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Sanni Haahti

# Insights into Butyrate Production via Fermentation from Food Waste in a Biorefinery Development Process

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This thesis was made for the VTT Technical Research Center of Finland as a part of a biorefinery development project BioChem, funded by the Academy of Finland. The project aims to exploit food industry side-streams to produce value-added chemicals via microbial fermentation. Butyric acid was the desired compound. Previous accomplishments within the BioChem project were used as a basis for this study. This study focused on i) applying both biological and mechanical preprocessing methods on the provided side stream cabbage (Vaissi Oy, Finland) and ii) examining the functionality of a process simulation modeling the kinetics and metabolic networks of the involved micro-organisms. The objective of this thesis was to determine a cost-efficient preprocessing step and to provide data considering involved operational parameter settings, in order to obtain butyric acid as the dominant fermentation product.

Determining the preprocessing method was done by enzymatic hydrolysis experiments with various operational conditions and enzyme products. Mechanical pretreatment was furthermore applied. The food waste hydrolysate was used in anaerobic bioreactor fermentations of a co-culture of *Megasphaera cerevisiae* and *Pediococcus Pentosaceus* as media carbon source. The results were evaluated for the final concentrations of glucose, fructose, and the produced organic acids of which especially butyrate was of interest. The MATLAB® simulation was operated to find potential initial concentration and operational parameter values for the bioreactor experiments. The simulation output values were compared to the most relevant results obtained from the conducted fermentations. Literature review including anaerobic metabolism and modeling fermentation processes was adopted.

According to this study, enzymatic hydrolysis using cellulases and pectinases on mechanically pretreated cabbage could be applied as a preprocessing step for biorefinery purposes. A sufficient amount of released sugars along with a completely liquefied hydrolysate was obtained within an hour. The highest obtained concentration of butyrate by using the cabbage hydrolysate in the fermentation media, was 16, 4 g/l. Furthermore, the simulation provides a promising platform for the planning of the experiments to favor the desired product within the process in question. A higher initial concentration of *M. cerevisiae* along with adjusted sugar concentrations could provide a cost-efficient method to increase butyrate production.

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| Keywords | butyric acid, enzymatic hydrolysis, fermentation, fermentation kinetics, fermentation parameters, food waste, *M. cerevisiae*, metabolic network simulation, *P. pentosaceus* |

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| Tekijä<br>Otsikko<br><br>Sivumäärä<br>Aika | Sanni Haahti<br>Katsaus biojalostamon kehitysprojektiin: voihapon tuotto elintarvikejätteestä fermentoimalla<br>70 sivua + 6 liitettä<br>8.5.2020 |
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Tämä opinnäytetyö tehtiin VTT Teknologian tutkimuslaitokselle osana Suomen Akatemian rahoittamaa biojalostamon kehitysprojekti BioChemia. Projektin tavoitteena on hyödyntää elintarviketeollisuuden sivuvirtoja arvokemikaalien tuottamiseen fermentoimalla. Ensisijaisesti haluttu lopputuote oli voihappo. Projektin aiempia saavutuksia on käytetty pohjana tälle työlle. Tässä työssä keskityttiin i) soveltamaan sekä mekaanista että biologista esikäsittelymenetelmää projektiin tarkoitetulle sivuvirtakaalille (Vaissi Oy, Suomi) sekä ii) tutkimaan projektin aiemman datan pohjalta tehdyn prosessin kinetiikkaa ja aineenvaihduntareittejä hyödyntävän simulaation toimivuutta käytännössä. Työn tavoitteena oli kehittää kustannustehokas esikäsittelyvaihe ruokajätteelle sen hyödyntämiseksi hiilenlähteenä fermentoinnissa, sekä saada tietoa eri prosessiparametrien vaikutuksesta voihapon tuottoon.

Esikäsittelymenetelmän määrityksessä tehtiin entsyymihydrolyysikokeita erilaisissa olosuhteissa sekä eri entsyymituotteilla. Mekaaninen käsittely lisättiin menetelmän tehostamiseksi. Saatua hydrolysaattia käytettiin *Megasphaera cerevisiaen* ja *Pediococcus pentosaceuksen* yhteisviljelmän anaerobisissa bioreaktorikokeissa fermentointialustojen hiilenlähteenä. Kasvatusten tuloksia tarkasteltiin glukoosin ja fruktoosin kulutuksen sekä syntyneiden orgaanisten happojen, ja erityisesti voihapon, kannalta. Fermentointikokeissa käytettävät alkukonsentraatiot sekä olosuhteisiin vaikuttavat parametrit säädettiin vastaamaan valittuja arvoja, jotka olivat MATLAB®-simulaation mukaan projektin kannalta kiinnostavia. Fermentointien merkittävimpiä tuloksia verrattiin simulaatiosta samoilla alkuparametreilla saatuihin lopputuotearvoihin. Kirjallisuuskatsauksessa keskityttiin matemaattisiin malleihin prosessin optimoinnin apuna sekä mikrobien anaerobiseen aineenvaihduntaan ja fermentointikinetiikan mallintamiseen.

Tässä työssä selvisi, että sellulaasien ja pektinaasien synergialla saa mekaanisesti murskatusta kaalista lyhyessä ajassa täysin nesteytyneen hydrolysaatin, jossa on riittävästi vapautuneita sokereita sen hyödyntämiseksi fermentoinnissa. Kaali-hydrolysaatin käyttö fermentointialustassa tuotti parhaimmallaan 16.4 g/l voihappoa. MATLAB®-simulaatio on lupaava pohja fermentointikokeiden suunnittelulle haluttujen lopputuotteiden saamiseksi kyseisessä prosessissa*. M. cerevisiaen* suurempi alkukonsentraatio voisi sokerikonsentraatioiden optimoinnin myötä toimia kustannustehokkaana menetelmänä kasvattaa voihapon saantoa.

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| Avainsanat | aineenvaihduntareittien simulointi, elintarvikejäte, entsyymihydrolyysi, fermentointi, fermentointikinetiikka, fermentointiparametrit, *M. cerevisiae*, *P. pentosaceus,* voihappo |

## Contents

## List of Abbreviations

| | |
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| AD | Anaerobic Digestion |
| DOE | Design of Experiments |
| VFA | Volatile Fatty Acid |
| MCF | Mixed Culture Fermentation |
| FW | Food Waste |
| HPLC | High-Performance Liquid Chromatography |
| OD | Optical Density |
| RBO | Reverse β- oxidation cycle |

# 1 Introduction

Approximately 88 million tons of food waste (FW) is generated in EU-28 countries annually with an associated value of around 143 billion euros. The waste is accumulated at each step of the supply chain, households being responsible for over 50%, followed by side streams from food processing covering around 20 % of the total amount. [1] The most common procedures to dispose of food wastes are in landfills or by sending them to be incinerated. Both of these options result in almost no recovery of resources whereas high amounts of toxic compounds and greenhouse gases are emitted in the atmosphere and soil. [2] The simultaneously increasing concerns of global warming, poverty, and famine drive both: the overall reduction of food waste and the development of sustainable alternatives to recycling it.

Food wastes have high organic contents including mainly carbohydrates, proteins, and lipids. In addition, they contain nitrogen and phosphorus that are essential for microbial metabolism. [2, p.281] Due to these features, a promising approach is to exploit food wastes in anaerobic digestion (AD) to produce Volatile Fatty Acids (VFAs) [3]. These organic compounds including butyric acid, acetic acid, propionic acid, and valeric acid are widely used in many industries but currently produced from nonrenewable natural resources through chemical synthesis deriving from petroleum processing [2]. Natural microbial VFAs production occurs in anaerobic digestion. AD generally involves i) enzymatic hydrolysis of the organic matter ii) acidogenesis, where VFAs are produced as intermediate metabolites by acidogenic fermentation and iii) acetogenesis, where VFAs are converted to acetic acid, carbon dioxide and hydrogen and iv) methanogenesis, where biohydrogen is generated. [4, p. 395–396; 5, p. 113] The challenge is to create a process that provides suitable anaerobic fermentation conditions along with a large scale and cost-efficiency to compete with the crude oil-based processes [6].

BioChem, a project funded by the Academy of Finland, aims to develop a biorefinery platform to produce value-added chemicals from low-grade biomass. One of the goals within BioChem is to investigate the possibilities of butyrate production via microbial fermentation from the food industry side-streams. Butyric acid is a 4-carbon short-chain fatty acid that appears as a colorless liquid with an unpleasant odor. Its main application

is in the manufacture of plastics, but its esterified forms can be found in cosmetics, textiles, different pharmaceutical products, and as flavoring agents in foods [6]. Among other VFAs, butyrate is also a substrate for producing biofuels such as methane, biopolymers such as polyhydroxyalkanoates (PHAs), and hydrogen [2].

Rosaliina Turunen [7] examined potential microbial strains for the purpose of the Bio-Chem project in her thesis at VTT. All the included strains were natural, of group one biological factors obtained from VTT strain collection. She observed that co-culture fermentation of two specific strains of *Megasphaera cerevisiae* and *Pediococcus pentosaceus* resulted in higher butyric acid production rates compared to *M. cerevisiae* single strain cultivations. The strains were selected for further use within this project according to promising yields and their sufficient tolerance towards butyrate. Thus, these strains and their metabolic pathways and fermentation kinetics with respect to butyrate production are further investigated in this thesis. Turunen also determined the ideal media for the fermentation process, by using the food industry side-streams such as apple, cabbage, and their mixtures as carbon sources. She furthermore investigated that media enriched with acetate buffer promoted the butyrate production by the co-culture.

One of the issues in the exploitation of biomass is to develop the most time- and cost-efficient pre-processing method to obtain the highest possible amounts of suitable substrates for the microbes to ferment [8]. Pretreatment methods for food wastes can be divided into three main categories: biological, chemical, and physical (mechanical), of which biological and mechanical are involved in this study. Mechanical pretreatment aims to reduce the particle size; hence, the degree of polymerization while the surface area is increased. Biological pretreatment involves the degradation of complex molecular structures releasing monomeric substrates by enzymatic hydrolysis, for instance. The food waste used in this thesis is provided by Vaissi Oy (Finland) and constitutes of white cabbage residue parts. Cabbage contains approximately six grams of carbohydrates (per hundred) of which about 50 % is monomeric glucose and fructose and the rest in forms of different fibers. [9, p. 597–599; 10]

Mathematical modeling provides important tools for understanding, developing, and optimizing biotechnological processes such as fermentation. Different models can be applied to processes or systems operating on multiple levels from the function of metabolic

pathways within a cell to the operational conditions supporting the cell growth in a bioreactor. [11] Some models such as The Design of Experiments (DoE) focus on the experimental set-up within a process to find the optimum operational conditions. It enables the investigation of the impact of different factors, involved in a system, on desired results simultaneously [12].

As a part of the BioChem project, a MATLAB® model is being developed by Alberte Reguiera and Miguel Mauricio Iglesias at the *University of Santiago de Compostela, Spain,* to simulate the process. The model is based on data generated from previous experiments conducted by Rosaliina Turunen within this project. It aims to predict the process based on the fact that the system can be manipulated by designing it to target specific products. Hence, it enables product selectivity by changing the initial operational conditions such as the substrate and inoculum concentrations as they impact the compound concentrations at the end of the process. It provides essential information about how butyrate and other organic acids are produced by the co-culture; what are the concentrations of butyrate and other metabolites obtained, in which ratios are the main substrates consumed, and what are the involved metabolic pathways. Each reaction rate with required coefficients has been determined independently for ten apropos reactions that occur during the process. Two of these reactions are performed by *P. pentosaceus* and eight are performed by *M. cerevisiae*.

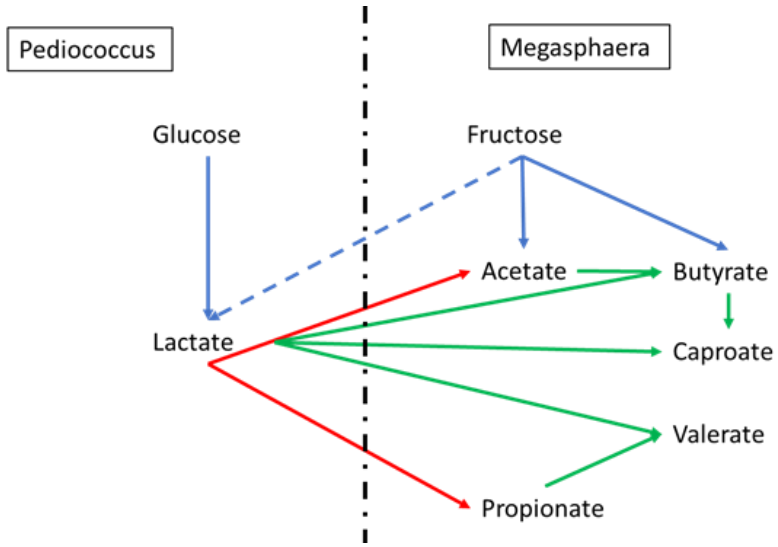


Figure 1. Metabolic network demonstrating the co-culture system of *Megasphaera cerevisiae* and *Pediococcus pentosaceus*. The arrows indicate the reactions from different substrates by the bacteria. *Pediococcus* uses preferentially glucose and then fructose (dashed arrow) to produce lactate. Red arrows indicate lactates further use *Megasphaera.* *Megasphaera* uses preferentially fructose to produce butyrate and acetate. Green arrows represent reactions performed by *Megasphaera* from previously generated compounds in the system. Illustration by Alberte Requiera (University of Santiago de Compostela).

The metabolic network of the system is illustrated in Figure 1. *P.pentosaceus*, referred to as *Pediococcus*, consumes glucose as its primary carbon source to produce lactate. After glucose is consumed, it starts consuming fructose. *M. cerevisiae*, referred to as *Megasphaera* in Figure 1, uses fructose as its main carbon source but also lactate to produce VFAs and acetate. Butyrate is produced by *Megasphaera* via reactions illustrated in Figure 2. All involved catabolic reactions by the metabolic network of *P. pentosaceus* and *M. cerevisiae* are described in Appendix 1.

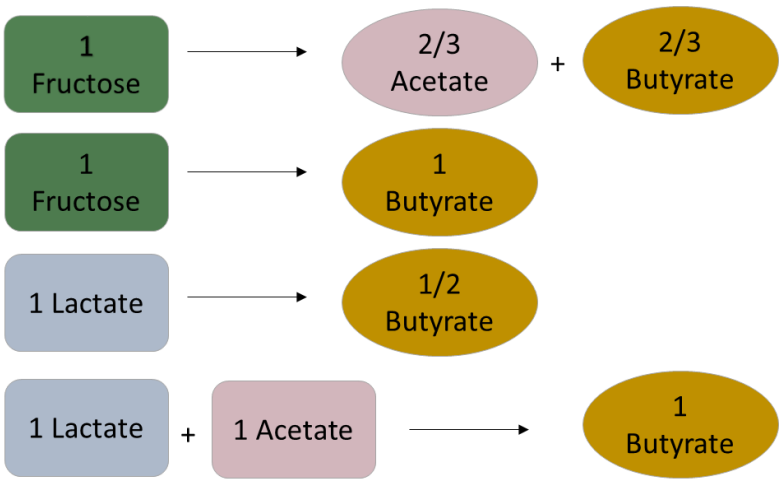



Figure 2. Butyrate production by *Megasphaera cerevisiae* from fructose and lactate produced by *Pediococcus pentosaceus.* The numbers represent molar amounts. From fructose, *M. cerevisiae* can produce either both butyrate and acetate (top) or only butyrate (second). One mol of lactate can be utilized alone to produce 0.5 mol of butyrate, or with one mol of acetate to generate one butyrate. Reactions are determined by Alberte Reguiera and are displayed in Appendix 1.

To model the co-culture, the biomass growth is determined individually for each microorganism. The biomass growth rate (μ) depends on the substrate consumption rate (q). For biomass growth, *P. pentosaceus* consumes glucose and fructose, referred to as sugars, whereas *M .cerevisiae* consumes fructose and lactate, respectively. As for *P. pentosaceus,* the biomass growth is proportional to the sugar consumption reactions; the

specific growth rate $\mu_{Ped}$ is derived from the sum of the sugar consumption reactions multiplied by the yield coefficient $Y_{sugars}$. *M. cerevisiae* derives its biomass from the consumption of fructose and lactate via different reactions, as follows:

$$\mu_{Mega} = Y_{sugars} \cdot \left(q_{Mega,1} + q_{Mega,2} + q_{Mega,8}\right) + Y_{lac} \cdot \sum_{j=3-7} q_{Mega,j} \quad (1)$$

The sub-indices of the consumption rates refer to reaction numbers determined in Appendix 1 and $Y_{lac}$ is the biomass yield of lactate. In terms of kinetics, the reaction rate determinations follow the Monod equation (19). For better description of the results, all reactions are multiplied by an inhibition term given in equation (2).

$$I_{VFA} = \frac{1}{1+\frac{\sum[VFA]}{K_{VFA}}} \quad (2)$$

The inhibition term $I_{VFA}$ describes the inhibition caused by accumulated VFAs and is determined at each step of the simulation with the sum of all VFA concentrations and a VFA inhibition constant $K_{VFA}$. Another inhibition term simulating the inhibition in fructose consumption by *P. pentosaceus* when glucose is available is defined in equation (3). It enhances the model by the information that *P. pentosaceus* consumes glucose as the preferential substrate and fructose only after glucose is finished. The $I_{Glucose}$ represents a so-called non-competitive inhibition term and is multiplied by the fructose consumption by *P. pentosaceus*.

$$I_{Glucose} = \frac{1}{1+\frac{[Glucose]}{K_I}} \quad (3)$$

In this study, this model, along with studied metabolic pathways and kinetics, is used to optimize the fermentation process parameters for higher yields of butyrate. Permission to use the simulation in this thesis has been obtained from its creators.

The objective of this thesis can be divided into two sections: 1) Developing and optimizing a cost and time-efficient pre-processing method for the provided food waste. This includes determining suitable enzymatic solutions including optimal enzyme product concentrations and other operational conditions for the hydrolysis. The Design of Experi-

ments will be exploited and necessary mechanical methods will be investigated. 2) Optimizing fermentation parameters such as substrate and acetate concentrations, the initial bacterium strain ratio and pH, using metabolic pathways, and the MATLAB® simulation as a basis for the experiments. This section includes i) a literature review about the influence of different operational parameters and factors on microbial butyrate production, ii) using the simulation, and finding interesting initial conditions to iii) conduct fermentation experiments with.  A process where each step is well organized and optimized will contribute to the development of a continuous process and the scale-up in the future.

