</tr>
<tr>
<td>Chemical/ Physico-chemical</td>
<td>Alkali/ Thermo-alkali</td>
<td>Sodium hydroxide</td>
<td>- Low requirement on equipment</td>
<td>- Irrecoverable salts formed and incorporated into biomass</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Potassium hydroxide</td>
<td>- Moderate temperature</td>
<td>- Not effective for high lignin biomass (for some alkali)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Magnesium hydroxide</td>
<td>- Cost can be reduced depending on choice and combination of chemicals</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Calcium hydroxide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Method</td>
<td>Benefits</td>
<td>Drawbacks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>--------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| **Ammonia recycle percolation (ARP)** | - Removal of lignin  
- Produce sulphur and sodium free lignin | - Need to clean hydrolysate or remove ammonia |
| **Acid**                       | Sulphuric acid  
Hydrochloric acid  
Phosphoric acid | - High reaction rate  
- Significant hydrolysis of (hemi)cellulose  
- Equipment corrosion  
- Formation of inhibitory compounds |
| **Supercritical**              | Carbon dioxide  
Water | - Significant hydrolysis or dissolution of biomass  
- High equipment cost  
- Discontinuity of process  
- Difficulty in upscaling  
- Safety issues |
| **Explosion**                  | Steam explosion | - No recycling or environmental costs  
- Limited use of chemicals  
- Avoids excessive dilution of sugars  
- Low energy input  
- Hydrolyses hemicellulose  
- Partial destruction of xylan fraction  
- Incomplete disruption of the lignin carbohydrate matrix  
- Produces compounds inhibitory to microorganisms |
| Ammonia fibre explosion (AFEX) | - Short residence time  
- High selectivity for reaction with lignin  
- Does not produce inhibitors for downstream processes  
- Moderate temperature  
- Ability to recycle ammonia  
- Removal of lignin | - Not efficient for biomass with high lignin content  
- Environmental concerns |
| CO₂ explosion                  | - Increases accessible surface area | - High equipment cost |
| SO₂ explosion                  | - Relatively cost-effective  
- Does not cause formation of inhibitory compounds | |
| SO₃ explosion                  | - Ambient pressure | - Handling of corrosive chemicals |
| **Oxidizing agents**           | Hydrogen peroxide | - Fractionation of biomass at ambient pressure and low temperature  
- Produce inhibitory compounds  
- High oxidants cost |
| Wet oxidation                  | - Removal of lignin  
- Hydrolyses hemicellulose | - Production of inhibitory compounds is possible |
<p>| Ozonolysis                     | - Effectively removes lignin | - Large amount of ozone required |</p>
<table>
<thead>
<tr>
<th>Method</th>
<th>Solvents / Agents</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent extraction (Organosolv)</td>
<td>Ethanol-water</td>
<td>- Does not produce toxic residues</td>
<td>- High cost</td>
</tr>
<tr>
<td></td>
<td>Benzene-water</td>
<td>- Reactions are performed at ambient temperature and pressure</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Butanol-water</td>
<td>- High quality lignin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ethylene-water</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Swelling agents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biological</td>
<td>White/Brown rot fungi</td>
<td>- Hydrolyses lignin and hemicelluloses</td>
<td>- Solvents need to be drained from the reactor, evaporated, condensed, and</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>recycled</td>
</tr>
<tr>
<td></td>
<td>E. coli, etc.</td>
<td></td>
<td>- High cost</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerobic fungi</td>
<td></td>
<td>- Degrades lignin and hemicelluloses</td>
<td>- Long retention time</td>
</tr>
<tr>
<td>Anaerobic fungi</td>
<td></td>
<td>- Low energy requirements</td>
<td></td>
</tr>
<tr>
<td>Manure/Rumen fungi</td>
<td></td>
<td>- Available in nature</td>
<td>- Long retention time</td>
</tr>
<tr>
<td>Bacteria</td>
<td></td>
<td>- Genetic modification of bacteria can improve pretreatment efficiency</td>
<td>- More research needed</td>
</tr>
<tr>
<td>Enzymes</td>
<td></td>
<td>- Short retention time</td>
<td>- High cost</td>
</tr>
</tbody>
</table>
1.4.2 Current production from grass

It is well known that lignocellulosic biomass including grass can be utilized in anaerobic digestion process to produce methane, which can be used for electricity and heat generation. However, the price of electricity is low at this moment due to the low price of shale gas and fossil fuels. Hence, the carbon and hydrogen molecules from biomass can be valorized for more valuable chemical production. There has also been attempted to produce hydrogen from lignocellulosic biomass, which can be used in hydrogen powered vehicle. Although much research is still needed to improve the efficiency and cost in order to justify an economic process.

Another notable product which can be produced from fermentation of lignocellulosic biomass is ethanol. Great success has been achieved at commercial scale with corn in the U.S. and sugar beet in Brazil. Now, there is even a commercial plant which utilizes cellulosic feedstock – corn stover, operated by DuPont in Nevada, Iowa, with the capacity to produce 30 million gallons ethanol per year. Also, switchgrass is also one of the potential substrate under consideration (Mitchell et al., 2012).

Another interesting technology is pyrolysis, a thermochemical technique which decomposes organic matter at elevated temperature in the absence of oxygen. This technique can be applied on biomass including grass to produce biofuel and biochar (Gupta & Demirbas, 2010; Saikia et al., 2015). This technology can also generate syngas, which contains mainly hydrogen and carbon monoxide. Syngas can then be used to produce biofuels such as ethanol, or used in chemical industry to produce chemicals such as methanol.

1.4.3 New products from grass

The carboxylate platform has been the emerging path during the past few years (Figure 1.8). The carboxylate platform consists of short chain carboxylates as intermediate feedstock chemicals, which are derived from industrial and agricultural wastes, using hydrolysis and fermentation with undefined mixed cultures in engineered systems under anaerobic conditions (Agler et al., 2011). Production of carboxylic acids from lignocellulosic biomass has drawn major interest, with some of the main products include lactic acid, propionic acid, butyric acid, succinic acid, levulinic acid, caproic acid, as such. Many of these products have significant industrial importance and there is a huge market for their uses and applications.
Via the carboxylate platform, a wide variety of carboxylic acids and derivatives could be produced from grass. One of the many routes would be towards lactic acid production. Lactic acid is an important chemical with wide applications in industrial sector, food and beverage, pharmaceuticals, personal care and others (Market Research Report, 2012). Other than that, it can serve as precursor for many other chemicals synthesis such as polylactic acid (PLA) and its ester – ethyl lactate. PLA is
a biodegradable polymer which has applications in packaging industry and other sectors such as textile industry, in which PLA demand is expected to rise significantly in the coming years.

Chemicals obtained from biological conversion or extraction step can be further used to synthesize other high value product such as caproic acid. Caproic acid is a product which can be produced from lactic acid through biological conversion. Caproic acid can be used as antimicrobial agent in animal feed, and it can also be used to produce ester. Another possibility is to synthesize energy dense liquid fuels. For instance, lactic acid can also be used in catalytic upgrading pathway to produce fuels and chemicals by dehydration/hydrogenation and C–C coupling reactions (Carlos Serrano-Ruiz & Dumesic, 2009).

Apart from fuels synthesis, by applying appropriate conversion and extraction technology, lactic acid produced from lignocellulosic biomass can also be used to synthesize its polymer – polylactic acid (PLA) (Figure 1.9). PLA has attracted a substantial attention due to its properties and widespread applications. Its biodegradable and biocompatible properties allow its use in many fields such as medical and food industry. Furthermore, PLA can also be utilized as feedstock in 3D-printing technology, which opens up a great deal of opportunities for its utilization. The lactic acid market worth is expected to reach 3.82 Billion USD while PLA will reach 5.16 Billion USD by the year 2020 according to (Markets and markets, 2015). Currently, the use of PLA is still hampered due to the cost of its precursor, hence cheaper source of feedstock is one of the main driver to unlock its full potential. Apart from PLA, grass can also be utilized to produce medium chain length polyhydroxyalkanoate (mcl-PHA) (Cerrone et al., 2014), which is also a biodegradable plastic.

Figure 1.9 (a) Lactic acid solution, (b) PLA pellet, (c) PLA filament
Apart from lactic acid, it is also possible to produce other carboxylic acids such as propionic acid, citric acid, butyric acids, etc. from grass through bioprocesses. In order to fully explore the potential of grass, one can look at the intrinsic properties of grass, as well as the possible conversion and secondary conversion of products which can be obtained from grass. A list of potential utilization of components in grass is portrayed in Table 1.2.

<table>
<thead>
<tr>
<th>Component</th>
<th>Technology</th>
<th>Product/Application</th>
<th>Estimated TRL level</th>
<th>Limitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lignin</td>
<td>Ionic liquid extraction</td>
<td>Highly pure lignin</td>
<td>2 – 3</td>
<td>High extraction cost</td>
</tr>
<tr>
<td></td>
<td>Controlled-rise moulding</td>
<td>Polyurethane foam</td>
<td>2 – 3</td>
<td>High production cost</td>
</tr>
<tr>
<td></td>
<td>Melt-spinning, oxidation, carbonization</td>
<td>Carbon fibre</td>
<td>2 – 3</td>
<td>High precursor and production cost</td>
</tr>
<tr>
<td>(Hemi-) Cellulose</td>
<td>Fermentation</td>
<td>Carboxylic acids: lactic acid, caproic acid</td>
<td>3 – 4</td>
<td>High pretreatment and extraction cost</td>
</tr>
<tr>
<td></td>
<td>Fermentation/Polymerization</td>
<td>Bio-plastic: polylactic acid, polyhydroxyalkanoates (PHA)</td>
<td>2 – 3</td>
<td>Downstream processing cost</td>
</tr>
<tr>
<td></td>
<td>Cellulose extraction</td>
<td>Pharmaceutical application: cellulose ester, bio-adhesive, coating</td>
<td>2 – 3</td>
<td>High extraction cost</td>
</tr>
<tr>
<td>Mixed</td>
<td>Paper and pulp technology</td>
<td>Cardboard, carton, paper</td>
<td>9</td>
<td>Potentially low price of products</td>
</tr>
<tr>
<td></td>
<td>Injection molding</td>
<td>Bio-plastic (Arboform)</td>
<td>9</td>
<td>Some forms are not water resistant; 60% higher cost compared to polypropylene</td>
</tr>
<tr>
<td></td>
<td>Pyrolysis</td>
<td>Biochar, carbon fibre, biofuel, syngas</td>
<td>8 – 9</td>
<td>Potentially low price of products</td>
</tr>
<tr>
<td></td>
<td>Syngas fermentation</td>
<td>Liquid fuel</td>
<td>2 – 3</td>
<td>Potentially low price of products</td>
</tr>
</tbody>
</table>

Grass can also contain a significant amount of lignin depending on the species and time harvested. Lignin spans a wide range in terms of industrial application such as dispersants in high performance
cement applications, water treatment formulations and textile dyes, bio-sorbent for water and soil purification, additives in specialty oil field applications and agricultural chemicals. It also serves as raw materials for chemicals such as vanillin, DMSO, ethanol, xylitol sugar, and humic acid. Lignin can also be used as environmentally sustainable dust suppression agent for roads and leather tanning agent. Development of extraction technology of lignin can allow the utilization of lignin component in grass.

1.4.4 Finding the right conditions

For biological conversion, the process can be done either with a pure culture bacteria or mixed community. Pure culture fermentation involves only a single species of microorganisms, while mixed culture community can consist of a synthetic mix of several microorganisms species, open culture or naturally occurring microorganisms under non-sterilized condition. Pure culture process is more common on industrial biochemical production, as it results in only a single high purity product, which ease downstream processing significantly. However, sterilization is required and this leads to process cost, and there is a risk of contamination. Mixed culture process can often avoid the need of sterilization, however the process needs to be well understood and controlled to obtain a satisfactory purity of desirable product.

Conversion processes can also be performed under different operating conditions such as different temperatures, pH, salinity and presence of oxygen. Temperature wise, extreme temperatures such as thermophilic and hyperthermophilic operations can often limit the diversity of bacteria community, which can lead to a lower risk of contamination. This can play a big role in terms of product purity, with the possibility to aid downstream processes and product extraction which are the major limitations of pushing technologies towards a higher TRL level as shown in Table 1.2. Higher temperature also has the advantage of higher hydrolysis rate of biomass. In terms of pH, extremely low or high pH can often inhibit undesirable microorganisms or biochemical routes. For biorefinery processes, it is worth to bear in mind that many enzymes work best around neutral or slightly acidic pH, although that is also dependent on the type of processes, microorganisms and enzymes used. Metabolic engineering can also drastically change the game, as microbes which unusual characteristics can be made to tolerate extreme conditions, or knockout of genes to produce certain metabolites. Nowadays, it is even possible to construct cells with entirely synthetic genomes as the
computing power and genetic technology advance (Gibson et al., 2010), and this can potentially have a huge impact on biorefinery (Ghim et al., 2010).

Apart from this, conversion can also be performed under liquid submerged state or solid state. Most fermentation processes are performed under submerged state due to easier control of operating parameters such as temperature and pH, and handling of reactor content. While solid state processes are harder to operate, they offer the advantage of giving a higher concentration of product since dilution is reduced or limited. An example of solid state operation is the DRANCO process, where anaerobic digestion is performed at dry matter content of up to 40% in reactor. For some conversion processes such as fermentation, the process can be single outcome (homo-fermentation) or multiple outcome (hetero-fermentation). For instance in lactic acid fermentation, homo-fermentation produces lactic acid as sole product, while in hetero-fermentation, each mole of lactic acid produced is accompanied by 1 mole of carbon dioxide and ethanol or acetic acid. Homo-fermentation is often more desirable as it results in higher purity of product. Reactor operation regime can also be a crucial element. For instance in a mixed culture PHA production, a strategy of feast and famine feeding regime is used to select and enrich bacteria which are good at storing substrate (Reis et al., 2003). Finding the right conditions for intended product can be a challenging process as it requires a combination of good knowledge of biochemical pathways, microbiology and reactor operations.

1.4.5 Getting the product: The extraction challenge

Last but not least, one also needs to ponder how to get the product after one has produced it. Extraction process plays a cardinal role in biorefinery processes, it has been, and still is, one of the biggest challenges in both chemical and biochemical industry. It does not only give a direct outcome in the form of product obtained, it also affects the biological conversion process indirectly by reducing product inhibition. The most common extraction technologies include distillation, liquid-liquid extraction, adsorption, evaporation, filtration, crystallization, precipitation, ion exchange, electrochemical and membrane extraction (Jiang & Zhu, 2013; Van Hecke et al, 2014).

When lignocellulosic biomass is used as feedstock for fermentation, it is often not economical to sterilize the low cost substrate. With the non-sterile operation, a mixed spectrum of products can often be expected. Hence, the downstream extraction technology chosen will have to be able to deal with the complexity of the stream. And in some cases, selectivity of extraction can be desirable. This can
be the case if the lactic acid produced is intended for uses such as polylactic acid production. Lactic acid is often accompanied with by-products such as acetic acid in a mixed culture fermentation. In lactic acid polymerization, the total amount of impurities (e.g. acetic acid and citric acid) should preferably be lesser than 0.05 mol% (Inkinen et al., 2011). While in other cases, selectivity is not an issue such as pure culture fermentation, or if the carboxylic acids can be utilized as a whole.

The main challenges associated with extraction technology are often its energy demand, equipment requirement and process complexity. While distillation can function as an effective extraction method for ethanol, it is still nonetheless a very energy intensive method. Extraction processes in both academia and industry are still going through revolutionary change. Emerging technologies comprise ionic liquid extraction and molecularly imprinted polymer that are being developed to pave alternative routes to product extraction (Flieger & Czajkowska-Żelazko, 2011; Pratiwi & Matsumoto, 2014; Martín-Esteban, 2001; Puoci et al., 2012). In industry, an example would be the transition of lactic acid extraction technology. Conventional extraction of lactic acid is performed with first addition of calcium carbonate to neutralize the pH of fermentation broth. The calcium lactate formed is then re-acidified with the addition of sulphuric acid. Lactic acid is produced along with the formation of unwanted by-product – calcium sulphate, which is also known as gypsum. This has been the standard extraction method for lactic acid since the first industrial production of lactic acid in the U.S. by microbial process in 1881. However, it is only until recently that ion exchange has become the new norm for lactic acid extraction technology.

Extraction can be performed ex-situ or in-situ. Ex-situ extractions are more common as they are easier to control and the extraction occurs separated from the biological conversion system. This is often translated into a batch or semi-batch operation. In a biorefinery process, continuous operation is often desirable as it gives a higher volumetric production and lesser costly plant down time. Continuous process can still be achieved with ex-situ extraction, with multiple duplication of extraction line, which also means higher investment cost on equipment. While in-situ extraction can be more complicated and requires sophisticated process control, it allows removal of product as it is produced during conversion step. This can potentially improve volumetric productivity by alleviation of production inhibition, and avoid further side reactions of target product especially in a mixed culture system.

Extraction can also be incorporated strategically with the previous conversion step. For instance, lactic acid is difficult to extract due to its high boiling and hydrophilic property. Production of caproic
acid from lactic acid can ease the extraction process due to the hydrophobicity of the longer chain carboxylic acid. The caproic acid can then be extracted and used for further synthesis.

1.5 Objectives and thesis outline

Currently, there is no process line taking grass to valuable products, making it an underused feedstock in biorefinery. The objective of this study is hence to open up a process pipeline of chemicals production from grass through the production of lactic acid and its derivatives (Figure 1.10). First, grass is pretreated to improve biodegradability of biomass, which is tested through methane production in anaerobic digestion process and fermentation to lactic acid. From here, lactic acid is used as an intermediate chemical with the aim to produce caproic acid. The caproic acid is then further converted into decane via Kolbe electrolysis. Extraction technologies for lactic acid are also investigated.

Figure 1.10  Process pipeline of chemicals production from grass
This research is organized into seven chapters and two appendices.

**Chapter 1** gives a general introduction to the background, objectives, and outline of this thesis.

**Chapter 2** looks into the potency of mechanical and chemical pretreatment for further utilization of biomass in terms of biogas production. Extrusion and calcium hydroxide pretreatment are included in this chapter.

**Chapter 3** tests the effects of extrusion and calcium hydroxide on storage for a 3 month period. The effects are evaluated through changes in biomass characterization, methane production anaerobic digestion, and carboxylic acids production through fermentation.

**Chapter 4** focusses on lactic acid fermentation from grass under different operational conditions. Effect of acetate accumulation on lactic acid fermentation is also investigated, both from a functional and from a microbial community standpoint. Thermophilic fermentation are also performed.

**Chapter 5** compares the current state of art extraction technology and some of the most promising extraction technology under development for lactic acid recovery from aqueous broth which contains acetic acid as impurity. Effect of pH on extraction of lactic acid and acetic acid is also tested.

**Chapter 6** shows the production of caproic acid and decane via elongation of lactic acid and Kolbe electrolysis. Semi-continuous chain elongation process is shown. Maximum production rate and concentration of caproic acid are also tested.

**Chapter 7** discusses the overall process. It includes conclusions, recommendation for future research and perspectives.
Pretreatment of Biomass
Chapter 2 Pretreatment of Biomass

2.0 Abstract

Ca(OH)$_2$ treatment was applied to enhance methane yield. Different alkali concentration, incubation temperature and duration were evaluated for their effect on methane production and COD conversion efficiency from (non-)extruded biomass during mesophilic anaerobic digestion at lab-scale. An optimum Ca(OH)$_2$ pretreatment for grass is found at 7.5% lime loading at 10 °C for 20 h (37.3% surplus), while mild (50 °C) and high temperatures perform sub-optimal. Ca(OH)$_2$ post-treatment after fast extrusion gives an additional surplus compared to extruded material of 15.2% (grass), 11.2% (maize straw) and 8.2% (sprout stem) regarding methane production. COD conversion improves accordingly, with additional improvements of 10.3% (grass), 9.0% (maize straw) and 6.8% (sprout stem) by Ca(OH)$_2$ post-treatment. Therefore, Ca(OH)$_2$ pretreatment and post-treatment at low temperature generate an additional effect regarding methane production and COD conversion efficiency. Fast extrusion gives a higher energy efficiency ratio compared to slow extrusion.

![Image](image.png)

**Figure 2.1** Pilot scale biomass extruder and illustration of its internal twin screw structure

2.1 Introduction

Over the past decades, lignocellulosic biomass has become an increasingly important renewable resource for biogas and biochemicals. Today, grass is as yet unused for this despite its wide availability in temperate regions. Grass can be available from nature, agriculture and roadside clipping, although the latter is less attractive due to the impurities which require prior removal steps before further processing can take place. By considering the economic and environmental impacts, previous study has also shown that grass is a suitable feedstock for biogas production (Prochnow et
al., 2009). Next to grass, maize straw and sprout stem are of major interest due to the big scale plantation of the crops, hence a potential input for energy and chemical production.

Depending on the type of feedstock, 40–65% of the chemical oxygen demand (COD) of typical plant biomass is converted to biogas when digested without pretreatment (Weiland, 1993), whereas digestate exiting the anaerobic digester can be composted and thereby returned as fertilizer and soil improvement to arable land, the effective economic value tends to be low. The biodegradability of plant biomass is limited due to the recalcitrant nature of lignin and its structural carbohydrates. Therefore, pretreatment decreasing particle size and hydrolysing (part of) the lignocellulosic biomass is considered critical. While the effects of different pretreatment methods with various feedstock on anaerobic digestion have been compared (Carlsson et al., 2012), chemical alkali treatment and thermomechanical extrusion are the main focuses of this study.

Chemical pretreatment typically aims at depolymerisation of the biomass. The approaches most commonly researched are the use of acids, bases and peroxides. Alkali substances have been found the most promising for soft wood (Mosier et al., 2005) and it has been shown to be able to remove lignin and release acetate groups from hemicellulose, increasing the vulnerability of the hemicellulose and cellulose structures to enzymatic attack (Chang and Holtzapple, 2000). Among these, sodium hydroxide is the most effective, but the chemical is costly when applied on large scale. Therefore, Ca(OH)$_2$ seems to be an alternative worthwhile investigating, as it relies on the same working principle but is 8–10 times cheaper. However, its effectiveness does require an adequate temperature and contact time. Thermo-chemical pretreatments are often carried out at high temperature (80–120 °C) (Gonzalez et al., 2013; Sierra et al., 2009), while mild (50 °C) and ambient (21 °C) temperatures have also been investigated (Xu et al., 2010). However lower temperature ranges such as 10 °C mimicking the ambient temperature of north and central Europe for extensive time intervals have not been tested before. This could be interesting, as calcium hydroxide has a different property where the solubility of the chemical increases with decrease in temperature (Grieve et al., 2011).

Combination of alkali and extrusion have also been investigated in the past by Kang et al. (2013) for ethanol production and Zhang et al. (2012) for glucose yield. Sodium hydroxide was added before the extrusion, resulting in an increase of 20–60% glucose yield versus untreated corn stover. In contrast to alkali treatment, extrusion is a thermo-mechanical operation which results in reduction of biomass particle size as well as depolymerisation of lignin, hemicellulose and cellulose. The process involves compression at the centre of a barrel and expansion at the end,
which breaks down the biomass structure due to the shearing, friction and pressure release. To our knowledge, the effect of Ca(OH)$_2$ has not yet been tested on extruded biomass. Moreover, the combination of alkaline treatment after extrusion (alkaline post-treatment) is novel, although the alkaline treatment could benefit from the increase in surface ratio of the extruded biomass. As both pretreatments have different working principles, added value is expected.

The objective of the current study is to investigate the optimal effect of Ca(OH)$_2$ in terms of a broad temperature range, duration and concentration for methane conversion on biomass. Furthermore, this study wants to pinpoint if Ca(OH)$_2$ after extrusion of biomass is still effective in giving a methane surplus. In the first phase Ca(OH)$_2$ dosing was optimized using a CCD (Central Composite Design) in terms of temperature, duration and lime loading for grass. Secondly, the effect of extrusion on three biomass (grass, maize straw and sprout stem) was determined. Finally Ca(OH)$_2$ was applied on these extruded biomass to estimate the additional effect of combined (mechano-thermo-chemical) treatment. The energy input for extrusion of biomass was also measured and related to the energy surplus obtained from extra methane production to assess the energetic feasibility of the pretreatment process.

2.2 Materials and Methods

2.2.1 Materials

Biomass including maize straw, grass (from landscape, extensive management) and sprout stem (Brassica oleracea convar. oleracea var. gemmifera) were harvested during October 2012 and kindly provided by Inagro vzw (West Flanders, Belgium). All biomass was manually size reduced to less than 5 mm with scissors before storage. Grass, used for the Ca(OH)$_2$ Central Composite Design (CCD) experiment was stored in vacuum bags at 4 °C until usage. For the extrusion test, biomass was stored in vacuum bags at −20 °C until usage.

2.2.2 Pretreatment of grass with Ca(OH)$_2$

A 3 factor 3 level Central Composite Design (CCD) (Box et al., 1978) was carried out to determine the optimum condition for grass pretreatment with Ca(OH)$_2$. Alkali loadings, times and temperatures were arrayed using central composite design. Controls without Ca(OH)$_2$ addition or extrusion were included. All Ca(OH)$_2$ treatments were charged with a water loading of 10 g water per g total solid (Chang et al., 1997). Design Expert® 9.0.2.0 (Statease, Inc. Minneapolis, USA) was used to generate the experimental runs and the experimental data was analysed using response surface methodology (RSM) (Box et al., 1978). Experiments were carried out in two triplicated
blocks. In total, 15 conditions were tested, including the centre point. The control (non-treated) was run simultaneously in triplicate (Table 2.1). One way analysis of variance (ANOVA) was used to test the variation in mean values of methane produced and the fitness of model for increase in methane production.

**Table 2.1** Experimental results of a three factor (lime loading, temperature and time) Central Composite Design. The increase in methane production is calculated versus non-treated grass.

<table>
<thead>
<tr>
<th>Run No. replicate</th>
<th>Lime loading (g / 100 g TS)</th>
<th>Temperature (°C)</th>
<th>Time (minutes)</th>
<th>% increase in methane production</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>2.5</td>
<td>10</td>
<td>300</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>7.5</td>
<td>10</td>
<td>300</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>2.5</td>
<td>92</td>
<td>300</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>7.5</td>
<td>92</td>
<td>300</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>2.5</td>
<td>10</td>
<td>1150</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>7.5</td>
<td>10</td>
<td>1150</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>2.5</td>
<td>92</td>
<td>1150</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>7.5</td>
<td>92</td>
<td>1150</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>0.8</td>
<td>51</td>
<td>725</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>9.2</td>
<td>51</td>
<td>725</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>5.0</td>
<td>-18</td>
<td>725</td>
</tr>
<tr>
<td>12</td>
<td>3</td>
<td>5.0</td>
<td>120</td>
<td>725</td>
</tr>
<tr>
<td>13</td>
<td>3</td>
<td>5.0</td>
<td>51</td>
<td>10</td>
</tr>
<tr>
<td>14</td>
<td>3</td>
<td>5.0</td>
<td>51</td>
<td>1440</td>
</tr>
<tr>
<td>15</td>
<td>6</td>
<td>5.0</td>
<td>51</td>
<td>725</td>
</tr>
</tbody>
</table>

### 2.2.3 Extrusion and Ca(OH)$_2$ post-treatment of grass, maize straw and sprout stem

A pilot scale twin-screw extruder (model MSZK, Laborextruder 4 kW, Lehmann Germany) was kindly provided by Bioliquid (Raalte, the Netherlands). About five kilograms of fresh biomass (grass, maize straw and sprout stem respectively) were extruded at fast rate (60 rpm and 90 °C) and slow rate (15 rpm and 60 °C). The temperature rise was purely due to the friction between the screw and the biomass during the extrusion operation and no extra heating was applied. Non-extruded controls were included for each biomass. Part of the extruded biomass were further post-treated with 7.5% Ca(OH)$_2$ at low temperature (10 °C) and mild temperature (50 °C) for 24 hours.

### 2.2.4 Biochemical methane potential (BMP) test

BMP test was carried out to investigate the effect of the pretreatments on methane yield from the feedstock. The inoculum was acquired from a 200 m$^3$ co-digestion plant (Inagro vzw) digesting cow manure and grass, assuring the inoculum was well adapted for plant feedstock. The batch assays
were set up with reference to the protocol from VDI 4630 (2006). Glass reactors of 500 mL were filled with 300 mL of inoculum, and a corresponding amount of lignocellulosic biomass to achieve a loading ratio of 0.5 g VS inoculum per g VS feedstock. Negative controls (inoculum without feedstock) were also included. The biogas produced was collected in graduated glass cylinders filled with an acidified barrier solution (sulphuric acid at pH 2). The BMP test was carried out at 37 °C and lasted 30 days to evaluate the pretreatment efficiency. Both gas production and gas composition were measured two to three times weekly and the values were normalized to standard temperature and pressure (STP). The rate of biomethanation is represented as the ratio between total methane production of the treated biomass and the total methane production of the untreated control at day 5.

For experiments with Ca(OH)₂ pretreatment and post-treatment, the pH was not adjusted prior to digestion as this incurs an unrealistic cost towards application. Moreover, the buffering capacity of the sludge was able to maintain the pH between 7 and 8 throughout the digestion period. The Ca(OH)₂ pretreatment and post-treatment were carried out in triplicate hence only one BMP test was performed on each replicate. Extrusion was run in batch for grass, maize straw and sprout stem respectively, and hence BMP was tested in triplicate for each batch of biomass. COD conversion efficiency is calculated based on the ratio of COD converted into methane versus initial feedstock COD, with assumption that 1 g of COD gives 350 mL of methane.

2.2.5 Kinetic modelling

A first-order kinetic model - modified Hill model, was used to model the methane production (Kiely et al., 1997). The model was deemed suitable for this study as there was no lag phase in the anaerobic digestion and the experimental data points appeared to follow the curve of first order reaction. The modified Hill model for methane production is described as:

\[ y(t) = y_{\text{max}} \times \frac{(b \cdot c^b \cdot t^{-1})}{(c^b + t^{-b})^2} \]

where \( y_{\text{max}} \) is the maximum methane yield (mL CH₄ per g VS), t is time (days), and b and c are equation coefficients.

2.2.6 Analytical methods

Total solid (TS), volatile solid (VS), ash content, pH, soluble chemical oxygen demand (sCOD) and total Kjeldahl nitrogen (TKN) were measured using the standard method (APHA et al., 1995). The biogas composition was analysed with gas chromatography (GC, Agilent 6980A) with a thermal conductivity detector (TCD) with helium as carrier gas at flow rate of 46.7 mL per minute. Injection
temperature (280 °C), oven temperature (60 °C) and detector temperature (250 °C) were used. The volumes of methane produced were normalized to STP condition (273 K and 101.3 kPa). COD for solid feedstock were measured in accordance to (Zupančič & Roš, 2011) using an electrochemical system due to the difficulty in determining the end point with the dark colour of solution using Ferroin indicator.

The structural carbohydrates and lignin in raw biomass were determined according to Laboratory Analytical Procedures (LAP) developed by National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008). Sugar compounds (mainly glucose and xylose) were analysed using high performance liquid chromatography (HPLC, Agilent Varian ProStar 220 SDM, USA) with a refractive index detector and Rezex H+ column (Aminex) to estimate the hemicellulose and cellulose component of the raw feedstock according to NREL method. Mobile phase of 5 mM H₂SO₄ at flow rate of 0.6 ml per minute and column temperature of 60 °C were used. All analysis were carried out in triplicate.

2.2.7 Power measurement

Power consumption for extrusion was measured with a Fluke 434 (Fluke Industrial B.V., Netherlands) and kindly provided by Lemcko, UGent. The device monitored current, voltage, power and energy throughout the extrusion process. Energy surplus was calculated from the difference between the energy input for extrusion and the extra methane production in terms of energy (40% energy conversion efficiency is assumed). An energy efficiency ratio (energy surplus / energy input) is then calculated to evaluate the performance of extrusion.

2.3 Results and Discussion

2.3.1 Low temperature 10 °C Ca(OH)₂ pretreatment

*Research Question 1: How effective is low temperature calcium hydroxide pretreatment on biomass?*

A temperature as low as 10 °C for the Ca(OH)₂ pretreatment leads to the best result in this study (37.3% increase, run 6) although a rather extensive treatment time is required (19.17 h). An explanation could be the higher solubility of Ca(OH)₂ at lower temperature, which allows more Ca(OH)₂ to dissolve and react with the biomass. Whereas many published studies are performed at room (20 °C), mild (50 °C) and high temperatures, no comparable reference was found in literature for lime pretreatment at 10 °C. Xu et al. (2010) tested lime pretreatment of switchgrass at 21 °C and
found that 96 hours were required to achieve maximum reducing sugar yields, which is 3.43 times that of untreated biomass, while at 50 °C it took only 24 hours (3.61 times) and for 121 °C, 0.5 hour (3.46 times) was needed. In view of our results, this may imply that an even higher increase for run 6 would be possible with a longer treatment. Nevertheless, 37% increase as a result of lime pretreatment, regardless of pretreatment time, is in a good range compared to literature, as other authors have found increases as high as 38.9% with sodium hydroxide pretreatment on grass silage (Xie et al., 2011).

2.3.2 Pretreatment temperature and time effect

When comparing low temperatures combined with short incubation times (run 1 and 2) vs high temperatures with the medium incubation time (run 3 and 4), a higher temperature correlates positively with methane yield (Table 2.1). This is expected as reaction kinetics are slower at low temperatures. However, for long incubation times, low temperatures (run 5 and 6 at 10 °C) result in different outcome versus high temperatures (run 7 and 8 at 92 °C and 120 °C respectively); increased duration at high temperatures decreases the methane production. Run 12 was performed with the highest temperature of the test (120 °C) for a long period, but resulted in only 8% increase. Other authors have described decreasing benefits and even decreases in parameters such as methane production, hydrolysis rate and preservation of carbohydrate compounds due to extreme temperature conditions (Kaar & Holtzapple, 2000). It is well known that high temperature treatments for extended duration can release inhibitory compounds like hydroxymethylfurfural (HMF) and phenols that inhibit the anaerobic digestion process (Oefner et al., 1992). The inoculum for this research was well adapted as it was obtained from a plant which co-digests manure and lignocellulosic biomass such as grass and maize silage. Therefore, no lag phase was observed under normal conditions. After high temperature treatment, the lag phase also remained absent. This might therefore indicate an effect of re-condensation of the biomass after chemo-thermal treatment to less biodegradable structures (Bobleter & Concin, 1979) rather than the production of inhibitory compounds due to “over-treatment” of the biomass. The risk of “over-treatment” of the biomass is present at high temperatures, but these chances are lower or negligible when low temperatures are employed.

The -18 °C treatment results in 20.7% increase, which is the highest for all treatments by the same lime loading, but not in the overall test. Zhao et al. (2008) treated spruce with 7% NaOH / 12% urea solution at -15 °C and obtained up to 70% glucose yield after enzymatic hydrolysis, compared to 13% of untreated spruce. In this study however, the activity of Ca(OH)₂ has completely halted at
Chapter 2

this temperature, so the treatment was solely due to the temperature effect. This was confirmed with a triplate of controls without addition of Ca(OH)$_2$. Although freezing appears to be a good pretreatment as well as preservation method, implementation is complicated due to the high energy demand and logistic challenges of treating the materials.

2.3.3 Ca(OH)$_2$ loadings effect

When comparing lime loadings at equal treatment times and temperatures, a higher lime concentration results in better methane production. Lime loading at 0.8% (run 9) increases the methane production by 4%, 5% lime loading (run 15) increased by 8% and 9.2% (run 10) results in 14% increase of methane release. This trend also consistently occurs in all the pairings, run 1 and 2, 3 and 4, 5 and 6, and 7 and 8 (Table 2.1). Song et al. (2014) compared Ca(OH)$_2$ pretreatment at different concentrations (4%, 6%, 8% and 10%) on corn straw and found that lime loadings up till 10 g per 100 g TS have increasing beneficial effect regarding hydrolysis. In their study, compared to non-treated maize straw, percentage increase in methane yield are 59% (for 4% lime loading), 79% (6%), 106% (8%) and 100% (10%), thus subscribing a similar dose-response effect of lime. In this study, in terms of lime loading, there is no lime overload effect which resulted in a negative response observed due to the optimum range of lime loading chosen (0.8 g to 9.2 g lime per 100 g feedstock).

2.3.4 Statistical analysis and result validation

According to the response surface methodology, points facing outward of the centre point (Run 12, 5 g Ca(OH)$_2$ per 100 g TS, 51 °C, 725 min) have increased methane production (Table 2.1). Only two conditions result in lower or similar methane production than the centre point (run 9 and 12). Therefore, the response surface methodology results in a set of hyperbolic figures with a trough around the centre point. The final equation (actual factor) for the percentage increase in methane production as a function of lime loading (LL), temperature (T) and time (t) is:

\[
\% \text{ increase in methane production} = 11.8 - (1.2 \times LL) + (0.1 \times T) - (0.02 \times t) - (0.03 \times LL \times T) - (0.0004 \times T \times t) + (0.4 \times \text{LL}^2) + (0.003 \times T^2) + (0.00002 \times t^2) \quad -- \quad \text{Eq. 1}
\]

All three factors (lime loading, temperature and time) show significant (p < 0.01) effect on the percentage increase in methane production, as well as the interaction terms between lime loading and temperature, and temperature and time respectively. The response surface model of percentage increase in methane production has a $R^2 = 0.85$ and a significance of fit < 0.0001, indicating that regression fits the data well. To illustrate the hyperbolic nature of the CCD model and the effects as
described below, the hyperbolic plot at highest increase (7.5 g Ca(OH)$_2$ per 100 g TS) is shown (Figure 2.2).

![Response Surface Plot](image)

**Figure 2.2** Response Surface Plot showing the percentage increase (%) in methane production in relationship to time (minutes) and temperature (°C) at lime loading of 7.5 g Ca(OH)$_2$/g volatile solid.

To validate the model derived from the CCD analysis, the observed experimental responses were plotted against the predicted responses for the percentage increase in methane production (Figure 2.3). In general, there is a good correlation found between the observed and predicted values. All three parameters (lime loading, temperature and time) have substantial effect on the response on the outcome. The interactions between these factors are significant and therefore careful consideration is needed when choosing the conditions for maximum efficiency and economic return. As discussed above, both temperature and pretreatment time are of imperative importance towards methane yield, while lime loading is rather concentration dependent with no inhibition effect within the range tested here.
Figure 2.3  Observed experimental responses versus CCD predicted responses of percentage increase in methane production for all experimental points performed. The solid line (˗˗˗) indicates the linear trend found ($R^2 = 0.87$) with dashed lines (---) as 95% confidence interval and dotted lines (···) as 95% prediction interval of predicted values. The symbol of triangles (▲) represent the data points.

Non-treated grass produces $196 \pm 3$ mL CH$_4$ per g VS, which is within the expected range of reported production in anaerobic digestion (Nallathambi Gunaseelan, 1997). A characterisation of the biomass used in this study reveals typical values for grass biomass (Nizami et al., 2009) (Table 2.2). In general, all lime pretreatments perform better than the non-treated grass regarding methane production.

Table 2.2  Characterisation of raw biomass

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Maize Straw</th>
<th>Grass</th>
<th>Sprout Stem</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lignin (%)</td>
<td>18.9 ± 5.8</td>
<td>19.9 ± 2.0</td>
<td>11.3 ± 1.8</td>
</tr>
<tr>
<td>Hemicelluloses (%)</td>
<td>14.3 ± 0.3</td>
<td>12.9 ± 1.0</td>
<td>15.6 ± 1.0</td>
</tr>
<tr>
<td>Cellulose (%)</td>
<td>22.8 ± 0.8</td>
<td>23.7 ± 0.3</td>
<td>21.0 ± 1.0</td>
</tr>
<tr>
<td>Total solid, TS (%)</td>
<td>38.7 ± 0.7</td>
<td>43.6 ± 1.0</td>
<td>20.0 ± 1.2</td>
</tr>
<tr>
<td>Volatile solid, VS (%)</td>
<td>34.5 ± 1.7</td>
<td>35.9 ± 1.2</td>
<td>18.6 ± 0.1</td>
</tr>
<tr>
<td>Chemical Oxygen Demand, COD (mg per g VS)</td>
<td>1122 ± 118</td>
<td>1035 ± 62</td>
<td>987 ± 40</td>
</tr>
<tr>
<td>Total Kjeldahl Nitrogen, TKN (% TS)</td>
<td>0.8 ± &lt;0.1</td>
<td>1.8 ± &lt;0.1</td>
<td>1.6 ± 0.1</td>
</tr>
</tbody>
</table>
2.3.5 Extrusion and subsequent Ca(OH)$_2$ post-treatment on different feedstock

Research Question 2: How much does extrusion improve the digestibility of the biomass?

Methane production increases significantly (26 to 49%) after extrusion and increases even further when post-treated with Ca(OH)$_2$ in most cases. The overall highest total increase (extrusion and Ca(OH)$_2$ post-treatment) is found for grass (53.8% ± 4.3, Figure 2.4), maize straw (47.6 ± 7.1%, Figure 2.4) and sprout stem (38.6 ± 6.2%, Figure 2.4).

After extrusion, Ca(OH)$_2$ post-treatment at 10 °C and 50 °C only lead to a moderate increase in methane production. Ca(OH)$_2$ post-treatment on fast extruded grass obtains the highest value of 15.2 ± 2.1% more surplus with respect to the extruded biomass (Figure 2.4). The post-treatment effect sometimes appears to compensate for a lack of extrusion effect – at least with grass and sprout stem – as there is a higher methane production increase after Ca(OH)$_2$ post-treatment for extruded material with lower yield, resulting in similar combined efficiency (Figure 2.4). With maize straw, post-treatment only results in a positive outcome when the biomass is extruded in a fast operation. The Ca(OH)$_2$ post-treatment further enhances the rate of biomethanation at both temperatures tested, for grass (1.15 ± 0.04 times versus extruded, Figure 2.5a) and maize straw (1.20 ± 0.03 times, Figure 2.5b). The surplus is modest for sprout stem (1.08 ± 0.01 times) with slow extrusion followed by 50 °C Ca(OH)$_2$ post-treatment (Figure 2.5c), but this can be expected as the initial methane production rate is already very high. An additional effect of Ca(OH)$_2$ in surplus to the extrusion indicates that Ca(OH)$_2$ treatment is of a different nature than extrusion. The change in structure after extrusion, most likely the increased surface area, benefits the action of Ca(OH)$_2$. Extrusion makes the biomass and its organic acids more accessible. Ca(OH)$_2$ tends to neutralise the organic acids in a saponification reaction, further enhancing the biomass accessibility to microbial breakdown (Tarkow & Feist, 1969).

To test whether there was a different outcome for post-treatment regarding the applied temperature, extruded biomass was post-treated at two different temperatures. The Ca(OH)$_2$ post-treatment at 50 °C and 10 °C lead to similar methanogenesis, except for fast extruded sprout stem and slow extruded maize straw (Figure 2.4). Clearly, the importance of temperature on the Ca(OH)$_2$ efficiency is less important when applied after extrusion, as both 10 °C and 50 °C after extrusion and Ca(OH)$_2$ treatment result in comparable outcome.

Regarding the methane production curve, an increase of methane production is typically seen throughout the digestion period in comparison to the non-extruded material (Figure 2.5). Extrusion
and post-treatment result in a high increase of rate of biomethanation (mL CH₄ per g VS per day) for grass (1.46 ± 0.05 times, Figure 2.5a) and maize straw (1.65 ± 0.06 times, Figure 2.5b) versus non-treated.

For sprout stem, when compared to the untreated biomass, the increase in rate of methane production is more subtle, with only 1.18 ± 0.06 times faster compared to non-treated sprout stem (Figure 2.5c). Sprout stem contains less lignin (Table 2.2) and is therefore assumed to be already fairly accessible for microbial breakdown, explaining the more subtle increase.

For all biomass used, extrusion severely impacts the biomass structure by significantly reducing particle size, as seen by visual observation. When comparing fast versus slow extrusion (Figure 2.4), grass results in higher methane production for slow extrusion (48.8 ± 2.1% increase versus non-treated) versus fast extrusion (35.1 ± 0.8%). There is no significant difference between fast (36.4 ± 6.9%) and slow (36.3 ± 4.7%) extrusion for maize straw, and for sprout stem (29.6 ± 3.2% versus 26.2 ± 5.9% for fast and slow respectively). The rate of methane production increases by slow extrusion for grass (1.27 ± 0.01 versus non-treated), maize straw (1.37 ± 0.04) and sprout stem (1.09 ± 0.05) respectively. Hjorth et al. (2011) extruded various lignocellulosic biomass including grass and barley straw, and found that the methane yield increased by 18 to 70% after 28 days of anaerobic digestion, while extrusion increased methane yields by 8 to 27% after 30 days of anaerobic digestion with grass and maize on industrial scale (Brückner et al., 2007), therefore confirming the ranges found in previous studies. Extrusion screw speed has in the past been studied extensively, with limited agreement on results (Karunanithy et al., 2013). Here, two extrusion rates were tested and no significant differences for maize straw and sprout stem were found while slow extrusion yielded higher methane production than fast extrusion for grass. Therefore, this study is also not conclusive regarding extrusion speed.

Figure 2.4 Effect of extrusion and Ca(OH)₂ post-treatment on percentage increase in methane production
Figure 2.5 Typical methane production curves (models) for (a) grass (b) maize straw and (c) sprout stem with no extrusion (▪ ▪ ▪), slow extrusion (- - -) and slow extrusion with calcium hydroxide post-treatment at 50 °C (---). For reason of clarity, standard deviations are not shown. The symbols of squares (■) represent the data point for non-treated feedstock, triangles represent
(▲) extruded-only feedstock, and circles (●) represent extruded and calcium hydroxide post- treated feedstock.

The COD conversion efficiency corresponds well with the increase in methane yield, indicating a good agreement for both parameters (Figure 2.6). The overall highest total increase in COD conversion efficiency for combined treatments are 82.9 ± 2.2%, 89.3 ± 3.0% and 92.7 ± 4.2% for grass, maize straw and sprout stem, respectively. The soluble COD did not fluctuate much between the beginning and end of the anaerobic digestion test, confirming the conversion of biomass into biogas instead of solubilizing without conversion. The pH of the digester content was between 7 and 8 before starting and at the end of the anaerobic digestion test thus well within the range of methane production.

**Figure 2.6** Total methane production vs total% COD converted ($R^2 = 0.82$) for extruded feedstock with and without calcium hydroxide post-treatment

### 2.3.6 Energetic assessment of extrusion

**Research Question 3: Is the energy gain higher than the energy input for extrusion pretreatment?**

While it is difficult to quantify the calcium hydroxide treatment in the form of energy input, the energy consumption during extrusion was measured for only grass and an energy efficiency ratio was calculated (Table 2.3). Clearly, fast extrusion consumed less energy than slow extrusion, hence resulting in a higher energy efficiency ratio. As grass contains the highest solid content, the energy consumption on grass was used as a maximum reference for estimation of energy efficiency ratio.
for extrusion. Fast extrusion consumed 0.0867 ± 0.0021 kWh per kg grass while slow extrusion required 0.140 ± 0.009 kWh per kg grass. The trend is consistent with the study of Deng et al. (2014) and the energy consumption is also within the range and magnitude of previous study (Chen et al., 2011). Slow extrusion delivers a better increase in methane yield for grass, while there is no difference for maize straw and sprout stem. By considering the process time and return on energy input, fast extrusion would be preferred in this study for pretreatment of the biomass tested. On practical consideration, extrusion which results in significant particle size reduction (by visual), allows more condensed storage and efficient use of space, and eventually lowers transportation cost if extrusion is carried out at the biomass production or collection site. Whereas physical pretreatments to decrease particle size are effective, they are generally highly energy intensive (Bruni et al., 2010). Extrusion has been found to be less relatively less energy demanding compared to milling (Chen et al., 2014). In comparison to the other physical processes, Cadoche & López (1989) found that energy input for particle size reduction between 3 to 6 mm can be maintained under 30 kWh per tonne for a multi-feedstock process plant. Hence, the surpluses found in this study agree well with the previous studies.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Extrusion</th>
<th>Energy input (kWh / kg biomass)</th>
<th>Energy surplus (kWh / kg biomass)</th>
<th>Energy efficiency ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grass</td>
<td>Fast</td>
<td>0.0867 ± 0.0021</td>
<td>0.115 ± 0.005</td>
<td>1.326</td>
</tr>
<tr>
<td></td>
<td>Slow</td>
<td>0.140 ± 0.009</td>
<td>0.160 ± 0.014</td>
<td>1.143</td>
</tr>
<tr>
<td>Maize straw</td>
<td>Fast</td>
<td>ND*</td>
<td>0.097 ± 0.018</td>
<td>1.119</td>
</tr>
<tr>
<td></td>
<td>Slow</td>
<td>ND*</td>
<td>0.097 ± 0.013</td>
<td>0.693</td>
</tr>
<tr>
<td>Sprout stem</td>
<td>Fast</td>
<td>ND*</td>
<td>0.052 ± 0.005</td>
<td>0.600</td>
</tr>
<tr>
<td></td>
<td>Slow</td>
<td>ND*</td>
<td>0.034 ± 0.010</td>
<td>0.243</td>
</tr>
</tbody>
</table>

* ND = not determined.

Out of the three substrates tested in this study, grass shows the most significant improvement in biodegradability with the pretreatment methods tested. Considering the scale and potential of grass, it is chosen as the substrate for the next phase storage and conversion test.
Storage of biomass
Chapter 3 Storage of biomass

3.0 Abstract
Grass is an abundant feedstock which is commonly used as animal fodder. It can also be utilized for bio-production, however its availability is generally not consistent through time. Storage is therefore an economic necessity to ensure continuous, steady supply for bioprocessing. Extrusion tests were performed, before or after Ca(OH)$_2$ addition (75, 100 or 200 g kg$^{-1}$ total solid (TS) of grass Ca(OH)$_2$), with the objective to achieve 3 months storability without major carbon loss. The performances were determined by the changes in biomass characterisation, methane production through anaerobic digestion and carboxylate production through fermentation, in three conditions: fresh, and after ensiling or wilting for 3 months. For wilting, addition of 100 and 200 g kg$^{-1}$ TS Ca(OH)$_2$ before extrusion, and 200 g kg$^{-1}$ TS Ca(OH)$_2$ after extrusion significantly preserved the biomass. For ensiling, the biomass were well preserved. Ensiling with addition of 100 g kg$^{-1}$ TS Ca(OH)$_2$ before extrusion was the optimum preservation method in this study, with methane production of 237 ± 10 (test) vs 265 ± 29 (initial control) cm$^3$ g$^{-1}$ VS$_{initial}$ CH$_4$, and carboxylate production of 124 ± 8 (test) vs 109 ± 4 (initial control) mg g$^{-1}$ VS$_{initial}$ Carbon. Especially when storage conditions were sub-optimal (wilting), addition of 100 g kg$^{-1}$ TS Ca(OH)$_2$ before extrusion outperformed the non-treated, wilted condition to a large extent (178 ± 18 vs 50 ± 8 cm$^3$ g$^{-1}$ VS$_{initial}$ CH$_4$ and 86 ± 17 vs 18 ± 4 mg g$^{-1}$ VS$_{initial}$ Carbon. Overall, combined extrusion and alkali pretreatment improves grass storability.

3.1 Introduction
Lignocellulosic biomass can be a renewable resource for biogas and biochemicals such as carboxylic and hydroxycarboxylic acids (Agler et al., 2011). Grass, as one of the most abundant plant families on Earth, has become an interesting and fitting target for this purpose. It is a readily available waste stream with low economic value and limited applications. In Flanders alone (north part of Belgium), grasslands represent an estimated $42 \times 10^6$ kg of available biomass annually (Verbeke, 2012). Despite the advantages, the supply of the biomass occurs decentralized and production is not consistent throughout the year, with most of them harvested only twice a year. A preservation method would play an important role to ensure a continuous and thus more economic supply towards the bio-industry, including the possibility to store grass locally until needed for centrally processing.
In the past, several methods have been investigated including ensiling, drying and pelletizing, use of chemicals such as acid and alkali and among others on farm storage systems (EPA, 2007). The most common approach to store grass is ensiling which is nowadays commonly assisted by biological additives such as lactic acid bacteria (Pakarinen et al., 2008). During ensiling, lactic acid produced lowers the pH to around 4 and inhibits the growth of other microorganisms, hence preserving the biomass (Bolsen et al., 1996). If optimal conditions for ensilage are not met, e.g. there is a low density of biomass or a high buffer capacity present, microbial degradation may occur in the silage due to bacterial and fungal growth. A high moisture content of biomass induces a secondary *Clostridia* based fermentation, producing butyric acid, which leads to a poor quality ensilage (Muck et al., 2001). Key parameters that have to be taken into consideration for ensiling include dry matter content, buffering capacity, water soluble carbohydrates and water activity (Pahlow et al., 2003).

Next to ensiling, harvested biomass can be left in open-air condition to wilt for a short period to reduce the moisture content of biomass in order to improve storage. However, if wilting is prolonged, biomass can be degraded biologically. Rigdon et al. (2013) found that biomass which is stored uncovered for 6 months led to a dramatic decrease in total solid content from 0.88 to 0.60 g total solid g\(^{-1}\) sorghum.

Herrmann et al. (2012) investigated the effect of particle size reduction on ensiling for biogas production and found that short chopping length (6 to 33 mm) gives a maximum increase in methane yield afterwards. Extrusion also leads to particle size reduction by shear, which may lead to an improved biodegradability and easier growth of microorganisms beneficial for biomass conservation such as lactic acid bacteria, hence improving the efficiency of ensiling for storage purpose (Müller et al., 2009). It is currently a promising pretreatment method for lignocellulosic biomass due to its versatility and ease of process modification, the continuous process operation and ease of scaling up, and lower energy consumption compared to other comminution technologies (Hjorth et al., 2011).

Next to physical methods such as extrusion, acid and alkali addition have also been widely studied for their applications in biomass treatment (Agbor et al., 2011). Sulphuric acid and calcium hydroxide have both been tested on perennial grass as on-farm pretreatment for storability and both showed promising results, acid treatment gave a conversion of cellulose to ethanol of 0.16 to 0.83 mol mol\(^{-1}\) ethanol over cellulose, while alkali treatment yielded 0.18 to 0.55 mol mol\(^{-1}\) ethanol over cellulose (Digman et al., 2010). Sodium hydroxide is also often used as a chemical pretreatment (Wang et al., 2010). Calcium hydroxide would be considerably cheaper, but is more difficult for
application due to it being solid and having a low water solubility (1.6 kg m\(^{-3}\) at 293 K and 0.7 kg m\(^{-3}\) at 373 K) (Oates, 2007).

Considering the above, the objective of this study was to investigate the possibility of combining extrusion and Ca(OH)\(_2\) pretreatment to improve storability and availability for biodegradation after storage of the lignocellulosic biomass (grass). Three Ca(OH)\(_2\) concentrations (i) 75, (ii) 100 or (iii) 200 g kg\(^{-1}\) total solid of grass Ca(OH)\(_2\) were added either (i) before or (ii) after extrusion, and these were compared to a set of controls with no treatment and extrusion-treated only biomass. The biomass were scored under three conditions, namely (i) fresh, (ii) ensiling and (iii) wilting. The (i) biomass compositions, (ii) methane yield and (iii) carboxylate production were recorded initially and after 3 months of storage to compare the effectiveness of the pretreatment.

3.2 Materials and Methods

3.2.1 Materials

Lignocellulosic biomass – grass (from landscape, West Flanders, extensive management, harvested on September 2014) was harvested using a flail mower and kindly provided by Inagro (West Flanders, Belgium). The grass was coarsely sieved to remove fine particles and the length distributions of grass were 2 to 5 cm. The grass was stored in vacuum bags at 4 °C until usage.

3.2.2 Process overview

In this study, the grass was subjected to a series of process steps including pretreatment, storage, anaerobic digestion and fermentation. The overall process scheme is presented in Figure 3.1, and each of the processes are described in detail in the following sections.

![Figure 3.1](image_url)
3.2.3 Pretreatment
The pilot scale twin-screw extruder (MSZ B 22e, 2 x 11 kW) was kindly supplied by Lehmann-maschinenbau (Pohl, Germany). The grass was split into two batches, one batch was not extruded, and the other batch was extruded at rotation speed of 6.28 rad s\(^{-1}\), with or without alkali addition. 5 kg of grass was used in each treatment to ensure that the extrusion chamber was filled and biomass was extruded. The mass flow of grass through the extruder was approximately 0.2 kg s\(^{-1}\). Three concentrations of Ca(OH)\(_2\), (i) 75, (ii) 100, and (iii) 200 g kg\(^{-1}\) total solid Ca(OH)\(_2\) of grass, were applied as dry solid on grass and mixed thoroughly by manual operation, either (i) before or (ii) after extrusion. After pretreatment, treated and non-treated grass were further split into three sub-batches. One batch was incubated for 24 hours to allow for hydrolysis before anaerobic digestion and fermentation (for fresh condition). The other two sub-batches were used for storage test.