## 2 Design of Experiments (DoE)

Design of Experiments is a statistical approach to experimental design. It is a mathematical tool used to determine and to understand how factors such process parameters affect response variables determined for the examination. Its objective is to provide a framework for an experimental process as illustrated in Figure 3. [12, p.27]


Figure 3. Framework for experimental design. The objective of the Design of Experiments (Doe) statistical tool. [12, p. 27]

The fundamental advantage of DoE is that all selected factors are varied systematically and simultaneously. It will then define which factors, variables or their interactions are either significant or insignificant in contributing to the measured effect and finally to the particular systems being examined. [12, p. 6; 13]

The Design of Experiments can be used for system development, industrial research and production in different industries such as the biotech-, chemical-, food-, and pharmaceutical industries as well as marketing. DoE is applicable for purposes, including

- process and product development
- process and product optimization
- optimization of analytical instruments
- quality optimization
- minimization of production costs
- robustness testing of methods or products. [12, p. 8]

An alternative to DoE is COST (Change One Separate Factor at a Time), also referred to as the *intuitive* approach. In COST, the effect of each variable is determined by changing the value of each factor one at a time, until all values of all factors have been contrasted with each other. Comparing the two approaches, DoE handles three critical problems better than COST. First, with DoE the interactions between factors can be estimated. Second, the designed set of experiments enables the estimation of systematic variability, i.e. *effect* and unsystematic variability, i.e. *noise.* The third aspect has to do with reporting and visualizing the results; a designed system of experiments is more likely to give a reliable and meaningful map representing the investigated system. [12, p. 3]

One of the purposes of the DoE is to provide the required information while minimizing the number of needed experiments. As methods for this, it uses experimental limits, specific experimental conditions, and mathematical analysis for response prediction at any point within the determined limits [13].

## 2.1 Model

The mathematical approach to DoE is based on two types of variables: factors and responses. Responses describe the properties and conditions of the examined system or process. Responses can be objectives such as pH-change or the substrate consumption rate during a fermentation process. Factors are assumed to influence the system and may, therefore, be manipulated according to the wanted response [12, p. 19].

In DoE, understanding and finding the correlations between factors and responses is done mathematically by connecting factorial changes to those in response values. The data from DoE-based experiments are typically fitted on a polynomial linear model. With three factors, the linear model can be written as follows:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_1 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \varepsilon, \quad (4)$$

where y is the response and x's are the effects of the factors 1, 2 and 3, $\beta_0$ is the constant term, β's are model parameters, and the ε the residual response variation not explained by the model, i.e. experimental error or *noise*. The first three coefficients tell the *real effect* of each factor. The last three coefficients show the possible interactions among the factors. The constant $\beta_0$ is the response y when all effects are zero. [12, p.13]

## 2.2 Experimental objectives

DoE is applicable to six types of problems, referred to as experimental objectives:

(i) familiarization

(ii) screening

(iii) finding the optimal region

(iv) optimization

(v) robustness testing, and

(vi) mechanistic modeling,

of which screening, optimization, and robustness testing are the main ones. When selecting the experimental design, questions such as “Why is the experiment conducted?” “What is the purpose?” and “What is the desired result?” should be asked. [12, p. 27–28]

*Familiarization* is used when the objective of the experiment involves an entirely new application or equipment not previously used. Hence, this stage is often skipped and the experiment is started at *screening* step instead. Familiarization aims to define what is, and is not experimentally possible and whether the desired results can be achieved with this application or equipment. Familiarization is often accomplished intuitively using very simple designs such as two factorial design. From such design it is intended to confirm that, 1) replicated center-point runs give similar results and that 2) different results are obtained from the corner points. Familiarization is also used to verify that the response measurement techniques work in practice. [12, p. 28]

*Screening* is used to identify the factors that are the most influential considering the experiment in question. Multiple factors can be included in this step to determine the most dominating ones and their optimal ranges. According to an Italian economist Vilfred Paretos principle, 20% of the investigated factors cause 80% of the effects on the responses. Noise is considered in this principle. Identifying the strongest factors applies well to the screening step of the DoE; hence, it is a good theoretical goal of this stage. Another method to put screening into practice is to ascribe the determined *factors* the same chance to affect the responses, whereas after conducting and evaluating the set of experiments, only the most influential ones remain. Screening is typically performed using a fractional factorial design type. Simple linear or interaction polynomials are sufficient to model the objective. [12, p. 28 & 101–107]

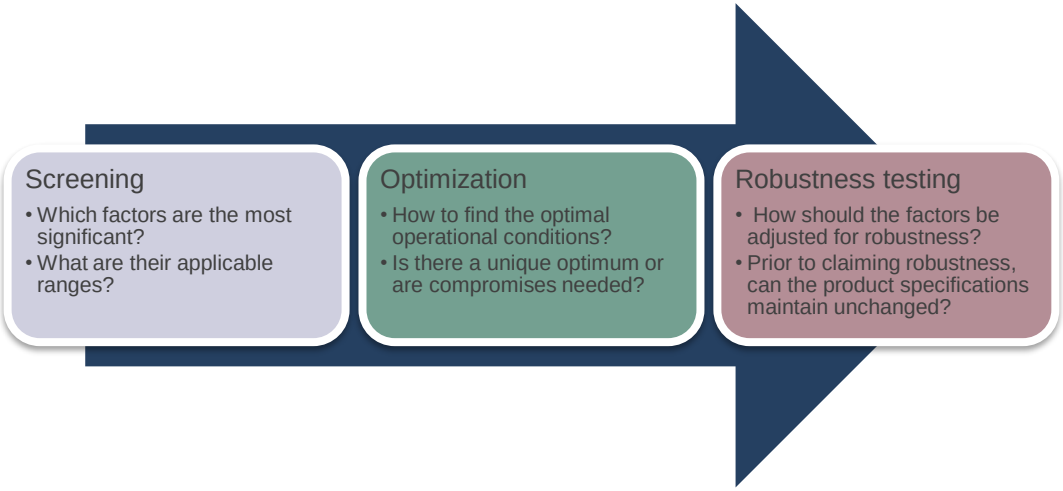



Figure 4. Main problems i.e. experimental objectives in the DoE; Screening, Optimization, and Robustness testing, and their specifications. The arrow indicates the total experiment as a process. [12, p. 3]

If the post-evaluations show that screening has not resulted in the region that includes the optimal condition, the domain of the experimental design has to be moved. For this, factor settings must be altered. [12, p. 125]

After screening, the most important factors have been identified and the region for the experiment has been set so that it is assumed to include the optimal point. In *optimization*, the aim is to find the optimal point by examining the remaining factors' correlation to the responses. Thus, comparing to screening, the optimization step includes more experiments with fewer factors to gain information about the dominating factors affecting the system. In optimization, composite factorial designs are used for the experiments and the data are fitted in quadratic polynomial models type

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 + \cdots + \varepsilon, \quad (5)$$

where three to five levels of each factor are encoded. The fitted model is usually approached with *response surface modeling, RSM,* where a response surface plot displays an approximation of the 'true' relation between the factors and the responses. [12, p. 31–32]

*Robustness testing* is performed to ensure quality before releasing the examined system or product. The objective is to minimize the variation by identifying and regulating all the

factors that, in addition to the normally considered factors such temperature and time, might have little impact on the system, yet possibly causing unwanted spread around the optimal result. Examples of such factors are humidity and variability in raw material composition. Robustness testing may be performed in different ways, of which a fractional factorial design and Plackett-Burman design are the generally used. [12, p. 32]

# 3 Microbial Butyrate Production

Butyric acid, also known as butanoic acid or butyrate, is a linear 4-carbon short-chain fatty acid (SCFA) and one of the so-called Volatile Fatty Acids (VFAs). It is a common product of important fermentative systems such as animal and human guts as well as of microbial butyrogenesis. [14] Among other VFAs, butyrate is also an intrinsic intermediate of anaerobic digestion (AD), where organic matter, including carbohydrates, lipids, and proteins, is degraded into carbon dioxide and methane in the absence of oxygen. Therefore, butyrate is also a valuable substrate for a renewable energy source (biomethane). AD is furthermore a typical example of Mixed Culture Fermentation (MCF) [15, p. 275]. Mixed cultures include more than one microbial species, either known or unknown. Typically, used mixed cultures for fermentations are derived from sludge, where the bacterial biomass is responsible for various natural anaerobic processes. In that case, the cultures are trusted to be stable microbial populations and can be used in non-sterile fermentation processes without significant contamination risks. [15] Other advantages of mixed culture fermentations include better utilization of substrates of wider spectrum, possibly higher yield and growth rates, and a variety of applicable metabolic pathways of which many are based on different chain-elongation reactions [16]. The co-culture of *P. pentosaceus* and *M. cerevisiae* operated within this thesis is an artificially mixed community of two initially pure cultures.

## 3.1 Butyrate-producing microbes

Butyrate-producing bacteria can be found in various environments of which the most common are the rumen, the mouth, and large intestine [17]. Most of them are not potential for industrial applications due to low production rates and have only recently gained

attention as important factors maintaining epithelial integrity in the colon. In their research, Vital et al. [17] screened over 3000 sequenced bacterial genomes from the Integrated Microbial Genome Database and identified genomes of 225 potential butyrate-producing bacteria. Of the identified bacteria, those belonging to *Firmicutes* phyla formed the major group.

For industrial scale, some over ten bacteria from different genera including *Clostridium, Butyrvibrio, Butyribacterium, Eubacterium, Fusobacterium Megasphera,* and *Sarcina* have been investigated as potential butyrate producers. Of the previously mentioned, *Clostridium* strains have already been used for industrial production and are the most studied. The studies have focused on species such as *C. butyricum, C. populeti,* and *C. tyobutyricum* and *C. thermobutyricum*. These are all gram-positive, spore-forming obligatory anaerobes that, apart from *C. thermobutyricum* (that prefers 55 °C), optimally culture in around 30– 37 °C and in an environment of pH at range 5.0 –7.5. [6]

The *Megasphaera* genus, also part of the phylum *Firmicutes* and class *Clostridia*, is mostly recognized for including three common beer spoilage strains *Megasphaera cerevisiae* (‘of beer’), *Megasphaera paucivorans* (‘user of a few substrates’) and *Megasphaera sueciensis* (‘of Swedish origin’) [18]. Another species, *Megasphaera elsdenii*, isolated from rumen or human gut, has been studied for its potential in industrial VFAs production. All *Megasphaera* strains are anaerobic, gram-negative cocci and can produce acetic, butyric, valeric, and propionic acids in their metabolism. Their optimal growing conditions vary depending on the species. *M. cerevisiae* grows at temperature range 15–37 °C ( 28 °C considered optimal) and is inhibited in an environment with low pH (<4.1) and high ethanol concentration (> 2.1 vol %) [19]. The properties and optimal growing conditions of butyrate-producing cultures will be discussed later in chapter 4.

## 3.2 Metabolic pathways in butyrate production

In the direct conversion from glucose to butyrate, two mol of $H_2$ per mol of produced butyrate is generated according to the equation:

$$C_6H_{12}O_6 \rightarrow C_4H_8O_2\ (butyric\ acid) + 2H_2 + 2CO_2 \quad (6)$$

Butyrate is synthesized via a series of enzymatic reactions in four main pathways: the glutarate, acetyl-CoA, lysine, and 4-aminobutyrate pathways, of which the acetyl-CoA is the most common. The glutarate, lysine, and succinate/4-aminobutyrate pathways have generally been omitted as potential butyrate-producing ways, due to the gastric environments where they occur. They use amino acids as major substrates and can be found in phyla such as *Firmicutes, Fuscobacteria,* and *Bacteroidetes.* [17, p.2]

Another way to divide butyrate production pathways is to direct and indirect reaction routes (Figure 5). Direct pathways refer to carbohydrate conversion into butyrate via butyrate kinase. Indirect pathways include the following interconversion reactions before the final conversion to butyrate via butyrate kinase:

- acetate via butyryl-CoA:acetate-CoA transferase
- lactate via lactate dehydrogenase
- succinate via succinyl-CoA synthase [14, p. 12–13].

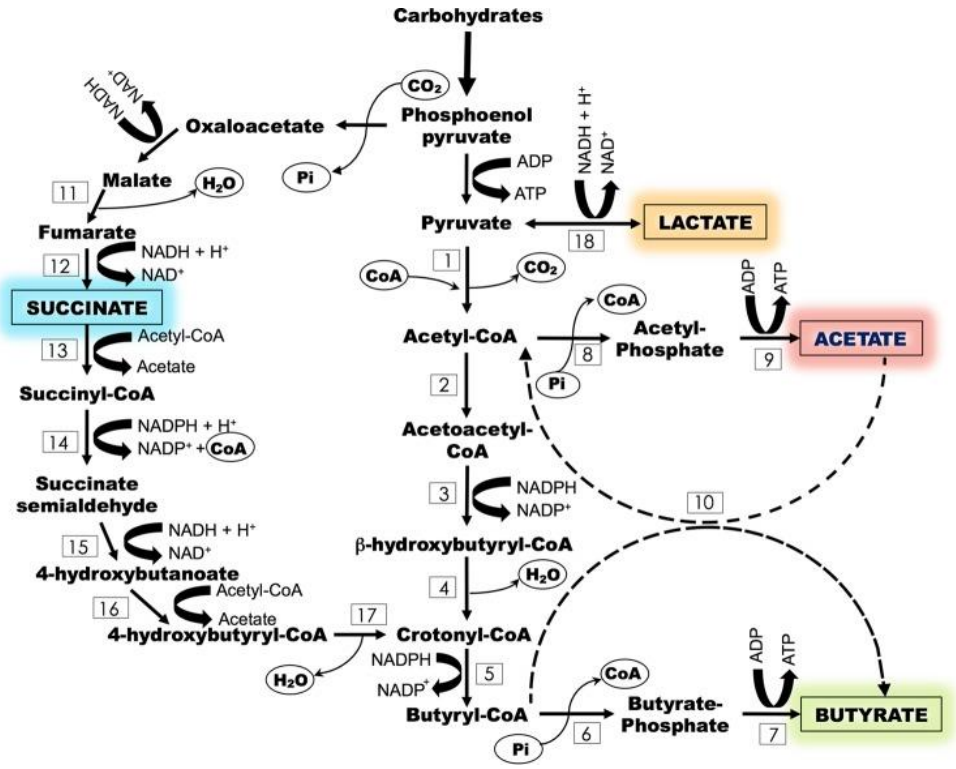


Figure 5. Metabolic pathways for butyrate production divided into i) direct from carbohydrates converted to butyrate via butyrate kinase) and ii) indirect pathways from lactate (via lactate dehydrogenase), acetate (via butyryl-CoA:acetate-CoA transferase) and succinate (via succinyl-CoA synthase), and butyrate kinase. The involved enzymes are numbered: 1=pyruvate-ferredoxin oxidoreductase, 2=acetyl-CoA-acetyltransferase, 3= *β*-hydroxybutyryl-CoA dehydrogenase, 4=4-hydroxybutyryl-CoA dehydratase, 5=butyryl-CoA dehydrogenase, 6= phosphotransbutyrylase, 7=butyrate kinase, 8=phosphotransacetylase, 9=acetate kinase, 10=butyryl-CoA:acetate-CoA transferase, 11=malate dehydrogenase, 12=fumarate reductase, 13=succinyl-CoA synthase, 14=succinate semialdehyde dehydrogenase, 15=4-hydroxybutyrate dehydrogenase, 16=4-hydroxybutyrate CoA transferase, 17=4-hydroxybutyryl-CoA dehydratase, 18=lactate dehydrogenase. [14, p.13]

In anaerobic glucose fermentation, glucose is first converted to pyruvate in glycolysis via the Embden-Meyerhof-Parnas (EMP) pathway. The EMP generates two ATP molecules and NADH, respectively. In the acetyl-CoA pathway, pyruvate is converted to acetyl-CoA by pyruvate-ferredoxin oxidoreductase, producing $CO_2$ and reduced ferredoxin $FdH_2$. $FdH_2$ is re-oxidized to Fd by hydrogenase, forming $H_2$ -gas as a by-product [6]. A four-step pathway, where acetyl-CoA is converted to butyryl-CoA, follows [16]. First, acetyl-CoA is converted to acetoacetyl-CoA by thiolase via acetyltransferase. Acetoacetyl-CoA is then transformed in a reaction by *β*-hydroxybutyryl-CoA dehydrogenase to 3-hydroxy butanoyl-CoA, which crotanase then converts to crotonyl-CoA. All four butyrate-producing pathways (the glutarate-, acetyl-CoA-, lysine-, and succinate/4-aminobutyrate-) merge here, as crotonyl-CoA is further converted to butyryl-CoA via butyryl-CoA dehydrogenase which is catalyzed by ferredoxin reduction with NADH. [17; 20; 21]

The final transformation to butyrate has two alternative pathways: i) via butyrate kinase catalyzed by phosphorylated butyryl-CoA (encoded by *buk)* or ii) via acetate-CoA transferase catalyzed by butyryl-CoA (encoded by *but*). The latter is an interconversion reaction since it requires external acetate being simultaneously converted to acetyl-CoA. Other corresponding interconversion reactions are lactate and succinate conversions to butyrate (Figure 5). [14, p. 12–13]

At least *Megasphaera elsdenii* and *Megasphaera micronuciformis* of the *Megasphaera* genus, as well as many Clostridia, produce butyrate via butyryl-CoA:acetate-CoA transferase (*but*) [17]. This requires simultaneous production of acetic acid in a branched pathway as shown in Figure 5.

## 3.3 Butyrate production via chain elongation

Some microorganisms, including *Clostridium sp*. and *Megasphaera elsdenii*, can produce volatile fatty acids through chain elongation. In *n*-butyric and *n*-caproic acid formation, a pathway called reverse β-oxidation (RBO) cycle (Figure 6), is the core process. In RBO, two short-chain carboxylates such as lactate and acetate or ethanol elongate with two carbons into longer carboxylates such butyrate. [20] Chain elongation of acetate and lactate to *n*-butyrate via reverse *β*-oxidation by *M. elsdenii* can be expressed as follows:

$$Lactate^- + acetate^- + H^+ \rightarrow n-butyrate^- + CO_2 + H_2O \quad (7)$$

The presence of easily degradable compounds to form acetyl-CoA, energy, and reducing equivalents is required to maintain the RBO cycle. Lactate and ethanol are such compounds and supply electrons during carboxylic acid chain-elongation. [22, p. 108]

In the RBO cycle, lactate is first converted to acetyl-CoA via pyruvate conversion where ATP and NADH are generated. The acetyl-CoA then enters a cyclic pathway where it provides the carbon atoms that are needed to elongate acetate to *n*-butyrate. The RBO cycle is illustrated in Figure 6.