3.2.4 Storage
The two sub-batches of grass were stored at room temperature (average 18 ± 3 °C) for 3 months (90 days) for storability test. Two storage conditions were used – (i) ensiling and (ii) wilting. For ensiling, 5 g of grass were stored in 8 cm\(^3\) glass vials to ensure a minimum density of 600 kg m\(^{-3}\) fresh material (180 kg m\(^{-3}\) dry matter, which was well within the recommended range of 140 – 260 kg m\(^{-3}\) dry matter required for ensiling in the study of Honig et al. (1991). The glass vial was sealed with butyl rubber to minimize oxygen penetration. While for wilting, 5 g of grass were stored in 100 cm\(^3\), non-gas-tight, transparent plastic containers, which was 50 kg m\(^{-3}\) fresh material. Weights of grass were measured gravimetrically before and after storage to determine the weight losses, this was expressed as volatile solid loss, in g g\(^{-1}\) VS\(_{\text{initial}}\). For each combination of pretreatment and storage, a triplicate test was performed. As 24 conditions (resulting from combination of pretreatment and storage method, including non-treated grass) with 3 replicates were tested, an overall total of 72 tests were performed.

3.2.5 Biochemical Methane Potential (BMP) test
BMP test was performed to study the effect of the pretreatment and storage on methane yield from the substrates. The performance was scored in three conditions: fresh (24 hours after pretreatment or non-treated), and after ensiling or wilting for 3 months. The inoculum (total solid 58.2 ± 3.2 g kg\(^{-1}\) inoculum, volatile solid 38.8 ± 3.4 g kg\(^{-1}\) inoculum) was obtained from a 200 m\(^3\) co-digestion plant digesting cow manure and grass, assuring the inoculum was well adapted for lignocellulosic substrates. The batch assays were set up according to Khor et al. (2015). Glass bottles of 500 cm\(^3\) were filled with 300 cm\(^3\) of inoculum, and a corresponding amount of lignocellulosic biomass to
achieve a loading ratio below 0.5 g g\(^{-1}\) VS inoculum over VS substrate. Negative controls (only inoculum and water, without feedstock) were also included to take into account the biogas production from the VS of inoculum itself. The biogas produced was collected in graduated glass cylinders filled with an acidified barrier solution (sulphuric acid at pH 2). The BMP test was carried out at 37 °C and lasted 30 days to evaluate the pretreatment efficiency. Both gas production and gas composition were measured two to three times weekly. The methane production was always compared to the initial biomass (before pretreatment and storage) to take into account VS loss, and it was expressed as cm\(^3\) g\(^{-1}\) VS\(_{\text{initial}}\). The anaerobic digestion test was terminated after 30 days as the daily biogas production rate was less than volume fraction of 1% of the total biogas produced, and to allow comparison between the tests before and after storage.

### 3.2.6 Fermentation test

Batch fermentation was performed simultaneously to investigate the fermentability of the treated biomass to carboxylates. The performance was scored in three conditions: fresh (24 hours after pretreatment), and after ensiling or wilting for 3 months. Fermentation was carried out in 20 cm\(^3\) serum bottles for 8 days, for each of the pretreatment and storage replica. Each serum bottle was filled with 1 g of substrate, 9 cm\(^3\) of M9 medium and 1 cm\(^3\) of rumen fluid. Compositions of M9 medium were 8.5 kg m\(^{-3}\) Na\(_2\)HPO\(_4\), 3.0 kg m\(^{-3}\) KH\(_2\)PO\(_4\), 0.5 kg m\(^{-3}\) NaCl, 1.0 kg m\(^{-3}\) NH\(_4\)Cl, 0.24 kg m\(^{-3}\) MgSO\(_4\), 0.011 kg m\(^{-3}\) CaCl\(_2\). Negative controls (inoculum without substrate) were also included. The bottles were flushed with nitrogen and then placed on a shaker at 30 °C. Pressure inside the bottles was measured, and liquid and gas samples were taken on day 0, 2, 4 and 8. pH was not controlled throughout the experiment, except the bottles with 200 g kg\(^{-1}\) TS Ca(OH)\(_2\), where the pH was adjusted to 7 at the beginning of experiment. The carboxylate production was always compared to the initial biomass, and it was expressed as mg g\(^{-1}\) VS\(_{\text{initial}}\).Carbon.

### 3.2.7 Analytical methods

Total solid (TS), volatile solid (VS), ash content, soluble chemical oxygen demand (sCOD), solid COD, soluble ammonium ion (NH\(_4^+\)), and biogas composition were measured using gas chromatography (GC, Agilent 6980A), according to Khor et al. (2015). The GC was equipped with a thermal conductivity detector (TCD) with helium as carrier gas at flow rate of 0.778 cm\(^3\) s\(^{-1}\). Injection temperature (280 °C), oven temperature (60 °C) and detector temperature (250 °C) were used. The volumes of methane produced were normalized to STP condition (273 K and 101.3 kPa). Changes in biomass weight and biomass VS were expressed in term of g g\(^{-1}\) VS\(_{\text{initial}}\). The TS measurement was corrected according to volatilization coefficient for grass silage proposed by
Porter et al. (2001). pH before and after storage were measured by adding 0.4 g of grass in 10 cm$^3$ of distilled water. Determination of fermentation products including organic acids (lactic acid, formic acid, acetic acid, propionic acid, butyric acid), was performed with 930 Compact ion chromatography (IC) Flex system (Metrohm, Switzerland) with inline bicarbonate removal. The IC is equipped with Metrosep organic acids (250/7.8) column and Metrosep organic acids (4.6) guard column, and an 850 IC conductivity detector. Detection was enhanced using a chemical suppression module to replace protons with Li-cations (0.5 mol dm$^{-3}$ LiCl as regenerant for suppressor). The eluent was 1 mmol dm$^{-3}$ H$_2$SO$_4$ at flow rate of $8.33 \times 10^{-3}$ cm$^3$ s$^{-1}$ and the oven temperature was set at 35 °C. Ethanol and glycerol were analysed using 930 Compact ion chromatography (IC) Flex system (Metrohm, Switzerland), equipped with a Metrosep Carb 2 (250/4.0) column, a Metrosep Trap 1 100/4.0 guard column and an IC amperometric detector. The oven temperature was set at 35 °C, the eluent was 20 mmol dm$^{-3}$ NaOH at flow rate of $1.33 \times 10^{-2}$ cm$^3$ s$^{-1}$, and 0.5 mol dm$^{-3}$ LiCl was used as regenerant for suppressor.

### 3.2.8 Statistical analysis

One-way ANOVA was used to determine the significant differences in the mean values of the VS loss, methane production and carboxylate production. Tukey’s range test was performed as a post-hoc analysis to pinpoint the mean values which are significantly different from each other between each treatment. The analysis was carried out with a 95% confidence interval.

### 3.3 Results and Discussion

#### 3.3.1 Effect of pretreatment on the biomass storability in terms of biomass properties

Research Question 4: How do extrusion and calcium hydroxide pretreatment affect the biomass characterization and storability?

After 3 months of wilting, treatments with 100 and 200 g kg$^{-1}$ TS Ca(OH)$_2$ before extrusion, and 200 g kg$^{-1}$ TS Ca(OH)$_2$ after extrusion, showed significantly lower VS loss compared to the other experiments ($p < 0.05$, Table 3.1). This indicates that a minimum dose threshold of Ca(OH)$_2$ is required for preservation of biomass under wilting condition. Regarding carboxylates, only the treatments with 100 and 200 g kg$^{-1}$ TS Ca(OH)$_2$ before extrusion and 200 g kg$^{-1}$ TS Ca(OH)$_2$ after extrusion retained the carboxylates (Table 3.2), which agrees with the result of VS loss.
Ensiling performed better than wilting for every treatment considering VS loss, with or without Ca(OH)$_2$ addition (Table 3.1). For each treatment followed by ensiling, after 3 months of storage, the biomass had minimal VS loss, compared to its starting point (Table 3.1). The pretreatment combinations did not show much difference in terms of VS loss, also when compared to non-treated grass (Table 3.1, $p = 0.13$), also the treatments did not diminish the biomass VS in ensiling condition. The changes in weight were negligible for all conditions with ensiling, which was shown with the changes in VS (Table 3.1), this agrees with the study of Kreuger et al. (2011). In terms of carboxylate production, all treatments had an increase in carboxylate concentration after 3 months of ensiling (Table 3.2). For treatments without Ca(OH)$_2$ addition, the normal ensiling process occurred, where organic acids were produced, resulting in a drop in pH and growth inhibition of microorganisms (Pahlow et al., 2003). While for the treatment with Ca(OH)$_2$ addition, Ca(OH)$_2$ addition increased the release of organic acid (especially acetic acid), as in the study of Digman et al. (2010).

In terms of pH, for wilting, final pH of non-treated and extrusion-only grass increased versus initial pH (Table 3.1). This indicated the biological activity and organic acids were metabolized. For 75 and 100 g kg$^{-1}$ TS Ca(OH)$_2$ after extrusion, the pH change was not only due to the Ca(OH)$_2$ addition, but also associated to biological activity as there was a high VS loss (Table 3.1). For 100 and 200 g kg$^{-1}$ TS Ca(OH)$_2$ before extrusion and 200 g kg$^{-1}$ TS Ca(OH)$_2$ after extrusion, the decrease of pH was due to alkaline hydrolysis since the VS was preserved to a certain extent (Table 3.1). For ensiling, there was a drop in pH for all treatments. For non-treated and extruded-only grass, organic acids were produced during ensiling, lowering the pH and therefore preserving the biomass by preventing the growth of other microorganisms. In ensiling of grass, typically a pH of 4.2 or less indicates good quality (De Man, 1952). Comparing between the grass with no treatment and with only extrusion, the extruded grass achieved a lower pH ($4.1 \pm 0.1$) than the non-treated grass ($4.8 \pm 0.1$) (Table 3.1), which indicated the improvement in biodegradability of the biomass due to pretreatment as more volatile fatty acids were produced (Agbor et al., 2011). For the treatments with Ca(OH)$_2$ loading, the pH remained alkaline (Table 3.1) although there was a pH drop due to chemical hydrolysis.

Some pretreatments can induce losses in TS and VS due to aggressive reactions such as high temperature, high sheer, or chemical reactions (Ariunbaatar et al., 2014). Before storage, combinations of pretreatment did not change the TS and VS of the biomass significantly (Table 3.1), indicating no significant loss of organic matter by the pretreatments. The treatments increased
the soluble chemical oxygen demand (sCOD) (Table 3.1), which indicated solubilisation of (hemi)cellulose into lower molecular weight organic compounds. The initial pH of extrusion-only grass was also lower than that of no treatment, suggesting the release of intracellular organic acids from acetyl groups of hemicellulose fraction (Table 3.2) (Karunanithy & Muthukumarappan, 2011), while the initial pH of all treatments with Ca(OH)\(_2\) remained alkaline. In terms of NH\(_4^+\) concentration, there were not much changes before and after pretreatment combinations. This served as a good indicator for the treatment intensity, as there was no significant increase in NH\(_4^+\) the biomass was not over-treated and protein was not degraded (Papadopoulos & McKersie, 1983). The increase in sCOD and constant NH\(_4^+\) concentration showed that the pretreatment was effective in making organic compounds accessible due to solubilisation while maintaining the biomass protein composition. There was also an increase in initial total carboxylate content in all treatment combinations compared to the non-treated grass (Table 3.2).

Addition of alkali before extrusion was more successful in preserving the biomass, by considering addition of 100 g kg\(^{-1}\) TS Ca(OH)\(_2\) before or after extrusion. This was likely due to intense mixing of the chemical with the biomass, as mentioned before a typical issue with Ca(OH)\(_2\) is its low solubility which makes good distribution complicated. Extrusion increased the surface area of biomass, thus making it more available for interaction with the chemical; while addition of Ca(OH)\(_2\) helped to prevent microbial growth by raising the pH and effectively preserve the biomass quality. The solubilisation of Ca(OH)\(_2\) was done with just the moisture of biomass itself and no additional water was added, hence a lower chemical activity was expected since previous research has shown that 10 g g\(^{-1}\) water over Ca(OH)\(_2\) is the optimum amount (Chang et al., 1997). This may explain the difference in pretreatment effectiveness in this study. The rationale of using 75 g kg\(^{-1}\) TS Ca(OH)\(_2\) was based on the optimal result obtained in previous pretreatment study of Khor et al. (2015), 100 g kg\(^{-1}\) TS Ca(OH)\(_2\) was selected to allow for depletion during the storage period, while 200 g kg\(^{-1}\) TS Ca(OH)\(_2\) was chosen as an overdose to investigate the effect on storage.
### Table 3.1 Characterization of biomass before (fresh) and after (final) storage (in triplicate). (Tukey’s range test, \( p < 0.05 \), superscript ‘a’ and ‘b’ mean statistically significant when compared within each condition).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TS fresh (g g(^{-1}) grass)</th>
<th>VS fresh (g g(^{-1}) grass)</th>
<th>sCOD fresh (g g(^{-1}) VS)</th>
<th>NH(_4^+) fresh (mg g(^{-1}) TS)</th>
<th>VS loss (g g(^{-1}) VS(_{\text{initial}}))</th>
<th>pH fresh</th>
<th>pH final</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>0.30 ± 0.01</td>
<td>0.24 ± 0.02(^\ast)</td>
<td>0.13</td>
<td>3.3</td>
<td>0.06 ± 0.03</td>
<td>7.2</td>
<td>4.8 ± 0.1</td>
</tr>
<tr>
<td>Extrusion only</td>
<td>0.29 ± 0.02</td>
<td>0.24 ± 0.02</td>
<td>0.17</td>
<td>2.7</td>
<td>0.03 ± 0.01</td>
<td>6.0</td>
<td>4.1 ± 0.1</td>
</tr>
<tr>
<td>75 g kg(^{-1}) TS Ca(OH)(_2) + extrusion</td>
<td>0.31 ± 0.02</td>
<td>0.24 ± 0.01</td>
<td>0.18</td>
<td>2.2</td>
<td>0.03 ± 0.01</td>
<td>9.6</td>
<td>8.4 ± 0.2</td>
</tr>
<tr>
<td>100 g kg(^{-1}) TS Ca(OH)(_2) + extrusion</td>
<td>0.32 ± 0.03</td>
<td>0.24 ± 0.03</td>
<td>0.20</td>
<td>2.7</td>
<td>0.02 ± 0.01</td>
<td>10.2</td>
<td>8.8 ± 0.1</td>
</tr>
<tr>
<td>200 g kg(^{-1}) TS Ca(OH)(_2) + extrusion</td>
<td>0.31 ± 0.01</td>
<td>0.22 ± 0.01</td>
<td>0.21</td>
<td>3.1</td>
<td>0.02 ± 0.01</td>
<td>12.1</td>
<td>11.5 ± 0.3</td>
</tr>
<tr>
<td>Extrusion + 75 g kg(^{-1}) TS Ca(OH)(_2)</td>
<td>0.31 ± 0.02</td>
<td>0.23 ± 0.02</td>
<td>0.16</td>
<td>2.9</td>
<td>0.04 ± 0.02</td>
<td>10.9</td>
<td>10.1 ± 0.3</td>
</tr>
<tr>
<td>Extrusion + 100 g kg(^{-1}) TS Ca(OH)(_2)</td>
<td>0.31 ± 0.01</td>
<td>0.24 ± 0.02</td>
<td>0.16</td>
<td>3.5</td>
<td>0.03 ± 0.01</td>
<td>12.1</td>
<td>11.4 ± 0.1</td>
</tr>
<tr>
<td>Extrusion + 200 g kg(^{-1}) TS Ca(OH)(_2)</td>
<td>0.32 ± 0.02</td>
<td>0.24 ± 0.02</td>
<td>0.19</td>
<td>2.7</td>
<td>0.02 ± 0.01</td>
<td>12.2</td>
<td>11.8 ± 0.4</td>
</tr>
</tbody>
</table>

TS = total solid; VS = volatile solid; sCOD = soluble chemical oxygen demand; \(^\ast\) represents VS\(_{\text{initial}}\)

### Table 3.2 Total carboxylate concentration of grass before and after storage (before fermentation test) (Tukey’s range test, \( p < 0.05 \), superscript ‘a’ ‘b’ and ‘c’ mean statistically significant when compared within each condition).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total carboxylate concentration (mg g(^{-1}) VS(_{\text{initial}}) Carbon)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh</td>
</tr>
<tr>
<td>None</td>
<td>6</td>
</tr>
<tr>
<td>Extrusion only</td>
<td>25</td>
</tr>
<tr>
<td>75 g kg(^{-1}) TS Ca(OH)(_2) + extrusion</td>
<td>25</td>
</tr>
<tr>
<td>100 g kg(^{-1}) TS Ca(OH)(_2) + extrusion</td>
<td>26</td>
</tr>
<tr>
<td>200 g kg(^{-1}) TS Ca(OH)(_2) + extrusion</td>
<td>25</td>
</tr>
<tr>
<td>Extrusion + 75 g kg(^{-1}) TS Ca(OH)(_2)</td>
<td>30</td>
</tr>
<tr>
<td>Extrusion + 100 g kg(^{-1}) TS Ca(OH)(_2)</td>
<td>31</td>
</tr>
<tr>
<td>Extrusion + 200 g kg(^{-1}) TS Ca(OH)(_2)</td>
<td>29</td>
</tr>
</tbody>
</table>
3.3.2 Effect of pretreatment and storage on methane production during anaerobic digestion

Research Question 5: How do extrusion and calcium hydroxide pretreatment and storage affect the potential of biogas production?

Fresh, non-treated grass produced $265 \pm 29 \, \text{cm}^3 \, \text{g}^{-1} \, \text{VS}_{\text{initial}} \, \text{CH}_4$ (this was taken as the base reference point) (Figure 3.2). Extrusion gave a methane production of $301 \pm 20 \, \text{cm}^3 \, \text{g}^{-1} \, \text{VS}_{\text{initial}} \, \text{CH}_4$. Both methane production of fresh non-treated and extruded grass were in the same range as in the studies of Prochnow et al. (2009) and Nizami et al. (2009). Addition of Ca(OH)$_2$ did not increase the methane production further ($p = 0.53$), which was also due to the chemical activity as mentioned previously.

After wilting, all treatments had a lower methane production than the initial. It is well known that wilting can induce losses in both carbohydrates contents and volatile fatty acids (Dewhurst, 1998). The wilted, non-extruded biomass retained only one fifth of the methane production ($50 \pm 8 \, \text{cm}^3 \, \text{g}^{-1} \, \text{VS}_{\text{initial}} \, \text{CH}_4$), while extruded grass gave $70 \pm 15 \, \text{cm}^3 \, \text{g}^{-1} \, \text{VS}_{\text{initial}} \, \text{CH}_4$. The effect is similar to the study of Rigdon et al. (2013), where ethanol production decreased from a concentration 0.20 to 0.02 kg m$^{-3}$ after wilting for 6 months. Treatments with 100 and 200 g kg$^{-1}$ TS Ca(OH)$_2$ before extrusion, and 200 g kg$^{-1}$ Ca(OH)$_2$ TS after extrusion, showed significantly higher methane yield ($178 \pm 18$, $166 \pm 12$ and $159 \pm 18 \, \text{cm}^3 \, \text{g}^{-1} \, \text{VS}_{\text{initial}} \, \text{CH}_4$ respectively) compared to other treatments ($p < 0.05$, Figure 3.2), this is in agreement with the result of VS loss.

After 3 month ensiling, non-treated and extruded-only grass retained the same methane production, $189 \pm 11$ vs $200 \pm 15 \, \text{cm}^3 \, \text{g}^{-1} \, \text{VS}_{\text{initial}} \, \text{CH}_4$ respectively ($p = 0.46$), compared to $265 \pm 29 \, \text{cm}^3 \, \text{g}^{-1} \, \text{VS}_{\text{initial}} \, \text{CH}_4$ of fresh, non-treated grass. The preservation was similar as in the study of Pakarinen et al. (2008), where the methane yield of grass (initial TS of 0.156 g g$^{-1}$ grass) decreased from 360 cm$^3$ to 281 cm$^3 \, \text{g}^{-1} \, \text{VS}_{\text{initial}} \, \text{CH}_4$ after 6 months of ensiling. Although the VS was preserved during ensiling, it does not always translate directly to methane production, this was also observed in the study of Whittaker et al. (2016). When comparing extruded grass and extrusion with Ca(OH)$_2$, the preservation was significantly improved with 100 and 200 g kg$^{-1}$ TS Ca(OH)$_2$ before extrusion and 100 and 200 g kg$^{-1}$ TS Ca(OH)$_2$ after extrusion (Figure 3.2). Increase in surface area of biomass due to extrusion resulted in biomass more vulnerable to microbial degradation. Furthermore, the addition of Ca(OH)$_2$ before extrusion led to a better coverage of Ca(OH)$_2$ on biomass and effective hydrolysis.
By comparing the 100 g kg\(^{-1}\) TS Ca(OH)\(_2\) addition before and after extrusion, it was shown that the Ca(OH)\(_2\) was more effective when added before extrusion and resulted in better preservation, confirming the results in Table 3.1 and Table 3.2. Increasing Ca(OH)\(_2\) concentration (up to 100 g kg\(^{-1}\) TS Ca(OH)\(_2\) for ensiling and up to 200 g kg\(^{-1}\) TS Ca(OH)\(_2\) for wilting) led to better preservation and hydrolysis of biomass and hence also better methane production (Figure 3.2). From a practical standpoint, addition of 100 g kg\(^{-1}\) TS Ca(OH)\(_2\) before extrusion was the best option as it preserved the biomass well without major chemical demands. Ca(OH)\(_2\) treated grass can be co-digested as the anaerobic digestion process produces acids. At normal operation, there is a large buffer capacity in the digester, pH perturbation can be avoided or kept at a minimum by limited feeding and good operational practice. Therefore, both digestion process and usage of the digestate are not impaired by this pretreatment.

Both the experimental and statistical analysis for ensiling and wilting pointed towards the 100 g kg\(^{-1}\) TS Ca(OH)\(_2\) before extrusion as the optimal treatment for storage for biogas production in this study. Addition of Ca(OH)\(_2\) before extrusion seemed to shield the biomass from both VS loss and methane decrease due to wilting conditions.

**Figure 3.2** Methane yield of anaerobic digestion test before and after storage for all treatments (Tukey’s range test for each condition – fresh, ensiling and wilting, \(p < 0.05\); ‘a’, ‘b’ and ‘c’ mean statistically significant when compared within each condition).
3.3.3 Effect of pretreatment and storage on carboxylate production during fermentation

Research Question 6: How do extrusion and calcium hydroxide pretreatment and storage affect the potential of carboxylate production?

Similarly to digestion, the (non-)treated grass was subjected to fermentation before and after storage (Figure 3.3). Before storage, fresh non-treated grass gave a carboxylate production of 109 ± 4 mg g\(^{-1}\) VS\(_{\text{initial}}\) Carbon (this was taken as the base reference point), while extruded-only grass yielded 121 ± 3 mg g\(^{-1}\) VS\(_{\text{initial}}\) Carbon.

After storage under wilting condition, 100 and 200 g kg\(^{-1}\) TS Ca(OH)\(_2\) before extrusion, and 200 g kg\(^{-1}\) TS Ca(OH)\(_2\) after extrusion, the grass retained a significantly higher carboxylate production (86 ± 17, 72 ± 21 and 91 ± 28 mg g\(^{-1}\) VS\(_{\text{initial}}\) Carbon respectively, \(p < 0.05\)), which again fits well with the result of VS loss and anaerobic digestion. While wilting can lead to significantly carbohydrate loss (Sanderson et al., 1997), the treatment in this study resulted in a production level comparable to ensilage of non-treated and extruded-only biomass (Figure 3.3). This is important for a biorefinery process as much of the volatile solids can still be utilized for carboxylate production. Again, by comparing the 100 g kg\(^{-1}\) TS Ca(OH)\(_2\) addition before extrusion (86 ± 17 mg g\(^{-1}\) VS\(_{\text{initial}}\) Carbon) and after extrusion (31 ± 2 mg g\(^{-1}\) VS\(_{\text{initial}}\) Carbon) to the reference (initial carboxylate production of 109 ± 4 mg g\(^{-1}\) VS\(_{\text{initial}}\) Carbon for non-treated grass), it was shown that the Ca(OH)\(_2\) addition before extrusion was more effective at preserving the biomass.

After 3 month of ensiling, 100 g kg\(^{-1}\) TS Ca(OH)\(_2\) before extrusion also gave the best performance for ensiling, with carboxylate production of 124 ± 8 mg g\(^{-1}\) VS\(_{\text{initial}}\) Carbon. Oxidative lime pretreatment (with presence of oxygen) was more effective for woody biomass while non-oxidative lime treatment is more suited for herbaceous biomass (Sierra et al., 2009). In this study, herbaceous biomass – grass was tested, in combination with the long storage time of 3 months, the ensiling method (non-oxidative) was more effective as a storage method compared to wilting (oxidative). The non-treated grass retained 75 ± 4 mg g\(^{-1}\) VS\(_{\text{initial}}\) Carbon meaning a loss of one third, while extruded-only grass gave 84 ± 6 mg g\(^{-1}\) VS\(_{\text{initial}}\) Carbon, the performance was improved with Ca(OH)\(_2\) addition as the carboxylate productions increased (Figure 3.3). For ensiling, 100 and 200 g kg\(^{-1}\) TS Ca(OH)\(_2\) before extrusion, and 75,100 and 200 g kg\(^{-1}\) TS Ca(OH)\(_2\) after extrusion showed significant effect compared to non-treated. 100 g kg\(^{-1}\) TS Ca(OH)\(_2\) before extrusion was significantly higher compared to other treatments (Figure 3.3). The decline observed at higher
Ca(OH)$_2$ loading was because of inhibitory effect on microbial enzyme due to calcium acetate formed (Nagwani, 1992).

Before storage, extrusion-only grass gave a carboxylate production of 121 ± 3 mg g$^{-1}$ VS$_{\text{initial}}$ Carbon. Contrary to the methane production (Figure 3.2), where the addition of Ca(OH)$_2$ did not result in much effect increase in methane production, adding Ca(OH)$_2$ increased the carboxylate production further during fermentation (Figure 3.3). The reason was likely a combination of chemical hydrolysis by Ca(OH)$_2$ with better pH buffering. During fermentation, the retention time was lower (8 days) compared to anaerobic digestion (30 days), and the pH was also lower (pH 5.5 – 6.5 vs pH 7 – 8). Therefore, a lower microbial enzymatic hydrolysis was expected during fermentation and the chemical hydrolysis became more important. This resulted in a higher carboxylate production when Ca(OH)$_2$ was added.

Assuming Ca(OH)$_2$ price of 0.06 € kg$^{-1}$, addition of 100 g kg$^{-1}$ TS Ca(OH)$_2$ would lead to a cost of 0.006 € kg$^{-1}$ TS treated. If green electricity price of 0.125 € kWh$^{-1}$ and acetic acid price of 0.60 € kg$^{-1}$ are assumed, the value of the additional CH$_4$ or carboxylates retained are approximately a factor of 2.5 and 8, respectively. After biogas production, the digestate can be applied as fertilizer on soil with high acidity to raise the pH, which is an added value to this process.
Figure 3.3 Carboxylate production of fermentation test before and after storage for all treatments (Tukey’s range test for each condition – fresh, ensiling and wilting, $p < 0.05$; ‘a’, ‘b’ and ‘c’ mean statistically significant when compared within each condition).
Lactic acid fermentation
Chapter 4 Lactic acid fermentation

4.0 Abstract

Lactic acid is a high-in-demand chemical, which can be produced through fermentation of lignocellulosic feedstock. However, fermentation of complex substrate produces a mixture of products at efficiencies too low to justify a production process. We hypothesized that the background acetic acid concentration plays a critical role in lactic acid yield, therefore its retention via selective extraction of lactic acid or its addition would improve overall lactic acid production and eliminate net production of acetic acid. To test this hypothesis, we added 10 g/L of acetate to fermentation broth to investigate its effect on products composition and concentration, and bacterial community evolution using several substrate-inoculum combinations. With rumen fluid inoculum, lactate concentrations increased by 80 ± 12% (corn starch, \( p < 0.05 \)) and 16.7 ± 0.4% (extruded grass, \( p < 0.05 \)) while with pure culture inoculum (\( L. \) delbrueckii and genetically modified (GM) \( E. \) coli), 4 to 23% increase was observed. Using rumen fluid inoculum, the bacterial community was enriched within 8 days to >69% lactic acid bacteria (LAB), predominantly \( Lactobacillaceae \). Higher acetate concentration promoted a more diverse LAB population, especially on non-inoculated bottles. In subsequent tests, acetate was added in a semi-continuous percolation system with grass as substrate. These tests confirmed our findings producing lactate at concentrations 26 ± 5% (\( p < 0.05 \)) higher than the control reactor over 20 days operation. Overall, our work shows that recirculating acetate has the potential to boost lactic acid production from waste biomass to levels more attractive for application.

4.1 Introduction

Lactic acid is a compound with versatile applications in many industries including food production, chemistry, textile, pharmaceutics and cosmetics. The global lactic acid market is forecasted to reach 367.3 kton by 2017, primarily due to the drive and demand from industry and new applications (GIA 2012). During the past decades, interest has grown in polylactic acid as a renewable and biodegradable plastic. However, due to the high cost of its precursor, lactic acid, use of this polymer has been limited. Lactic acid is currently produced at industrial scale through pure culture fermentation using filamentous fungi (e.g. \( Rhizopus \) spp.), bacteria (e.g. \( Bacillus coagulans \)) or yeast, and using costly feedstock such as glucose (Taskila & Ojamo, 2013). Lactic acid accumulation acidifies the fermentation broth, therefore much research has been carried out to genetically engineer microorganisms that are more tolerant of acids and low pH (below pH 4), but also utilize both pentose and hexose sugars, which are commonly found in lignocellulosic biomass (Taskila & Ojamo, 2013).
Depending on the product application, mixed culture fermentation of lignocellulosic biomass can be an attractive alternative since it would eliminate the need for sterilization and utilizes a cheaper substrate.

Lactic acid bacteria (LAB) usually ferment glucose into lactic acid. During homolactic fermentation two molecules of lactate is produced per molecule of glucose via the formation of two molecules of pyruvate. During heterolactic fermentation, which typically occurs under substrate limitation, one molecule of lactate is produced via pyruvate, while one molecule each of ethanol and carbon dioxide are produced via Acetyl-CoA. At either low pH or high substrate concentration bacteria will undergo the shorter pathway of homolactic fermentation, to decrease demand for reducing power (Thomas et al., 1979). Many LAB are also able to degrade lactic acid to acetate under anoxic conditions in the presence of alternative electron acceptors or even under strict anaerobic conditions without supporting cell growth (Oude Elferink et al., 2001). When oxygen is present, pyruvate may be converted directly to acetate to benefit the cell (Quatravaux et al., 2006). Temudo et al. (2007) investigated open mixed culture fermentation of glucose under different pH to direct specific product formation since normally a mixture of formate, acetate, butyrate and ethanol is produced. Acetate is typically the main side-product because production of acetate is energetically more favourable than lactate production (Hunt et al. 2010). Typically acetate concentrations vary widely in mixed culture fermentation (Elsden, 1945; Wang et al., 2012). To completely eliminate acetate production, research has been done to genetically modifying microorganisms such as E. coli, however small amounts are still detected even when the gene for acetate production is knocked out (De Mey et al., 2007). Bobillo & Marshall (1992) found that addition of salt (6% NaCl) could inhibit acetate production in Lactobacillus plantarum at pH 4.5, without inhibiting lactate production. It has also been demonstrated that acetate can inhibit bacterial growth when present at high concentrations (>5 g/L) and low pH (<7) (Luli & Strohl, 1990; Roe et al., 2002; Russell, 1992), depending on the bacteria strain and operating conditions. If high but not toxic concentrations of acetate are present in open culture, it is possible that acetate accumulation will direct the biochemical processes towards other reactions. This may be a possible strategy to steer the bacterial community towards net lactate production. To enable this, a process which is able to extract and separate lactic acid and acetic acid is necessary.

Regarding the separation of lactic acid from acetic acid, numerous separation technologies have been investigated including electrodialysis, ion exchange, extractive distillation. Separation of lactic acid from acetic acid has also been demonstrated using a four zone simulated moving bed process (Lee et
al., 2004). Recently a membrane electrolysis approach was developed that provides both specific
 extraction of unbranched fatty acids and pH control of the fermentation broth without chemical dosing
 (Andersen et al., 2014). This approach might be used to boost lactate production by separating acetate
 and lactate downstream of the fermentation, and returning the acetate.

The objective of the current study was thus to investigate the effect of high acetate concentration on
 the fermentation products (both batch and semi-continuous mode) and bacterial community (batch
 mode) with the aim of driving the fermentation to lactic acid. A target of 10 g/L acetate was chosen
 as a realistic value to reach considering typical production values in mixed cultures (2.5 g/L acetate
 in the study of Wang et al. (2012)), and an extraction system where lactate is removed and acetate is
 recycled back to the reactor. To compare the performance between different substrates and inocula,
 fermentation of both a simple (corn starch) and complex (extruded grass) substrate were tested using
 three different inocula: a pure culture of \textit{L. delbrueckii}, a genetically modified (GM) \textit{E. coli} and a
 mixed microbial community from rumen fluid.

\section*{4.2 Materials and Methods}

\subsection*{4.2.1 Substrate}

A readily fermentable substrate such as corn starch (total solid (TS) 86.77 ± 0.05\%, volatile solid
 (VS,\% of fresh mass) 86.35 ± 0.01\%, sold under the commercial name Basak) and a more
 lignocellulosic and complex substrate such as extruded grass (TS 64.14 ± 0.04\% and VS 60.35 ±
 0.01\%) were used. Landscape grass was harvested on October 2012, kindly provided by Inagro vzw
 (West Flanders, Belgium). To make the grass more accessible for fermentation, it was extruded with
 a pilot scale twin-screw extruder (model MSZK, Laborextruder 4 kW, Lehmann, Germany), provided
 by Bioliquid (Raalte, the Netherlands), as performed by Khor et al. (2015). No further hydrolysis was
 performed. Substrates were stored at 4 °C until used.

\subsection*{4.2.2 Microorganisms and cultivation}

\textit{Lactobacillus delbrueckii} LMG 6412 strain was obtained from the Belgian Co-ordinated Collections
 of Micro-organisms (BCCM) and grown in 1 L ‘de Man, Rogosa and Sharpe’ (MRS) medium at 37
 °C. GM \textit{Escherichia coli} strain (3KO: \textit{E. coli} K12 MG1655 δ (ackA-pta) δ (poxB), where the carbon
 flow to acetate is directly reduced), was provided by Marjan de Mey (InBio, Ghent University) and
 grown in 1 L Luria Bertani (LB) medium (De Mey et al., 2007) at 37 °C. Both strains were chosen as
 they have the ability to utilize both pure sugars and more complex substrates (Dumbrepatil et al.,
Prior to inoculation, the cells were washed with M9 medium and concentrated to 50 mL through centrifugation (1500 x g for 5 min) (De Weirdt, 2013). Optical density (OD_{610}) was 30.1 for L. delbrueckii and 10.2 for GM E. coli. M9 medium was composed of 8.5 g/L Na_{2}HPO_{4}, 3.0 g/L KH_{2}PO_{4}, 0.5 g/L NaCl, 1.0 g/L NH_{4}Cl, 0.24 g/L MgSO_{4}, and 0.011 g/L CaCl_{2}. Rumen fluid (0.06% bacterial protein) was provided by Institute for agricultural and fisheries research (ILVO), Ghent University. It was sieved and stored in a thermostash before use on the same day.

### 4.2.3 Batch fermentation

Fermentations were carried out in 120 mL serum bottles for 8 days. In total, 20 different conditions and corresponding negative controls without substrate, each of them in triplicate, were performed, as shown in Table S4.1. For pure culture tests, each serum bottle contained 49 mL of M9 medium, 1 mL of concentrated bacteria (for L. delbrueckii and GM E. coli), 5 g of substrate (corn starch or extruded grass) and 0.03 g of sodium bicarbonate to mitigate the stripping of CO_{2} into headspace. For mixed culture tests, each serum bottle was filled with 40 mL of M9 medium and 10 mL of rumen fluid. For tests with acetate addition, 683.5 mg sodium acetate (10 g/L acetate) was added. During the addition of substrate and inoculum, bottles were sparged with nitrogen to ensure anaerobic condition. The bottles were then flushed with nitrogen, except for a subset that were flushed with hydrogen. Gas samples were taken from the headspace of each bottle immediately after flushing to confirm that oxygen was removed. The pH was initially adjusted to either 5.5 for tests with rumen fluid and L. delbrueckii, or 7 for tests with GM E. coli. The pH was not controlled throughout the experiment, but was measured at the end. As controls, auto-fermentation of extruded grass without inoculum addition was evaluated at pH 5.5 and pH 7, with and without acetate addition. All bottles were placed on a shaker (130 rpm) at 30 °C for 8 days. Gas production was monitored by means of pressure measurements, and liquid and gas samples were taken and analysed on day 0, 1, 2, 4 and 8 for each replicate. Samples for bacterial community analysis were taken on day 8.

### 4.2.4 Semi-continuous fermentation

Two vertical up-flow tubular acrylic glass reactors of 400 mL were packed with 30 g of extruded grass each and inoculated with 60 mL of rumen fluid. They were run under anaerobic condition for 50 days in a 20 °C room. M9 medium was circulated through the reactors at a flow rate of 0.1 mL/min. Half of the reactor outlet was recycled back to the reactor while the other half was purged as effluent (2 days of hydraulic retention time). For the test reactor, concentrated sodium acetate (100 g/L sodium acetate) was added in the inlet at a rate of 5 µL/min to replenish the loss of acetate in the effluent, achieving 10 g/L acetate in the reactor, while for the control reactor there was no acetate addition.
Half of the substrate was replaced with fresh substrate every two days, the substrate was not mixed to ensure four days solid retention time. The reactors were sparged continuously with nitrogen during substrate replacement to ensure anaerobic environment. Sampling for chemical analysis was performed before the substrate was replaced (every two days) and average concentrations and standard deviations were calculated for all collected values.

4.2.5 DNA extraction

DNA extraction was performed using the FastPrep method described by Vilchez-Vargas et al. (2013). Samples were taken of inoculum, substrate and end of fermentation broth for analysis. Samples of 0.5 mL were centrifuged at 11 000 g for 5 min in a 2 mL Lysing Matrix E tube (Qbiogene, Alexis Biochemicals, Carlsbad, CA). Cell pellets were re-suspended in 1 mL of lysis buffer containing Tris/HCl (100 mM pH 8.0), 100 mM EDTA, 100 mM NaCl, 1% (w/v) polyvinylpyrrolidone and 2% (w/v) sodium dodecyl sulphate. Cells were lysed in a Fast Prep-96 homogenizer (40 s, 1600 rpm). Samples were then centrifuged at 18 000 g for 1 min at room temperature and washed once with one volume phenol/chloroform (1:1) and the second time with one volume chloroform. After centrifugation, nucleic acids (aqueous phase) were precipitated with one volume of ice-cold isopropanol and 1:10 volume of 3 M sodium acetate. After centrifugation and washing with 80% ethanol, the pellet was re-suspended in 20 µL of milliQ water. The quality and quantity of the DNA samples were analysed on 1% agarose gels.

4.2.6 DNA sequencing and bioinformatics processing

The V3–4 region of the bacterial 16S rRNA gene was sequenced by Illumina sequencing Miseq and v3 Reagent kit (http://www.illumina.com/products/miseq-reagent-kit-v3.ilmn, by LGC Genomics GmbH, Berlin, Germany) using 2 x 300 bp paired-end reads and primers 341F (5’-NNNNNNNNTTACGGGNGGCAG-3’) and 785R (5’-NNNNNNNTGACTACHVGGGTATCTAAKCC) described in Stewardson et al. (2015). Each polymerase chain reaction (PCR) included approximately 5 ng of DNA extract and 15 pmol of each forward and reverse primer, in 20 µL volume of MyTaq buffer containing 1.5 units MyTaq DNA polymerase (Bioline) and 2 µl of BioStabII PCR Enhancer (Prokopenko et al., 2013). For each sample, the forward and reverse primers had the same 8-nt barcode sequence. PCRs included a pre-denaturation step of 2 min at 96 °C pre-denaturation step; followed by 30 cycles of the following: 96 °C for 15 s, 50 °C for 30 s, and 72 °C for 60 s. DNA concentration of the amplicons of interest were determined by gel electrophoresis. About 20 ng of amplicon DNA from each sample were pooled for a total of 48 samples each carrying different barcodes. PCRs showing low yields were further
amplified for 5 cycles. The amplicon pools were purified with one volume AMPure XP beads (Agencourt) to remove primer dimer and other small mispriming products, followed by an additional purification on MinElute columns (Qiagen). About 100 ng of each purified amplicon pool DNA was used to construct Illumina libraries using the Ovation Rapid DR Multiplex System 1-96 (NuGEN). Illumina libraries were pooled and size selected by preparative gel electrophoresis. Sequencing was done on an Illumina MiSeq using v3 Chemistry (Illumina).

Bioinformatics was conducted with 16S rRNA targeted metagenomics analysis (QIIME; (Caporaso et al. 2010)). Data were pre-processed by first de-multiplexing of all samples using Illumina’s CASAVA data analysis software version 1.8.4. The reads were then sorted by amplicon inline barcodes, no barcode mismatches were allowed. The barcode sequence was clipped from the sequence after sorting and reads with missing barcodes, one-sided barcodes or conflicting barcode pairs were discarded. Sequencing adapters in all reads were removed and reads with final lengths below 100 nt were discarded (Clipping of Illumina TruSeq™ adapters in all reads). Combination of forward and reverse reads was done using BBMerge 34.30 (http://bbmap.sourceforge.net/). The sequence fragments were turned into forward-reverse primer orientation after removing the primer sequences. FastQC report was creating for every FASTQ files. 16S pre-processing and OTU picking from amplicons were performed using Mothur v1.33 software package (Schloss et al. 2009). Sequences containing ambiguous bases (Ns), with homo-polymer stretches of more than 8 bases or with an average Phred quality score below 33 were removed. Reads were aligned against the 16S Silva reference alignment release 102. Truncated and unspecific PCR products were removed. 15 000 sequences per sample were subsampled. Pre-clustering allowed up to 3 differing bases in a cluster. Chimera were removed using uchime algorithm (Edgar et al., 2011). Taxonomical classification of sequences and removal of non-bacterial sequences were done using the Silva database. OTU were picked by clustering at the 97% identity level using the cluster split method.

The raw sequence dataset was deposited on the European Nucleotide Archive (ENA) of European Bioinformatics Institute, with accession number LT006862-LT009376 (http://www.ebi.ac.uk/ena/data/view/LT006862-LT009376). Information regarding the OTUs are included in the Supplementary Material – OTU table (xls format).

4.2.7 Statistical analysis

Representation of Principal Coordinate Analysis (PCoA) of Bray-Curtis dissimilarity indexes was performed. The Vegan package in R (R version 2.13.2, http://www.r-project.org/) was used to
calculate Bray-Curtis dissimilarity matrices (vegdist function) and the data was represented by PCoA function using the ape package.

4.2.8 Analytical methods

Determination of fermentation products including lactate, acetate, propionate and butyrate, was performed with Dionex ion chromatography equipped with IonPac ICE-AS1 column (Dionex) using 4 mM H$_2$SO$_4$ as eluent at a flow rate of 0.8 mL/min and an ED50 conductivity detector. The gas phase composition was analysed with a compact gas chromatography (Global Analyser Solutions, Breda, The Netherlands), equipped with a Molsieve 5A pre-column and Porabond column (for CH$_4$, H$_2$ and N$_2$) and a Rt-Q-bond pre-column and column (for CO$_2$). Nitrogen was used as carrier gas for H$_2$ analysis while helium was used for CH$_4$ and N$_2$. Concentrations of gases were determined by means of a thermal conductivity detector. Bacterial protein of rumen fluid was analysed according to Makkar et al. (1982). Optical density (OD$_{610}$) of pure culture inoculum was measured with a UV-VIS spectrophotometer (ISIS 9000, Dr Lange, Germany) at 610 nm.

4.3 Results

4.3.1 Impact of acetate addition on lactic acid concentration – batch mode

Research Question 7: What is the effect of acetate accumulation on batch lactic acid fermentation?

Figure 4.1 depicts the results of fermentation tests with different inocula and substrates. When acetate was not added (w/o acetate), it was still detected as an intermediate at the end of the tests. However, in the test with acetate addition (w/ acetate), a smaller acetate production over the initially supplied was obtained (the difference between initial and end point was smaller than in w/o acetate tests) in all combinations. Using corn starch, there was no statistical difference in terms of lactic acid production between tests with and without acetate addition when pure cultures were used as inoculum. However, with rumen fluid as inoculum, the final lactic acid concentration was 80 ± 12% ($p < 0.05$) higher when acetate was added vs. control bottles. In experiments with extruded grass, acetate addition increased the lactic acid concentration after 8 days of fermentation by 16.7 ± 0.4% ($p < 0.05$) with rumen fluid inoculum, and by 4 to 23% with either pure culture or genetically modified inoculum. There was no statistical difference in lactate production for non-inoculated extruded grass tests at pH 5.5 ($p > 0.05$) with or without acetate addition (Table 4.1), due to . Although initially the pH was adjusted to 5.5, the final pH of all bottles supplemented with acetate reached an average of 4.4, while those without acetate addition reached 3.9, which roughly corresponds to the pKa of the respective major species present in solution (pKa acetic acid 4.75: pKa lactic acid 3.8).
The highest final lactate concentration (14.7 ± 0.3 g/L) was achieved with rumen fluid inoculum and extruded grass as substrate, coupled with 2.7 ± 0.1 g/L acetate production over that supplied (Figure 4.1; Table S4.2). Elevated acetate concentrations did not alter the spectrums of other fermentation products such as butyrate and propionate (Figure S1). The initial rates of lactate production did not differ much with acetate addition (Figure S2). No methane was detected in all tests with nitrogen in the headspace.

Table 4.1  Lactate concentration of extruded grass fermentation with or without inoculum at different pH

<table>
<thead>
<tr>
<th></th>
<th>pH 5.5</th>
<th></th>
<th>pH 7.0</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>w/o acetate</td>
<td>w/ acetate</td>
<td>w/o acetate</td>
<td>w/ acetate</td>
</tr>
<tr>
<td>No inoculum</td>
<td>4.6 ± 0.3</td>
<td>5.6 ± 0.2</td>
<td>4.3 ± 1.6</td>
<td>9.1 ± 0.6</td>
</tr>
<tr>
<td>Rumen culture</td>
<td>12.6 ± 0.1</td>
<td>14.7 ± 0.3</td>
<td>N/T</td>
<td>N/T</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>N/T</td>
<td>N/T</td>
<td>7.4 ± 0.2</td>
<td>9.0 ± &lt;0.1</td>
</tr>
</tbody>
</table>

N/T: not tested

Figure 4.1  Lactate and acetate concentrations after 8 days of fermentation batch test. (Error bars represent standard deviation between experimental replicates; ‘grey’ horizontal lines represent the starting lactate concentration; ‘black’ horizontal lines represent the starting acetate concentration)
4.3.2 Impact of acetate addition on lactic acid concentration – semi-continuous mode

Research Question 8: What is the effect of acetate accumulation on semi-continuous lactic acid fermentation?

A semi-continuous lactic acid fermentation of extruded grass gave results similar to those observed in batch mode, where supplemental acetate resulted in a higher final lactate concentration (Figure 4.2). Reactors were started up allowed to acclimate during the first 30 days after which operation continued until day 50. On average over the latter fermentation period (20 days), the lactic acid concentration was $26 \pm 5\%$ ($p < 0.05$) higher in the reactor with acetate addition, compared to that of the control reactor. The spectrums of fermentation products were similar to those observed in the batch fermentation tests, where they did not differ with or without the addition of acetate. Also, no methane was detected during the fermentation. Since the purpose of this test was to investigate the effect of elevated acetate concentration, process optimization was not performed, hence the low conversion and production rate. The average conversion achieved was $0.14 \pm 0.01$ g lactate/g VS extruded grass fed for the control reactor and $0.17 \pm 0.01$ g lactate/g VS extruded grass fed for the test reactor ($p < 0.05$). The average production rate was $2.0 \pm 0.1$ g/L.d for control reactor and $2.6 \pm 0.2$ g/L.d ($p < 0.05$) for test reactor. The operational conditions for the semi-continuous reactors were defined from the batch test outcomes. A solids retention time (grass) of 4 days was chosen since lactate concentration had reached a plateau after 4 days of fermentation in batch tests (Figure S2). Propionate and butyrate profiles are presented in Figure S3.

![Figure 4.2](image) Lactate and acetate profile of fermentation semi-continuous test.
4.3.3 Effect of operational parameters on lactic acid production

Research Question 9: What is the effect of pH, headspace gas and inocula on batch lactic acid fermentation?

Additional batch tests were carried out to evaluate the effect of operational conditions on lactate production, including headspace composition, pH, and inoculum choice. First, hydrogen presence to create reductive conditions was tested. Corn starch was used as substrate and rumen fluid as inoculum, with and without acetate addition, with either nitrogen or hydrogen in the headspace (Table 4.2). Hydrogen addition did not enhance lactate production but, even with a low working pH of 5.5, generated methane with and without acetate addition (3.18 ± 0.12 mmol and 3.26 ± 0.36 mmol, respectively).

To evaluate pH and inocula, tests with extruded grass were performed with different inocula and their respective optimal pH (5.5 or 7). Controls without inoculum addition at pH 5.5 and 7 were also included to evaluate the performance of the autochthonous bacteria in the grass (Table 4.1). No differences in lactate (only about 4 g/L) were detected in the bottles without inoculum at different pH. However, when acetate was supplied, lactate only increased about 1 g/L at pH 5.5 (not statistically significant) while it improved by nearly 5 g/L at pH 7, which accounted for an increase of 111.1 ± 42.4%. Thus, lactate titres were improved at higher pH, either with or without inocula. In most cases, inoculated bottles reached higher lactic acid concentration compared to the non-inoculated ones. When acetate was supplied, lactate production was again improved. Fermentation of extruded grass by inoculated with either rumen microbial community or *L. delbrueckii* maintained higher lactic acid concentrations than with GM *E. coli*. When looking at production rates (Figure S2), a 1-day lag time was observed before lactate concentration increased significantly in tests without inoculum, while lactate increased from day 0 in the inoculated bottles. Apart from the lag phase, the rate of production for both inoculated (3.9 ± 0.1 g/L.d, between day 0 and day 1) and non-inoculated bottles (4.1 ± 0.3 g/L.d, between day 1 and day 2) were similar.

Table 4.2  Lactate concentration of corn starch fermentation inoculated with rumen fluid under different headspace

<table>
<thead>
<tr>
<th>Headspace gas</th>
<th>Lactate concentration (g/L)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>without acetate</td>
<td>with acetate</td>
</tr>
<tr>
<td>N₂</td>
<td>6.7 ± 0.1</td>
<td>14.0 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>H₂</td>
<td>6.7 ± 0.6</td>
<td>13.5 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>
4.3.4 Community shift with different substrates

Research Question 10: What is the effect of substrate on batch lactic acid fermentation?

The effect of elevated acetate on bacterial community of bottles inoculated with rumen fluid (both extruded grass and corn starch) or without any inoculation (extruded grass only) was investigated and compared. A mixed microbial community such as ones found in rumen fluid can be modified from its initial structure by application of different operational conditions. Firstly, we measured the impact of acetate addition on fermentation of corn starch and extruded grass as substrates (Figure 4.3). *Lactobacillus* spp. became the most dominant species, with and without acetate addition for both substrates tested. *Lactobacillus* spp. is a lactic acid bacterium (LAB) able to convert sugars such as glucose and xylose, present in lignocellulosic biomass, to lactic acid. When no acetate was added, there was no significant differences in the microbial community between the tests with corn starch and extruded grass (Figure 4.3a versus Figure 4.3c). When acetate was supplemented, the bacterial diversity increased, resulting in a decrease in the relative abundance of *Lactobacillus* spp. (e.g. 69 ± 3%, Figure 4.3b versus 84 ± 4% in control bottles, Figure 4.3a, p < 0.05). Also with acetate addition, a higher relative abundance of *Prevotella* spp. was found in tests with corn starch (20%) compared to extruded grass (2%). Furthermore, when the two substrates both with acetate addition were compared, extruded grass gave a more diverse lactic acid producers compared to corn starch at the end of fermentation batch test (e.g. Figure 4.3b and Figure 4.3d), which likely correlates to higher feed complexity.

In rumen fluid inoculum itself, *Prevotellaceae* family was the most dominant (e.g. 85% relative abundance, Figure S4a). *Acidaminococcaceae* (6%) and *Ruminococcaceae* (2%) were also present in lower abundance (Figure S4a). While on the extruded grass itself, a very diverse inherent bacterial community was found, with *Curvibacter* spp. (23%), *Massilia* spp. (14%) and *Bacillus* spp. (9%) being the three most abundant species (Figure S4d). After 8 days of batch fermentation, most test and control bottles consisted mainly of LAB, especially *Lactobacillus* spp. (69% to 99%), while its presence in both the rumen inoculum (0.03%) and extruded grass (Figure S4d, below detection limit) were extremely low.
4.3.5 Effect of headspace composition on the microbiome

Research Question 11: What is the effect of headspace gas on microbiome of lactic acid fermentation?

The bacterial community distribution in fermentations carried out on corn starch as substrate and inoculated with rumen fluid was analysed under different headspace gas composition and acetate presence (Figure 4.4). As we observed with different substrates, gas composition did not have any effect on the community structure and *Lactobacillus* spp. was by far the dominant species without acetate addition. Also in this case, regardless of the gas composition in the headspace, the addition of acetate increased the microbial diversity with the presence of 3-4% *Prevotella* spp. (Figure 4.4b and 4d). Primers used in these tests were specific to bacterial DNA, hence methanogens were not detected in the sequencing.
Figure 4.4  Bacterial community of batch fermentation of corn starch at pH 5.5, inoculated with rumen fluid. (a) N$_2$ headspace; (b) N$_2$ headspace with acetate addition; (c) H$_2$ headspace; (d) H$_2$ headspace with acetate addition

4.3.6 Bacterial community under different pH and acetate presence

*Research Question 12: What is the effect of pH and acetate accumulation on microbiome of lactic acid fermentation?*

Chemical analysis already showed a lower lactic acid production in non-inoculated bottles compared to the inoculated during fermentation test with extruded grass. The bacterial community under fermentation at different pH and acetate concentrations was evaluated (Figure 4.5). During fermentation at pH 5.5 with extruded grass and no inoculum, a more diverse bacterial community was obtained compared to samples inoculated with rumen fluid (e.g. Figure 4.5a vs Figure 4.3c). Initially on extruded grass, the *Comamonadaceae* family (26%, Figure S4d) dominated the diverse mix bacterial community but over time, the fermentation resulted in a bacterial community mainly composed of LAB (52 ± 25%, Figure 4.5a). When acetate was elevated, LAB abundance increased further to 82 ± 29% (Figure 4.5b).
In batch experiments where extruded grass was fermented at pH 7 without inoculum, lower abundance of LAB was obtained, with *Lactobacillus* spp. Making up 17 ± 15% (Figure 4.5c) and *Leuconostoc* spp. Making up 4 ± 4% (Figure 4.5c). When acetate was supplemented, multiple strains of LAB dominated the bacterial community (89 ± 76%, Figure 4.5d). Apart from *Lactobacillus* spp., the other LAB detected were *Leuconostoc* spp., *Lactococcus* spp. and *Pediococcus* spp. By comparing the non-inoculated extruded grass fermentation, *Leuconostoc* spp. (LAB) represented a much higher proportion (34 ± 25%, Figure 4.5a) at pH 5.5 compare to at pH 7 (4 ± 4%, Figure 4.5c). Presence of *Leuconostoc* spp. was further stimulated by decrease of pH with the control bottles (Figure 4.5b vs Figure 4.5d), while the phenomenon was different for *Lactobacillus* spp., which was not perturbed by the pH (15 ± 3% at pH 7, Figure 4.5a versus 17 ± 15% at pH 5.5, Figure 4.5b). Data regarding alpha and beta diversity are presented in the supplementary material (Figure S5 and S6).

**Figure 4.5** Bacterial community of batch fermentation extruded grass under N₂ headspace, without inoculation. (a) pH 5.5; (b) pH 5.5 with acetate addition; (c) pH 7; (d) pH 7 with acetate addition
4.4 Discussion

4.4.1 Impact of acetate addition on lactic acid concentration

In most cases, elevated acetate concentrations decreased the net production of acetic acid. When different metabolic pathways are possible (i.e. lactate or acetate production), high product concentration will thermodynamically favour alternative pathways according to the Van’t Hoff equation ($\Delta G = \Delta G^\circ + RT\ln Q$). With regards to physico-chemical properties, acetate addition will increase the conductivity of the system and high salt concentration can inhibit the growth of microorganisms (McCarty & McKinney, 1961). Furthermore, acetate addition will bring the pH of the system towards its acidic buffer capacity (pKa 4.75), thus possibly favouring the route of the lactic acid production by LAB. However, a high proportion of the total acetate will be in equilibrium with the acid form of acetic acid, which may inhibit some microorganisms. By considering these effects, addition of acetate should have a positive impact on lactic acid fermentation if toxic concentrations are not reached. Takahashi et al. (1999) found that increasing acetate concentration (from 2 g/L to 12 g/L) reduced the GM $E. coli$ biomass concentration, however the fermentation product (ethanol) concentration was not affected. In this study, addition of 10 g/L sodium acetate did not negatively affect the lactic acid production rate. On the contrary, initial rates seemed to be the same (Figure S2) and the addition of acetate increased the final lactic acid concentration. In the fermentation pathway, lactate production takes place via a relatively short and simple pathway to reduce pyruvate, generating one NAD$^+$ for each pyruvate reduced. This pathway can quickly relieve the cell of reducing power by using lactate as electron sink, e.g. when substrate is over-abundant or during pH perturbation, although at the cost of lower energy gain (2.50-2.66 ATP per molecule glucose reduced) compared to acetate production. While producing acetate can yield more energy (4 ATP per molecule glucose reduced), lactate production can become the preferred pathway of microorganisms under these circumstances. Addition of acetate may perturb the reducing state of the environment, and hence stretch the lactate production further, as in this study.