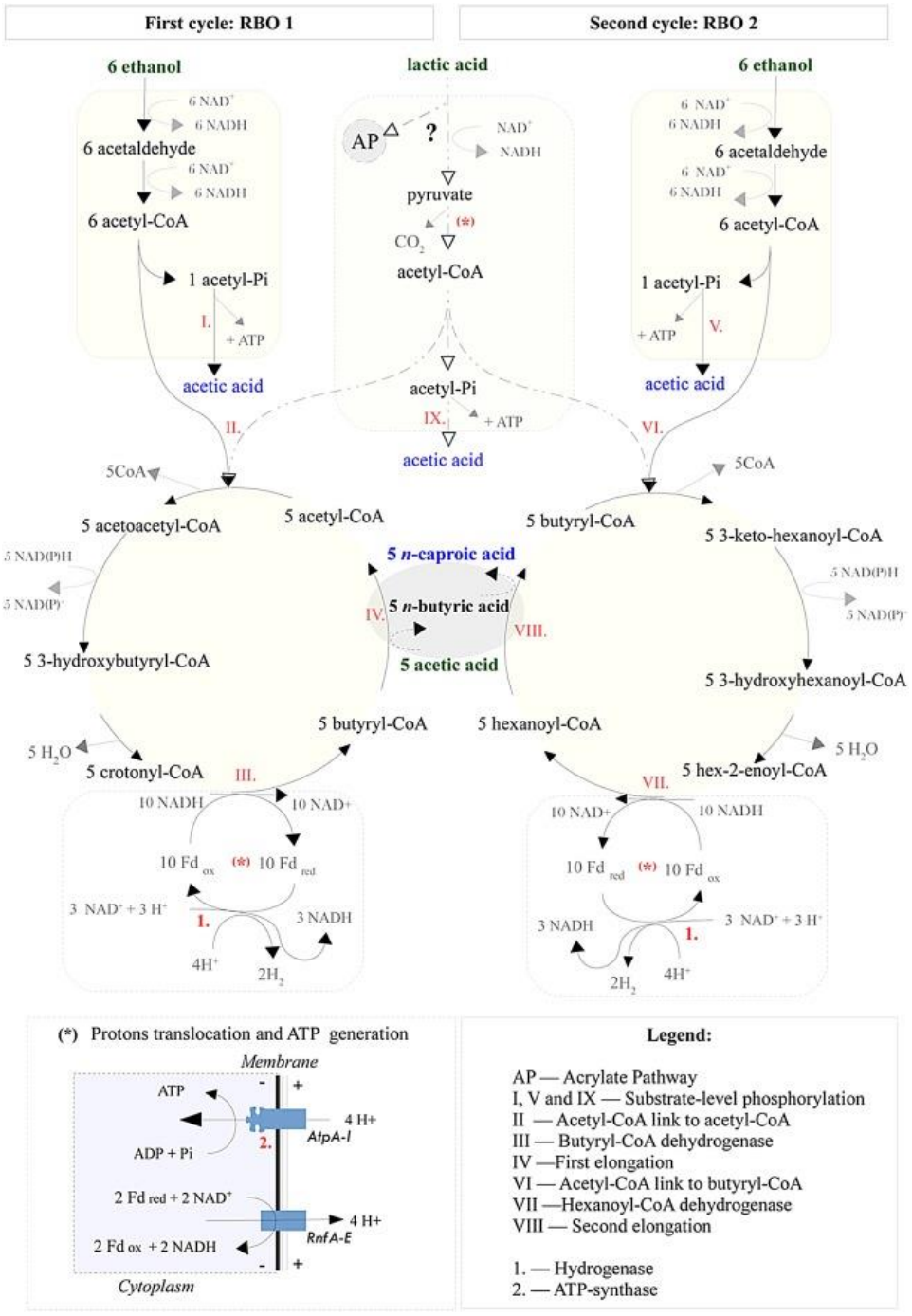



Figure 6. Reverse *β*-oxidation cycle by *Clostridium kluyveri* including metabolic chain elongation pathways from acetate to *n*-butyric and *n*-caproic acids. The pathways are shown as red numbers, which are explained in the legend. Substrates are shown in green and products in blue. Butyric acid is generated in the first elongation RBO1- IV from lactic and acetic acids via the acetyl-CoA pathway. [22, p. 109]

With ethanol as electron donor, part of the ethanol is first converted to acetate in an ATP-producing reaction. The rest of the ethanol is then converted to acetyl-CoA, which promotes the chain elongation from acetic acid to *n*-butyrate by coupling. Acetyl-CoA is finally used to elongate *n*-butyric to *n*-caproic acid. [20; 22, p. 108]

# 4 Fermentation Parameters Affecting Volatile Fatty Acids (VFAs) Production

Different fermentation parameters describe the process' environmental and operational conditions [23]. Parameters, such as pH, temperature, and hydraulic retention time (HRT) as well as the carbon-source-nature, substrates, and their concentrations and involved microbial cultures influence the quantity and the composition of obtained products [2, p. 279]. Even though these parameters have a synergetic effect on the cellular metabolism of the involved micro-organisms, their impacts can be investigated individually [2, p. 282]. In this chapter, some of these parameters, with respect to butyrate production, are reviewed. Volatile fatty acids are short-chain compounds of 2–6 carbon atoms. Here, the considered VFAs are acetic acid (acetate), butyric acid (butyrate), valeric acid (valerate), propionic acid (propionate), and caproic/hexanoic acid (caproate). Studies focusing on anaerobic fermentation processes using different food waste (FW) or kitchen waste as fermentation stuff (carbon source) and different inoculum including activated sludge and anaerobically digested sludge, count among relevant.

## 4.1 pH

pH is a significant factor in microbial VFAs production as it affects the amount of obtained fermentation products and the distribution among them [15; 4; 24]. Considering the optimum range, studies show different results depending on the other process parameters. However, all of them accentuate the relationship between pH and fatty acid synthesis. Most of the VFA producing microbes cannot survive in extremely acidic (pH 3) or alkaline (pH 12) environments, which can explain some of the correlation [5]. Many conducted studies suggest that the ideal pH to produce some specific VFA, including butyrate, acetate, and propionate, is highly dependent on the used biomass or waste as well as the

main substrate [15; 13]. This can be explained by the product formation pathways: acetate, propionate, and butyrate can be formed directly of proteins, carbohydrates, and lipids whereas *n*-valeric acid is mainly produced from degraded proteins. High carbohydrate content in food waste is suggested to correspond to a high VFAs production rate. The pH also affects kinetic parameters such as the growth rate, carbon source utilization, and substrate conversion efficiency. [4]

Jankowska et al. [15] studied the VFA production in a mixed culture fermentation under different pH values between 4 and 12 with different retention times. The highest VFA concentrations were achieved at pH 10 after 15 days and promising yields at pH 11, indicating that alkaline conditions would be optimal in VFA production. At pH 12 the growth was inhibited. [15] Dahiya et al. [5] got similar results by fermenting food waste in an acidogenic process at different pH values (5–11), gaining the highest VFA concentration at pH 10 (6.3 g/l). At alkaline environment (pH 10), compositional observations showed a higher percentage of acetate and lower of butyrate in both studies. Butyrate was the main product at pH 5.0 (1.8 g/l). However, due to methane production that can occur in alkaline environments by certain archaebacteria, the produced VFAs are consumed. Hence, the VFA concentrations are higher before the conversion; thus, it cannot be concluded that alkaline pH is ideal for VFAs production [2; 5].

A study carried out by Jiang et al. [25] demonstrated VFA production from synthetic kitchen waste under pH values 5.0, 6.0, and 7.0. The highest VFA concentrations were observed at pH 6.0 (39.46 g/l) and pH 7.0 (37.09 g/l). At pH 6.0, butyrate was the main fermentation product covering 52 % of the total production, followed by acetate (24 %), propionate (13.5 %), and valerate (9.5 %). In an uncontrolled reactor within the same study, the pH dropped quickly to 3.0 and the VFA concentration maintained low. Such a reaction is caused by the undissociating VFAs passing the cell membranes inhibiting cell growth [25]. Other studies show similar results. Wang et al. [4] noticed that under a pH 6.0, VFAs production increased by 17 times and VFAs yield by 7.5 times, with respect to pH 4.0. They also discovered that pH 6.0 resulted in the highest VFAs production, despite the used inoculum. Composition analysis revealed that butyrate was the dominant VFA followed by acetate and propionate. In a study carried out by Lim et al. [26], organic acids were produced in a semi-continuous process from FW at pH values 5.0, 5.5, and 6.0. The productivity and yield were the highest under pH 6.0 (5–5.5 g/l) and nearly the

same under pH 5.5 (4.72–5.10 g/l) with HRT of 8 days and temperature at 35 °C. Valerate was not produced at pH 5.5. [26] Hence, based on literature, pH 6.0 is the most suitable for butyrate production from food waste. This is likely due to the hydrolytic enzymes' optimum activities at pH 6.0 and the studies showing that acid-neutral conditions have a positive effect on the fermentative production of microbial metabolism. [2]

## 4.2 Temperature

Temperature is one of the key parameters in acidogenic fermentation since it is directly involved in both microbial growth and metabolism. Each microbial taxon has a range of optimal temperatures for its replication. [2] When considering anaerobic digestion in food waste fermentation, the temperature has a significant role affecting i) the degree of hydrolysis and ii) the acidification. The former can be explained by microbial enzyme activity, and the latter is due to the altering microbial structure in a mixed culture, caused by the change in temperature [2]. Enzyme activity affects the chemical oxygen demand (COD) solubility but is inhibited at higher temperatures. However, hydrolysis occurs in high temperatures as well but is often due to the chemical-physics effect. [27] Several studies have reported that the increase of temperature (55 °C <) systematically increases the SCOD concentration indicating higher solubilization [25; 27; 28], which is a rate-limiting step in anaerobic fermentation process [27], as it provides fermentative microbial metabolism with available hydroxylates [2, p. 283].

He et al. [29] conducted an acidogenic fermentation experiment where food waste was fermented by sludge for nine days in three different temperatures. They observed that by increasing the temperature from mesophilic (35 °C) to thermophilic (55°C), the maximum total VFAs production decreased from 17 g/l to 11 g/l, respectively. The pH was uncontrolled. Increasing the temperature further to 70 °C resulted in approximately 13 g/l of VFAs. Consistent results were achieved by Jiang et al. [25], when they observed notably lower concentration of VFAs at 55 °C (14.90 g/l) compared to that at 35 °C (41.34 g/l) and 45 °C (47.89 g/l). The effect of temperature on overall VFAs concentration also depends on the retention time: After seven days, the VFAs concentration dropped in the fermentations of 55 °C and 70 °C, while the concentration still increased in that of 35 °C.

Concerning butyrate production, the results differ depending on the study. Komemoto et al. [27] examined the effect of temperature on acidogenesis at six different temperatures ranging from 15 °C to 65 °C. They observed that butyrate concentrations started to increase at temperatures 35 and 45 °C after four days and peaking on the seventh day at 6.2 and 5.6 g/l, respectively, remaining then high until the end of the experiment. On the other hand, in the study by He et al. [29] the highest butyrate concentrations were achieved at temperatures 70 °C, and 55 °C, respectively, and only little butyrate was obtained at 35 °C. Butyric acid concentration increased proportionally with acetic acid concentration. Garcia-Aguirre et al. [30] showed similar results by fermenting organic waste streams at different temperatures and pH values, obtaining the highest butyrate concentrations at 55 °C and pH 5.5. Adding pH control when altering the temperature, has a significant impact on the VFAs production rate and distribution [30]. Increasing the VFAs production by adjusting temperature can only be done according to the ideal growing temperature of the bacteria involved in the process. [2]

### 4.3 Food waste as carbon source and influence of additional acetate

The food waste composition influences the quantity and the chemical distribution of VFAs production via biosynthesis [2, p.282]. The main groups of macromolecules found in FWs are carbohydrates, proteins, and lipids. Additionally, food wastes include various nutrients that microbes use as substrates and are hence ideal for VFAs production in biorefineries. [31, p. 21713–21714; 2, p.281] In general, carbohydrates and proteins are more suitable for fermentation than lipids. This is due to lipids' slower biodegradation kinetics, whereas carbohydrates are readily converted into glucose by enzymes. Long-chain fatty acids (LCFAs) generated from hydrolyzed lipids can, furthermore inhibit anaerobic metabolism. Proteins are also considered to have lower biodegradability due to their complex structures; their hydrolysis limits the acidogenic fermentation rates. [2, p. 282]

Concerning butyrate production, carbohydrates seem to be the most significant substrates. Yin et al [31] investigated the influence of carbohydrates, proteins, and lipids on acidogenic fermentation using pure glucose, peptone, and glycerol as substrates, respectively. For each substrate, they examined the involved metabolic pathways and the amounts of produced VFAs as well as the distribution among them. As a result, it was observed that for glucose fermentation, the main product was butyrate (50%), followed

by propionate (30 %), acetate (17 %) and valerate (3 %), whereas for peptone fermentation, acetate was the main VFA covering 70 % of the produced VFAs. Butyrate accounted for approximately 10 %, propionate for 15 %, and valerate for approximately 5 % of the total VFAs production. For carbohydrates and proteins both, the origin affected the VFAs production. [2, p. 282] In general, the conversion to VFAs was the fastest for glucose, followed by peptone and glycerol [31, p.21818].

*Megasphaera cerevisiae* is a strict anaerobe species and selective regarding substrates. It uses mainly fructose and some lactate and pyruvate as the carbon source to produce acetic, propionic, butyric, valeric and caproic acids, $H_2$ and $CO_2$. Some strains can also ferment arabinose. The relative rates of products produced from different substrates by *M. cerevisiae* are displayed in Table 1. Comparing to ruminal *M. elsdenii* of the same genus, *M.cerevisiae* does not ferment glucose and maltose. [19, p. 126]

Table 1. Organic acids and metabolites produced by *M.cerevisiae* from fructose and lactate. The amounts are in mmol produced product per mmol of used substrate. [19, p. 126]

| *Substrate* | *Acetic acid* | *Butyric acid* | *Propionic acid* | *Valeric acid* | *Caproic acid* | *$H_2$* | *$CO_2$* |
|---|---|---|---|---|---|---|---|
| *fructose* | 0.10 | 0.27 | 0.07 | 0.17 | 0.24 | 0.38 | 0.65 |
| *lactate* | 0.15 | 0.17 | 0.11 | 0.42 | 0.03 | 0.04 | 0.22 |

*Megasphaera* species can also grow in sugar-free media utilizing protein hydrolysates but the growth, in such case, can be characterized as poor [19, p. 126].

Acetic acid is a typical by-product of microbial butyrate fermentation. For many *Clostridium,* acetate is further used by the metabolism to produce butyrate [6, p. 659]. Additional acetate in the medium has been studied to promote glucose consumption, resulting in higher cell growth rates and increased amounts of biomass and butyrate at the end of the process. [32, p. 153–154] The advantages depend on the used microbes' tolerance towards acetate, and for some strains; it may result in an extended lag phase, slowing the process down. [33, p. 343] The simultaneous production of acetate slows down the butyrate production as well as the downstream processing which makes adding acetate in the medium controversial; it results in higher butyrate yield and biomass growth but increases the costs of refinery and product restoration [6, p. 659].

# 5 Modeling Fermentation Kinetics

Fermentative processes can be represented with kinetic models that give mathematical insight to the reactions during fermentation. Kinetic models describe the connections between the concentration rate $r_s$ of the substrate S, the formation rate $r_x$ of the biomass X and the formation rate $r_p$ of the product P. The modeling aims to demonstrate the process in the most simple, yet the most specific way including all relevant phenomena to describe and to predict the process. Kinetic models also allow the process' larger-scale simulations. The models describing fermentation kinetics contain three sections; substrate consumption model, biomass growth model, and product formation model represented in equations (5…7).

$$\frac{dS}{dt} = -r_s \tag{8}$$

$$\frac{dX}{dt} = r_x \tag{9}$$

$$\frac{dP}{dt} = r_p \tag{10}$$

Kinetic models describe the correlation between involved compounds, their concentrations, and reaction rates so that when combined to mass balance, they can help in predicting the substrate conversion and product yield in operational conditions that differ from those where they were initially developed. [23, p. 43]

There are many ways to classify kinetic models. One of these is the classification to empirical and mechanistic models. The former is based on empirical research and the latter on the assumption of reaction mechanisms. In empirical models, the parameters are adapted and all of them do not necessarily have physical significance in the process. Mechanistic models are used when the mechanisms behind the process are examined. Another way to bifurcate kinetic models is into structured and unstructured models. Structured models demonstrate systems where the microbial culture consists of multiple components whereas in unstructured models the system involves only one component. [23, p. 43–44; 34, p.84–85] Here, the discussed kinetics are delimited to those relevant considering batch reactor operations and that are also applicable to the Monod model,

with respect to the process in question. Of the mentioned above, the Monod model represents an empirical, unstructured model [23, p. 48]

## 5.1 Specific rates and yield coefficients

To develop a kinetic model of a process, the stoichiometry of the chemical reactions, reaction rates, and yield coefficients must be identified. Furthermore, the structural formulas of the substrate, biomass, and the metabolites involved in the process, must be understood. The cellular production of biomass and metabolites is due to many biochemical reactions catalyzed by various enzymes. However, when modeling a fermentation process where the biomass growth and the product yield are the main subjects of interest, describing singular reaction kinetics is not relevant. [23, p. 44]

Specific growth rate variable μ ($l/h^{-1}$) describes the cell growth per a biomass unit. It can be expressed with equation (11).

$$\mu = \frac{dX}{dt} \cdot \frac{1}{X} \tag{11}$$

Specific product yield rate q represents the metabolite production per a biomass unit and can be formulated with equation 12).

$$q = \frac{dP}{dt} \cdot \frac{1}{X} \tag{12}$$

The amount of carbon in the substrate converted to the metabolite of interest indicates the overall yield and can be represented with yield coefficient Y. It describes the formation rate of the observed component over a selected reference component. When the reference component is the substrate, the yield coefficients are given by equations (13…14).

$$Y_{PS} = \frac{r_P}{r_S} \tag{13}$$

$$Y_{XS} = \frac{\mu}{r_S} \tag{14}$$

With the described yield coefficients, the substrate consumption rate can be expressed with equation (15). [22, p. 45]

$$\frac{dS}{dt} = -\left(\frac{1}{Y_{XS}}\frac{dX}{dt}\right) - \left(\frac{1}{Y_{PS}}\frac{dP}{dt}\right) \tag{15}$$

## 5.2 Black Box Model

A simple way to illustrate cell growth stoichiometry is the black-box model, where all the cellular reactions are merged into a single reaction. The stoichiometric coefficients correspond to the components yield coefficients towards the biomass. Therefore, the overall reaction can be presented with equation (16).

$$X + \sum_{i=1}^{M} Y_{xp_i} P_i - \sum_{i=1}^{N} Y_{xs_i} S_i = 0 \tag{16}$$

In the black-box model, the yield coefficients are presumed constant, which does not correspond to reality. Thus, correlations between substrate uptake and specific growth rate, for instance, cannot be estimated with the black-box model. However, the black-box model is useful for validating the consistency of experimental data because it can represent the balance between the elements involved in the conversion reactions that occur in the system. [23, p. 45–46]

## 5.3 The Monod model

Fermentation kinetics have been demonstrated in numerous different models of which the Monod model works as the basis for almost all the rest. The Monod model is one of the so-called unstructured models that can be used to predict the specific growth rate when the reaction is presumed to follow the black box stoichiometry. Unstructured models demonstrate cellular operations of basic biological reactions without taking cell structure into account. The Monod model describes the process kinetics on a simple level. The presumption is that change in the limiting substrate concentration during the process directly correlates to the reaction rate. Nevertheless, as a result of various biochemical reactions within biomass, microbial growth reacts to changes with a delay. Thus, unstructured kinetic models underestimate the time constant of dynamic change and might not be sufficient for more complex systems or a larger scale. [23, p.48; 33, p. 84–85]

The Monod model is based on empirical study, according to which in a steady flow stage with small substrate concentrations, as substrate concentration approaches zero, the specific biomass growth rate is linearly proportional to the glucose concentration. With high concentrations of the substrate, the specific growth rate does not depend on the substrate concentration. The reaction limiting factor is an essential substrate. The Monod equation shows cell growth as follows:

$$r_x = \frac{\mu_m S}{K_s + S} x_{v,} \tag{17}$$

where $r_x$ is the cell growth rate (kg cells $m^{-3}h^{-1)}$, $\mu_m$ is the maximum specific growth rate ($h^{-1}$), S is the limiting substrate concentration (kg substrate $m^{-3}$), $K_s$ is the saturation constant (kg substrate $m^{-3}$) and $x_v$ is the concentration of viable cells (kg cells in $m^{-3}$). From this, the specific growth rate μ (S), often abbreviated to μ ($h^{-1}$), when

$$r_x = \mu(S) x_v, \tag{18}$$

can be expressed as

$$\mu = \mu_{max} \frac{S}{K_S+S}. \quad (19)$$

[33, p. 91]

In the basic form of the Monod model, the reactor is assumed well mixed so that the substrate availability is equal within the reactor. Furthermore, all cells are presumed vital and the possible death during fermentation is not considered. No gradients are presumed to be present.