Lactic acid can be degraded to acetate by many LAB, requiring electron acceptors and producing hydrogen (Quatravaux et al., 2006; Thauer et al., 1977). Thus, hydrogen presence should, by thermodynamically increasing the energy requirements of acetate production (Van’t Hoff equilibrium), assist in the shift from acetate to lactate production. However, no improvement was observed in the tests performed. While acetoclastic methanogenesis was inhibited during the fermentation tests under low pH (5.5), presence of carboxylates, or short experimental time (8 days), low concentrations of methane were detected when hydrogen was supplied. Costa (2013) studied the electron flow and energy conservation in hydrogenotrophic methanogens and showed that they are...
capable of using other substrates such as formate for growth, independently of hydrogen. Hence, this may result in substrate competition for lactate production and thus not notably increase its production.

Short hydraulic retention time (2 days) in combination with low pH (5.5) also allowed the suppression of methanogenesis. The tested reactor mimicked a continuous recirculation of acetate while lactate was harvested in the effluent. This is the first proof of concept that a selective extraction of lactate from acetate will enhance the titres and make the fermentation from (ligno)cellulosic biomass more feasible towards lactate production.

4.4.2 Effect of operational parameters: pH, inoculum and headspace gas composition

Fermentations inoculated with rumen culture or *L. delbrueckii* inoculum were set at pH 5.5 while those inoculated with GM *E. coli* were set at pH 7, based on the optimal working pH for the inoculum. This difference in pH could have an effect on the kinetics of the fermentation and caused the difference in lactic acid concentration (Figure 4.1). In the test without inoculum, the end lactate concentration was similar without acetate addition at both pH tested. Lactic production was higher at pH 7 compared to pH 5.5 when acetate was added (Table 4.1), likely due to enhanced bacterial growth.

The pH only had a major impact when inoculum was not supplied. When the pH was low, more carboxylic acids (e.g. acetic acid and lactic acid) are in their protonated form. This allows the compounds to diffuse more easily into the bacteria cells, lowering the inner pH of bacterial cell. To combat this, bacteria have to invest more energy in cell maintenance and transporting the compounds out of the cell to maintain the inner cell pH. This caused energy loss which could otherwise be used to grow. Inoculation ensured that the fermentation was not inhibited due to low number of bacteria cells initially. Inoculation shortened the lag phase and allowed immediate conversion of readily digestible substrates into lactic acid (Figure S2). Comparing the three inoculums tested, rumen fluid inoculum had the best performance in terms of lactate concentration for both corn starch and extruded grass as substrate. The mixed culture inoculum proved to be more effective for lactic acid fermentation on this complex substrate, probably due to a higher capacity to deal with the complex substrate.
4.4.3 Impact of acetate addition on bacterial community structure

Free acid inhibition of biochemical reactions is well documented (Colin et al., 2001). At high concentrations and low pH, carboxylate equilibrium shifts to the protonated form, which can penetrate the cell membrane and reduce the activity of most bacteria. Many studies have been performed on single strains of bacteria, such as Clostridium (Tang et al., 1989), and models have been developed to study the inhibitory effect of acetate (Zeng et al., 1994). However, the effect can be different for mixed microbial communities. In a study of a mixed community consisting of methanogens and acetogens, Fotidis et al. (2013) increased the acetate concentration stepwise up to 9 g/L and found that the growth of some cultures was inhibited, while that of others was either similar or significantly higher. In this study, a similar behaviour may occur as some bacteria only perform homolactic fermentation while many others can undergo heterolactic fermentation, and even lactate degradation, that will be affected by acetate concentration. Elevated acetate levels during fermentation of corn starch promoted the presence of a more diverse bacterial community. A more specialized bacterial community, mainly composed of LAB, was found in the bottles without acetate addition (Table S4.3). However, the lactate concentration was higher in the bottles with acetate addition. This indicates that with acetate addition, more capacity for lactic acid production arose.

For fermentation of extruded grass without rumen fluid inoculum, an elevated amount of acetate promoted the presence of multiple species of LAB at pH 7, but not at pH 5.5 which may be due to the aforementioned higher relative proportion of its free acid form. Bobillo & Marshall (1992) studied the effect of acidic pH and salt on acid end products and found that Lactobacillus plantarum is capable of altering its metabolic pathways of acid production as the environmental pH changes. Here we observed rather a shift towards other bacteria maintaining the lactic acid fermentation. During fermentation of corn starch, Lactobacillus spp. dominated the bacterial community and elevated acetate concentration gave rise to a more diverse bacterial community. Also, when rumen inoculum was used with extruded grass, the diversity of the bacterial community increased with elevated acetate concentration. Although the experiments performed here were likely too short to draw many conclusions on diversity in the longer term, the fact that similar chemical results were obtained for the semi-continuous operation could indicate that this likely remains the case in longer term. In the rumen fluid inoculum, Prevotellaceae family was the most dominant, followed by Acidaminococcaceae and Ruminococcaceae. These bacteria are commonly found among the species belonging to the rumen fluid core microbiome (Huws et al., 2015). Some Prevotella species have been reported to breakdown carbohydrates such as in Kabel et al. (2011), and they may have an important role in ruminal biohydrogenation (Huws et al., 2011).
Overall, it can be concluded that the addition of acetate to mixed culture fermentations geared at lactic acid production improves the rate and yield of the process while selecting for a community dominated by LAB. Although addition of acetate on mono-cultures fermentation increased lactate production, the difference was not significant compared to bottles without acetate addition in some cases. The findings might be related to the capacity of mixed-cultures to undergo different reactions and the thermodynamic impediment caused by acetate addition to heterolactic fermentation or lactic acid degradation.

Since it is shown that lactic acid can be produced from lignocellulosic biomass – grass, and selective extraction of lactate has the potential to improve lactic acid fermentation, the next course of action is naturally to investigate the extraction of carboxylic acids.
Extraction of carboxylic acids
Chapter 5 Extraction of carboxylic acids

5.0 Abstract

Lactic acid (LA) is a bulk chemical, used as such or as building block for e.g. polymers. In recent years, its production from waste feedstock via mixed culture fermentation has been considered. This production generally comes with a mixture of side products, mainly acetic acid (AA). These side products can hamper further utilization of the LA, hence product extraction with a certain selectivity is necessary. The traditional approach is filtration and precipitation as calcium lactate, which is a chemical intensive approach leaving waste and not delivering the target, LA. Here, compared alternative extraction strategies including membrane electrolysis, nanofiltration, pertraction, ion exchange and ionic liquid extraction to extracting LA from a mixture of LA and AA, and the options towards further usage. An important parameter affecting extraction efficiency and selectivity is the difference in pKa between LA (3.86) and AA (4.76), enabling different ratios of protonized and deprotonized species. Pertraction with a pH above 4 allows the extraction of acetic acid into draw solution and not LA, which is a suboptimal outcome, but at least AA and LA are separated. Membrane electrolysis does not permit a selective extraction, however it concentrates the solutes as separated acid in concentrations up to 100 g L\(^{-1}\) without requiring any chemical dosing. With ionic liquids used on e.g. this extract, more organic acids were extracted into the ionic liquid phase when the pH decreased, the extracted organic acids can be directly converted to esters. Nanofiltration and ion exchange using resin offer a limited degree of selectivity, however they are currently some of the most economically feasible and highly efficient methods for organic acids extraction. The different extraction strategies provided a better understanding on the characteristics of each system, selection of extraction technologies greatly depends on operation purpose and the end use of the product.

Figure 5.1 Membrane electrolysis with anion exchange membrane for lactic acid extraction
5.1 Introduction

LA is an important chemical precursor in industry with wide application. It is a naturally produced biochemical for example during ensiling, a key bottleneck for such mixed population processes is its extraction. The conventional method of extracting LA is the addition of calcium carbonate, precipitating a calcium lactate, followed by re-dissolution in sulphuric acid. The issue with this approach is the production of calcium sulphate as waste product which is undesirable. Alternative extraction technologies such as membrane technology (Garde, 2002; Saxena et al., 2007), liquid-liquid extraction (Tik et al., 2001), solid adsorption, reactive distillation, as such, aim to maximize extraction efficiency while minimizing waste production and cost. In mixed culture fermentation of complex substrate such as lignocellulosic biomass, a certain fraction of side products is difficult to avoid. For instance, fermentation of LA will often be accompanied by AA (Khor et al., 2016). this highlights the need for selective extractions. Some studies have been done on extraction of organic acids in multicomponent system (Reisinger & Marr, 1992), however more work is still needed to improve the understanding of the principles behind selective extraction. With pK$_a$ values of lactate (3.86) and acetate (4.76) having a difference of 1 magnitude, there may be room to engineer extraction process towards a certain selectivity.

Some of the most promising technologies which have been tested for extraction of LA include electrodialysis (Garde, 2002; Hábová et al., 2004; Kim & Moon, 2001), nanofiltration (Timmer et al., 1993) (González et al., 2008; Xiong, Richard, & Kumar, 2015), ion exchange (Garrett et al., 2015; John et al., 2008; Srivastava et al., 1992), pertraction (Huang et al., 2004; Ramchandran et al., 2012; Sirman et al., 1991; Tong et al., 1998) (Harington & Hossain, 2008) and ionic liquid extraction (Lateef et al., 2012; Martak & Schlosser, 2007; Tonova et al., 2015). Ion exchange is often considered the best state of the art technology for LA recovery. It is efficient and generates less waste (small amount of NaCl solution, if the resin is activated as its Cl$^-$ form regenerated with HCl solution) compared to the former recovery method, where calcium hydroxide and sulphuric acid were dosed, generating gypsum. However, it is uncertain whether the recovery of organic acids from mixed culture fermentation is feasible in terms of selectivity and efficiency.

Also electrodialysis offers the advantage of avoiding additional chemical dosage for product extraction and pH adjustment by generation of OH$^-$ ions at the cathodic chamber, and hence avoiding waste and by-product generation. During electrodialysis, electromigration drives the ions across ion selective membranes allowing separation of the product in a concentrate. Apart from electrodialysis, a similar but modified electrochemical approach is membrane electrolysis, where only one membrane
Chapter 5

is used. An anion exchange membrane is often used to allow migration of carboxylate across the membrane into a clean, acidified extract stream while regulating the pH in the production broth. While there have been plenty of studies on LA extraction using electrodialysis (Hábová et al., 2004), only few studies focus on the extraction of LA with a single membrane (Saxena et al., 2007), and none on binary systems of LA and AA.

Selection of extraction technology is therefore not always straightforward as different criteria have to be taken into consideration. Depending on the end use of product e.g. bulk chemical use, polymer production, ester production or others, one technology can be more suitable compared to other, and different technologies can often be combined to improve extraction efficiency.

This comparative study aims to compare state of art extraction technology with particular focus on selectivity, efficiency, concentration and end use of LA recovery from aqueous broth which contains AA as impurity. The purpose of this study is not to perform thorough investigation into each extraction techniques, but rather a comparative study between different techniques under the same applied conditions to access the advantages and disadvantages of each techniques. Effect of pH (3, 4, 4.8, 5.5 and 7) on extraction of LA and AA was also tested. Extraction technologies tested in this study included (1) Membrane electrolysis with anion exchange membrane, (2) Nanofiltration system, (3) Pertraction system which involved use of hollow fibre membrane, (4) Ion exchange resin, (5) Ionic liquid extraction.

5.2 Materials and Methods

5.2.1 Materials

Synthetic solution containing equi-molar of lactic acid (150 mol m⁻³) and acetic acid (150 mol L⁻³) was prepared by using DL-lactic acid (~ 90%, Sigma Aldrich), acetic acid (≥ 99.7%, Fluka) and distilled water. pH of synthetic solution was adjusted to 3, 4, 4.8, 5.5 and 7 with 3 M NaOH solution.

5.2.2 Membrane electrolysis

The electrochemical cell made of acrylic glass consisted of an anode chamber (8 cm³, height 8 cm × length 1 cm × width 1 cm) and a cathode chamber (8 cm³, height 8 cm × length 1 cm × width 1 cm), separated by an anion exchange membrane (fumasep FAB, FumaTech GmbH, Germany) with a surface area of 8 cm². The cathode was an AISI Type 316L stainless steel felt with 1 mm wire thickness, 6 cm², (LierFilter Ltd., China), the anode was an IrOx-MMO coated titanium electrode
(IrO$_2$/TiO$_2$: 0.65/0.35), 0.0006 m$^2$, with a centrally attached, perpendicular current collector (Magneto Special Anodes BV, The Netherlands). The anolyte was made up of 5 mM sodium sulphate solution, pH was adjusted to 2 with 0.5 M HCl. The catholyte was synthetic solution containing lactic acid and acetic acid. The anion exchange membrane was pretreated with 5 M NaCl solution 24 hours prior to use. The extraction cell was run as in continuous, single pass mode at flow rate of 0.167 cm$^3$ s$^{-1}$ at 20 °C. Potentiostat (VSP, Biologic, France) was run in chronopotentiometry mode at three different current densities (10, 20 and 30 A m$^{-2}$). Samples were taken from the anodic chamber for carboxylate analysis after the system was stabilized for 20 minutes. All tests were performed in triplicate.

Concentration operation was performed with a current density of 0.4 A m$^{-2}$. Samples were taken at time 0, 3, 8 and 48 h for carboxylate analysis.

### 5.2.3 Nanofiltration

The filtration cell consists of two compartment (8 cm$^3$ each) separated by a nanofiltration membrane (8 cm$^2$). Two nanofiltration membrane, NF270 and NF90 (Dow Filmtec) were kindly provided by PalInT (Particle and Interface Technology group), Ghent University. The membranes were chosen to test for different molecular weight cut-offs, where NF90 is the tightest with 200 Da, and NF270 is at the opposite end with molecular weight cut-off of 400 Da and. A positive displacement pump (Qdos 60 manual, Watson-Marlow) was used to generate a 3 bar (for NF270 operation) and 5 bar (for NF90 operation) pressure on the system with flow velocity of 2 m s$^{-1}$, at 20 °C. Nanofiltration was performed as a continuous, single pass operation. Samples were taken from permeate and retentate for carboxylate analysis. The system was allowed to stabilize for 20 minutes before taking samples. All tests were performed in triplicate.

### 5.2.4 Pertraction

A tubular hollow fibre membrane (Accurel PP S6/2, Membrana GmbH, Wuppertal, Germany) was kindly provided by LEQUIA (Institute of the Environment, University of Girona). The membrane has a wall thickness of 450 µm, inner diameter 1.8 mm, and pore size 0.2 µm. The pertraction system consisted of a 12 mL penicillin bottle filled with 10 mL of synthetic solution. One hollow fibre membrane tube of 10 cm in length (resulting in an internal surface area of 5.65 cm$^2$) was submerged in the synthetic solution. Distilled water was pumped through the hollow fibre membrane as draw solution in a single pass continuous mode, at a flow rate of 0.083 cm$^3$ s$^{-1}$ and temperature of 20 °C. Samples were taken from synthetic solution daily for carboxylate analysis for 30 days. All tests were performed in triplicate.
5.2.5 Ion exchange
Ion exchange resin Amberlite IRA 67 free base (Sigma Aldrich, total exchange capacity $\geq 1.6$ eq L$^{-1}$ in its free base form) was used, the resin was pretreated according to the study of John et al. (2008). To obtain the resin in its Cl$^{-}$ form, the resin was washed sequentially with 1 N HCl solution, distilled water, 1 N NaOH solution, distilled water, 1 N HCl solution and distilled water until pH reached 7. Moisture content of the resins was also determined by oven drying at 105 °C for 24 hours. 1.0 g of wet resin was added to 10 cm$^3$ of solution, the mixture was mixed well and left standing at 20 °C for 24 hours. Samples were taken from the solution for carboxylate analysis to determine the amount of carboxylic acids absorbed. All tests were performed in triplicate.

5.2.6 Ionic liquid extraction
The ionic liquid trihexyl tetradecyl phosphonium bis(2,4,4-trimethylpentyl) phosphinate ($\geq 95.0\%$, Sigma Aldrich) was used for the extraction test. 1 mL of ionic liquid was added to 1 mL of synthetic mixture solution. The mixture was vortexed vigorously and left standing at 20 °C for 24 hours. Samples were taken from the water fraction and analysed to determine the concentration of carboxylic acids extracted into the ionic liquid phase. All tests were performed in triplicate.

5.2.7 Analytical methods
Determination of organic acids (lactic acid and acetic acid) was performed with Metrohm ion chromatography equipped with Metrosep organic acids column and Metrosep organic acids guard column, and an ion chromatography conductivity detector, using 1 mM H$_2$SO$_4$ as eluent at flow rate of 0.0083 cm$^3$ s$^{-1}$, oven temperature at 35 °C, and 500 mM LiCl as regenerant for suppressor.

5.3 Results and Discussion
5.3.1 To achieve selectivity during extraction
Research Question 13: How to achieve selectivity in extraction?
Acetate could be separated from the lactate-acetate binary solution with the hollow fibre membrane and distilled water as draw solution. At pH values between 4 to 7, mainly acetate passes through the membrane and not lactate, as at that pH it was not protonated. The lactate flux was close to 0 while acetate flux was between $0.05 \pm 0.02$ to $0.34 \pm 0.04$ (Table 5.1). The hollow fibre membrane used had a pore size of 0.2 µm, hence the selectivity was not due to the size of the ions, but rather the effect of interaction between membrane surface charge and ions charge. The disadvantage of the approach here is that no up-concentration of the acetate is possible. After removing acetate with distilled water
as draw solution, the remaining LA can be extracted with the use of organic solvent such as tri-n-octylmethylammonium chloride dissolved in oleyl alcohol as shown in literature (Tong et al., 1998), hence separation and extraction of lactate and acetate may be achieved by combining these two methods. This can open up possibility of selective extraction in a mixture solution containing lactate and acetate.

Membrane electrolysis exhibit little selectivity towards LA or AA extraction, although a minor effect on the selectivity was observed due to the difference in pKₐ value of lactate and acetate. At pH of 4 and 4.8, more lactate than acetate was transported across the membrane, where lactate flux is 1.5 times higher than acetate flux at pH 4 (Table 5.2). Acetate is mostly in its protonated form (85% at pH 4 and 48% at pH 4.8), which makes it not or only to a limited extent subject to the electromigration force. For pH 5.5 and 7, both carboxylates are mostly dissociated hence acetate passed through the membrane at a higher rate due to its smaller size at current density of 10 A m⁻², while there was no selectivity towards acetate or lactate at higher current density of 20 A m⁻² and 30 A m⁻². At pH 3, both lactate and acetate were mostly protonated, hence there was no selectivity between LA and AA.

The ionic liquid tested in this study exhibit a higher affinity towards acetate, with the selectivity increased (from 1.3 to 18 times) with increasing pH from 3 to 4.8 (Table 5.3). As the ionic liquid is more hydrophobic, protonated ions can migrate easier into the ionic liquid phase. Having a smaller molecular weight and smaller valence number, acetate can be extracted into the ionic liquid more easily (Canle et al., 2014; Flieger & Czajkowska-Żelazko, 2011).

The ion exchange resin only gave a certain selectivity towards LA or AA depending on the operating condition. The extraction process was more selective towards lactate at low pH of 3 and 4 (1.9 to 2.4 times), but favours acetate at pH of 5.5 and 7(1.4 to 1.9 times) (Table 5.4), thus in those conditions it can be used to clean up the stream of acetate. The adsorption process in ion exchange resin favours ions with high valence and low molecular weight. The degree of hydration of an ion is directly proportional to its valence and inversely proportional to its hydrated radius. For ions of a given radius, selectivity of a resin for an ion is inversely proportional to the ratio of the valence/ionic radius (Dechow, 1989). By combining this theory with the effect of pH, the selectivity of ion exchange resin for lactate and acetate obtained in this study may be explained.

Nanofiltration gave a limited selectivity for lactate and acetate, although a higher selectivity was achieved with NF90 membrane compared to NF270 membrane. This could be due to the lower
molecular cut-off value of the NF90 membrane. For NF270 from pH 3 to 7, lactate rejection was between 22 ± 1 to 44 ± 2% and acetate was 2 ± 1 to 16 ± 2% (Table 5.5). For NF90, lactate rejection was between 2 ± 1 to 27 ± 1% while acetate was 3 ± 1 to 15 ± 2%. Difference in pH had an effect on the selectivity of extraction, where the lactate rejection decreased by half while acetate rejection decreased by eight fold from pH 7 to pH 3.

5.3.2 To achieve a high extraction efficiency

Research Question 14: How to achieve a high extraction efficiency?

The extraction efficiency of membrane electrolysis increases with increasing pH (from 16.54 ± 0.65% to 87.44 ± 1.21% for current density of 10 A m⁻²) (Table 5.2), this was expected as there were more dissociated ions, which were able to participate in the process. Current density did not have a significant effect on the extraction selectivity. Although higher applied current can allow a faster extraction rate, the coulombic efficiency will drop. Higher current density allows for more hydroxyl production at the cathode, leading to more deprotonated carboxylates which improves the extraction.

For nanofiltration, the flux of lactate and acetate decreased as pH increased from 3 to 7 (Table 5.5). For NF90 membrane, lactate flux decreases from 2.19 ± 0.04 to 1.58 ± 0.07 mmol m⁻² s⁻¹, while acetate flux from 4.71 ± 0.08 to 4.05 ± 0.02 mmol m⁻² s⁻¹. At higher pH, there were more charged molecules and hence they are repulsed by the charged surface of the membrane. The result of this study is consistent with literature (Freger et al., 2000; Timmer et al., 1993), where the increase in pH resulted in increased lactate rejection. The permeate flux achieved in this study is of the same magnitude as earlier studies (Bouchoux et al., 2006), with a lactate recovery rate of 6.94 ± 0.56 mmol m⁻² h⁻¹. Addition of salts such as Na₂SO₄ can result in a negative retention of lactate at low permeation fluxes (Umpuch et al., 2010), and operational parameters such as pH, salts concentration, temperature, etc can directly influence the properties of membranes and solutes, hence affecting extraction efficiency (Freger et al., 2000), therefore these effects need to be taken into consideration when dealing with real fermentation broth.

For pertraction with the hollow fibre unit, increasing pH from 3 to 7 resulted in decreased extraction rate. Lactate flux decreased from 0.32 ± 0.02 to 0 mmol m⁻² s⁻¹, while acetate from 0.37 ± 0.03 to 0.05 ± 0.02 mmol m⁻² s⁻¹. This showed that undissociated ions can migrate more easily across the membrane, which is consistent with literature (Huang et al., 2004). The extraction rate is a function of feed flow rate and not extractant flow rate (Huang et al., 2004). The effect of feed flow rate was not tested in this study but it is worth mentioning that the extraction rate can be further improved.
For ion exchange operation with resin, higher pH improved the extraction efficiency of acetate, while lactate extraction was the highest at pH 4. Adsorption of lactate increased from $0.29 \pm 0.02$ to $0.73 \pm 0.11$ mol kg$^{-1}$ dry resin from pH 3 to 4, and decreased to $0.49 \pm 0.08$ mol kg$^{-1}$ dry resin at pH 7. For acetate, adsorption increased from $0.12 \pm 0.02$ to $0.91 \pm 0.10$ mol kg$^{-1}$ dry resin from pH 3 to 7. The combined carboxylates adsorbed were $1.23 \pm 0.12$ mol kg$^{-1}$ dry resin at pH 4, and $1.21 \pm 0.07$ mol kg$^{-1}$ dry resin at pH 5.5. Selection of ion exchange resin IRA 67 in its Cl$^-$ form was based on the study of John et al. (2008), where lactate adsorption of 126 mg g$^{-1}$ wet resin (2.15 mol kg$^{-1}$ dry resin) was obtained after direct contact of wet resin and aqueous LA solutions of different concentrations at 25 °C and pH 5 for 12 hours, hence the result obtained in this study were reasonable.

For ionic liquid, the extraction efficiency decreases with increasing pH. Combined carboxylates extracted was from $223.6 \pm 1.3$ to $23.0 \pm 1.7$ mol m$^{-3}$ IL. The observation can be explained by the increase in charged molecules at higher pH. At pH 5.5 and 7, the concentration of carboxylate even increased, this indicated that water was lost, possibly due to the forming of reverse micelle (Martak & Schlosser, 2007).

5.3.3 To achieve a high concentration extract

*Research Question 15: How to obtain a high concentration in extraction?*

When one considers mixed culture fermentation or fermentation using a waste substrate, a low concentration of carboxylic acids can be expected as tested here (0.150 M). Electrochemical extraction techniques hold the advantage of being able to concentrate the product to levels of around 7 to 10 times the input stream. Membrane electrolysis was able to achieve a high extract concentration of up to 100 g L$^{-1}$ LA (Figure 5.2), with the anion exchange membrane tested. This is fairly reasonable compared to the reported concentration achieved by electrodialysis, up to 175 g L$^{-1}$ (Hábová et al., 2004). However, electrodialysis can only concentrate the product as it is whereas the membrane electrolysis generates a concentrate of the acid form.
For ion exchange resin, depending on the regeneration solution used, the eluate concentration can be as high as 50 g L\(^{-1}\) LA. Regeneration purity of LA can decrease with increasing normality of the acid regenerant (Bishai et al., 2015), while regeneration with alkali regenerant often results in a lower recovery efficiency (Cao et al., 2002). Ionic liquid extraction can potentially give a higher concentration of LA in the ionic liquid layer with distribution coefficients for aqueous systems above 40 reported (Martak & Schlosser, 2007). Nanofiltration does not give a concentrate of LA or AA, while pertraction often results in a lower extract concentration due to the hydrophilic nature of LA and AA.

### 5.3.4 Product utilization

The different methods used here give either concentrates of lactate or LA, or solutions with diminished acetate/acetic content. This implies that the suitability of the resulting stream will depend on the target further product. Extraction of LA using ionic liquid can allow a direct utilisation of carboxylic acid extracted. The phosphinate based ionic liquid was chosen for LA extraction in this case based on the study of Martak and Schlosser (2007), where a high distribution coefficients for aqueous systems of above 40 was observed. Recently, the study of Andersen et al. (2016) shows that extraction and esterification of low-titre short chain volatile fatty acids (AA) from anaerobic fermentation can be performed simultaneously with ionic liquids extraction and addition of ethanol, forming ethyl acetate. Thus, with ionic liquids the products of the extraction can be an ester and a LA solution. If combined with membrane electrolysis, the LA can be at high concentration.
Membrane electrolysis gives products of carboxylic acids in their acid form, effectively avoiding the acidification step hence less chemical dosing is required. For pertraction, the acid form of carboxylic acids can also be obtained after removal of the solvent used for extraction. While for nanofiltration and ion exchange resin, carboxylic acids often remain in their salt forms.

5.3.5 Comparison of extraction methods

Research Question 16: How do the extraction methods tested compared to each other?

Depending on the purpose, process design and end use of product, different extraction method may be more suitable based on their nature and properties. The most essential criteria are summarized in Table 5.6.

Selection of extraction technology can be largely dependent on the nature of solution to be extracted. A typical mixed culture fermentation broth without pH control will have a total carboxylic concentration of lower than 20 g L$^{-1}$ (Khor et al., 2016), compared to pure culture fermentation with pH control where concentration of more than 100 g L$^{-1}$ can be obtained. For electrochemical approach, extraction of carboxylate is feasible as long as the concentration is above 3 g L$^{-1}$, of which the extraction efficiency drops tremendously below that. For very low solute concentration, other extraction method such as adsorption or ion exchange may be more appropriate. As both LA and AA are not volatile, extraction technologies such as gas stripping, pervaporation and vacuum were not considered. In membrane electrolysis, decrease in pH resulted in a low carboxylate flux as shown in Table 5.3, especially when the pH is lower than the pK$_a$ of the carboxylate. The results of this study fit well with the decision tree developed in Van Hecke et al. (2014) for-in situ product recovery, which considers product characteristics such as inhibitory effect, stability, volatility and pK$_a$.

Practical issues have to be taken into consideration as well in extraction processes, especially when it is intended to be coupled to real biological system such as fermentation. Extraction technique such as membrane electrolysis may have less problems at solid handling as the driving force is electric current. While with nanofiltration, the driving force is pressure, hence the configuration and set-up have to be optimized to mitigate clogging problem. Cost is also a main criterion during selection of extraction techniques, for electrochemical extraction, membrane and electrodes can often be expensive as they require a more sophisticated specification to avoid scaling and reduce resistance. Mitigations such as removal of cations before contact with electrochemical system can reduce scaling. While ion exchange resin, nanofiltration and hollow fibre membrane can often be cheaper as there are less requirements, and they are already manufactured and used commercially. Practical hurdles
such as manufacturing cost, biocompatibility and environmental toxicity also exist in ionic liquid application, which are gradually being overcome as more research is done to discover different classes of ionic liquid or ways to synthesize ionic liquid in order to fully exploit its potential.