For a system, where more than one limiting substrate is present, the Monod model can be modified into equation (20).

$$\mu = \mu_{max} \frac{S_1}{K_1+S_1} \frac{S_2}{K_2+S_2} \dots \quad (20)$$

The Monod model is applicable to batch processes including one exponential stage or to a stable continuous process. The model does not consider environmental changes affecting the cells. The Monod model is not ideal for scale-up processes due to its lack of a mechanical basis.

### 5.4 Multiple strain culture kinetics

For a microbial culture, where more than one species is growing, the kinetic functions describe how the different microorganisms involved interact with each other. Compared to pure strain kinetics, state variables are changing with respect to each other and to at least one independent variable such as time. For well-mixed cultivation including two different strains at population densities X1 and X2, and in the absence of immigration or mutation, the system can be represented with Lotka-Volterra equations (21…22), that can be used to investigate multispecies ecosystems.

$$\frac{dX_1}{dt} = (\alpha_1 + \beta_{11}X_1 + \beta_{12}X_2)X_1 \quad (21)$$

$$\frac{dX_2}{dt} = (\alpha_2 + \beta_{22}X_2 + \beta_{21}X_1)X \quad (22)$$

When the $\alpha_i$ terms are considered constant, they can be regarded as functions of specific growth rate; hence, implied to the Monod equation (19). The $\beta_{ij}$ terms represent factors that regulate the population density in ways that depend on the type of interaction. [35, p. 25–26]

The interaction between species can be determined as direct or indirect. In indirect interaction, no physical contact between species is involved but some non-living parts of the environment such as a product of one, for instance, is an essential intermediary. These interactions can further be defined either positive or negative depending on whether the effect of the other species promotes or inhibits the growth of the other. The describing terms for different associations are *commensalism*, *competition*, *mutualism*, *predation,* and *parasitism.* [35, p. 27–35]

In the case of commensalism, one of the populations benefits from the presence of the other. When the association is indirect, some substance that promote the growth of the other population may be produced by the other species. In terms of Monod kinetics, the increase of the specific growth rate of the first species ($\mu_1$) can be represented as the function of the concentration of the product manufactured by the other species ($P_2$) according to the equation (23). The specific growth rate of the second species ($\mu_2$) remains, as shown in equation (24), where S is some externally supplied limiting substrate. [35, p. 34]

$$\mu_1 = \frac{\mu_{max1}P_2}{K_1+P_2} \quad (23)$$

$$\mu_2 = \frac{\mu_{max2}S}{K_2+S} \quad (24)$$

If the association is mutual, the specific growth rate $\mu_2$ could be formulated as a function of $P_1$, the concentration of the metabolite produced by the first species, as in equation (25).

$$\mu_2 = \frac{\mu_{max2} P_1}{K_2 + P_1} \quad (25)$$

Other interactions occur, for example, when one of the species uses both the supplied substrate and the manufactured product of the other species for its cell growth. In some cases, this can lead to competition, and the process should be operated with respect to the desired outcome. [35, p. 35]

## 5.5 Inhibition

Fermentation and cell growth can be inhibited by biomass, substrate, fermentation products and possible contaminants. Operational conditions such as pH and temperature affect the amplitude of the inhibition. Thus, the effect can be decreased by adjusting such parameters. [23] Growth can be inhibited by various factors. An increase in inhibitor concentrations can be reflected with enzyme reaction inhibitors; hence, cell growth inhibition can be applied to models describing enzyme inhibitions, such as equation (26)

$$\mu = \mu_{max} \frac{S}{K_s + S} (1 - K_i I), \quad (26)$$

where *I* represents the concentration of the inhibitor. [34, p.93] Substrate inhibition may occur in a system where the substrate concentrations exceed the levels that are characteristic for the species. The higher the substrate concentrations are, the more likely they are to cause inhibition and the more powerful the effect may be. Substrate inhibition is modeled by equation (27). [23, p. 37; 34, p.93]

$$\mu = \mu_{max} \frac{S}{K_s + S + (K_i S)^2} \quad (27)$$

The butyrate-producing pathway is inhibited when a certain amount of butyrate has been accumulated in the process. The butyrate penetrates the membrane of the bacteria, dissociating inside the cell. It hence alters the pH gradient of the transmembrane lowering the energy yield needed for biomass growth. [6, p. 659]

# 6 Materials and Methods

## 6.1 Common

In the experimental part of this thesis, a pre-processing method was designed for the provided food waste. The aim was to optimize the hydrolysis in order to derive the best sugar concentration in the fermentation media as a part of the process. Different enzymes and parameters were applied and the Design of Experiments-model was used to find the ideal conditions. The hydrolysate was then exploited as carbon source media reagent to conduct fermentation experiments.

The fermentation experiments aimed to optimize some of the process's operational parameters. The experiments were designed based on a MATLAB®-simulation that is being developed to model the process kinetics and the metabolism of the involved microbial strains. The experiments were conducted in bioreactors of different working volumes, using a co-culture of *Megasphaera cerevisiae* and *Pediococcus pentosaceus.* All the fermentations were anaerobic and conducted at 30 °C. The main objective of the fermentations was to steer the metabolic reactions to obtain butyrate as the dominant fermentation product. This was done by manipulating the fermentation parameters according to the simulation outputs.

## 6.2 Food waste and the hydrolysis experiments

The food waste cabbage was obtained from Vaissi Oy (Finland). The production of cabbage rolls generates different parts of cabbage: top leaves, stems and inner parts referred to as *mass cabbage*, as industry side streams. These different parts were examined with enzymatic hydrolysis in order to determine the amounts of released sugars. White cabbage contains approximately 5.0 grams of carbohydrates (per 100 g) including 2.3 grams of fibers such as cellulose, and one gram of water-soluble polysaccharides such as pectin. [9]

0.1 M citrate buffer (pH 5.0) was used in the first experiments to estimate the need for pH-control during hydrolysis. The used enzyme products and their optimal conditions were

- Cellic® Ctec2 (Novozymes, Denmark), cellulase complex
  - 45–50 ° C, pH 5.0–5.5
- Cellic® Htec2 (Novozymes, Denmark), endoxylanase
  - 45–50 ° C, pH 5.0
- Pectinex® Ultra SP-L (Novozymes, Denmark), a mixture of pectinases
  - 50 °C, pH ~4.5 .

All hydrolysis experiments were conducted with HT Infors incubator shakers (Infors AG CH-4103 Bottmingen, Switzerland) in sterile laboratory flasks. Sample preparation, used enzymes, stirring, flask volume, temperature, and duration of the experiments differed, depending on the objectives and the experimental set-ups described below (linked to Table 2, Headers 1–5):

1. Comparing different cabbage parts for released sugars with pH control; examining two different enzyme dosages.

2. Comparing leaf and mass parts; examining the effect of enzyme synergy of Ctec2 and Htec2 and comparing to hydrolysis without Htec2; examining different enzyme dosages; aiming to maintain pH at 5.0.

3. Examining the effect of enzyme synergy of Ctec2 and Htec2 and comparing to hydrolysis without Htec2; examining the effect of mechanical pre-treatment; examining whether buffer is needed or not.

4. Comparing different concentrations of Ctec2 on mass cabbage, with and without mechanical pretreatment.

5. DoE (screening, by MODDE® software) to examine the effects and interactions of factors (at the range):

- enzyme dosages of Ctec2 (3–14 %) and Pectinex (0–1.4 %)
- stirring (150–250 rpm)
- temperature (35–55 °C)
- time (24–72 h).

Full factorial design, where possible *corners* including minimum and maximum factorial values are investigated and experiments of triplicate center points are conducted, was used.

Table 2. Operational conditions for different hydrolysis experiments numbered 1–5. Different cabbage samples l (leaf), m (mass), s (stem) and M (mushed mass) are color-coded indicating which parts were examined within each experiment or with single condition. Enzyme dosages and synergies (2–3) are represented in braces, respectively. Experiments 1–4 were carried out triplicate. Flask volumes were 500 ml (exp. 1) and 250 (exp. 2–5). The used buffer was 0.1 M citrate.

| *Conditions* | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| *Samples* | leaf = l<br>mass = m-<br>stem = s | l<br>m | m<br>mushed cab-<br>bage = M * | m<br>M | M |
| *Enzyme product and dosage (% [w/w])* | - Ctec2<br>(1.5 l m ; 3<br>l m s) | - Ctec2<br>(6 ;2.7; 5.4)<br>- Htec2<br>(0; 0.3; 0.6) | -C-tec2<br>(6; 5.4)<br>-Htec2<br>(0; 0.6 ) | -Ctec2<br>(6; 7.7; 12) | -Ctec2<br>(3; 8.5; 14)<br>-Pectinex®<br>(0; 0.7; 1.4) |
| *Stirring (RPM)* | 150 | 200 | 200 | 200 | 150<br>200<br>250 |
| *T (°C)* | 50 | 50 | 50 | 50 | 35<br>45<br>55 |
| *Duration (h)* | 138 | 48 | 48 | 48 | 24<br>48,<br>72 |
| *Buffer (ml)* | 20 | 40 | 5 (20 % of dry weight) | - | - |

**Mechanically pre-treated with Bamix® or food processor.*

The unit for the enzyme dosages was percentage w/w (g enzyme/g cellulose). The dosages and experimental conditions were estimated according to literature findings and recommendations by the product manufacturer [35; 36]. The dosage of each enzyme for each experiment and sample was calculated according to protein concentrations determined at VTT and the dry weight measurements of each cabbage part. The mechanical pretreatment aimed to mush the cabbage and was carried out by using either a food-grade Bamix® M140 (Switzerland) or a Braun Combimax 600 food processor (Germany).

## 6.3 Using the MATLAB® simulation

A simulation modeling the process is in development by Alberte Regueira and Miguel Mauricio Iglesias at the University of Santiago de Compostela, Spain. The modeling is a part of the BioChem project, with the main idea to design processes that target specific VFAs. The used computing environment and programming language is MATLAB® by MathWorks. All parameters and kinetics have been determined according to the data generated from the previous experiments within the project. Initial operational parameters are due to previous optimizations by Rosaliina Turunen (VTT). The simulation is at its validation phase; thus, designing the experiments according to the simulation settings was carried out in co-operation with its creators via email correspondence.

The provided model includes multiple files of code and an Excel source file holding the process parameters. The model has a separate *simulate.m* file (Listing 1), where the user can input the volume of the reactor, estimated duration of the process and initial concentrations of compounds such substrates and the bacteria.

```
%% INPUTS OF THE MODEL

% Set reactor parameters
V = 1;                      % Volume of the reactor (L)
pH = 6.5;                   % Reactor pH

% Set the initial concentration in the reactor

C_ini(1) = 20.9;               % Glucose initial concentration (g/L)
C_ini(2) = 12.8;              % Fructose initial concentration (g/L)
C_ini(3) = 8.7;             % Acetate initial concentration (g/L)
C_ini(4) = 0;               % Propionate initial concentration (g/L)
C_ini(5) = 0.117;            % Sugar degrader initial concentration (g/L)
C_ini(6) = 0.043;            % Lactate degrader initial concentration (g/L)

% Set the simulation time
t = 100;                    % Reaction time (h)

% Select graphs at the end of the simulation
flag_graph = 1;             % 1= graphs are activated, 0= graphs are deac-
tivated
```

Listing 1. Section of the *simulate.m* input file including process parameter values. Reactor volume (V), pH, the initial concentrations (C_ini 1–5, explanations written in green at the right) of involved compounds and reaction time (t) can be set by the user. The user can also choose whether graphs of the process will be printed as output. Coded using MATLAB®.

After running the file, the simulation returns the accumulated product and biomass concentrations at the end of the process as output. When the simulation is run with following default parameter values:

- V =1 l
- pH 6.5
- Initial substrate concentrations (g/l)
    - glucose 20.9
    - fructose 12.8
    - acetate 8.7
    - propionate 0
- *P. pentosaceus:* 0.117 g/l
- *M. cerevisiae* 0.043 g/l
- t (reaction time h) 100,

as they have been after optimizing the process and the scale-up by Rosaliina Turunen, the output shows the final concentrations of each involved compound with values shown in Listing 2.

```
The concentration of the compounds at the end of the batch are (g/L):
  Columns 1 through 8

    'Glucose'    'Fructose'      'Lactate'       'Acetate'    'Propionate'
    [      0]    [4.4977e-99]    [1.0833e-98]    [ 4.9200]    [    4.8418]

     'Butyrate'    'Valerate'    'Caproate'
   [ 10.9875]    [  3.8150]    [  0.2314]

 Columns 9 through 10

    'Pediococcus'    'Megasphaera'
    [     2.5373]    [     1.3712]
```

Listing 2. Output from the simulate.m file with default input values: V=1l; pH=5.5; [glucose]= 20.9 g/l; [fructose]= 12.8 g/l; [acetate]= 8.7; [propionate]=0; [*P.pentosaceus*]=0.117 g/l and [*M.cerevisiae*]=0.043 g/l.

Additionally, the program prints an optional line chart-graph for each involved compound, demonstrating the transformations against time (example found in Appendix 3, Figures 1–6).

## 6.4 Hydrolysate preparation for the fermentation media

The cabbage was melted, manually squeezed of extra water and mashed with Braun Combimax 600 kitchen processor (Germany). The hydrolysis took place in HT Infors incubator shakers (Infors AG CH-4103 Bottmingen, Switzerland) in the following conditions:

- flask volume of 1000-5000 ml
- the temperature at 50 °C
- a stirring of 200 rpm
- duration of 60–90 minutes
- used enzymes were Cellic® Ctec2 and Pectinex® in dosages 6 % (w/w) and 1 % (w/w), respectively.

The hydrolysates were mixed and centrifuged with Thermo Scientific™ Sorvall BIOS 16 centrifuge (United States) at 3800 rpm for 30 minutes, after which the clarified supernatant was poured through a sieve to separate the maintained solid parts of the cabbage (Figures 7a and b).

a) b)


Figure 7. Mass cabbage hydrolysate before (a) and after (b) centrifuging.

The amount of obtained hydrolysate towards cabbage was estimated according to hydrolysis experiments, where one gram of cabbage dry matter resulted in approximately 15 ml of hydrolysate.

## 6.5 Minimal media preparation

The minimal media included the cabbage hydrolysate, vitamin and mineral solutions, acetate, yeast extract, and *L*-cysteine hydrochloride. The solutions were separately prepared and sterilized either in an autoclave or by filtration. The hydrolysate was autoclaved with yeast extract and acetate after pH-adjustment. The precise ingredients of

other media components are described in Appendix 2. The mineral solution was prepared without $CaCl_2$ and $FeOH^-$ to avoid sedimentation; separate stock solutions of these compounds were prepared.

## 6.6 Fermentations

Anaerobic co-culture fermentations were conducted to examine the effect of different fermentation parameters on butyrate production and to investigate the functionality of the MATLAB®-simulation in practice.

### 6.6.1 Inoculum

All fermentations were co-cultivations of butyrate-producing strain *Megasphaera cerevisiae* VTT E-981087 and lactate producing strain *Pediococcus pentosaceus* VTT E-153483. The strains were obtained from VTT strain collection for this project, they were natural and not gene-modified.

The strains were stored at –80 °C as frozen stock cultivations. Both cultures were revived as an amount of a needle head into sterile RCM broth (5 ml, recipe found in Appendix 2). *M. cerevisiae* was incubated in an anaerobic cabin at 30 °C for approximately three days. *P. pentosaceus* was incubated at 37 °C aerobically and the incubation time was 4–5 days. Both strains were transferred further into larger volumes (3 vol %) for pregrowth if needed.

The inoculum volume was determined by measuring the optical density (OD) with a spectrophotometer (with Shimadzu UV-1201 & UV-Vis (Australia)) at wavelength 630 nm. The needed amounts were calculated with equation $c_1V_1=c_2V_2$, where $c_1$ represents the inoculum OD, $V_1$ the needed inoculum volume, $c_2$ the desired OD in the reactor and $V_2$ the reactor volume. The initial *M. cerevisiae* concentration in the reactor depended on the objective of the experiment. For *P. pentosaceus* the concentration was always calculated to correspond to OD 0.1.

### 6.6.2 Bottle fermentation conditions

The experiment took place in an anaerobic chamber in triplicate Scotch bottles. RCM-broth was the used media. The conditions were the following:

- the volume of the media 20 ml
- the temperature was 30 °C
- time of the experiment was 24 hours
- the pH of the media was set to 6.5
- sampling after inoculation (0) and after 7 and 24 hours
- sample volume ~2ml.

### 6.6.3 Bioreactor fermentations

The fermentations were conducted as batch operations with Multifors 2 (Infors AG, Bottmingen, Switzerland) table bioreactors and with Biostat® CT-2 (Sartorius AG, Germany) bioreactors. The operational conditions were the following:

- the volume of the media 400- 1500 mL
- anaerobic ($N_2$ inlet gas flow to the upper state of 0.2 lpm)
- pH 6.0–7.0
- a stirring of ~150 rpm
- the growing temperature at 30 °C.

The retention times vary from two to four days during which 2–3 samples per day were taken. The sample volumes were 5–15 ml. The media was prepared as described in chapters 6.4 and 6.5. The bioreactors were sterilized in an autoclave (Multifors) or in position (Biostat® CT2). The anaerobic environment was maintained by nitrogen ($N_2$) sparging into the over part of the reactor at 0.2 l/min during the entire fermentation. The pH was automatically controlled with 4 M sodium hydroxide (NaOH) and 15 % phosphoric acid ($H_3PO_4$). The pH- and $pO_2$- values and the outlet-gas $CO_2$-percentage were observed and considered as indicators of the process propagation.

## 6.7 Analytical methods

The different cabbage parts were determined for dry matter weights. The hydrolysis experiment samples were analyzed for released monomeric sugars (glucose and fructose), pH and by evaluating the liquidity of the hydrolysate. All fermentation samples, including those from bottle experiments, were analyzed for cell growth, pH, substrate consumption and product formation. From the experiments conducted in bioreactors, the data containing molar percentages of oxygen, carbon dioxide and nitrogen in the outlet gas was acquired during the process.

### 6.7.1 Food waste dry matter weight determination

Dry matter weights were determined with triplicates of each residue part. The food waste cabbage was not mechanically pre-processed. Samples of 2–10 grams were weighed and rinsed with Bühner-filtering on separately weighed filter papers. Samples were then oven-dried at 105 °C for approximately 45 hours and cooled down in a desiccator before levelling.