Combination of different extraction technologies to improve extraction efficiency can also be an option. For example, the combination of pertraction or ionic liquid to remove acetate from a lactate-acetate mixture solution, followed by subsequent electrochemical extraction or ion exchange processes to obtain a concentrate of LA.

**Table 5.1** Pertraction of synthetic binary mixture containing lactate and acetate

<table>
<thead>
<tr>
<th>pH</th>
<th>Rate of lactate extracted (mmol m$^{-2}$ s$^{-1}$)</th>
<th>Rate of acetate extracted (mmol m$^{-2}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.32 ± 0.02</td>
<td>0.37 ± 0.03</td>
</tr>
<tr>
<td>4</td>
<td>0.01 ± 0.01</td>
<td>0.34 ± 0.04</td>
</tr>
<tr>
<td>4.8</td>
<td>0.00 ± 0.00</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>5.5</td>
<td>0.00 ± 0.00</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>7</td>
<td>0.00 ± 0.00</td>
<td>0.05 ± 0.02</td>
</tr>
</tbody>
</table>

**Table 5.2** Membrane electrolysis extraction lactate and acetate from synthetic binary mixture

<table>
<thead>
<tr>
<th>pH</th>
<th>Lactate flux (mol m$^{-2}$ s$^{-1}$)</th>
<th>Acetate flux (mol m$^{-2}$ s$^{-1}$)</th>
<th>Cathodic efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Current density (A m$^{-2}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>0.80 ± 0.03</td>
<td>0.69 ± 0.05</td>
<td>16.54 ± 0.65</td>
</tr>
<tr>
<td>4.0</td>
<td>1.55 ± 0.05</td>
<td>1.04 ± 0.03</td>
<td>28.93 ± 0.65</td>
</tr>
<tr>
<td>4.8</td>
<td>3.48 ± 0.02</td>
<td>2.45 ± 0.04</td>
<td>46.23 ± 0.50</td>
</tr>
<tr>
<td>5.5</td>
<td>2.93 ± 0.07</td>
<td>4.82 ± 0.11</td>
<td>86.62 ± 1.46</td>
</tr>
<tr>
<td>7.0</td>
<td>2.96 ± 0.06</td>
<td>4.87 ± 0.09</td>
<td>87.44 ± 1.21</td>
</tr>
</tbody>
</table>
### Table 5.3 Ionic liquid extraction of lactate and acetate from synthetic binary mixture

<table>
<thead>
<tr>
<th>pH</th>
<th>Lactate extracted (mol m⁻³ IL)</th>
<th>Acetate extracted (mol m⁻³ IL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>98.7 ± 0.9</td>
<td>124.9 ± 0.9</td>
</tr>
<tr>
<td>4.0</td>
<td>46.4 ± 2.1</td>
<td>80.9 ± 2.1</td>
</tr>
<tr>
<td>4.8</td>
<td>1.2 ± 1.2</td>
<td>21.8 ± 1.2</td>
</tr>
<tr>
<td>5.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7.0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

### Table 5.4 Ion exchange of lactate and acetate from synthetic binary mixture

<table>
<thead>
<tr>
<th>pH</th>
<th>Lactate extracted (mol kg⁻¹ dry resin)</th>
<th>Acetate extracted (mol kg⁻¹ dry resin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.29 ± 0.02</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>4</td>
<td>0.73 ± 0.11</td>
<td>0.38 ± 0.06</td>
</tr>
<tr>
<td>4.8</td>
<td>0.63 ± 0.09</td>
<td>0.60 ± 0.08</td>
</tr>
<tr>
<td>5.5</td>
<td>0.49 ± 0.05</td>
<td>0.72 ± 0.05</td>
</tr>
<tr>
<td>7</td>
<td>0.49 ± 0.08</td>
<td>0.91 ± 0.10</td>
</tr>
</tbody>
</table>

### Table 5.5 Nanofiltration of synthetic binary mixture containing lactate and acetate

<table>
<thead>
<tr>
<th>pH</th>
<th>Lactate flux (mmol m⁻² s⁻¹)</th>
<th>Acetate flux (mmol m⁻² s⁻¹)</th>
<th>Lactate rejected (%)</th>
<th>Acetate rejected (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF270 &amp; 3</td>
<td>2.77 ± 0.03</td>
<td>4.67 ± 0.07</td>
<td>2 ± 1</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>&amp; 4</td>
<td>2.59 ± 0.12</td>
<td>4.62 ± 0.02</td>
<td>8 ± 2</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>&amp; 4.8</td>
<td>2.32 ± 0.04</td>
<td>4.53 ± 0.07</td>
<td>18 ± 1</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>&amp; 5.5</td>
<td>2.16 ± 0.11</td>
<td>4.36 ± 0.09</td>
<td>23 ± 2</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>&amp; 7.0</td>
<td>2.05 ± 0.04</td>
<td>4.10 ± 0.04</td>
<td>27 ± 1</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>NF90 &amp; 3</td>
<td>2.19 ± 0.04</td>
<td>4.71 ± 0.08</td>
<td>22 ± 1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>&amp; 4</td>
<td>2.03 ± 0.02</td>
<td>4.63 ± 0.04</td>
<td>28 ± 1</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>&amp; 4.8</td>
<td>1.81 ± 0.07</td>
<td>4.51 ± 0.09</td>
<td>36 ± 2</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>&amp; 5.5</td>
<td>1.61 ± 0.06</td>
<td>4.27 ± 0.07</td>
<td>43 ± 2</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>&amp; 7.0</td>
<td>1.58 ± 0.07</td>
<td>4.05 ± 0.02</td>
<td>44 ± 2</td>
<td>16 ± 2</td>
</tr>
</tbody>
</table>
# Table 5.6 Comparison of extraction technologies

<table>
<thead>
<tr>
<th>Methods</th>
<th>Concentration factor*</th>
<th>Selectivity</th>
<th>Energy investment</th>
<th>Cost</th>
<th>Solid handling</th>
<th>End use of product</th>
<th>Pretreatment requirement</th>
<th>TRL level</th>
<th>Concentration level</th>
<th>Energy input</th>
<th>Chemical use</th>
<th>Equipment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrodialysis</td>
<td>Up to 15 times</td>
<td>Low</td>
<td>Moderate</td>
<td>High</td>
<td>Poor</td>
<td>Mixed products</td>
<td>Yes</td>
<td>8-9</td>
<td>High (maximum 200 g L⁻¹)</td>
<td>Electrical</td>
<td>Salts for conductivity</td>
<td>Normal pump, membrane, power supply</td>
</tr>
<tr>
<td>Membrane electrolysis</td>
<td>Up to 10 times</td>
<td>Low</td>
<td>Moderate</td>
<td>High</td>
<td>Good</td>
<td>Mixed products</td>
<td>No (salt precipitation can be done to reduce scaling)</td>
<td>3-4</td>
<td>High (maximum 100 g L⁻¹)</td>
<td>Electrical</td>
<td>Salts for conductivity</td>
<td>Normal pump, membrane, power supply</td>
</tr>
<tr>
<td>Nanofiltration</td>
<td>~1</td>
<td>Low – moderate</td>
<td>Moderate</td>
<td>Average</td>
<td>Poor</td>
<td>Mixed products</td>
<td>No (pre-filter can be done to reduce clogging)</td>
<td>8-9</td>
<td>Low – moderate</td>
<td>High pressure flow</td>
<td>None</td>
<td>High pressure pump, membrane</td>
</tr>
<tr>
<td>Ion exchange resin</td>
<td>Up to 5 times</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>Good</td>
<td>Mixed products</td>
<td>No</td>
<td>9</td>
<td>High</td>
<td>Low flow</td>
<td>Regeneration chemicals for adsorption and desorption</td>
<td>Normal pump, resin</td>
</tr>
<tr>
<td>Pertraction</td>
<td>&lt; 1</td>
<td>Low – moderate</td>
<td>Low</td>
<td>Low</td>
<td>Average</td>
<td>Mixed or Pure products - polymerization</td>
<td>No</td>
<td>9</td>
<td>Low</td>
<td>Low flow</td>
<td>Draw solution – tap/distilled water/low salt concentration solution</td>
<td>Normal pump, membrane</td>
</tr>
<tr>
<td>Ionic liquid extraction</td>
<td>&gt; 50 times (depending on solubility of water in ionic liquid)</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
<td>Good</td>
<td>Mixed products - esterification</td>
<td>No</td>
<td>2-3</td>
<td>High</td>
<td>Low mixing</td>
<td>Regeneration chemicals</td>
<td>Mixing device, decanter</td>
</tr>
</tbody>
</table>


* concentration factor considering initial concentration of 10 g L⁻¹
Alkane production from grass
Chapter 6 Alkane production from grass

6.0 Abstract

Energy dense alkanes are classically obtained from fossil fuels, but can also be generated electrochemically from carboxylic acids. Caproic acid is a six carbon microbial metabolic product that can be converted to decane via Kolbe electrolysis. Here, we achieved the fastest caproic acid production described thus far from a lignocellulosic biomass (grass, via a coupled process), resulting in a >0.7 g g$^{-1}$ purity solution. Grass was first fermented in a leach-bed-type reactor to lactic acid, and used as an intermediate to form caproate in a secondary fermenter. The lactic acid concentration in the fermentation system was 9.36 ± 0.95 g L$^{-1}$ over a 33-day semi-continuous operation, and converted to caproic acid at a concentration of 4.09 ± 0.54 g L$^{-1}$ during stable production of semi-continuously system. In a separate test, the highest rate of caproate production (from lactate) obtained was 0.99 ± 0.02 g L$^{-1}$ h$^{-1}$, the highest reported production rate to date from a real substrate. The culture was capable of producing a maximum caproate concentration of 10.92 ± 0.62 g L$^{-1}$. The produced broth was transferred to membrane electrolysis to extract and concentrate the caproate as caproic acid, and the hydrophobic acid then phase-separated with a purity of >70%. This extracted caproic acid was upgraded into energy dense fuel, decane, through Kolbe electrolysis, with an approximate energy investment of 19.2 kWh kg$^{-1}$, under non-optimized proof-of-concept condition. Notably, the pipeline was almost completely powered through electrical inputs, and thus could potentially be driven from sustainable energy without need for chemical input.

Figure 6.1 Graphical abstract

6.1 Introduction

Alkane production from carboxylic acids by Kolbe electrolysis was first described in 1849, and has recently come back into focus following increased attention for carboxylic acids as products from biomass (Kolbe, 1849; Frontana-Uribe et al., 2010; Wang et al., 2016). Caproic acid is a key example, as an energy dense medium-chain carboxylic acid. The demand for caproic acid has been growing due to its application as chemical commodity, feed additive and more recently as bio-based fuel.
precursor. The medium-chain carboxylic acid market (lauric acid, caprylic acid, caproic acid, and capric acid) will reach USD 1.25 billion globally by 2020 (Zion, 2014). Generally these medium chain fatty acids are derived from natural fatty acids such as coconut and palm oil by fractional distillation, ozonolysis or catalytic reduction processes (Larranaga et al., 2016). Apart from the conventional processes, medium chain fatty acids can also be microbially synthesized from alcohols and carboxylates through fermentation. It was recently shown that a solution of lactic acid could enable caproic acid production through the microbial reverse β-oxidation pathway where substrate is elongated, as opposed to the well-known β-oxidation pathway where substrate is catabolized. Within a context of second generation bio-refining and the initial production of lactic acid, grass comes to the fore.

Grass is a widely available substrate presently underused. In the US, grasslands represent an estimated $2.51 \times 10^6$ km$^2$ of available biomass (FAOSTAT, 2016), which is either ensiled or wasted. A grass based fermentation can lead to the formation of lactate and acetate. In a pH uncontrolled, mesophilic fermentation of grass, a concentration of 12.6 g L$^{-1}$ lactic acid can be reached, along with 2.0 g L$^{-1}$ of acetic acid (Khor et al., 2016). Lactic acid is highly soluble in water, which makes the downstream extraction of lactic acid difficult. Therefore, it is desirable to create a more hydrophobic product such as caproic acid. $n$-caproate can be produced from reverse β-oxidation pathway with ethanol as the most common reducing substrate (Angenent et al., 2016) and acetate as the electron acceptor. Studies have been carried out with pure cultures such as Clostridium kluyveri (Ding et al., 2010; Barker et al., 1945) and Megasphaera elsdenii (Choi et al., 2013). Although caproate production from lignocellulosic material (Xiong et al., 2015) and a route using lactate was considered long ago (Elsden et al., 1956; Zhu et al., 2015; Sträuber et al., 2012), only recently this process was considered feasible through the reverse β-oxidation pathway (Sträuber et al., 2016; Spirito et al., 2014; Andersen et al., 2015), and it has been demonstrated in mixed culture communities (Kucek et al., 2016).

The hydrophobicity and low water solubility of caproic acid allows extraction of caproic acid from the fermentation broth through phase separation. If the concentration of caproic acid exceeds the solubility limit (11.0 g L$^{-1}$ at 20 °C), caproic acid will form a miscible layer and phase separate from the fermentation broth. Recently, Xu et al. (2015) managed to extract n-caproate from a bioreactor broth using an in-line membrane electrolysis system, creating at an anode an acidified broth with the caproic acid.
The acidified broth could be further converted via Kolbe electrolysis (Kolbe, 1849). This is the process of electrochemically converting carboxylic acids into alkanes through the decarboxylative dimerization of two carboxylic acids or carboxylate ions. The process generates alkanes as main products, and the anodic reaction can be represented by $3 \text{R}_1\text{COO}^- + 3 \text{R}_2\text{COO}^- \rightarrow \text{R}_1\text{R}_1 + \text{R}_1\text{R}_2 + \text{R}_2\text{R}_2 + 6 \text{CO}_2 + 6 e^-$. Conditions for Kolbe electrolysis are well documented in the literature (Schäfer, 1990), and Kolbe electrolysis of carboxylic acids have also been studied in depth (Stang & Harnisch, 2016). Caproic acid has been tested for generation of hydrocarbons in the past (Levy et al., 1984; Wadhawan et al., 2001), but it has not been tested on extracts from fermentation. And even though each of the process steps has been reported in the past in literature, the pipeline approach is a novel and challenging approach, showing plenty of room for further optimization and research.

### 6.2 Materials and Methods

#### 6.2.1 Substrate, microorganisms and cultivation

Farmland grass was harvested from a meadow on January 2016 (East Flanders, Belgium). The grass was air-dried at 28 °C to total solid of $0.836 \pm 0.006$ g g$^{-1}$ grass (volatile solid $0.728 \pm 0.011$ g g$^{-1}$ grass) and stored at 4 °C until used. To make the grass more accessible for fermentation, size reduction was performed with a blender (Philips Daily HR2100/90) before fermentation. The bacterial culture for lactic acid fermentation was native to the grass itself without extra inoculation. The microbiome for elongation, dominated by *Clostridia* and *Lactobacillus* spp. related species, was obtained from a continuous reactor producing caproic acid from thin stillage containing both ethanol and lactate (Andersen et al., 2015). 25 mL of thin stillage reactor effluent was collected and centrifuged at 5000 g for 300 seconds. The supernatant was removed and the pellet obtained was washed 3 times with tap water and dissolved in 10 mL tap water before it was used as inoculum for elongation test.

#### 6.2.2 Semi-continuous fermentation

The semi-continuous fermentation test for grass to lactic acid consisted of a 100 mL Schott bottle filled with 50 mL of tap water and 10 g (wet weight) of grass. The grass was packed in 2 bags made of AISI 316 stainless steel mesh (44 µm mesh size, 33 µm wire thickness), each containing 5 g (wet weight) of grass. The experiment was run under anaerobic condition for 33 days at 32 °C. The substrate in one of the stainless steel bag was replaced every day (2 days of solid retention time). Half of the liquid phase was removed and refilled with tap water (2 days of hydraulic retention time). The system was sparged with nitrogen each time during substrate and liquid replacement and flushed with...
nitrogen after substrate replacement to ensure anaerobic condition. Samples were collected every day for carboxylic acids analysis. pH was between 4.8 and 5.8, and it was not adjusted or controlled throughout the experiment.

6.2.3 Semi-continuous microbial elongation

The semi-continuous elongation test consisted of a 100 mL Schott bottle filled with 50 mL of elongation broth. The experiment was run under anaerobic condition for 30 days at 32 °C. Half of the solution was replaced with effluent from the fermentation test every day (2 days of hydraulic retention time). The system was sparged with nitrogen each time during substrate replacement and flushed with nitrogen after substrate replacement. Samples were collected every day for carboxylic acids analysis. pH was between 5.5 and 6.3, and it was not adjusted or controlled throughout the experiment. At the end of experiment, the elongation broth was split into six parts, which served as the inoculum for independent experiments to determine maximum caproic acid concentration (n = 3) and maximum rate of caproic acid production (n = 3).

6.2.4 Maximum rate of caproic acid production test

This test was performed (n = 3) in 50 mL Falcon tubes, each containing 30 mL of elongation broth. The Falcon tubes were centrifuged at 5000 g for 300 seconds to retain the cells in the elongation broth. Half of the supernatant was replaced with effluent from the fermentation test every day, for each replacement it was expressed as 1 cycle. Normal operation (without cells retention) was performed for the first 4 cycles, which served as the baseline. From the 5th to 8th cycle, cells were retained. The tubes were then left unattended for 1 week before resuming the 9th to 14th cycle, with cell retention. Samples were collected at 0, 1 and 24 hours for carboxylic acids analysis. 1 mL of sample was collected for volatile suspended solid analysis, before the solution was centrifuged and replaced.

6.2.5 Maximum concentration of caproic acid test

The test was performed (n = 3) in 50 mL Falcon tubes, each containing 30 mL of elongation broth. 1.135 mL of 50% sodium lactate (VWR) solution was added to the elongation broth to give an extra 20 g L⁻¹ lactate in the tubes as no on-line recovery was installed in the semi-continuous elongation operation. In addition to the lactate coming from the lactic acid fermentation, the resulting lactate concentration in the tubes was approximately 25 g L⁻¹. Samples were taken at 0, 2, 4, 6, 8, 12, 24 and 48 hours for carboxylic acids analysis.
6.2.6 Electrochemical extraction

The electrochemical cell was constructed from Perspex™, consisting of an anode chamber (20 × 5 × 0.3 cm³) and a cathode chamber (20 × 5 x 2.6 cm³), separated by an anion exchange membrane (Type 2 membrane, Fujifilm Manufacturing B.V., Netherlands) with a surface area of 0.01 m². The cathode was an AISI Type 316L stainless steel felt (20 × 5 x 0.15 cm³) with 1 mm wire thickness (LierFilter Ltd., China), and the anode was a (mixed metal oxide) iridium oxide coated titanium electrode (IrO₂/TaO₂: 0.65/0.35), 20 cm × 5 cm, with a centrally attached, perpendicular current collector (Magneto Special Anodes BV, The Netherlands). The cathode chamber was filled with effluent from the elongation system, the anode chamber was filled with tap water. The extraction was performed in batch, a recirculation flow rate of 1.67 mL s⁻¹ was maintained for both cathode and anode chamber to ensure mixing. 0.4 A of current was applied using a potentiostat (VSP, Biologic, France) in chronopotentiometry mode to drive the extraction operation, resulting in a current density of 25 A m⁻². The pH of cathode chamber was controlled at 5.5 ± 0.3 by electrochemical water reduction and dosing of 2 M sulphuric acid solution with a pH controller to maximize recovery in this experiment.

6.2.7 Kolbe electrolysis

The Kolbe electrolysis process was performed according to the study of Wadhawan et al. (2001). 50 mL one chamber dimerization reactor consisted of a spiral wire platinum anode (0.5 mm diameter, 3 cm² surface area, Bio-Logic, France), a stainless steel plate cathode (12.5 cm² surface area, height 5 cm × length 2.5 cm, and width 0.5 cm), and an Ag/AgCl reference electrode (filled with 3 M KCl solution). Platinum was chosen as anode material, stainless steel as cathode material. The chamber was filled with either control (0.5 M sodium sulphate in distilled water), synthetic solution (0.1 M caproic acid and 0.5 M sodium sulphate), or real solution (electrochemical extraction anolyte, containing 26 mM caproic acid), with pH was adjusted to 7. Before electrolysis, cyclic voltammetry (CV, 10 mV s⁻¹), with ohmic drop compensation, was performed on the broth using a potentiostat (SP-50, BioLogic, France), at 25 °C. Kolbe electrolysis experiments were performed at chronopotentiometry mode at a fixed current density of 0.133 A cm⁻² (i = 0.4 A) for 24 h. Liquid phase of reactor was sampled at time 0, 2, and 24 for analysis. The overall process is presented in Figure 6.2.

![Figure 6.2 Overall process](image-url)
6.2.8 Analytical methods

Determination of fermentation products including organic acids (lactic acid, acetic acid, propionic acid, butyric acid) was performed with Metrohm ion chromatography equipped with Metrosep organic acids column and Metrosep organic acids guard column, and an ion chromatography conductivity detector, using 1 mM H_2SO_4 as eluent at flow rate of 0.0083 mL s\(^{-1}\), oven temperature at 35 °C, and 500 mM LiCl as regenerant for suppressor. Caproic acid in aqueous phase was measured by gas chromatography (GC-2014, Shimadzu®, The Netherlands) with DB-FFAP 123-3232 column (30 m x 0.32 mm x 0.25 µm; Agilent, Belgium) and a flame ionization detector. Total solids (TS), volatile solids (VS) and volatile suspended volatile (VSS) were measured via the standard method (APHA, 1995). Phase separated caproic acid and decane were measured using nuclear magnetic resonance (NMR) spectroscopy. \(^1\)H NMR and \(^13\)C NMR were performed at 400 MHz and 100 MHz respectively on a Bruker Avance III Nanobay 400 MHz spectrometer. 400 µL DMSO-d6 was added to 25 mg of caproic acid and decane sample, stirred and transferred to an NMR tube. Quantification was performed relative to caproic acid and decane as standard (0.2 M in DMSO-d6), contained in a NORELL 100 µL capillary insert. \(^1\)H NMR experiments were run with 8 scans and 1 second relaxation delay.

6.3 Results and Discussion

6.3.1 Semi-continuous fermentation of lactic acid from grass

Research Question 17: How to produce caproic acid from grass?

Semi-continuous fermentation of lactic acid using grass as substrate was performed for 33 days and the lactic acid concentration stabilized at 9.36 ± 0.95 g L\(^{-1}\), with the acetic acid concentration at 0.90 ± 0.14 g L\(^{-1}\) (Figure 6.3a). The pH of the fermentation broth was between 4.5 and 5. The lactic acid concentration rapidly reached its final concentration within the first day of fermentation, indicating that the native microorganisms of grass were active as there was no lag time for lactate production. Lactic acid and acetic acid were the main products during fermentation due to low pH and short retention time of 2 days.

The conversion of organic compounds to lactic acid was low relative to our earlier study (Khor et al., 2016) (0.056 g g\(^{-1}\) versus 0.128 g g\(^{-1}\) grass total solid) and the rate of lactic acid production was 0.197 g L\(^{-1}\) h\(^{-1}\). The conversion rate and efficiency of biomass to lactic acid was low in this study, which is likely due to the limited pretreatment and reactor design involving a higher ratio of solution volume relative to mass of grass. Pretreatment can be performed to improve the biomass biodegradability,
while taking into consideration the cost-benefit-sustainability nexus. For instance, mild pretreatment methods such as lime pretreatment can be applied without introducing excessive cost, to improve conversion efficiency and production rate, and minimize carbon loss (Khor et al., 2015).

![Figure 6.3](image)

**Figure 6.3** (a) carboxylate profile of fermentation system, and (b) bacterial community of fermentation system; (c) carboxylate profile of elongation system, and (d) bacterial community of elongation system

### 6.3.2 Semi-continuous elongation of caproic acid through lactic acid

When the grass fermentation effluent was fed into the elongation system, lactic acid was consumed, producing mainly caproic acid (C6), as shown in Figure 6.3b, along with butyric acid (C4) and acetic acid (C2). Enanthic acid (C7) and caprylic acid (C8) were also tested for but were not detected unlike in other chain elongation systems (Andersen et al., 2015). Caproate concentration was $4.09 \pm 0.54$ g L$^{-1}$ ($9.03 \pm 1.19$ g COD L$^{-1}$) and the selectivity for caproate was $49 \pm 9\%$ during the stable phase (30 day operation, Figure 6.3b). The pH of the elongation broth was between 5.5 and 6.2, showing the pH increasing effect of chain elongation.

As proposed in the study of Zhu et al. (2015), the stoichiometry for caproic acid formation via lactic acid was:

$$3 \text{CH}_3\text{CHOHCOO}^- + 2 \text{H}^+ \rightarrow \text{CH}_3(\text{CH}_2)_3\text{COO}^- + \text{H}_2\text{O} + 2 \text{H}_2 + 3 \text{CO}_2$$

$$\Delta G_r^0 = -123.1 \text{ kJ mol}^{-1}$$

This stoichiometry was followed to assess the caproic acid production in this study. In terms of lactic acid and caproic acid balance, on average $4.68 \pm 0.47$ g L$^{-1}$ ($53 \pm 5$ mM) of lactic acid was fed and the average caproic acid production was $2.05 \pm 0.27$ g L$^{-1}$ ($18 \pm 2$ mM). This fits very well with the stoichiometry of 3 mol lactate forming 1 mol caproate, or a consumption of $2.32$ g lactate per g caproate formed leading here to an efficiency of $101 \pm 17\%$. It is demonstrated here that lactic acid
which is generated from low value substrate, in this case grass, can act as an intermediary for caproic acid production. While caproic acid production can also be achieved with ethanol utilization, it tends to bind one to be connected with an integrated biorefinery. Under different circumstances, one can foresee that both pathways are interesting.

### 6.3.3 Maximum rate of caproic acid production and maximum concentration of caproic acid test

**Research Question 18:** What is the maximum concentration of caproic acid that can be achieved during lactic acid elongation?

**Research Question 19:** What is the maximum production rate of caproic acid that can be achieved during lactic acid elongation?

The highest rate of caproic acid production obtained was 0.99 ± 0.02 g L\(^{-1}\) h\(^{-1}\), when the cells were retained (Figure 6.4 – cycle 6). When the system was left unattended for 1 week to test the resilience of the elongation microorganisms, the production rate could recover to its maximum state after 4 cycles of operation (Figure 6.4 – cycle 9 to 12) showing the robustness of the culture. Table 6.1 compares different feed sources and maximum rates of medium chain fatty acids produced for microbial chain elongation process.

The maximum caproic acid production rate achieved in this study is high compared to the literature such as the study of Grootscholten et al. (2013b), where 4.5 g L\(^{-1}\) day\(^{-1}\) (0.19 g L\(^{-1}\) h\(^{-1}\)) was achieved with mixed culture up-flow anaerobic filter system using castor oil, at pH between 6.5 – 7. Similar rates as reported here were thus far only obtained with synthetic media, with the highest from acetate and ethanol to medium chain fatty acids, 57.4 g L\(^{-1}\) day\(^{-1}\) (2.39 g L\(^{-1}\) h\(^{-1}\)) (Grootscholten et al. 2013a). It is important to note that to achieve this a neutral pH value between 6.5 – 7.2 was needed, requiring dosing of base which was not done here.

Cell density is often unreported in other literature studies, and cell retention and maximizing the density in the fermenters can potentially improve conversion dramatically. Considering previous knowledge, it is suggested that the production rate achieved here is a result of high microbial density, lack of pH controlling agents such as caustic and the used substrate (real versus synthetic).

During maximum concentration of caproic acid test, an excess amount of lactic acid was dosed to avoid substrate limitation, and the maximum concentration of caproic acid achieved was 10.92 ± 0.62
g L$^{-1}$. The selectivity towards caproic acid was $69 \pm 5\%$ (Figure S6.2), compared to the $49 \pm 9\%$ selectivity of normal elongation operation. While chain elongation in most studies occurs at neutral pH ($6.5 - 7.2$), due to toxicity of caproic acid, the highest concentration achieved in this study was under acidic conditions (pH 5.5 – 6).