### 6.7.2 Liquidity observation

The liquidity of the hydrolysates was evaluated by rule of thumb according to the particles that could still be observed in the samples. For this a scale of 1–5, where 5 indicated the texture and the state of matter of the food waste as it was at the beginning of the hydrolysis, and 1 total liquidity so that no solid particles were longer observed, was determined.

### 6.7.3 pH and the optical density (OD)

pH was measured for each sample during fermentation experiments by using Norlab Knick 766 Laboratory pH-meter (Finland). Biomass production was detected with optical density (OD) measurements at wavelength 630 nm with Shimadzu UV-1201 & UV-Vis-spectrophotometer (Australia). Samples were homogenized using Vortex®, after which samples were appropriately diluted with ultrapure (Milli-Q) water in a cuvette, where they

were then measured at a volume of 1 ml. The optical density of the used medium was considered.

### 6.7.4 High-Performance Liquid Chromatography (HPLC)

The concentrations of glucose, fructose, butyric acid, acetic acid, and lactic acid were determined using High-Performance Liquid Chromatography (HPLC) (Waters Alliance 2695, Waters Corporation, Milford, USA) with following parameters:

- HPX-87H-column (Bio-Rad, Munchen, Germany)
- 5 mM sulphuric acid ($H_2SO_4$) as the mobile phase at flow rate 0.3 mL $min^{-1}$
- the temperature at 35 °C
- ultraviolet (UV) detector at wavelength 210 nm (Waters 2487)
    - butyric-, acetic- and lactic acids
- refractive index (RI) detector (Waters 410)
    - all compounds.

The samples were centrifuged at 13 000 rpm for 10 minutes with Heraeus® Biofuge® Fresco (Germany) in 2 ml Eppendorf® tubes to remove the cellular debris. For abundantly clear supernatant, the samples were centrifuged twice or filtered if necessary. The samples were diluted with 5 mM $H_2SO_4$. The data acquisition and analysis were performed with Empower™3 Chromatography Data software (Waters).

### 6.7.5 Cell mass dry weight determination

The inoculums were determined for cell concentration with dry weight measurements. One milliliter of inoculum was pipetted into a triplicate oven-dried and weighed Eppendorf® tubes and then centrifuged for 10 minutes at 13 000 rpm with Heraeus® Biofuge® Fresco (Germany). The cells were ‘washed’ twice by replacing the supernatant with two milliliters of ultrapure water and centrifuging before leaving the cells to dry at 105 °C overnight.

### 6.7.6 Gas Chromatography

Short-chain organic fatty acids including valeric, caproic, acetic, propionic and butyric acids, were detected by Gas Chromatography (GC) (Agilent 7890A) with the following parameters:

- mass selective detector (MSD) (Agilent 5975C)
- columns Agilent 160-2625-10 (25 µm x 200 µm x 0.3 and 1.6 x 150 µm x 0 µm) (Agilent Technologies, California, USA)
- helium as the carrier gas with a velocity of 1.2 ml/min
- 1 µl injection volume per sample, retention time of the solvent of 6 minutes
- column temperatures:
    I. 40 °C, retention time 1.5 minutes
    II. 160 °C with a rate of 10 °C/min
    III. 240 °C with a rate of 25 °C/min, retention time 3.3 minutes.

### 6.7.7 Mass spectrometry (MS)

The outlet gases for oxygen, nitrogen, and carbon dioxide were measured on-line on all bioreactors with QMG 421C-kvardrupol mass spectrophotometer (Malxers Pfeiffer Scandinavia AB, Sweden) and analyzed as molar percentages with Balzers Quadstar 422 software. Following the carbon dioxide content during the process, enabled real-time evaluation of the procession of the fermentation. Finishing the process was often based on the carbon dioxide curve. The outlet gas nitrogen and oxygen contents were observed to ensure anaerobic conditions in the reactor.

### 6.7.8 Acid hydrolysis and HPLC method for release of monosaccharides

The mass cabbage monosaccharides and their concentrations had been determined with acid hydrolysis and liquid chromatography with DIONEX ICS-5000 (column: CarboPac PA-20) and DIONEX-ICS-3000 (column: CarboPac PA1) liquid chromatography equipment, for this project at VTT. The sample contained of squeezed cabbage juice mixed with the residue cabbage matter from the juicing. In acid hydrolysis, the sample (liquid or fine-grained mass) polysaccharides were fractioned into monosaccharides with strong sodium acid (95–97 % $H_2SO_4$), after which the monosaccharides were separated

by HPLC with anion exchange columns and detected with a pulsed amperometric detector.

The units were mg of sugar/100 mg dry matter (acid hydrolysis) and mg/l (HPLC). The determined dry matter content for the mass cabbage was 7.3 %. The obtained monosaccharide concentrations were used as reference values for the enzymatic hydrolysis conducted in this thesis. This determination was not done for the cabbage leaf and stem.

# 7 Results

## 7.1 Enzymatic hydrolysis

A series of enzymatic hydrolysis with different conditions (see Table 2 in chapter 6.2) was conducted to determine the optimal parameters to obtain a completely liquefied hydrolysate with sufficiently released monomeric sugars to use further as fermentation media. A summarization of the enzymatic hydrolysis results after 42 –48 hours for Experiments 1–4 (see Table 2 in chapter 6.2) is found in Appendix 4. The cabbage stem and leaf parts were left out from further experiments, due to lower released sugar concentrations. The used cabbage is referred to as *mass* if no mechanical pre-treatment was applied, and *mush* if the cabbage was pre-treated as described in chapter 6.2. The released sugar concentrations are compared to *Reference* values obtained from the acid hydrolysis (chapter 6.7.8).

### 7.1.1 Released glucose and fructose

According to the acid hydrolysis (described in chapter 6.7.8) , the mass cabbage includes 2.6 mg of fructose per 100 mg of dry matter and 35 mg of glucose per 100 mg of dry matter. The corresponding concentrations according to anionic exchange HPLC method were 20.2 g/l and 16.6 g/l for glucose and fructose, respectively. The more specific results and data from the acid hydrolysis are not introduced in this report.

The enzymatically released glucose and fructose concentrations for the mass cabbage were compared to those obtained from the acid hydrolysis. The effects of i) pH control

with added 0.1 citric buffer at ii) different amounts displayed as percentages towards the dry matter amount and iii) the synergy of Ctec2 and Htec2 in different ratios were examined in experiments 1–3. According to the dry matter determination described in chapter 6.7.1, the mass cabbage contained 6.2 % ± 0.02 % of dry matter. The results are shown in Figure 8 and are compared to the *Reference*.

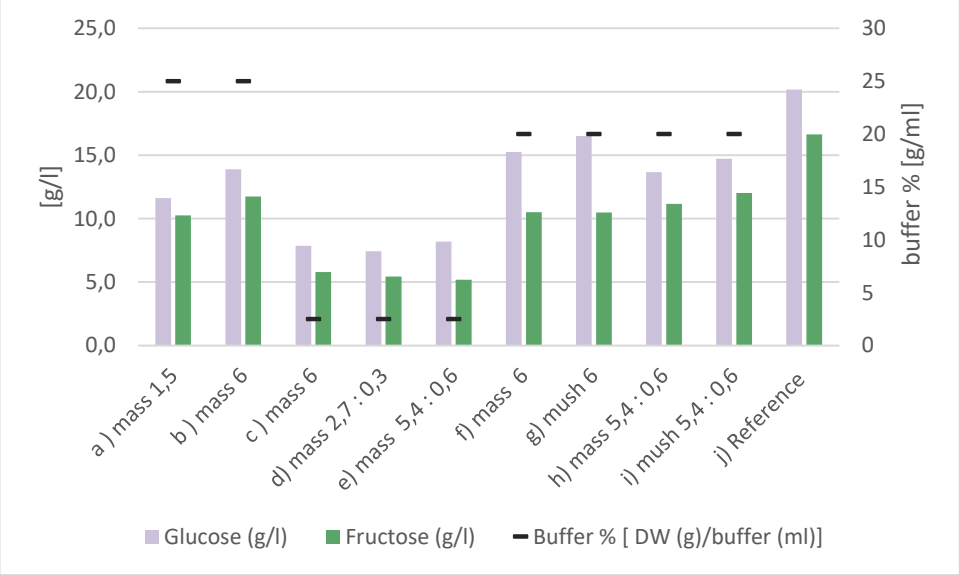



Figure 8. Released glucose and fructose of the hydrolysis experiments shown as g/l. Mass cabbage (from Vaissi Oy, Finland) *mass* and mechanically pre-treated mass cabbage *mush* hydrolysates (a-i) are compared to *reference* determined for mixed mass cabbage juice and mush. The used enzyme dosages (% w/w) are represented as ratios C-tec2: Htec2 for a-i. The reference j glucose (20.2 g/l) and fructose (16.6 g/l) have been determined at VTT by acid hydrolysis. The amount added buffer (0.1M citrate, black hyphen) is demonstrated as % of how much dry matter (g) the sample includes per ml of buffer. Operational conditions for a-i: T, 50°C; stirring, 200 rpm; t, 48 h.

The results show that the more buffer was used (Figure 8 samples c-e) the lower the final sugar concentrations were and that with additional buffer, the reference sugar levels were not reached. Comparing the samples with corresponding buffer additions, it can be noted that neither the addition of Htec2 (a & b, c-e, f-i) nor the increase in Ctec2 dosage (a-b), promoted the sugars release significantly. Mechanical pre-treatment had little impact.

Figure 9 shows the results from hydrolysis Experiment 4 (see Table 2 from chapter 6.2). The released sugars are shown for enzymatic hydrolysis experiments where buffer was

not used, comparing *mass* and *mush* samples at different Cellic® Ctec2 dosages to the *Reference* concentrations.

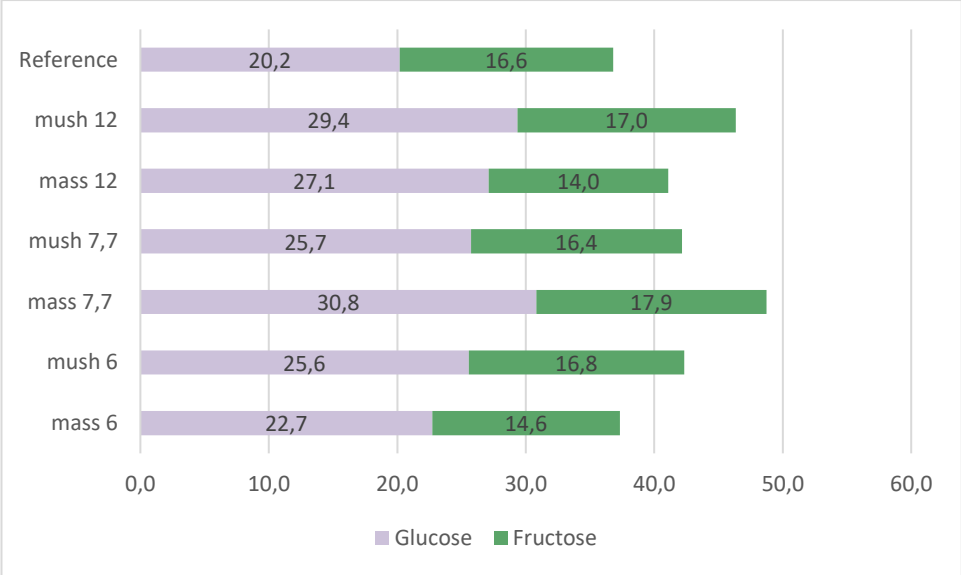



Figure 9. Released glucose and fructose concentrations as g/l from hydrolysis experiments (*mass* and mechanically pre-treated mass cabbage *mush*) using C-tec2 in three different enzyme dosages (6; 12 & 7.7 ) compared to the reference glucose (20.2 g/l) and fructose (16.6 g/l) concentrations determined of cabbage juice and mush at VTT by acid hydrolysis. Other conditions for the enzymatic hydrolysis: T, 50°C; stirring, 200 rpm; t, 48 h.

In Experiment 4, the *Reference* glucose concentration was exceeded by all enzymatic hydrolysis samples and fructose concentrations were higher than for the *Reference* in all samples apart from '*mass* 6' and *'mass* 12'. The Ctec2- dosage seems to correlate positively to the amount of released sugars, even though sample '*mass* 7.7' towards sample '*mass* 12' shows an exception.

The experimental set-up for hydrolysis Experiment 5 (see Table 2 in chapter 6.2) was conducted according to an output from MODDE® Design of Experiments software. The objective was to examine the effect of Pectinex® enzyme product of pectinases combined with Ctec2 dosages at large scale (3–14 % (w/w)). Also, as the DoE model enables, other factors with multiple values were included. Only the *center point*- experiments were conducted triplicate. The experimental conditions and the results are shown in Table 3.

Table 3. Experimental set-up derived from MODDE® Design of Experiments software and the corresponding results from each experiment (N1–19). N17–19 represent triplicate center-point experiments. The experimental factors (min-max) are Ctec2 (3–14 %(w/w)) and Pectinex® (0–1.4 % (w/w)) concentrations, time (24–72 h) and temperature (35–55 ° C) and the responses are liquidity (1–5, where 1 indicates completely liquefied hydrolysate) and glucose and fructose concentrations (g/l). Experiments 5–7 (marken in red) were contaminated. The experimental design is screening and the factorial design is full factorial design. The used cabbage was mechanically mushed.

| *Sample (N)* | *Ctec2 (% w/w)* | *Pectinex® (% w/w)* | *Time (h)* | *Temperature (°C)* | *Liquidity (1-5)* | *Glucose (g/l)* | *Fructose (g/l)* |
|---|---|---|---|---|---|---|---|
| *1* | 3 | 0 | 24 | 35 | 4.75 | 15.8 | |
| *2* | 14 | 0 | 24 | 35 | 4.25 | 31.0 | |
| *3* | 3 | 1.4 | 24 | 35 | 1.25 | 27.6 | 19.3 |
| *4* | 14 | 1.4 | 24 | 35 | 1 | 38.7 | 20.6 |
| *5* | 3 | 0 | 72 | 35 | 4 | 0.2 | |
| *6* | 14 | 0 | 72 | 35 | 3.75 | | |
| *7* | 3 | 1.4 | 72 | 35 | 1.25 | | |
| *8* | 14 | 1.4 | 72 | 35 | 1 | 14.2 | 13.7 |
| *9* | 3 | 0 | 24 | 55 | 4.75 | 15.1 | |
| *10* | 14 | 0 | 24 | 55 | 4 | 27.3 | 14.8 |
| *11* | 3 | 1.4 | 24 | 55 | 1 | 33.6 | 22.1 |
| *12* | 14 | 1.4 | 24 | 55 | 1 | 39.5 | 21.9 |
| *13* | 3 | 0 | 72 | 55 | 4 | 19.2 | 11.0 |
| *14* | 14 | 0 | 72 | 55 | 3.75 | 46.2 | 24.2 |
| *15* | 3 | 1.4 | 72 | 55 | 1 | 38.0 | 24.5 |
| *16* | 14 | 1.4 | 72 | 55 | 1 | 52.3 | 28.5 |
| *17* | 8.5 | 0.7 | 48 | 45 | 1 | 37.7 | 22.2 |
| *18* | 8.5 | 0.7 | 48 | 45 | 1 | 37.1 | 22.0 |
| *19* | 8.5 | 0.7 | 48 | 45 | 1 | 36.3 | 21.7 |

The concentrations for released sugars according to HPLC measurements (Table 3) are very high for the samples with high Ctec2-dosages (Cetec2 = 14 %) regardless of the Pextinex® dosage. The temperature at the selected scale does not seem to affect the release of the sugars.

### 7.1.2 Liquidity

Another objective of the hydrolysis was to obtain a perfectly liquefied hydrolysate that could be used as such for the fermentation medium. The hydrolysates were observed for solid particles and manually shaken to evaluate the viscosity. The state change (from the original sample to hydrolysate) quality was evaluated towards time. The observations for Experiments 1–4 (Table 2, chapter 6.2) are summarized in Appendix 4. It was observed, that the addition of Htec2 did not affect the liquidity, compared to hydrolysis including only Ctec2. In addition, increasing the Ctec2 dosage did not result in significant change considering the liquidity.

Some samples from Experiments 1–4 were almost at the desired liquid state after 48 hours whereas, in Experiment 5, the samples with Pectinex® were completely liquefied after 24 hours (Table 3 & Figure 10).

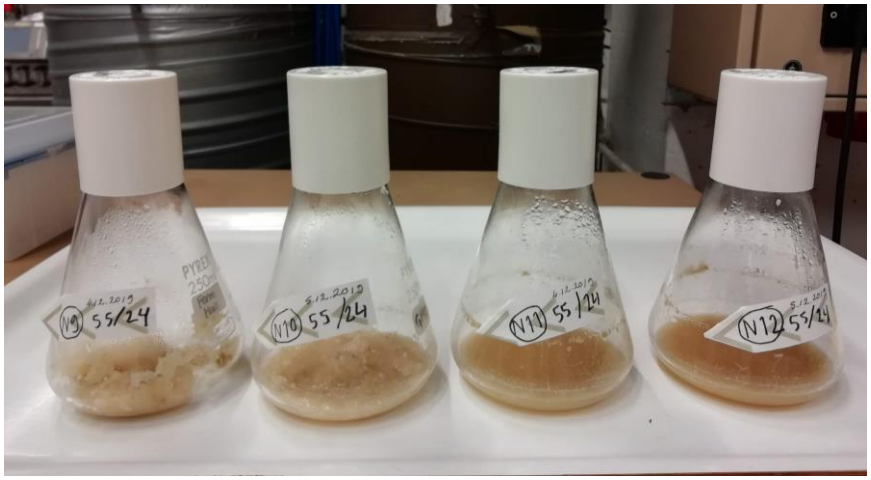



Figure 10. Hydrolysis samples N9–12, from left to right, respectively after 24 hours incubation at 55°C. Enzyme dosages %(w/w): N9, 3 Ctec2, 0 Pectinex® ; N10, 14 Ctec2, 0 Pectinex®; 11, 3 Ctec2, 1.4 Pectinex®; 12: 14 Ctec2, 1.4 Pectinex®.

For the fermentation media, the hydrolysis experiments were conducted using both Ctec2 and Pectinex® (chapter 6.4) and the incubation took approximately one hour.

## 7.2 Impact of pH

### 7.2.1 Bottle fermentation without pH control

A bottle fermentation experiment was conducted to learn how the pH would change in 24 hours without control. The objective was to determine whether further examinations without pH control would be possible. Materials and methods for the experiment are described in chapter 6.6.2. The media pH was adjusted to 6.5 before the experiment using NaOH. The pH dropped with approximately 1.8 for each sample i.e. the average pH-value after 24 hours, was 4.7. The HPLC results showed that without pH control, lactate (7.5 g/l) was dominant over butyrate (1.9 g/l).

### 7.2.2 Bioreactor fermentation at different pH-values

The effect of pH on butyrate production was examined by six in-line fermentations with table bioreactors (Multifors). Duplicate experiments were conducted with pH-values set to 6.0, 6.5 and 7.0. Chosen pH-values were based on literature findings (chapter 4.1). The objective was to obtain butyrate and to learn, whether a change in pH at a small scale would make a difference. The hydrolysate, the minimal media, and the inoculums were prepared as described in chapters 6.4, 6.5 and 6.6.1, respectively. The operational conditions corresponded to those described in chapter 6.6.3. The retention time was approximately 60 hours and eight samples including media samples were taken per each reactor. Offline pH-values measured for each sample are shown in Table 4.

Table 4. pH-values measured offline for each sample representing six co-culture fermentations. Duplicate processes are pH6.0_1 & _2, pH6.5_1&_2 and pH7.0_1&_2, according to initial media pH-value. Other conditions: V, 400 ml; T, 30°C, stirring, 150 rpm, anaerobic with $N_2$ gas flow 0.2 lpm.

| ***Time (h)*** | ***pH6.0_1*** | ***pH6.0_2*** | ***pH6.5_1*** | ***pH6.5_2*** | ***pH7.0_1*** | ***pH7.0_2*** |
|---|---|---|---|---|---|---|
| *0:00* | 5.85 | 5.87 | 6.2 | 6.31 | 6.79 | 6.7 |
| *13:12* | 5.66 | 5.54 | 5.92 | 6.11 | 6.4 | 6.2 |
| *19:23* | 5.72 | 6.04 | 5.95 | 6.21 | 6.52 | 6.25 |
| *23:27* | 5.84 | 6.04 | 6.13 | 6.3 | 6.82 | 6.3 |
| *37:21* | 5.98 | 6.3 | 6.15 | 6.58 | 6.92 | 6.58 |
| *43:30* | 6.11 | 6.36 | 6.29 | 6.56 | 7.05 | 6.92 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *60:56* | 6.05 | 6.27 | 6.2 | 6.46 | 7.04 | 6.85 |

As shown in Table 4, the pH-values were not maintained constant during fermentation. All pH-value set-points were adjusted after 13 hours when lowered pH-values were detected from samples taken at that time.

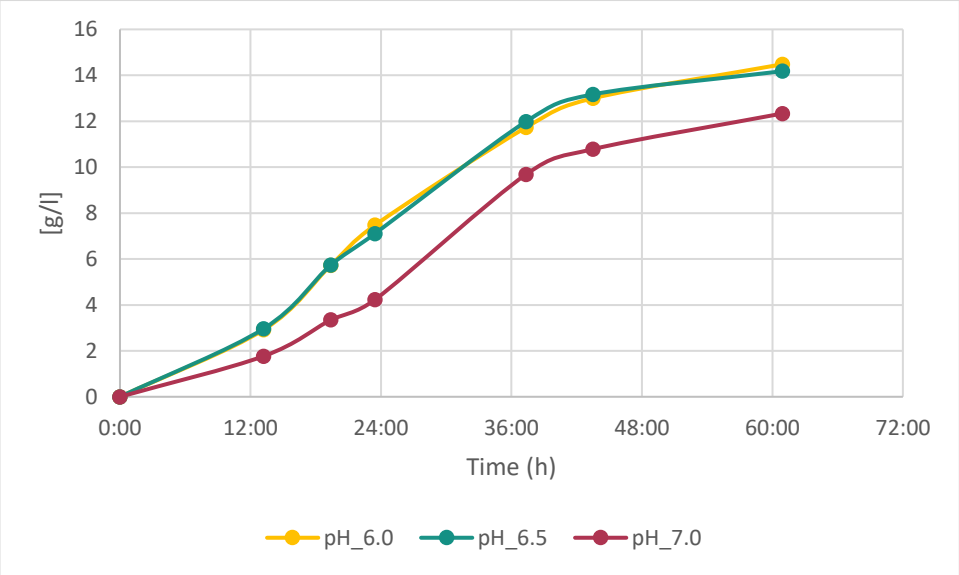



Figure 11. Butyrate production is shown as g/l towards time (h). Duplicate processes are represented as calculated averages for reactors of the same initial media pH- value; 6.0, 6.5, and 7.0. Other conditions: V, 400 ml; T, 30°C, stirring, 150 rpm, anaerobic with $N_2$ gas flow 0.2 lpm.

Figure 11 shows the produced butyrate concentrations for the duplicate fermentations as calculated averages. The highest amounts were observed in reactors where the pH was maintained below 6.5 during the process. The $CO_2$ - curves and the consumptions and productions of glucose, fructose, acetate, and lactate are found in Appendix 5, Figures 1–5. Glucose was consumed by the 37$^{th}$ hour for all reactors. Fructose was consumed by the end of the fermentation for reactors representing pH values 6.0 and 7.0. Acetate maintained higher and quite constant for the reactors representing pH 7.0 whereas for the others it lowered at a more constant rate for approximately 37 hours after which it either maintained the same (reactors pH_6.0) or slightly increased (reactors pH_6.5). According to inoculum dry weight measurements (found in Appendix 6), the initial concentration in the reactors (V= 400 ml) for *M. cerevisiae* was 0.033 g/l and for *P. pentosaceus* 0.037 g/l.

## 7.3 Simulation-based experiments

The simulation was used within this thesis to design experiments in order to obtain butyrate as the dominant fermentation product; hence, test the models' functionality in practice. 'Playing' with the simulation by running it over after changing the values of the parameters, enabled to detect those that favored butyrate production. Among these were i) increasing the initial concentration of *M. cerevisiae* and ii) the addition of either glucose or fructose or both. The reference values for the initial substrate concentrations were obtained from the previous fermentation zero samples (taken right after inoculation, chapter 8.2.2). The concentrations were calculated averages of glucose (23.16 ± 1.14 g/l), fructose (14.18 ± 0.73 g/l) and acetate (10.4 ± 0.48 g/l) (n=6) derived from HPLC measurements. The original values are found in Appendix 5, Table 1. pH was set at 6.5, and reaction time at 60 hours.

For the fermentation experiments, the hydrolysate, the minimal media, and the inoculums were prepared as described in chapters 6.4, 6.5, and 6.6.1, respectively. The operational conditions corresponded to those described in chapter 6.6.3. The fermentation lasted for approximately 60 hours and eight samples including media samples were taken per each reactor.

### 7.3.1 Impact of the increased initial concentration of *M. cerevisiae*

Increasing the initial concentration of *M. cerevisiae* showed only a small increase in butyrate production according to the simulation. As shown in Appendix 3, Listings 1 & 2, multiplying the *M. cerevisiae* concentration by five, only increased the final butyrate production by 0.08 g/l.

The effect of higher initial *M. cerevisiae* concentration was examined in practice by a fermentation experiment with two bioreactors (Biostat CT2) with media volumes of 1500 ml. The first reactor, referred to as **A** or the *reference*, was inoculated with an initial concentration of *M. cerevisiae*, corresponding to OD 0.1 in the reactor. The second reactor **B** was inoculated with five times more *M. cerevisiae* so that the initial reactor OD corresponded to 0.5. The initial *P. pentosaceus* concentrations were equivalent (OD 0.1) for both reactors.

The OD of *M. cerevisiae* inoculum was 3.6; hence, the volumes inoculated into the bio-reactors were 210 ml and 42 ml. According to dry weight measurements, the inoculum concentration of *M. cerevisiae* was 1.3 g/l; therefore, the initial concentrations in the reactors were 0.182 and 0.0364 g/l, respectively. However, for reactor B, the volume increased by the inoculum with ~200 ml; thus, the initial *M. cerevisiae* concentration for reactor B was ~ 0.16 g/l. For *P. pentosaceus,* the inoculum volume was 15.3 ml for both reactors. The concentration of *P. pentosaceus* inoculum was ~ 3.7 g/l; hence, the initial reactor concentrations for 1500 (A) and 1700 (B) ml were 0.037 and 0.033 g/l, respectively.

According to the fermentation carbon dioxide curves (Figure 12), it seems that the growth rate for the reactor with more *M. cerevisiae* has increased quickly, after which it has dropped and the cell mass has stayed stable, whereas the growth rate for the reactor with reference inoculum concentrations, has followed a more expected fermentation pattern.

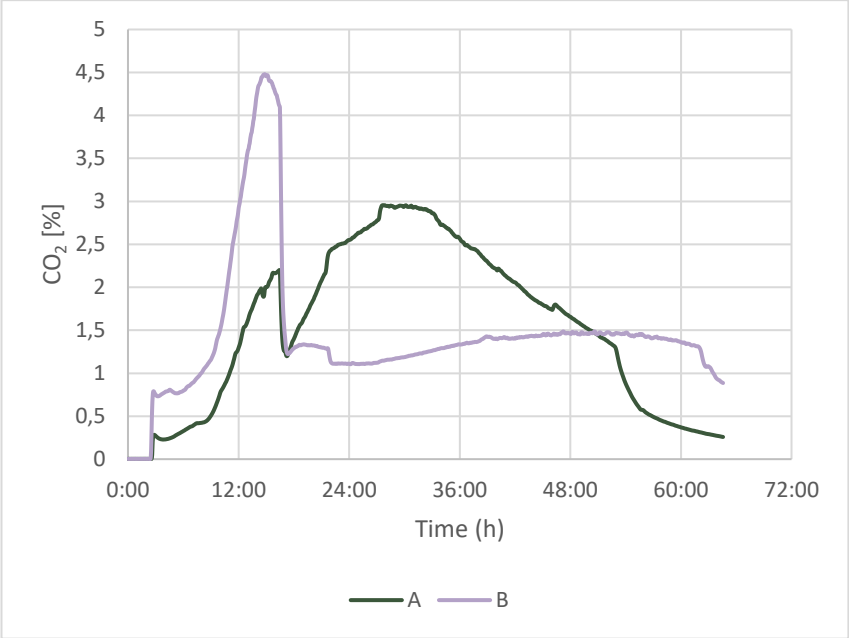



Figure 12. $CO_2$ -data acquired during the fermentation from the outlet gas as molar % against time (h, x-axis) for both reactors. Fermentations, A: reference, c [initial *M. cerevisiae*], 0.036 g/l; B: increased inoculum, c[initial *M. cerevisiae*], 0.16 g/l; c[initial *P. pentosaceus*], 0.037 g/l (A) & 0.033 g/l (B). Operational conditions: V, 1500 ml (A) and 1700 ml (B); T, 30° C; stirring,150 rpm; anaerobic, gas flow ($N_2$) ~0.2 lpm; media pH, 6.5.

Concentrations of involved compounds of interest towards time are shown in Figures 13 a and b. The media glucose and fructose concentrations before inoculation, for reactor A, were 26.3 and 15.5 g/l and for B 27.5 and 16.5 g/l, respectively.

a) b)

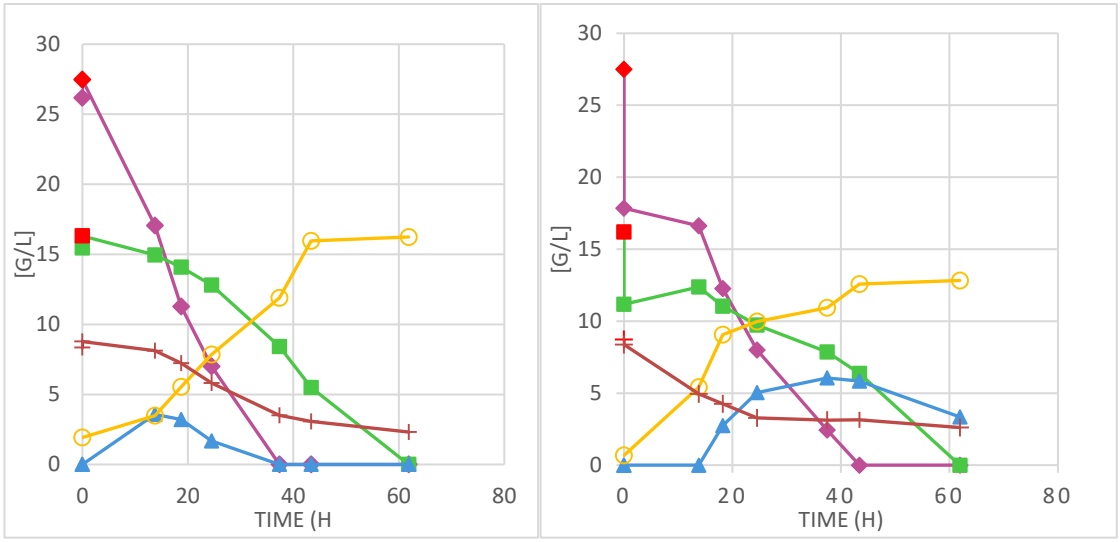



Figure 13. Compound concentrations for fermentations A (a) and B (b) towards time as g/l. A: reference, c [initial *M. cerevisiae*], 0.036 g/l; B: increased inoculum, c(initial *M. cerevisiae*), 0.16 g/l; c(initial *P. pentosaceus*), 0.037 g/l (A) & 0.033 g/l (B). Marker-codes at times of sampling: ▲ lactate, ■ fructose, ◆ glucose, ○ butyrate, + acetate. Operational conditions: V, 1500 ml (A) and 1700 ml (B); T, 30° C; stirring,150 rpm; anaerobic, gas flow ($N_2$) ~0.2 lpm; media pH, 6.5. Media samples are included and are marked red.

More butyrate was produced in reactor A, where glucose was consumed slightly faster compared to reactor B. For both reactors, butyrate production did not increase after 43 hours. As shown in Figure 13, the reactors show similar patterns for almost all compounds, despite the $CO_2$ curve (Figure 12). The most notable difference is observed between lactate concentrations. In reactor A, the lactate production starts from the beginning, is presumably under simultaneous consumption, and possibly finished already after 30 hours. For reactor B the lactate was not observed before ~14 hours, after which its concentration increased up to 6 g/l (~37 h) and was not fully consumed during the 60-hour operation. For reactor B the butyrate curve showed a similar pattern with lactate, only at slightly higher concentrations. In addition to these observations, OD for reactor B maintained lower during the entire process.

The ODs were measured at 630 nm and are shown in Figure 14. Because of the character of the cabbage hydrolysate, the media was not entirely clear and included particles that affected the absorbance. This was considered in the results so that the average media OD was set to 1.0 and was subtracted from the measured sample OD values.

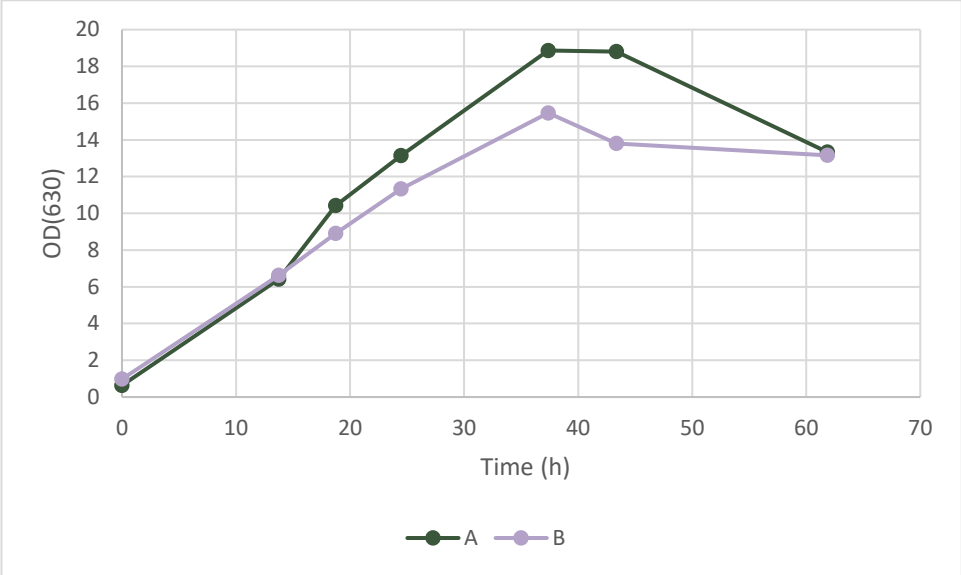



Figure 14. Optical Density (OD) - values measured at wavelength 630 nm for each sample (y-axis), shown against time (h, x-axis). Fermentations: A: reference, c (initial *M. cerevisiae*)= 0.036 g/l ; B: increased inoculum, c(initial *M. cerevisiae*), 0.16 g/l; c(initial *P. pentosaceus*), 0.037 g/l (A) & 0.033 g/l (B). Operational conditions: V, 1500 ml (A) and 1700 ml (B); T, 30° C; stirring,150 rpm; anaerobic, gas flow ($N_2$) ~0.2 lpm; media pH, 6.5.

The distribution of produced organic acids at the end of the fermentation is shown in Figure 15.

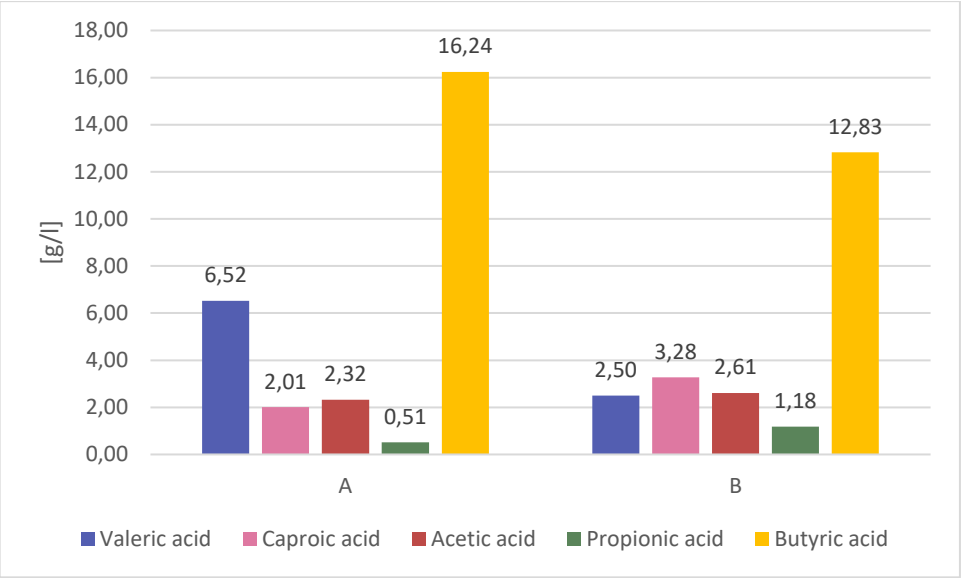



Figure 15. Organic acid compositions at the end of the processes shown as g/l (y-axis). Fermentations A: reference, c [initial *M. cerevisiae*] 0.036 g/l ; B: increased inoculum, c(initial *M. cerevisiae*), 0.16 g/l; c[initial *P. pentosaceus*], 0.037 g/l (A) & 0.033 g/l (B). Operational conditions: V, 1500 ml (A) and 1700 ml (B); T, 30° C; stirring,150 rpm; anaerobic, gas flow ($N_2$) ~0.2 lpm; media pH, 6.5.

Regarding the produced organic acids, approximately 4 g/l more valerate and butyrate were produced in the reference reactor A, whereas propionate and caproate concentrations were slightly higher (~0.7 / 1.3 g/l, respectively). Butyrate was the prevalent fermentation product for both reactors.