**Figure 6.4** Production rate of caproic acid (g L$^{-1}$ h$^{-1}$, red circles) and percentage volatile suspended solids (%VSS, yellow circles) of elongation broth during maximum rate of caproic acid production test (error bars represent the standard deviation of triplicates)
### Table 6.1 Microbial chain elongation operation and maximum rate of medium chain fatty acid production

<table>
<thead>
<tr>
<th>Feed source</th>
<th>Operation</th>
<th>Culture</th>
<th>pH</th>
<th>Maximum rate of production (g L(^{-1}) h(^{-1}))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthetic medium containing acetate, ethanol, and yeast extract, with CO(_2) gas flow</td>
<td>Continuous</td>
<td>Mixed (non-sterilized, mainly <em>Clostridium kluyveri</em>)</td>
<td>6.5 – 7.2</td>
<td>2.39 (medium chain fatty acids)</td>
<td>(Grootscholten et al., 2013a)</td>
</tr>
<tr>
<td>Grass (mixed source)</td>
<td>2 steps-semi-continuous</td>
<td>Mixed (non-sterilized, mainly <em>Clostridium cluster IV</em>)</td>
<td>5.5 – 6.2</td>
<td>0.99 (caproic acid)</td>
<td>This study</td>
</tr>
<tr>
<td>Synthetic medium containing galactitol, yeast extract, with in-situ extraction</td>
<td>Batch</td>
<td>Pure (<em>Clostridium sp. BS-1</em>)</td>
<td>6.5</td>
<td>0.34 (caproic acid)</td>
<td>(Jeon et al., 2013)</td>
</tr>
<tr>
<td>Dilute ethanol and acetate, with in-line extraction</td>
<td>Continuous</td>
<td>Mixed (non-sterilized, mainly <em>Clostridium spp.</em>)</td>
<td>5.2 – 5.5</td>
<td>0.33 (caprylic acid)</td>
<td>(Kucek et al., 2016)</td>
</tr>
<tr>
<td>Synthetic medium containing acetate and ethanol</td>
<td>Semi-continuous</td>
<td>Mixed (non-sterilized, mainly <em>Clostridium kluyveri</em>)</td>
<td>7</td>
<td>0.20 (caproic acid)</td>
<td>(Steinbusch et al., 2011)</td>
</tr>
<tr>
<td>Castor oil</td>
<td>Continuous</td>
<td>Mixed (non-sterilized, mainly <em>Clostridium kluyveri</em>)</td>
<td>6.5 – 7</td>
<td>0.19 (caproic acid)</td>
<td>(Grootscholten et al., 2013b)</td>
</tr>
<tr>
<td>Yeast fermentation beer</td>
<td>Continuous</td>
<td>Mixed (non-sterilized, mainly <em>Clostridium kluyveri</em>)</td>
<td>5.5</td>
<td>0.14 (caproic acid)</td>
<td>(Ge et al., 2015)</td>
</tr>
<tr>
<td>Diluted yellow water</td>
<td>Semi-continuous</td>
<td>Mixed (non-sterilized, mainly <em>Clostridium cluster IV</em>)</td>
<td>5.5 – 6.5</td>
<td>0.12 (caproic acid)</td>
<td>(Zhu et al., 2015)</td>
</tr>
</tbody>
</table>
6.3.4 Electrochemical extraction of caproic acid and fuel production through Kolbe electrolysis

Research question 20: Can caproic acid obtained from elongation process be turned into decane?

Effluent from the elongation system was sent to the cathodic chamber of an electrochemical system for extraction and concentration of caproic acid, with an applied current of 0.4 A (resulting in 25 A m\(^{-2}\)). When current was applied, the anion exchange membrane allowed only anions such as carboxylates, sulphate, chloride and phosphate to migrate from the cathodic to anodic compartment through the membrane to complete the circuit. The pH dropped at the anodic compartment as protons were generated due to water electrolysis, and carboxylates formed their undissociated counterparts and thus accumulated in the anodic compartment. Over time, the caproic acid phase-separated from the anodic broth as the concentration exceeded its maximum (11 g L\(^{-1}\) at 20 °C in water), forming a lower density, hydrophobic layer on top of the solution (Figure S6.3), similar to that of Xu et al. (2015).

Kolbe electrolysis was performed on the extracted product, introduced in anolyte again to a concentration of 3.03 g L\(^{-1}\) (26 mM). The electrolysis reaction resulted in decarboxylation and dimerization of caproic acid, leading to production of decane in the organic phase. Caproic acid was electrolyzed at a rate of 1.21 g L\(^{-1}\) h\(^{-1}\) (0.202 kg m\(^{-2}\) h\(^{-1}\)) for synthetic solution and 1.05 g L\(^{-1}\) h\(^{-1}\) (0.174 kg m\(^{-2}\) h\(^{-1}\)) for a real solution (Figure 6.5a). Compared to literature, where conversion rates of valeric acid were 1.9 kg m\(^{-2}\) h\(^{-1}\) assuming 100% Coulombic efficiency (Nilges et al., 2012). The coulombic efficiency was low in this study as the process was not yet optimized for Kolbe electrolysis, however optimized systems in literature have reached as high as 45% and 75% yield of Kolbe dimer product with hexanoic acid (Wadhawan et al., 2001). Increasing the current density beyond the mass transport limit can give rise to oxygen evolution rather than the desired Kolbe process, resulting in loss of efficiency. The synthetic solution CV was shifted earlier compared to the control, which suggested an anodic reaction apart than water electrolysis, while the real solution CV gave a distinctive peak for an unexpected anodic reaction (Figure 6.5b). A qualitative analysis was performed on the alkane formed, but a quantitative analysis was not possible due to small amount that could be sampled throughout the laboratory pipeline. NMR analysis reveals that 10% of the product was decane, and indicated that decane was not the sole product. The other products were unidentified at this stage. The aqueous phase contains more impurities such as Cl\(^{-}\), SO\(_4^{2-}\), acetic acid, butyric acid and others, which subsequently led to formation of other impurities and non-Kolbe products which warrants further investigation.
Figure 6.5 (a) Caproate concentration over the period of Kolbe electrolysis, and (b) Cyclic voltammetry of solution before Kolbe electrolysis. Control (red line) contained only 0.5 M Na$_2$SO$_4$ in water, synthetic solution (green line) contain 0.1 M caproic acid and 0.5 M Na$_2$SO$_4$ in water, and real solution (blue line) was the anolyte from electrochemical extraction system.

6.3.5 Preliminary assessment of turning grass into aviation fuel

Grass was used as substrate to produce chemicals such as lactic acid, caproic acid and decane. Here the process was performed in discrete steps, whereas in future phases the caproic acid fermentation and extraction will be directly coupled, and potentially the Kolbe electrolysis will be performed directly in the anode of the extraction cell. The system could thus be simplified to two fermenters and a coupled electrochemical system which will likely entail efficiency increases.

A rough estimate of economic outlook is made by considering the operating costs (Table 2). Pretreatment such as extrusion requires around 0.20 kWh kg$^{-1}$ TS of grass (Khor et al., 2015), and extraction using membrane electrolysis needs approximately 2 kWh kg$^{-1}$ carboxylic acid extracted. Kolbe electrolysis of valeric acid into octane requires around 1.64 kWh kg$^{-1}$ octane, assuming 100% selectivity (Nilges et al., 2012). Here, 50% conversion of caproic acid to decane is assumed. Electricity price is assumed to be 0.125 € kWh$^{-1}$. Production of 1 kg of lactic acid costs 0.33 €. When further converted into caproic acid, the production cost increased to 0.44 €. And conversion to 1 kg decane incurs a cost of 1.29 € kg$^{-1}$ decane produced. Comparing these numbers to the market selling price of the products, lactic acid (1 € kg$^{-1}$), caproic acid (1.60 € kg$^{-1}$), and decane (0.41 € kg$^{-1}$, as aviation fuel), one can see that caproic acid has a higher margin for profit. Lactic acid can also be an lucrative product, although further product purification may be needed. Decane is a less attractive end product due to its low economic value at this point of time. The calculations performed in this study only give an indication on the energy and costs associated with conversion of grass. A complete
economy analysis which takes into account the capital investment, maintenance, labor costs, and a more detailed operating costs need to be evaluated further in separate study.

Table 2  Material, energy and costs required to produce 1 kg of lactic acid, caproic acid or decane

<table>
<thead>
<tr>
<th>Material (kg TS)</th>
<th>Lactic acid</th>
<th>Caproic acid</th>
<th>Decane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grass</td>
<td>3.33</td>
<td>7.69</td>
<td>25.00</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Energy (kWh)</th>
<th>Lactic acid</th>
<th>Caproic acid</th>
<th>Decane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment</td>
<td>0.67</td>
<td>1.54</td>
<td>5.00</td>
</tr>
<tr>
<td>Extraction</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>Kolbe electrolysis</td>
<td>-</td>
<td>-</td>
<td>3.28</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>2.67</strong></td>
<td><strong>3.54</strong></td>
<td><strong>10.28</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cost (€)</th>
<th>Lactic acid</th>
<th>Caproic acid</th>
<th>Decane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment</td>
<td>0.08</td>
<td>0.19</td>
<td>0.63</td>
</tr>
<tr>
<td>Extraction</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Kolbe electrolysis</td>
<td>-</td>
<td>-</td>
<td>0.41</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>0.33</strong></td>
<td><strong>0.44</strong></td>
<td><strong>1.29</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Selling price (€ kg(^{-1}))</th>
<th>Lactic acid</th>
<th>Caproic acid</th>
<th>Decane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.00</td>
<td>1.60</td>
<td>0.41</td>
</tr>
</tbody>
</table>

In 2005, it was estimated that \(2.457 \times 10^{11}\) kg of municipal solid wastes were generated in the United States, where a significant fraction of the organic material was yard trimmings (U.S. Composting Council, 2005). This gives an estimation of \(1 \times 10^9\) kg \((1.35 \times 10^9\) L) of fuel which can be produced in a green manner, if 10% municipal solid waste is made up of yard trimming. This can be compared to \(7.8 \times 10^{10}\) L of aviation fuel consumption in year 2012 in the US (Davidson et al., 2014). Decane has an energy density of 44.2 MJ \(\text{kg}^{-1}\), if 1 kg of grass can be transformed into 0.08 kg of decane, an energy value of 3.49 MJ \(\text{kg}^{-1}\) grass can be obtained.

Today, fuel is clearly the least attractive endpoint economically and mechanisms such as \(\text{CO}_2\) taxes or biofuel labelling will be needed in years to come. However, the market size and need for liquid aviation fuel are likely to drive considerable development towards the fuel outcome. In the meantime, the developed pipeline here can also deliver lactic acid and caproic acid, both in demand as chemical building blocks or the latter also as feed additive. Further conversion through e.g. esterification in-situ can already create a wide array of outcome products (Andersen et al., 2016).
General discussion and perspectives
Chapter 7 General discussion and perspectives

7.1 Main findings

This thesis aimed to convert grass into valuable chemicals such as lactic acid, caproic acid and fuel. The process pipeline was investigated in several steps including pretreatment, storage, fermentation, anaerobic digestion, extraction, and product upgrading, and several major findings were obtained.

In Chapter 2, low temperature (10 °C) Ca(OH)$_2$ pretreatment on grass improves methane production up to 37%. After extrusion, Ca(OH)$_2$ post-treatment further enhances biogas production and the effect of temperature on Ca(OH)$_2$ treatment is less critical. When considering extrusion speed, fast extrusion is more energetically favourable for biomass pretreatment.

In Chapter 3, extrusion with Ca(OH)$_2$ addition improves grass storage, even when it is wilted. Ensiling grass with 100 g kg$^{-1}$ TS Ca(OH)$_2$ addition before extrusion is the optimum considering biomass characterization, methane production and carboxylate production. Carboxylate and methane production are well-retained after 3 months storage when using the right pretreatment combination.

In Chapter 4, lactate concentration increases with acetate accumulation on both batch and semi-continuous operation during fermentation. Given the substrates which are relatively more digestible (extruded grass and corn starch, compared to non-treated lignocellulosic biomass) at sufficient concentration, along with the operating conditions chosen (pH uncontrolled), the bacterial populations develop towards lactic acid producing community. This is essentially what happens in a cow stomach when one is overfed. Over-abundant substrates prompt lactic acid bacteria to take over the community and produce lactic acid to get rid of the reducing equivalent. This phenomenon is reproduced in a reactor under intended conditions and purposes to produce lactic acid. In this study, it is also found that higher acetate concentration promotes a more diverse lactic acid bacteria population, especially when the microbial culture is not inoculated.

In Chapter 5, pertraction with a pH above 4 allows the extraction of acetic acid into draw solution and not lactic acid from a mixture broth. After separation of acetic acid, it is possible to extract lactic acid using another solvent such as tri-n-octylmethylammonium chloride dissolved in oleyl alcohol (Tong et al., 1998). While membrane electrolysis is not selective towards acetate or lactate, it can concentrate solutes from low concentration broth to up to 100 g L$^{-1}$. Using ionic liquid, more organic acids are extracted when pH decreased, and it is possible to produce ester directly from the organic acids in the ionic liquid phase. Nanofiltration and ion exchange using resin offer a limited degree of selectivity, however they are currently some of the most economically feasible and highly efficient
methods for organic acids extraction. Overall, selection of extraction technologies greatly depends on the operation purpose and end use of the product.

In Chapter 6, it is shown that decane can be produced from grass, via production and conversion of lactic acid and caproic acid. Caproic acid up to a maximum concentration of $10.92 \pm 0.62 \text{ g L}^{-1}$ and rate of production of $0.99 \pm 0.02 \text{ g L}^{-1} \text{ h}^{-1}$ can be achieved. While the bacterial community of fermentation reactor is dominated by *Lactobacillus* spp. (lactic acid producer), the elongation reactor mainly consists of *Clostridium IV* (medium chain fatty acids producer) and *Lactobacillus* spp.. While previous study also reported *Megasphaera* spp. to be the main medium chain fatty acids producer in reactor bacterial community (Andersen et al., 2015), it is not detected in this study. This may indicate that reactor microbiome can develop towards different population while still achieving the same carboxylate products. However, more investigation will be needed to learn whether there is difference in functionality between the two microbiome.

### 7.2 Discussion

#### 7.2.1 Biomass preparation

Pretreatment via extrusion and calcium oxide addition clearly benefitted the further conversion of grass (Chapter 2 and 3). Although extrusion is an effective pretreatment, one should bear in mind that there is an associated equipment maintenance cost (~3000 € month$^{-1}$ for a 1 MW biogas plant), depending on the types and moisture content of substrates. We succeeded in creating a logistic chain for the pretreatment combination and storage. For woody biomass, it is possible to avoid storage by only cutting down the plant when needed. However, for other substrates such as grasses, storage is a necessity as they are normally not available or delivered continuously. Grass can grow at a rate of around 5 to 15 cm per month, depending on the species, time and weather. Considering the process cost of collecting grass, together with climate of location, grass storage period of at least 6 months to 1 year can be expected for continuous production in biorefinery assuming once or twice per year of grass harvesting. If other sources of grass such as from municipal solid waste is considered, the storage time can be reduced. Ideally, storage of biomass should preserve quality of biomass with minimal loss, and at the same time ‘prepare’ the biomass for further processing. The process should also not incur negative impacts such as inhibition on microbial activity. And essentially the storage process needs to be time, cost and energy efficient.

In the case where biomass is not stored, it is possible to use more than one type of feedstock when the other is not available. A biorefinery plant which co-digests more than one lignocellulosic biomass may mean that different pretreatment methods may be needed. This is often not possible as flexibility
is often not the case in biorefinery plant, since it is translated directly into equipment and process investment cost. But on the other hand, flexibility would make the biorefinery much more independent from feedstock supply and all its economics. Hence, this route is also possible if the substrates have similar properties whether or not after pretreatment, or a more robust and universal process chain is employed.

In the case of grass utilization, given enough scale of the substrate, it may be more beneficial to use only a single feedstock, as this minimizes issues with variability both processes and equipment wise. It is also demonstrated here that storage of grass can be integrated between pretreatment and conversion steps, to improve its biodegradability for further conversion, while preserving the biomass. Unlike other pretreatment such as acid pretreatment, use of Ca(OH)$_2$ also does not result in production of inhibitory compounds towards microbial activity. Calcium scaling is also not a big issue as fermentation process is performed at acidic pH.

In this study, it is shown that Ca(OH)$_2$ pretreatment at temperature as low as 10 °C can be effective for grass. This can be potentially interesting for locations within the temperate region. Grass can then be harvest during warm seasons, and stored while pretreated during cold season. Furthermore, in terms of storage, it is also found that wilting can be halted with Ca(OH)$_2$ given a high enough amount, which is between 10 to 20 g kg$^{-1}$ TS Ca(OH)$_2$ from this study. This makes a nice opportunity for high risk biomass storage, e.g. at high moisture content (>30%), or low sugar content. In terms of chemical use, instead of using Ca(OH)$_2$, there is also possibility to use other alkali such as CaO, which can be more reactive than Ca(OH)$_2$, and it has a drying effect which may assist storage. It is still necessary to take into account the low solubility of Ca(OH)$_2$ and CaO, which can be crucial to its pretreatment efficiency. Application in powder form can avoid the addition of moisture to the biomass, but homogenous mixing and solubilisation of the chemical are also more difficult in this case. In this study, the extrusion provides the mixing and moisture content coming from the grass solubilise the chemical. It may also be interesting to test the effect in combination with other chemicals such as glycerol, as Ca(OH)$_2$ is soluble in glycerol, and glycerol pretreatment was also reported to be efficient in delignification of biomass (Guragain et al., 2011). Glycerol is a common by-product from biodiesel industry with a reasonably low price at around 0.10 € kg$^{-1}$, hence the use of glycerol in pretreatment process would not add significant cost.

Apart from the advantages, there are also drawbacks regarding storage and Ca(OH)$_2$ treatment such as potential ammonium losses, e.g. $2 \text{NH}_4\text{Cl} + \text{Ca(OH)}_2 \rightarrow \text{CaCl}_2 + 2 \text{H}_2\text{O} + 2 \text{NH}_3$. However, these are of minor importance considering lactic acid production. Moreover, when storage is done at a biorefinery facility, adequate ammonium treatment facilities is often present such as ones at composting factories to deal with ammonium losses. Considering grass feedstock, it can come from
many different sources such as nature, agriculture, municipal solid waste, or roadside, which can be both a blessing and a burden. From a positive view, this makes the scale and opportunity of grass utilization bigger, but on the other hand, it becomes more difficult to choose what and when the feedstock should be used for. For instance, should agricultural grass be used for animal feed or biorefinery purposes? Although the economy is normally the ultimate criteria for decision making, however will that lead to a 2nd generation food versus feed debate? And this time it is the food for the animals? It is expected that when the demand for grass would increase, there arises pressure on previous non-cultured grasslands and even pristine grassland for exploitation. The logic question arises: how will that affect the ecosystem? What is the optimal pathway for grass? All these matters need to be further considered to ensure a balanced scenario between ecosystem, economy and society. In this study, protein degradation was not exclusively tested. If ammonium losses are minimal, there is a good chance that after lactic acid fermentation process, the solid residues can be used for animal feed.

### 7.2.2 Product conversion

At the moment, most commercial plants of ethanol and biodiesel productions are still working with rather clean feedstock such as dedicated crops. The main disadvantage is the use food-grade materials or land where food-grade materials can be grown on. The search for more complex solutions is inevitable as we step away from the food for fuel debate into the second generation of biorefinery, and the challenge is to simplify the process to a reasonable level for economical production.

In terms of fermentation, although it is known that high substrate concentration can induce lactic acid production, however the actual mechanism behind the trigger is still not fully clear. One of the explanations is the overabundance of reducing equivalent and bacteria can get rid of the reducing equivalent quickly by forming NAD$^+$ through lactic acid production, but the question follows is that why do the bacteria have to or respond in this way (by taking the lactate route) to dispose of the reducing equivalent quickly? Further investigation into redox potential, biochemical pathway, microbiology and genetics may help answering this question. Regarding the effect of acetate on lactic acid production, microbes can gain more energy by producing acetic acid and hence it is often the preferable pathway. Accumulation of acetate can shift the product equilibrium and reduce acetate production, which in turn more substrate can be directed towards lactic acid production. Under an ideal scenario, lactic acid as the sole product without any trace acetic acid would be preferred. However, this is often not possible and hence this strategy arose from the anticipation of acetate co-product which is not extracted. This will certainly add stress on the extraction system in terms of
selectivity. There is also a different approach where microbes which specifically consume acetate can be added to remove acetate co-product, however the effect on lactic acid production has to be tested.

During elongation of lactic acid to caproic acid, a maximum concentration of $11 \text{ g L}^{-1}$ was reached, which is also the maximum solubility of caproic acid in water coincidently. At a pH of between 5.5 to 6.2 in the elongation system in this study, only around 2 to 18% of caproic acid is in its protonated form. Hence caproic acid should still remain in the aqueous solution, and phase separation is not expected. At the moment, it is not completely well understood whether the protonated or non-protonated form, or both forms of the medium chain fatty acid contribute to the toxicity towards the microbes in the system. The toxicity effect of caproic acid and other carboxylic acids need to be further tested to understand their effect on the microbiome and the capacity of the caproic acid producer. In this context, flowcytometry analysis may shed light on the toxicity effect by measuring the live, dead and damaged cells with varying concentrations of short/medium carboxylic acids and exposure times. Isolation of the caproic acid producing bacteria in this system is also necessary to allow deep characterization of the bacteria functionalities. The highest caproic acid production rate of around $1 \text{ g L}^{-1} \text{ h}^{-1}$ was achieved in this study. This may not be actual maximum capacity of the microbiome as no additional nutrients such as yeast extract was added, except the grass itself. Furthermore, the production rate in this study was achieved without in-line extraction system. Integration of an in-situ extraction system may have a positive impact on the production rate.

Considering the microbial community of conversion processes, the community was tested for a period of around 30 days. On a longer term, community shifts might occur and that has to be verified in future work, although it is likely that product spectrum will not vary largely as in the study of Get et al., 2015.

In a mixed culture fermentation of complex substrate, it is expected to have multiple co-products or impurities along with the production of targeted compound. This can result in competition of substrates for products, and potential inhibitory effect of the co-product generated. In an undefined mixed culture fermentation, what really dictates the product outcome? Is it the kinetics, thermodynamics, operating parameters, inhibition, or others? While they are all interlinking factors which contribute to shape a fermentation process, it can be tedious to isolate factors and test the effect from each of them. Fermentation process in this study is directed towards lactic acid production, it is most likely an unrealistic to strive for pure outcome from a undefined mixed culture and so, extraction will be necessary.
7.2.3 Product extraction and synthesis

Product extraction is often a challenging and costly process. Ideally, a conversion process should aim to produce a single product of highest possible purity to reduce extraction and purification cost. Although in some cases a mixed spectrum of products is acceptable, however it also means a lower product price due to lower product purity. Hence it is almost always more desirable to obtain a single product of high purity. This can be tedious in a mixed culture fermentation process. The fermentation process in this study was tuned preferentially towards lactic acid production, nevertheless it is often accompanied by acetic acid as co-product. By assuming an extraction technology with selectivity towards lactic acid, the liquid recycle stream will contain residual acetic acid which will lead to an accumulation. From Chapter 4, it is shown that acetate accumulation resulted in a higher lactic acid concentration, in both batch and semi-continuous operation. This gives an indication that selective extraction of lactic acid and recycling of residue carboxylic acids to fermentation broth will not negatively impact lactic acid fermentation process. However, this is only provided that selective extraction can be achieved. In that sense, Chapter 5 gives a possible mean of selective extraction in a lactic acid and acetic acid mixture system using pertraction, although the mechanism is not yet fully understood.

Pertraction can allow an acetate residual concentration of around 200 mg L$^{-1}$ remaining in the broth. Compared to the concentration of lactic acid in fermentation broth which can be around 10 to 30 g L$^{-1}$ for mixed culture fermentation, it is already a good ratio to start with for further downstream processes. The flux of ions through of pertraction is low in this study ($< 1$ mmol m$^{-2}$ s$^{-1}$). However, this can be compensated by setup configuration and operating conditions, to bring rate to a factor of 100 times higher or more. The use of hollow fibre module with bundle configuration can increase contact surface area, without adding significant cost due to low price of hollow fibre membrane. Also, rather than a static broth in this study, a flowing broth in counter-current or cross-flow direction to the draw solution could also increase the flux of acetic acid through membrane (Schlosser et al., 2005).

It is possible to couple conversion process strategically with downstream processes to ease extraction. This can be done by taking advantage of target compounds which have specific properties, for example longer chain fatty carboxylic acids which are more hydrophobic, or esters which have lower boiling points. As such, producing caproic acid from lactic acid tackles the extraction issue by having a high value and easily extractable product. In this study, the route of lactic acid to caproic acid and decane was investigated. Even though three carbon molecules are converted to CO$_2$ for every molecule of caproic acid formed, it could potentially be a worthwhile approach considering the higher product price of caproic acid and the possibility to extract the product by phase separation. In this
study, caproic acid was extracted by membrane electrolysis, and the product was harvested at a separate anodic compartment. A question also arises whether caproic acid can be produced to an amount which exceeds the solubility limit of the system, so that it ‘auto-phase-separated’ from the elongation broth? If a pH of 5.5 is considered, caproic acid concentration of higher than 60 g L$^{-1}$ will be needed before caproic acid will start phase separating. For pH 4, the concentration needed will be 27 g L$^{-1}$, and for pH 3 it is 16 g L$^{-1}$. The limit of the caproic acid producing bacteria found in this study is not yet known completely, but there may be a possibility that this can be achieved through a well-designed process or metabolic engineering. By applying another extraction and synthesis strategy, it is also possible to produce ester from lactic acid and caproic acid. By applying the same principle as in the study of Andersen et al. (2016), a proper choice of ionic liquid can extract lactic acid and caproic acid into the ionic liquid phase, and the process can be tuned to form ethyl lactate and ethyl caproate by addition of ethanol.

Economic feasibility of fuel (decane) production from lignocellulosic biomass is also briefly discussed in Chapter 6. Membrane electrolysis is a costly operation, mainly due to the expensive anode (~500 € m$^{-2}$ for IrOx-MMO coated titanium electrode) and ion exchange membrane (~100 € m$^{-2}$), and energy requirement for applied current to drives the ions across ion selective membranes. The cost of membrane can be reduced significantly as the technology becomes more mature. As for anode, the situation appears to be the opposite, the price of anodes as currently manufactured will tend to increase due to the rising price of rare metals. There may be a need to look for alternative electrode materials, improve electrode efficiency, or optimize the electrochemical operation to increase the lifetime of the electrode. For example, integration of a layer of Bi-doped SnO$_2$ on anode can enhance the overall conductivity of the anode (Cho & Hoffmann, 2017). Advancement of material technologies such as polymer, nanoparticles and graphene could also potentially have significant impact on the breakthrough of extraction efficiency. For instance, development of nitrogen doped carbon nanotubes electrodes could potentially reduce the electrode cost as the synthesis technology matures. Prices of rare metals such as platinum is in the range of 25 to 35 USD per gram, and palladium at 19 to 25 USD, in their unfabricated state (APMEX® 2016). The bulk price of multi-walled carbon nanotubes (MWNTs) is now less than 0.1 USD per gram (De Volder et al., 2013), and for single-walled carbon nanotubes (SWNTs), it is possible to bring the price down to 15 to 35 USD per gram with new continuous production method (ScienceDaily® 2012), this can be further reduced with the increase in scale use and production capacity, and advance in synthesis technology. Development of new types of membrane electrolysis reactor such as the photocatalytic membrane reactor (Kim et al., 2016; Molinari et al., 2014) can also have potential application in the context of
Chapter 7

108

extraction. As already discussed, the energy demand for membrane electrolysis is not low, and the use of photocatalytic electrode can take advantage of solar energy as renewable energy source.

7.3 Grass biorefinery

Although a grass biorefinery has potential benefits and opportunities, it also comes with its disadvantages and threats. There are still issues which need to be addressed and overcome. In this study, a proof of concept to produce valuable chemicals such as lactic acid, caproic acid and decane was shown. For decane production from grass, although the proof of concept opens up possibilities towards production of energy dense fuel from lignocellulosic biomass, further optimization and technological breakthroughs may still be needed to allow a fully integrated process. Fuel may not be economically the most attractive end product of biorefinery at this point of time, considering the price of petroleum and abundance of shale gas. However, the process pipeline developed has the flexibility to deliver multiple products depending on the choice of operation, and it can be easily tuned towards fuel production when it is needed in the future. Also, there exists company such as Infinite Fuels GmbH (Germany) which aims to take carboxylic acid Kolbe electrolysis process to commercial scale. This indicates that it can still be a profitable business given that the process is executed efficiently.

As mentioned previously, there is a huge potential to synthesize biodegradable polymer from lignocellulosic biomass. However, the intermediate steps such as hydrolysis of biomass and extraction of carboxylic acids still need to be optimized to make the process economically sound. Research on that pipeline is also performed and still ongoing as part of this research project, although it is considered premature to be presented in this thesis at this stage.

At present, the yields of products and rates of conversions are low, and the costs associated are high, as the processes are not yet optimized. To improve the process efficiency and economic outlook, production rates need to be targeted at the level of industrial production of between 1 to 5 g L\(^{-1}\) h\(^{-1}\). A high conversion of substrate is also desirable. About 0.6 gram out of 1 gram of grass mass is constituted of cellulose and hemicellulose, hence it is fair to aim for at least 70% conversion to products, taking in account losses during pretreatment, hydrogen and carbon dioxide production during mixed culture biological conversion processes.

From a practical standpoint, low-level applications might also become ice-breakers for further technology development for grass utilization. For example, solution made from squeezing and compressing grass – ‘grass juice’ is used as an alternative for de-icing strategy (http://www.demorgen.be/wetenschap/het-nieuwe-strooizout-grassap-b7dc3823/). Also, grass fibres are utilized to produce innovative egg packaging (Figure 7.1). Apart from that, cell phone which is
made from grass blades and salvaged phone components has also been created (Figure 7.1). These prove that there is interest and potential in grass, and there are opportunities to look for innovative applications.

![Carton and phone made from grass](© GreeNest and O₂)

Apart from that, care has to be taken to ensure that the biodiversity and ecosystem surrounding the grassland is not disturbed by over-exploitation. SWOT analysis with particular dedication to grass biorefinery is summarized in Table 7.1.

**Table 7.1 SWOT analysis of grass biorefinery (adapted from Laub et al., 2016)**

<table>
<thead>
<tr>
<th><strong>Strengths</strong></th>
<th><strong>Weaknesses</strong></th>
<th><strong>Opportunities</strong></th>
<th><strong>Threats</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Grass is widely available almost everywhere on Earth</td>
<td>Collection of grass may be tedious and costly as it is widely spread throughout large areas</td>
<td>Some grass sources are easily accessible and they are yet to be used for biorefinery</td>
<td>Care has to be taken not to ensure that the biodiversity and ecosystem surrounding the grassland is not disturbed</td>
</tr>
<tr>
<td>Most EU countries have implemented favourable policy measures for AD, which resulted in a stable AD sector, consolidating its role as a favourable tool for sustainable waste management</td>
<td>Grass residues are legally classified as biowaste, therefore it has to be properly removed, treated and disposed, even though the risk is lower compared to biowaste such as manure. This is subjected to waste management system and resulting gate fee and transportation costs</td>
<td>Grass can be a renewable carbon source for biorefinery or act as a substitute for energy crop</td>
<td>More post-treatment may be required for the digestate from biorefinery of grass</td>
</tr>
<tr>
<td>Some regions provide subsidies to manage nature landscapes, which act as an incentive to harvest grass from nature areas</td>
<td>There is insufficient awareness and acceptance regarding technologies for mowing, storage and delivery of grass residues</td>
<td>Incentive instruments and initiatives for the acceleration of the grass valorisation chain can be improved</td>
<td>Biorefinery management system which focus on a reliable and consistent feedstock may contradict with the local roadside maintenance system which leans towards the most cost-effective methods</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Some countries differentiate in their support to AD from residual biomass compared to energy crops</td>
<td>Grass can be complex substrate with much impurities, hence extensive pre-treatment or pre-processing</td>
<td>Financial incentives are offered for the sustainable management of natural resources in rural areas in some countries</td>
<td>Transport cost and gate fees discourage grass collection and valorisation</td>
</tr>
<tr>
<td>Grass in a digester can reduce nitrogen concentrations in the digestate and decreases the potential for ammonia toxicity</td>
<td>There is no specific incentives that apply for grass in AD, no extra fee for biomass from nature conservation area, and no claim for a biomethane bonus</td>
<td>Urban and suburban areas for recreational purposes are rich in grass which are often harvested and the production cost is negligible. The grass is located within areas served by the collection of municipal solid wastes (MSW), creating an opportunity for a combined and more efficient collection and transport of grass</td>
<td>In some countries, incentives and support mechanisms for AD has been decreasing, which reduces the margins and impairs the use of grass as feedstock</td>
</tr>
<tr>
<td>In most European countries digestate is used on agricultural land OR as a soil enhancer for other types of end-use, closing the nutrient loop</td>
<td>The stakeholders throughout the grass value chain are mostly unaware of each other and do not work together</td>
<td>Grass from roadsides and watercourses is mostly left on site and is rarely transferred to a composting facility</td>
<td>---</td>
</tr>
</tbody>
</table>
7.4 Moving grass biorefinery forward

While waste or lignocellulosic biorefinery can bring forth plenty of benefits, there are still much challenges that needs to be tackled. It is shown in Chapter 6 that cell retention by centrifugation can improve the production rate of caproic acid significantly. However, this is economically demanding in a full sized continuous system. Cell immobilization by support materials may seem to be an energetically more favourable solution, but it is also difficult to achieve at larger scale due to several issues such as control of cell growth and mass transfer limitation on substrates and products. Moreover, at the inside of biofilms conditions may arise favourable for methanogens, diverting carbon away from products. One interesting approach could be the flocculating cells, where the microbes are engineered to flocculate and settle down (Zhao et al., 2014). Apart from that, a more efficient reactor design and process control may also assist cell retention.

Another main challenge comes with the substrate itself. As already mentioned, hydrolysis is still one of the major limitation when considering utilization of lignocellulosic biomass. Compared to pure sugars, lignocellulosic biomass or waste may be cheaper in terms of price, however it has also a lower density by considering both its physical and chemical components which can be utilized for conversion – lignin, cellulose, hemicellulose and extractives. Therefore, pretreatment employed needs to avoid further dilution of the substrate, while still hydrolyse the biomass efficiently and being cost effective. While steam/ammonia fibre explosion and alkali pretreatment are cost effective and currently employed in some commercial plant, more pretreatment methods are still developed to allow a more complete fractionation of biomass. This thesis exploits the use of calcium hydroxide at low temperature as pretreatment for grass and shows its potential. The pretreatment is low-tech, and effective with minimal drawbacks, even though it may not be suitable for all types of substrates. Other pretreatment methods such as the use of ionic liquid may be interesting, although the production cost and biocompatibility still need to be optimized for biorefinery processes. Ionic liquid pretreatment which is currently developed by Gschwend et al. (2016) can dissolve and extract lignin by precipitation, leaving hemicellulose and cellulose behind for conversion processes. Furthermore, production of the ionic liquid is estimated at a bulk price of 1.35 € kg$^{-1}$, which can have significant impact on making the use of ionic liquid economically feasible. Despite the advantages, recovery of ionic liquid and its influence on conversion process in terms of inhibition and toxicity still has to be considered.

It is undoubtedly that there is still much room for improvement in extraction technologies. As for membrane electrolysis which is mainly employed in this thesis, research is still needed to look into alternative electrode materials embedded with intended catalytic properties to increase process
efficiency and to overcome cost limitation. Development of extraction technology with product selectivity such as a more chromatography-like extraction, molecular imprinted polymer, and others may also be interesting not only to provide extraction process versatility, but also to improve our understanding towards selective extraction. New reactor design is also an important area which is often overlooked, but necessary to enable the transition into commercial scale. In line with this, process modelling can also help to simulate and provide preliminary assessment at larger scale, which can improve process design and avoid expensive modification cost.

Considering the overall process, production is split into multiple process steps in study. From a different biorefinery point of view, there is also a tendency to move towards a one pot process. One of such example is consolidated bioprocessing (Brethauer & Studer, 2014; Zhang et al., 2011). This process aims to combine enzymatic hydrolysis and fermentation into a single step, either by using multiple species of microbes or through genetic engineering of a single species. While process steps tend to be more bulky and requires a higher capital investment, they offer the advantage of a higher flexibility and more degree of freedom on process controls.

In order to achieve a conclusive economic outlook, complete utilization of biomass is necessary. While biological conversions mainly target cellulose and hemicellulose, the lignin fraction is often left untouched. To fully exploit the potential of biomass, the lignin component need to be extracted and utilized. Lignin can constitute a significant fraction of up to 30% in lignocellulosic biomass (McKendry, 2002), it is also a renewable feedstock with many potential applications. It has been shown that use of chemicals such as formadehyde stabilization is able to facilitate monomer production during biomass depolymerisation (Shuai et al., 2016). Monomer yields of 76 to 90 mol% can be achieved by depolymerizing cellulose, hemicellulates, and lignin separately. Even though an alternative chemical which is less toxic is needed for application at commercial scale. Leftover or unconverted substrate may be used for animal feed, if protein content of the biomass is well preserved. Otherwise, unconverted substrate may be composted for use as fertilizer and soil amendment. Other than that, leftover can also be used to produce biogas through anaerobic digestion. Last but not least, thermochemical conversion such as pyrolysis can also be an option.

While most parties are mainly focusing on the development of downstream processing, the predicaments of upstream may be more of an issue and they should not be underestimated. Scaling up of technology is complex, physical handling can be tedious at large scale, especially with solid biomass, which are some of the main issues encountered at large scale system at the moment. Upstream processing can also heavily influence the downstream options and technologies. Therefore, both research on improvement of unit technology, as well as a more general pipeline approach is necessary.
For biorefinery to move forward, not only technology breakthroughs are needed, one also need to consider the economy, social and environmental aspects. One has to consider how a proposed biorefinery process can be integrated into the surrounding systems, e.g. by creating job opportunities for the local community, or bringing in capital investment, and at the same time conserving the surrounding ecosystem. The society is nowadays under the control of heavy monetary based system, the rule applies also to the field of biorefinery. Return on investment has to be high before parties will consider investing. This may on one hand catalyse the development of applied research and processes, while it also runs the risk of neglecting the advancement of more fundamental knowledge, which can make a difference in the long term. Also, for many of the discussed chemicals, alternative processes to produce these chemicals exist, which mean that it is harder to introduce new renewable technologies. New technologies have not only to outperform the state-of-the art but also overcome the resistance of new investments while existing installations are not fully depreciated. On the other hand, markets do exist and this is a clear advantage as quality standards and prices are known.

It is also critical to have all the parties and stakeholders aware of each other and their functions. Along the same line, participation and involvement of key parties are also crucial. For instance, a farmer would want to know what the added values is for them and the risks associated before they will decide to participate in a biorefinery venture. Communication and negotiation among key parties including policy maker will become more prevailing in time, and the speed of decision making and vision of the future will make a huge difference in the game of biorefinery. In that sense, biorefinery is also a new concept, as it introduces new parties around the negotiation table.

New biorefinery process can adapt a few themes which are reported to consistently recur among top performing chemical companies (BCG, 2016). One would be the ability to embrace complexity, and even using it as a source of competitive advantage. Some successful companies also tend to differentiated their business models, of which some are generally considered deviate from industry best practice, to shield themselves from competition in a way that models more dependent on scale. The tendency to act against trends has also helped companies to stay one step ahead of their competitors. Over the past 5 years, focused specialties industry has also clearly outperformed the rest of the industry with median total shareholder return of almost twice that of industry median. Some example of specialty chemicals include adhesives, pesticides, elastomers, flavours, fragrances, polymers, lubricants, surfactants, as such. This gives an indication of the direction of development in chemical industry, and interesting product which can be produced from waste biomass.
Abstract
Abstract

Second generation biorefinery is a facility for biofuel and material production where both fuel and high value products are produced from waste feedstock such as lignocellulosic biomass. Hydroxycarboxylic acids such as lactic acid can be produced from lignocellulosic biomass, which can serve as precursor to its polymer polylactic acid (PLA), or precursor for aviation fuel. A widely abundant biomass feedstock which comes forth for this purpose is grass. To achieve this, the intermediary step processes have to be thoroughly investigated.

Before the biomass feedstock can be utilized, it has to be pretreated to improve its biodegradability. Hence, the first objective of this thesis was to perform pretreatment on the selected biomass – grass. Thermo-mechanical pretreatment – extrusion and chemical pretreatment – calcium hydroxide were employed to enhance the biodegradability of grass. The efficiency of the pretreatment was evaluated based on the methane production and chemical oxygen demand (COD) conversion through mesophilic anaerobic digestion.

Once pretreatment was confirmed to be effective in improving the biodegradability of biomass, fermentation process was performed to obtain lactic acid. Thermophilic fermentation was carried out to improve hydrolysis and obtain a relatively pure L-lactic acid for further processes. The native microbiome from grass involved in the fermentation was also investigated.

Extraction of lactic acid then follows after the fermentation process. Membrane electrolysis using anion exchange membrane was used to extract lactic acid from the fermentation broth. Through this process, the pH of the fermentation broth can be controlled without extra chemical addition, and lactic acid can be extracted and concentrated in a clear solution.

Due to the hydrophilic nature of lactic acid, a possible strategy is to convert lactic acid into a more hydrophobic product – caproic acid through microbial chain elongation which is also known as reverse beta oxidation. Caproic acid has a maximum water solubility of 11 g L⁻¹ at 20 °C, this allow an easier product separation from the fermentation broth. The extracted caproic acid can be further upgraded to fuel by Kolbe electrolysis.

For polymerization of lactic acid into PLA, further purification processes were needed. First esterification process was used to remove acetic acid from the extracted solution. Ion exchange resin was used to further remove impurities from the broth. Finally, pure lactic acid was obtained using
diethyl ether extraction. Polycondensation was performed to polymerize the lactic acid into the biodegradable plastic PLA.
Bibliography


European Commision, Technology readiness level (TRL)

Bibliography


Market Research Report. (2012). Lactic acid and poly lactic acid (PLA) market analysis by application (packaging, agriculture, transport, electronics, textiles) and segment forecasts to 2020.


References


http://www.inverde.be/content/pdf/graskracht_eindrapport_LR.pdf


Appendix A
Appendix A

Supplementary information for Chapter 4

Table S4.1  Experimental setup of fermentation batch test

<table>
<thead>
<tr>
<th></th>
<th>Corn starch</th>
<th>Extruded grass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N₂</td>
<td>N₂</td>
</tr>
<tr>
<td></td>
<td>pH 5.5 w/o acetate</td>
<td>pH 7 w/ acetate</td>
</tr>
<tr>
<td>No inoculum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. delbrueckii</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>E. coli</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Rumen fluid 1st batch</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Rumen fluid 2nd batch</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

The two batches of fermentation inoculated with rumen fluid were performed at different time points. 1st batch was the main test comparing corn starch and extruded grass, while 2nd batch was an additional test to confirm the reproducibility, and to test the effect of hydrogen. We obtained fresh rumen fluid each time we performed the test, Figure S4.4a, 4.4b and 4.4c showed that the community was similar.

Table S4.2  Change in acetate concentration in batch fermentation

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inoculum</th>
<th>Acetate addition</th>
<th>Change in acetate concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn Starch</td>
<td><em>L. delbrueckii</em></td>
<td>w/o acetate</td>
<td>0.21 ± 0.02</td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em></td>
<td>w/ acetate</td>
<td>-0.34 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>Rumen fluid</td>
<td>w/o acetate</td>
<td>1.69 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>w/ acetate</td>
<td>0.44 ± 0.98</td>
</tr>
<tr>
<td>Extruded Grass</td>
<td><em>L. delbrueckii</em></td>
<td>w/o acetate</td>
<td>0.65 ± 0.04</td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em></td>
<td>w/ acetate</td>
<td>0.98 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>Rumen fluid</td>
<td>w/o acetate</td>
<td>1.15 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>w/ acetate</td>
<td>1.50 ± 0.26</td>
</tr>
<tr>
<td></td>
<td>No inoculum (pH 7)</td>
<td>w/o acetate</td>
<td>0.37 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>w/ acetate</td>
<td>2.74 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>No inoculum (pH 5.5)</td>
<td>w/o acetate</td>
<td>0.15 ± 0.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>w/ acetate</td>
<td>-1.56 ± 1.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>w/ acetate</td>
<td>0.66 ± 0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>w/ acetate</td>
<td>-0.30 ± 0.26</td>
</tr>
</tbody>
</table>
Table S4.3  Total number of sequence of lactic acid bacteria (LAB) in each individual batch test

<table>
<thead>
<tr>
<th>Consensus lineage</th>
<th>CS</th>
<th>R1</th>
<th>N2</th>
<th>CS</th>
<th>R2</th>
<th>N2</th>
<th>CS</th>
<th>R2</th>
<th>H2</th>
<th>N2</th>
<th>CS</th>
<th>R2</th>
<th>Ex</th>
<th>N2</th>
<th>No I</th>
<th>EX</th>
<th>No I</th>
<th>EX</th>
<th>No I</th>
<th>EX</th>
<th>No I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillaceae_1: Bacillus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>165</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacillaceae: Enterococcus</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1004</td>
<td>478</td>
<td>57</td>
<td>1 031</td>
<td>480</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacillaceae: Lactobacillus</td>
<td>10 594</td>
<td>6 543</td>
<td>14 047</td>
<td>10 392</td>
<td>14 102</td>
<td>10 853</td>
<td>10 072</td>
<td>3 917</td>
<td>2 483</td>
<td>3 371</td>
<td>2 139</td>
<td>138</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacillaceae: Leuconostocaceae : Leuconostoc</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>568</td>
<td>3 978</td>
<td>4 965</td>
<td>12 206</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacillaceae: Leuconostocaceae : Weissella</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacillaceae: Streptococccaceae : Lactococcus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3 848</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacillaceae: Streptococccaceae : Streptococcus</td>
<td>81</td>
<td>22</td>
<td>29</td>
<td>119</td>
<td>25</td>
<td>135</td>
<td>506</td>
<td>6 383</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total LAB</td>
<td>11 136</td>
<td>6 667</td>
<td>14 084</td>
<td>10 526</td>
<td>14 137</td>
<td>11 000</td>
<td>11 583</td>
<td>10 778</td>
<td>3 278</td>
<td>13 636</td>
<td>7 718</td>
<td>12 350</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CS = corn starch; EX = extruded grass; R1 = first batch; R2 = second batch; N2 = nitrogen; H2 = hydrogen; A = acetate addition; 5.5 = pH 5.5; 7 = pH 7; corn starch tests were all inoculated; extruded grass tests were not inoculated, unless indicated with annotation ‘I’, where ‘I’ = inoculated
Figure S4.1  Carboxylates concentrations of batch fermentation. (Error bars represent standard deviation between experimental replicates)

Figure S4.2  Lactate profiles for batch fermentation tests with extruded grass. (Error bars represent standard deviation between experimental replicates)
Figure S4.3  Propionate and butyrate profiles for semi-continuous fermentation tests with extruded grass.
Figure S4.4  Initial bacterial community of batch fermentation test. (a), (b) and (c) represent the bacterial community for rumen fluid; (d) represents the bacterial community of grass initially.
Figure S4.5  Alpha diversity for lactic acid productions for batch experiments. (CS: corn starch; R1: first batch; R2: second batch; N2: nitrogen; H2: hydrogen; A: acetate addition)

Lactic acid concentration for each individual batch experiments series is plotted against alpha diversity value measured in individual sample in Figure S4.5. For fermentation of corn starch, elevated acetate concentration promotes higher bacterial alpha diversity and increases lactic acid concentration. Elevated acetate concentration gives rise to a more diverse bacterial community, as well as more variable of LAB.
Figure S4.6  Principle component analysis on beta-diversity of all batch experiments and controls (CS = corn starch ; EX = extruded grass ; R1 = Batch 1 ; R2 = Batch 2 ; N2 = nitrogen ; H2 = hydrogen ; A = addition of 10 g/L acetate ; C = initial ; 5.5 = pH 5.5 ; 7 = pH 7 ; corn starch tests were all inoculated ; extruded grass tests were not inoculated, unless indicated with annotation ‘I’, where ‘I’ = inoculated)

From the Bray-Curtis dissimilarity indexes (Figure S4.6) among all samples considered, the nature of lignocellulosic biomass (corn starch or extruded grass) was found to be the first factor which clustered the mix microbial community. Elevated acetate did not have an impact on bacterial community structure except in batch experiment 2 of corn starch. All 3 controls with rumen fluid as inoculum presented a very similar microbial community structure. For the control reactors with corn starch as substrate, the headspace gas (hydrogen or nitrogen) did not have any effect on the mix microbial community structure. However, there was a difference in microbial community structure between the first batch and second batch of corn starch fermentation with nitrogen headspace, this was due to the different species of dominant Lactobacillus. Lactobacillus porcinae was dominant in the first batch (Lactobacillus spp. abundance was 84 ± 4 %), while Lactobacillus mucosae was dominant in the second batch (Lactobacillus spp. abundance was 99 ± <1 %). The principle component analysis was performed based on the bacteria species and hence the difference was observed, despite a 93 % common base (397 out of 425) between the two Lactobacillus species.
Appendix B
Appendix B

Supplementary information for Chapter 6

Bacterial community analysis

DNA extraction

DNA extraction was performed as previously reported (Khor et al., 2016). Samples were taken from initial inoculum, initial substrate and end of fermentation broth for analysis. 1.5 mL samples were centrifuged at 11 000 g for 300 s in a 2 mL Lysing Matrix E tube (Qbiogene, Alexis Biochemicals, Carlsbad, CA). Pelleted cells were re-suspended in 1 mL of lysis buffer containing Tris/HCl (100 mM at pH 8.0), 100 mM EDTA, 100 mM NaCl, 1% (w vol\(^{-1}\)) polyvinylpyrrolidone and 2% (w vol\(^{-1}\)) sodium dodecyl sulphate. Cells were lysed using 0.2 cm\(^3\) beads of 0.1 mm size in a Fast Prep-96 homogenizer for 40 s at 1600 rpm twice. Samples were centrifuged at 18 000 g for 60 s at room temperature and washed with phenol/chloroform (1:1) and chloroform. After centrifugation, nucleic acids (supernatant) were precipitated with 1 volume of isopropanol at −20 °C and 1:10 volume of 3 M sodium acetate. After centrifugation and washing with 80% ethanol, the pellet was re-suspended in 20 µL of Milli-Q water. The quality and quantity of DNA samples were analysed using Illumina sequencing primers by polymerase chain reaction (PCR). Amplified sequences were separated by electrophoresis on 1% agarose gels.

DNA sequencing and bioinformatics processing

DNA sequencing was performed as previously reported (Khor et al., 2016). The V3–4 region of the bacterial 16S rRNA gene was sequenced with Illumina sequencing Miseq v3 Reagent kit (http://www.illumina.com/products/miseq-reagent-kit-v3.ilmn, by LGC Genomics GmbH, Berlin, Germany) using 2 x 300 bp paired-end reads, and primers 341F (5’-NNNNNNNTTCTACGGGNGGCWGCAG) and 785R (5’-NNNNNNNTGACTACHVGGGTATCTAAKCC). Each PCR included DNA extract (~ 5 ng), forward and reverse primer (~15 pmol for each) and MyTaq buffer (20 µL containing 1.5 units MyTaq DNA polymerase (Bioline) and 2 µL of BioStablII PCR Enhancer). 8-nt barcode sequence was performed for both forward and revers primers of each sample. PCRs were carried out for 96 °C pre-denaturation for 120 s and 30 cycles using the following parameters: 96 °C for 15 s, 50 °C for 30 s, and 72 °C for 60 s. DNA concentration of amplicons of interest was determined by gel electrophoresis. Amplicon DNA of each sample (~ 20 ng) were pooled for up to 48 samples carrying different barcodes. PCRs showing low yields were further amplified for 5 cycles. The amplicon pools
were purified with one volume AMPure XP beads (Agencourt) to remove primer dimer and other small mispriming products, followed by an additional purification on MinElute columns (Qiagen). Each purified amplicon pool DNA (~ 100 ng) was used to construct Illumina libraries using the Ovation Rapid DR Multiplex System 1-96 (NuGEN). Illumina libraries were pooled and size selected by preparative gel electrophoresis. Sequencing was done on an Illumina MiSeq using v3 Chemistry (Illumina).

16S rRNA sequences analysis was performed with mothur community pipeline and clustering into operational taxonomic units (OTUs). The analysis was initiated by clipping 16S rRNA sequences from primers. The fragments after removing the primer sequences were combined into forward and reverse primer orientation sequences. The sequences were then processed to remove wrong size sequence and identify unique sequences. Sequences which were not matched or overhung were removed by aligning with a V3–V4 customized SILVA database v123. Chimera were removed using UCHIME algorithm. Taxonomical classification of sequences and removal of non-bacterial sequences were done using Silva database v123. OTU were picked by clustering at 97% identity level using the cluster split method.

For lactic acid fermentation, samples were collected at the day 0 and 33 for bacterial community analysis.

For elongation operation, samples were collected at day 0, 9, 16, and 30 for bacterial community analysis.
Figure S6.1  Bacterial community for (a) fermentation system, (b) elongation system, (c) inoculum for elongation system, (d) maximum concentration test, (e) maximum rate test
For lactic acid fermentation, community analysis indicated that over time, the population became enriched with lactic acid bacteria such as *Lactobacillus* spp. (83% relative abundance), *Pediococcus* and *Weissella* spp. (Figure S6.1a). *Lactobacillus*, *Pediococcus* and *Weissella* species are known for their ability to utilize polysaccharides and monosaccharides with lactic acid as a key outcome.

In the elongation system, a different community developed from the initial fermentation, despite influx via the substrate. *Firmicutes* dominated the population (81% relative abundance), in which *Clostridium IV* and *Lactobacillus* related species were most prevalent. *Coriobacteriaceae* and *Anaerococcus* spp. were other highly abundant species (Figure S6.1b). *Clostridium* species including *Clostridium IV*, *Clostridium Sensu Stricto* and *Clostridium XIVa* spp. identified in this study, are the archetype organisms known to perform reverse β-oxidation (Wiegel et al., 2006; Schleifer, 2009), and also utilize lactic acid. Although being weakly fermentative, *Anaerococcus* spp. is known to be able to metabolize carbohydrate and produce butyric acid and lactic acid as major metabolic end products. While some genus have butyric acid and caproic acid as major end products (Schleifer, 2009), which fits very well in this context. *Coriobacteriaceae* spp. has been mostly reported to be saccharolytic, however its genus such as *Olsenella* spp. is able to convert glucose into lactic acid as major product (Clavel et al., 2014). Although they are present in only a small abundance, the family of *Actinomycetaceae* spp. are also able to produce lactic acid and acetic acid under fermentative condition (Schaal et al., 2006). The bacterial composition of the inoculum and the elongation system were similar (Figure S6.1c and Figure S6.1b). This showed that introduction of bacterial community from the fermentation system did not change the bacterial community of the elongation system significantly.

Interestingly, during the maximum rate of caproic acid production test with cell retention, *Anaerococcus* spp. (32% relative abundance) became the most dominant species (Figure S6.1d), followed by *Lactobacillus* spp., while *Clostridium IV* spp. was merely 6% relative abundance. While the low abundance does not translate directly to low quantity of *Clostridium IV* spp., since cells were retained during this operation, the emergence of *Anaerococcus* species was unexpected. These organisms are typically found in anaerobic conditions and were recently identified under methanogenic conditions (Li et al., 2013). They have not been implicated in chain elongation to our knowledge.

During the maximum concentration of caproic acid test where additional lactic acid was provided, *Clostridium IV* spp. remained the most dominant (Figure S6.1e), and other species reduced in terms
of abundance. This gives an indication that *Clostridium IV* spp. can tolerate a caproic acid concentration at least up to 10.92 g L\(^{-1}\).

The mixed population showed between experiments remarkable functional stability, while the community members differed greatly.

Figure S6.2  Carboxylate profile of maximum caproic acid concentration test – elongation under excess lactic acid condition (error bars represent the standard deviation of triplicates, and they are consistently less than 6% (n = 3) and obstructed by the medallions)

Figure S6.3  Phase separation of caproic acid and aqueous elongation broth
Curriculum Vitae
Curriculum Vitae

Name : Way Cern KHOR
Date & Place of birth : 17th February 1990, Penang, Malaysia
Email : waycern.khor@ugent.be
Phone : +32 (0)477 651 011
Affiliation : Ghent University, Faculty of Bioscience Engineering,
             Center for Microbial Ecology and Technology
             (http://www.cmet2.ugent.be/users/way-cern-khor)
Work address : Coupure Links 653, 9000 Gent, Belgium

Education

PhD in Applied Biological Sciences
Centre of Microbial Ecology and Technology, Ghent University, Belgium
Title: Production of lactic acid and derivatives from grass using mixed populations
2013 – 2017

Bachelor of Chemical Engineering
Newcastle University, Newcastle Upon Tyne, United Kingdom
Final year project: Underground coal gasification and methanol synthesis
2009 – 2012
Publications during candidature

Peer-reviewed journal papers


Article intended for publications


- **Khor, W. C.,** Verliefde, A., De Wever, H., Vervaeren, H., & Rabaey, K. Extraction technologies for lactic acid recovery compared. (manuscript under preparation)

- **Khor, W. C.,** Vervaeren, H., & Rabaey, K. Grass 2.0. (manuscript under preparation)

Conference presentations

- **Khor, W. C.,** Coma, M., Vervaeren, H., & Rabaey, K. Biorefinery of chemicals from lignocellulosic biomass. 12th International Conference on Renewable Resources and Biorefineries. 30th May – 1st June 2016, Ghent, Belgium. (Poster presentation)


- Andersen, S. J., Candry, P., **Khor, W. C.,** Coma, M., & Rabaey, K. Electro-fermentation production and recovery of volatile fatty acids from thin stillage with zero chemical input. 11th International Conference on Renewable Resources and Biorefineries. 3rd – 5th June 2015, Ghent, Belgium.