The compound concentrations at the end of fermentations A and B according to the simulation (run with initial reactor concentrations) were compared to those obtained by HPLC and CG measurements (Figure 15). Table 5 shows the real initial concentrations of sugars and acetate, measured from samples taken right after inoculation. Reactor A refers to the reference reactor and B to the one with increased *M. cerevisiae* concentration.

Table 5. Compound and bacteria concentrations as simulation input values (g/l) for fermentations A, reference, and B, increased initial *M. cerevisiae*. Values obtained from zero-sample HPLC measurements (glucose, fructose, acetate) and dry weight measurements (bacteria).

| INPUT | | | | | |
|---|---|---|---|---|---|
| | *Glucose* | *Fructose* | *Acetate* | *M. cerevisiae* | *P.pentosaceus* |
| A | 27.46 | 16.31 | 8.78 | 0.036 | 0.037 |
| B | 17.85 | 11.17 | 8.38 | 0.16 | 0.033 |

Table 6 shows the results and compares them with corresponding outputs obtained from the simulation when running it using input values displayed in Table 5.

Table 6. Compound concentrations at the end of fermentations A and B, shown as measured (R_) values and simulation (S_) output values. Compounds: glu, glucose; fru, fructose; ace, acetate; lac, lactate; but, butyrate; val, valerate; prop, propionate; cap, caproate. A: reference, c [initial *M. cerevisiae*], 0.036 g/l; B: increased inoculum, c[initial *M. cerevisiae*], 0.16 g/l; c[initial *P. pentosaceus*], 0.037 g/l (A) & 0.033 g/l (B). Operational conditions: V, 1500 ml (A) and 1700 ml (B); T, 30° C; stirring,150 rpm; anaerobic, gas flow ($N_2$) ~0.2 lpm; media pH, 6.5. Simulation input values are shown in Table 5.

| OUTPUT | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *R_glu* | *S_glu* | *R_fru* | *S_fru* | *R_ace* | *S_ace* | *R_lac* | *S_lac* |
| A | 0 | 0 | 0 | 0 | 2.32 | 4.22 | 0 | 0 |
| B | 0 | 0 | 0 | 0 | 2.61 | 5.22 | 3.34 | 0 |
| | *R_but* | *S_but* | *R_val* | *S_val* | *R_prop* | *S_prop* | *R_cap* | *S_cap* |
| A | 16.24 | 13.80 | 6.50 | 5.48 | 0.50 | 5.76 | 2.00 | 0.37 |
| B | 12.82 | 9.66 | 2.50 | 2.87 | 1.17 | 4.22 | 3.30 | 0.27 |

For both reactors, more butyrate and caproate was produced than what the simulation would have predicted whereas acetate and propionate concentrations were lower than according to the simulation. Final bacteria concentrations could not be measured, but according to the simulation, they were 1.783 and 3.145 g/l for A, and 1.358 and 2.019 for B, for *M. cerevisiae* and *P. pentosaceus,* respectively.

### 7.3.2 Impact of additional glucose, fructose, and acetate

According to the simulation, increased sugar concentrations in the media, correlate positively to the butyrate concentration at the end of the fermentation. Simulation inputs and outputs are found in Appendix 3, Listings 3–6. The input initial inoculum concentrations correspond to the default values described in chapter 6.3. Additionally, it was observed that increasing the initial acetate concentration along with the sugar concentrations, favored the butyrate production in particular whereas with less acetate, the concentrations of other VFAs were higher (Appendix 3, listings 3–4 compared to listings 5–6). Thus, fermentation experiments (C-H) with six in-line table reactors (Multifors) with following substrate additions were conducted.

- C & D: 10 g/l fructose addition, 15 g/l acetate in the media, duplicate.
- E & F: 10 g/l glucose addition, 15 g/l acetate in the media, duplicate
- G: 10 g/l fructose addition, 10 g/l acetate (reference) in the medium.
- H: 10 g/l glucose addition, 10 g/l acetate (reference) in the medium.

The inoculate volumes were calculated to correspond with OD 0.1. According to dry weight measurements, inoculum concentrations were 1.2 g/l for *M.* cerevisiae and 3.83 g/l for *P. pentosaceus*; hence, the initial reactor (V, 400 ml) concentrations for the strains were 0.0248 and 0.0345 g/l, respectively. The fermentation $CO_{2}$- curves are shown in Figure 16.

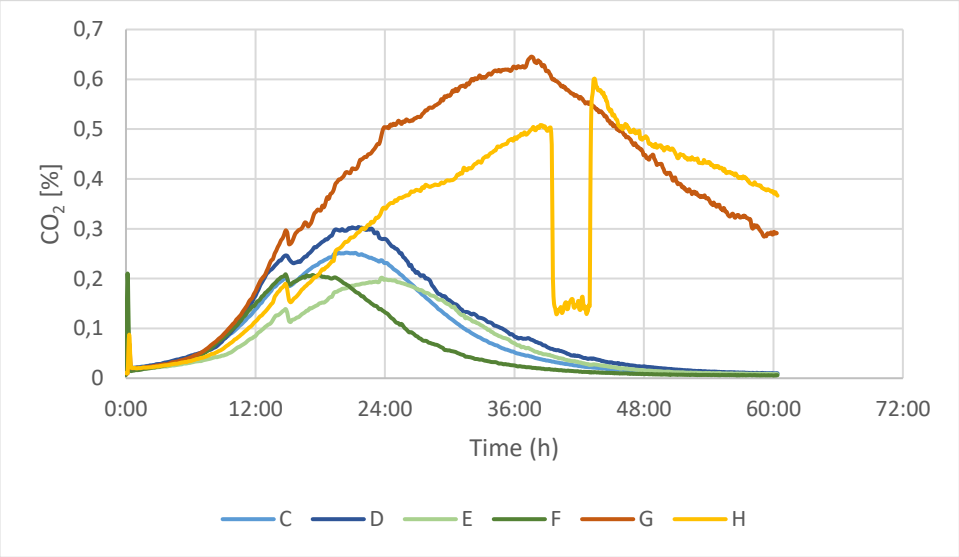
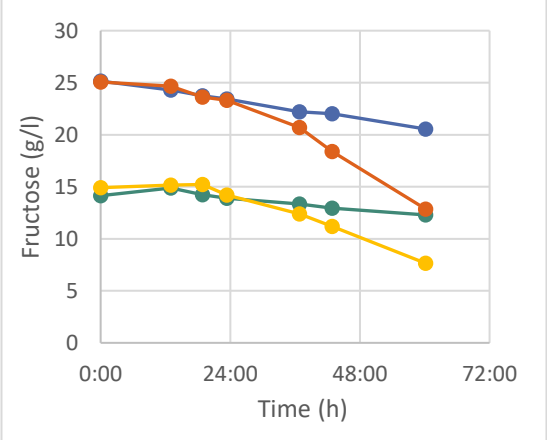


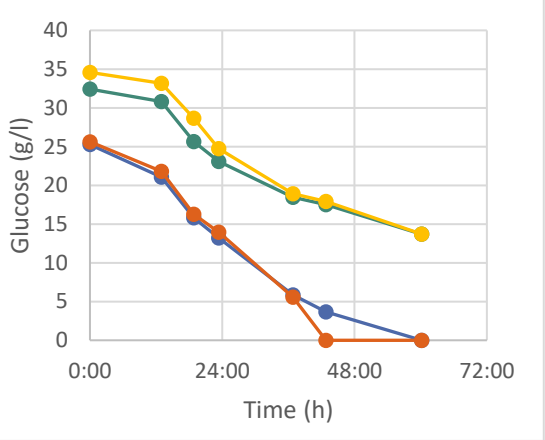
Figure 16. $CO_2$ - data as a molar percentage, acquired continuously from reactor outlet gases, towards time (h) (x-axis). Fermentations: C (light blue) & D (dark blue), 10 g/l of added fructose and 15 g/l acetate; E (light green) & F (dark green), 10 g/l of added glucose and 15 g/l of acetate; G (orange) 10 g/l added fructose and 10 g/l acetate; H (yellow), 10 g/l added glucose and 10 g/l acetate. The drop in the curve H is due to stopped stirring at ~39.3 hours and raised back as it was put back on. Cabbage hydrolysate was used in all media. Inoculum: initial c[*M.* cerevisiae], 0.0248 g/l and c[*P. pentosaceus*], 0.0345 g/l. Other fermentation parameters: V (reactor), 400 ml; anaerobic, $N_2$ inlet flow 0.2 lpm; T, 30° C; stirring, 150 rpm; media pH 6.5.

The $CO_2$ -curves for reactors C and D can be observed to reach slightly higher than those for E and F, whereas reactors G and H show a notably different pattern compared to the other reactors. Comparing the curves with the $CO_2$-curves obtained from previous experiments (Appendix 5, Figure 1) conducted with the same reactors (Multifors), it can be seen that the curves for experiments C-F maintain lower than was expected by these data. Glucose and fructose consumptions and acetate and lactate concentrations during each fermentation are illustrated in Figures and 17 a-d.

a)




b)




c)

d)

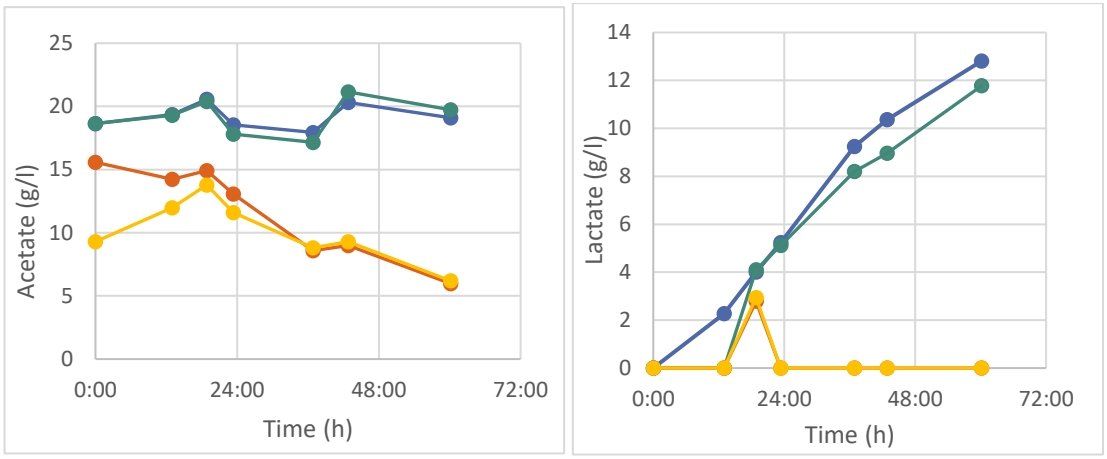



Figure 17. Fructose (a), glucose (b), acetate (c) and lactate (d) concentrations (g/l) measured for each sample by HPLC and GC (acetate), demonstrated towards reaction time (h). Fermentations: Duplicates, C & D (**blue,** added fructose 10 g/l, initial acetate 15 g/l) and E & F (**green,** added glucose 10 g/l, initial acetate 15 g/l) are represented as calculated average; G, **orange**, 10 g/l added fructose and 10 g/l acetate and H, **yellow**, 10 g/l added glucose and 10 g/l acetate. Cabbage hydrolysate was used in all media. Inoculum: initial c(*M.* cerevisiae), 0.0248 g/l and c(*P. pentosaceus*), 0.0345 g/l. Other fermentation parameters: V (reactor), 400 ml; anaerobic, $N_2$ inlet flow 0.2 lpm; T, 30° C; stirring, 150 rpm; media pH 6.5.

Glucose was fully consumed in the reactors where it had not been added into the media. Fructose was not entirely consumed in any reactor and its consumption was especially poor in processes where acetate was also added. Initial acetate concentrations obtained from GC measurements show high concentrations of acetate in processes C-F. Lactate concentration increases in processes C-F, whereas in G and H, it is almost absent.

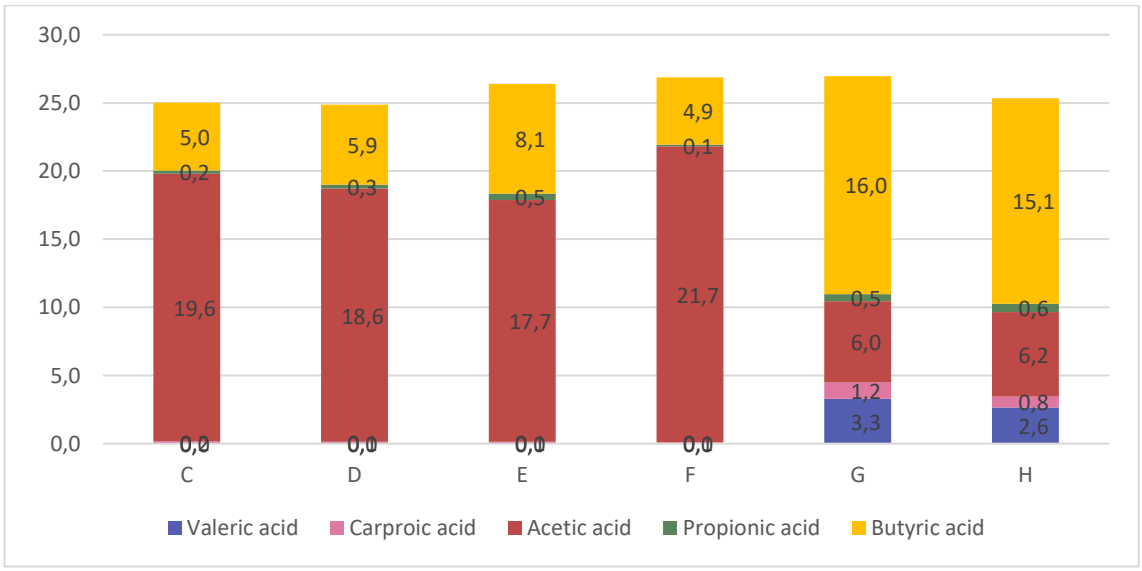


Figure 18. Distribution among produced organic acids shown as concentrations (g/l) for each process. Fermentations: C & D, 10 g/l of added fructose and 15 g/l acetate; E & F, 10 g/l of added glucose and 15 g/l of acetate; G, 10 g/l added fructose and 10 g/l acetate; H, 10 g/l added glucose and 10 g/l acetate. Inoculum: initial c[*M.* cerevisiae], 0.0248 g/l and c[*P. pentosaceus*], 0.0345 g/l. Other fermentation parameters: V (reactor), 400 ml; anaerobic, $N_2$ inlet flow 0.2 lpm; T, 30° C; stirring, 150 rpm; media pH 6.

The organic acids produced in each fermentation are shown in Figure 17. Acetate was the dominant acid in reactors C-F, whereas valerate was not present. Butyric acid was the dominant product in reactors G and H, where also 3.3 and 2.6 g/l of valerate, respectively, and small amounts of caproate were produced. Propionate was present in small amounts in all reactors. Fermentations C-F where interpreted as at least partially inhibited.

# 8 Discussion

This thesis was made as a part of the BioChem project funded by the Academy of Finland. The project aims to produce valuable organic acids from food industry side streams via microbial fermentation. The objective of this thesis was to 1) develop a cost-efficient pre-processing method on provided food waste to release monomeric sugars for fermentation and 2) to investigate and optimize the fermentation process and included parameters by using a simulation that is in development within the BioChem project. The experimental part of this thesis included various hydrolysis and fermentation experiments of which many were based on exploiting mathematical modeling.

The enzymatic hydrolysis was started by investigating the different cabbage residue parts, *leaf*, *mass* and *stem*, obtained for this project from Vaissi Oy (Finland). The desired result was a liquefied hydrolysate within a reasonable time and with at least the reference concentrations (20.2 g/l for glucose and 16.6 g/l for fructose) of monomeric sugars that were obtained from previous measurements by acid hydrolysis at VTT. Due to released sugars from HPLC measurements (Appendix 4), only the *mass* parts were included in the process. The leaf, however, showed potential considering the released sugars and liquefaction and could be further investigated in the future.

The problem with the first hydrolysis experiment was that the samples were not liquefied and the highest concentrations of glucose and fructose obtained for the mass cabbage

were 13.9 and 11.7 g/l, respectively. The further experiments, including investigations with different enzyme products (Ctec2 and Htec2), with and without buffer and with and without mechanical preprocessing led to following conclusions: i) Htec2 does not have a promoting effect on cabbage hydrolysis as it neither contributed the increase in the concentration of released sugars, nor improved the liquidity of the hydrolysate. ii) Mechanical pretreatment supports the hydrolysis, which is most probably due to broader surface area for the enzymes to access. iii) Buffer is not needed to maintain the pH as it only dilutes the hydrolysate sugar concentrations. The enzymes activity was not observed decreased in an environment where the pH-value was below their optimum. iv) The higher the Ctec2 dosage was, the higher glucose and fructose concentrations were measured for the hydrolysates. However, Ctec2 alone did not seem to be enough to liquefy the food waste so that it could be used for fermentation. The liquidity is important because solid particles of cabbage disturb the downstream processing.

The Design of Experiment software MODDE® was used to plan the experimental set up for one hydrolysis experiment. The idea was to examine the effect of temperature, enzyme dosages of Ctec2 and Pectinex®, and incubation time along with their interactions on the released monomers and state change during the hydrolysis. Temperature (35, 45, 55 °C) did not seem to have much impact, although it was observed that the samples incubated at 55 °C with Pectinex® seemed more clear compared to those at 35 °C. This might be due to the temperature that was below the optimal (55°C) determined for Pectinex®, and as of the characteristics of pectinases is to clarify the hydrolysate [36]. However, considering this project, the hydrolysate does not have to be clear. According to the HPLC measurements, the increased concentration of Ctec2 resulted in significantly high glucose and fructose concentrations. The used maximum dosage of Ctec2 was 14 % (weight /cabbage dry matter weight, w/w) which is an exceedingly high dosage of an enzyme, and should not be used in a process that aims for cost-efficiency. However, even the highest dosages of Ctec2 did not lead to a quickly liquefied result.

It was noted that Pectinex® was a key factor to obtain a completely liquefied hydrolysate that could be used in the fermentation media. Without better knowledge, the smallest *time* factor value was 24 hours, although a much shorter time could have been sufficient, as was proven in the fermentation media preparations: the cabbage was hydrolyzed within an hour with 1 % (w/w) of Pectinex® included. The results from the DoE screening

experiment (hydrolysis Experiment 5) were analyzed with MODDE® software, but scientific significance was not obtained and further experiments were requested. Because of time limitations, the process was not completely optimized and the DoE process (*screening-optimization-robustness testing*) was not completed. For future purposes, Pectinex® should be tried for smaller dosages to determine the minimum with which the hydrolysis with respect to liquidity, could be maintained a fast part of the process. Such a DoE experiment was planned, but not conducted within this thesis. The dosage of Ctec2 could also be optimized. In the media preparations the used Ctec2 dosage was 6 % (w/w).

As for the fermentation media preparations, the cabbage hydrolysate was centrifuged to obtain a clearer media to contribute the analysis. However, for scale-up, and as the process is optimized, this part could be left out as unnecessary. It is possible for the hydrolysis to continue during fermentation so that more sugars would be released. This of course, requires enzymes activity. Even though the cabbage was centrifuged and sieved, the sample ODs were difficult to calculate with reliable results; the media was left with cabbage particles and possibly denatured enzymes. The cell dry-weight determinations could only be done for inoculums due to the same reason.

As for all the hydrolysis experiments, due to the non-sterile food waste, contamination occurred quite frequently and many single experiments had to be excluded. This was especially the case for the first experiments where the retention times were longer (< 24 h). The contaminants were not identified, but HPLC data of such samples showed increased peaks for acetate and galactose whereas glucose and fructose were already consumed within short retention times. However, it would be of importance to maintain the retention time as short as possible to avoid contamination. The liquidity was quite difficult to evaluate consistently; thus, the results cannot be considered scientifically relevant. However, in order to exploit the cabbage in the fermentation experiments and considering the project in future, it had to be investigated. The process could be developed further by applying it with an alkaline pretreatment step. It has been studied that pretreating food wastes, especially those including lignin cellulose structures, with NaOH, for instance, results in reduced crystallinity in the hydrolysate, i.e. less solid particles [38].

The effect of pH was examined in a fermentation experiment where the process was conducted at three pH-values of 6.0, 6.5 and 7.0. However, from the very beginning, the pH was not maintained as desired. There are several possible reasons for this: i) the pH meters were not all in condition or the calibration did not work, as it should have. ii) As a result of the former, the adjustment before starting the process, did not go as planned. The media pH was adjusted before autoclave, where it dropped; thus, it had to be adjusted again. Then the pH was tested and the set-points for each reactor were defined according to the offline measurements. iii) The acidification results in the production of several different acids that lower the pH continuously during the process so that it is hard to control. This was, furthermore observed in later experiments, where the equipment was not to blame. The pH often set at its set-point value after the exponential state of the process. The problem here was that the set-point had often been adjusted so that the offline-pH would reach the desired value, and might have been higher than the desired value. Thus, at the end of the process, pH might have increased above the planned value. The results considering the influence of pH, are not consistent, and larger conclusions cannot be made. However, the reactors (6.0 & 6.5) where the pH maintained lower than 6.5 during the process (Table 4 in chapter 7.2.2 ) produced more butyrate than the two reactors where the pH was set to 7.0 and maintained slightly higher than in the rest (Figure 11 in chapter 7.2.2).

Promising conditions favoring butyrate production and to conduct fermentations with, were explored by running the MATLAB® simulation. The bioreactor experiments where the effect of increased initial *M. cerevisiae* was studied are referred to as A (reference, initial *M. cereivisiae* OD corresponding to 0.1) and B (increased *M. cerevisiae,* initial OD corresponding to 0.5). The experiments where the additions of glucose, fructose, and acetate were examined are referred to as experiments C & D (10 g/l added fructose, 15 g/l acetate), E& F (10 g/l added glucose, 15 g/l acetate), G (10 g/l added fructose, 10 g/l acetate) and H (10 g/l added glucose and 10 g/l acetate).

The effect of increased *M. cerevisiae* concentration; hence, different initial bacterium strain ratio was examined out of interest and based on the fact that *M. cerevisiae* is the butyrate-producing microbe. The simulation showed an only weak increase in the butyrate production (0.08 g/l, Appendix 3, listings 1–2), comparing to the previously used initial *M. cerevisiae* concentration. Nevertheless, in the case where increase was to occur, it

would be a simple way to promote butyrate production. However, the practice turned out problematic because concentrating the inoculum in the anaerobic chamber was not possible and the inoculum volume was proportional to the amount of *M. cerevisiae* that was inoculated in the reactor. This resulted in a diluted media, which affected the process so that the initial reactor conditions were not comparable as they were designed. According to inoculum dry-weight measurements the initial concentrations for *M. cerevisiae* and *P. pentosaceus* for reactor A were 0.036 g/l and 0.037 g/l and for b 0.16 g/l and 0.033 g/l. Thus, there were ~4.4 times more *M. cerevisiae* in reactor B at the beginning of the fermentation. Nevertheless, neither were the substrate consumption rates nor the concentrations of the end products higher for reactor B. It is highly probable that with proportionally equal amounts of fermentative sugars, reactor B would have produced more butyrate and other organic acids.

The results were compared to corresponding output values obtained from the simulation (Table 6 in chapter 7.3.1) by running it using the initial compound concentrations measured from the samples right after inoculations, as input values. The comparison showed similar patterns but also systematic differences between the results. Both butyrate and caproate were produced more than ought to according to the simulation. This might be partly compensated by acetate and propionate concentrations that were lower than expected by the simulation. For the reference reactor A, propionate, and acetate might have elongated into valerate in the presence of lactate, since its final concentration was 1 g/l higher than according to the simulation, and covered approximately 24 % of produced organic acids. Caproate is generated from lactate and butyrate and fructose and butyrate by *M. cerevisiae*. These results confirm that for reactor A, *P. pentosaceus* has actively produced lactate that has simultaneously been consumed. For reactor B, valerate and propionate concentrations are relatively more in accordance with the simulation outputs compared to reactor A. Observable is also that all lactate was not consumed, which is usually not the case. Additionally, the differences might be due to the cabbage hydrolysate, which may contain more nutrients and substrates that have been determined for the simulation.

The simulation gave promising outputs of sugar additions (Appendix 3, listings 3–6) promoting butyrate production. The addition of pure sugars had not been tried so far, possibly because of the objective of the BioChem project, where food waste is aimed to be

exploited as such. However, as *food waste* may correspond to a countless amount of different wastes, among them are those that consist of more sugars. Rosaliina Turunen (VTT) has previously proved the butyrate promoting effect of acetate addition which has furthermore support from the literature.

For all four reactors (C-F) with both increased sugar and acetate concentrations, the results indicate that *M. cerevisiae* was highly inhibited and that *P.pentosaceus* might have been partially inhibited. The inhibition of *M. cerevisiae* is well supported by the results: First, the consumption of fructose, the primary substrate of *M. cerevisiae,* was almost non-existent. Second, even though lactate was present, the production of propionate was very weak (< 0.5 g/l), which explains the absence of valerate that is only produced by lactate and propionate elongation. Usually, lactate has been consumed by *M. cerevisiae* as it has been produced by *P. pentosaceus*, but according to lactate data, shown in chapter 7.3.2, Figure 17d, its production was more active than its consumption and the end concentrations of lactate reached up to around 13 g/l. On the other hand, glucose was consumed much slower than in previous experiments, which indicates that *P. pentosaceus* might have been partially inhibited. Acetate was the dominant acid for reactors C-H, which indicates that acetate might have been the inhibitor. Small amounts of butyrate, however, were produced, presumably from lactate, which means that some of *M. cerevisiae* maintained active. Thus, further investigations about optimal initial sugar and acetate concentrations as well as their ratios should be conducted.

The two fermentations, where initial acetate concentration was 10 g/l (E & F), were contaminated. The reason they were included in this report was, that the distribution among the produced organic acids was comparable to previous fermentations, which brings another perspective to this work. The contaminant was never neither identified nor tracked but was justified by several matters. First, the only lactate data were obtained at 19 hours (approx. 2 g/l per reactor), which indicates that the contaminant either used lactate somehow or overruled or inhibited *P. pentosaceus*. On the other hand, according to the HPLC data, the lactate UV-channel values are not in correspondence with those observed in the RI-channel. Lactate among other organic acids should be observable at both. This, indicates that even the small peaks observed from the 19$^{th}$ hour samples, were possibly something else but lactate, just with similar retention times. Normally lactate is first produced by *P. pentosaceus*, after which *M. cerevisiae* ferments it into butyrate and other

VFAs. Hence, the contaminant must have either produced lactate or the same organic acids as *M. cerevisiae*, since valerate and propionate, for instance, are not produced without lactate. Another strange thing is that glucose and fructose consumptions did not significantly differ from previous fermentations by the co-culture. However, the $CO_2$ and OD data show that there was growth. Also, the OD measurements show that the initial OD for these reactors was lower than in the other reactors even though the inoculum volumes were the same for all reactors. Lastly, the reactors were determined contaminated by the smell that stood out from the process' characteristic butyrate-cabbage odor as lacquer-like pungent smell. If such, possible butyrate-producing contaminant was detected in the future, it could be tried to isolate and identify for further experiments.

Mathematical models enable a more systematic approach to optimization experiments when many factors and their interactions can be investigated at once. To determine the minimum dosage of needed enzymes without a decrease in the release of monomeric sugars, the Design of Experiments would offer a time-efficient way. Concerning the simulation, it provides an out-withstanding base with limitless amounts of opportunities to optimize and develop the process by. It also enables to adjust the process to promote other volatile fatty acids generated by *M. cerevisiae*, valeric acid for one. The simulation has already been modified and developed within the same project to model a semi-continuous process, estimating the effect of pulsing. Unfortunately, there was not enough time to get into that within this thesis, but for future purposes and considering a biorefinery, the possibilities for a semi-continuous process should be investigated. Separating butyrate by electrodialysis was successfully conducted last fall, by BioChem project partner Ludwig Selder, in the *Hamburg University of Technology*.

# 9 Conclusions

This thesis was a part of the BioChem project, funded by the Academy of Finland, which aims to develop a biorefinery to exploit food waste in the production of value-added chemicals via microbial fermentation. This study focused on two main aspects within the project in which both, mathematical-based modeling was involved. First, a preprocessing step for the project was developed. The provided food waste was white cabbage. The

preprocessing should consist of a cost- and time-efficient procedure, where complex molecules in the food waste would be degraded into monomeric compounds such glucose and fructose, for the microbes to ferment, also resulting in a liquefied hydrolysate. Second, by conducting literature research and by using a simulation modeling the process, fermentation experiments were conducted to gain information about the process and its parameters by aiming to favor butyrate production.

It was shown in this study that the design of experiments shows potential in optimization processes where the effects of many factors at different values must be examined and their interactions detected. Enzymatic hydrolysis using cellulases, hemicellulases, and pectinases works as a preprocessing step for white cabbage. With dosages, 6 %(w/w) and 1 % (w/w) of cellulases and pectinases, respectively, at a temperature of 45–55 °C, with stirring at 200 rpm, a liquefied hydrolysate, including a sufficient amount of released sugars to use in fermentation media, was obtained within an hour. Smaller dosages should be experimented with to optimize the step, resulting in an even more cost-efficient result. The amount of cellulase affected the final concentrations of monomeric sugars in the hydrolysate and pectinase affected the state of matter.

The kinetics and metabolic networks that have been determined for the modeling of the process, provide an important platform for understanding and estimating the reactions that occur during the fermentation. This enables the planning and optimizing and even modifying the process on another level. The simulation operated as such a tool for this study. Increasing the initial amount of the butyrate-producing microbe, showed promising results. However, for future experiments, the *M. cerevisiae* inoculum should be concentrated to corresponding volumes with the reference to create completely comparable processes and initial sugar concentrations should be adjusted for the best results. This would be a simple and cost-efficient way to promote butyrate production in the process. The cabbage hydrolysate seemed to provide an excellent media for the microbes to ferment; the highest amount of butyrate obtained was 16.4 g/l, covering 59 % of produced organic acids. Relatively high amounts of valerate were produced by the same experiment, which, according to the simulation, could be operated by the initial acetate concentration; acetate addition in the media favors butyrate production.

When the impact of increased initial acetate, glucose and fructose concentrations was studied, *M. cerevisiae* ended up highly inhibited and acetate was the prevalent organic acid throughout the entire fermentation. Thus, the tolerance for acetic acid as well as for other substrates such as lactic acid should be determined for *M. cerevisiae*. The importance of determining the final bacterium strain ratio in the co-culture fermentations was highlighted by this study. Further experiments are needed to optimize the process by determining optimal ratios between initial compound concentrations. By this, the highest possible yields of desired products, with respect to the objectives of the BioChem project, could be achieved. These results could be used with the simulation including the added aspect of *pulsing,* to develop the project even further towards a scale-up of a functioning semi-continuous biorefinery system.

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Appendix 1**. Metabolic Network of the Catabolism**

| | Gluc | Fruc | Lactate | Acetate | Propionate | Butyrate | Valerate | Caproate | Rate (mol·L$^{-1}$·d$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| **Glucose to lactate** | -1 | | 1 | | | | | | $q_{Ped\ 1,max} \cdot \frac{[Glu]}{K_{S,su} + [Glu]} \cdot X_{ped} \cdot I_{VFA}$ |
| **Fructose to lactate** | | -1 | 1 | | | | | | $q_{Ped\ 2,max} \cdot \frac{[Fruc]}{K_{S,su} + [Fruc]} \cdot X_{ped} \cdot I_{glucose} \cdot I_{VFA}$ |
| **Fructose to acetate and butyrate (EB)** | | -1 | | 2/3 | | 2/3 | | | $q_{Mega\ 1,max} \cdot \frac{[Fruc]}{K_{S,su} + [Fruc]} \cdot X_{mega} \cdot I_{VFA}$ |
| **Fructose to butyrate** | | -1 | | | | 1 | | | $q_{Mega\ 2,max} \cdot \frac{[Fruc]}{K_{S,su} + [Fruc]} \cdot X_{mega} \cdot I_{VFA}$ |
| **Lactate to butyrate** | | | -1 | | | 0.5 | | | $q_{Mega\ 3,max} \cdot \frac{[Lac]}{K_{S,Lac} + [Lac]} \cdot X_{mega} \cdot I_{VFA}$ |
| **Lactate and acetate to butyrate** | | | -1 | -1 | | 1 | | | $q_{Mega\ 4,max} \cdot \frac{[Lac]}{K_{S,Lac} + [Lac]} \cdot \frac{[Ac]}{K_{S,Ac} + [Ac]} \cdot X_{mega} \cdot I_{VFA}$ |
| **Lactate to acetate and propionate (Ac+Pro)** | | | -1 | 1/3 | 2/3 | | | | $q_{Mega\ 5,max} \cdot \frac{[Lac]}{K_{S,Lac} + [Lac]} \cdot X_{mega} \cdot I_{VFA}$ |
| **Lactate and propionate to valerate** | | | -1 | | -1 | | 1 | | $q_{Mega\ 6,max} \cdot \frac{[Lac]}{K_{S,Lac} + [Lac]} \cdot \frac{[Pro]}{K_{S,Pro} + [Pro]} \cdot X_{mega} \cdot I_{VFA}$ |
| **Lactate and butyrate to caproate** | | | -1 | | | -1 | | 1 | $q_{Mega\ 7,max} \cdot \frac{[Lac]}{K_{S,Lac} + [Lac]} \cdot \frac{[But]}{K_{S,But} + [But]} \cdot X_{mega} \cdot I_{VFA}$ |
| **Fructose and butyrate to caproate** | | -1 | | | | -2 | | 2 | $q_{Mega\ 8,max} \cdot \frac{[Lac]}{K_{S,Lac} + [Lac]} \cdot \frac{[But]}{K_{S,But} + [But]} \cdot X_{mega} \cdot I_{VFA}$ |

# Appendix 2. **Minimal Medium and RCM Recipes**

**The Minimal Medium**

| *Compound* | *Concentration* | *Unit* |
|---|---|---|
| L-cysteine hydrochloride | 0.5 | g L-1 |
| vitamin solution * | 1 | mL L-1 |
| mineral solution** | 80 | mL L-1 |
| yeast extract | 0.6 | g L-1 |
| | | |
| Glucose and fructose from cabbage hydrolysate | | |
| | | |
| * The vitamin solution contained: | | |
| biotin | 0.5 | mg L-1 |
| pyridoxine | 20 | mg L-1 |
| calcium pantothenate | 20 | mg L-1 |
| | | |
| ** The mineral solution contained: | | |
| CaCl2 | 0.125 | g L-1 |
| MgSO4·7H2O, | 0.125 | g L-1 |
| K2HPO4 | 1 | g L-1 |
| KH2PO4 | 1 | g L-1 |
| NaHCO3 | 5 | g L-1 |
| NaCl | 2 | g L-1 |
| (NH4)2SO4 | 2.5 | g L-1 |
| MnSO4·H2O | 0.05 | g L-1 |
| FeSO4·7H2O | 0.05 | g L-1 |
| ZnSO4·7H2O | 0.05 | g L-1 |
| CoSO4·6H2O | 0.005 | g L-1 |

**Reinforced Clostridia Medium (RCM) (pH 6.8 ± 0.2)**

| *Compound* | *Concentration* | *Unit* |
|---|---|---|
| yeast extract | 13.0 | g L-1 |
| peptone | 10.0 | g L-1 |
| glucose | 5.0 | g L-1 |
| soluble starch | 1.0 | g L-1 |
| sodium chloride | 5.0 | g L-1 |
| sodium acetate | 3.0 | g L-1 |
| cysteine hydrochloride | 0.5 | g L-1 |
| agar | 0.5 | g L-1 |

# Appendix 3. **MATLAB© Simulation Inputs and Outputs Regarding Sugar and Acetate Addition Experiments**

a) input

```
%% INPUTS OF THE MODEL

% Set reactor parameters
V = 1.5;                      % Volume of the reactor (L)
pH = 6.5;                    % Reactor pH

% Set the initial concentration in the reactor

C_ini(1) = 23.16;               % Glucose initial concentration (g/L)
C_ini(2) =14.18;               % Fructose initial concentration (g/L)
C_ini(3) = 10.4;              % Acetate initial concentration (g/L)
C_ini(4) = 0;                % Propionate initial concentration (g/L)
C_ini(5) = 0.117;             % Sugar degrader initial concentration (g/L)
C_ini(6) = 0.043;             % Lactate degrader initial concentration (g/L)

% Set the simulation time
t = 60;                     % Reaction time (h)
```

b) output

```
    'Glucose'        'Fructose'      'Lactate'
    [4.7109e-249]    [2.7257e-18]    [5.6527e-18]

  Columns 4 through 7

    'Acetate'    'Propionate'    'Butyrate'    'Valerate'
    [ 5.8046]    [    5.0866]    [ 12.6162]    [  4.2258]

  Columns 8 through 10

    'Caproate'    'Pediococcus'    'Megasphaera'
    [  0.2777]    [     2.7927]    [     1.5186]
```

Listing 1. The simulation input (a) and output (b) with reference amount of *M. cerevisiae*, so that the initial OD in the reactor corresponds to 0.1.

a) input

```
%% INPUTS OF THE MODEL

% Set reactor parameters
V = 1.5;                      % Volume of the reactor (L)
pH = 6.5;                    % Reactor pH

% Set the initial concentration in the reactor

C_ini(1) = 23.16;               % Glucose initial concentration (g/L)
C_ini(2) = 14.18;              % Fructose initial concentration (g/L)
C_ini(3) = 10.4;              % Acetate initial concentration (g/L)
C_ini(4) = 0;                % Propionate initial concentration (g/L)
C_ini(5) = 0.117;             % Sugar degrader initial concentration (g/L)
C_ini(6) = 0.215;             % Lactate degrader initial concentration (g/L)

% Set the simulation time
t = 60;                     % Reaction time (h)
```

b) output

```
  The concentration of the compounds at the end of the batch are (g/L):
Columns 1 through 3

  'Glucose'        'Fructose'      'Lactate'
  [1.4983e-242]    [1.7978e-24]    [2.3103e-24]

Columns 4 through 7

  'Acetate'    'Propionate'    'Butyrate'    'Valerate'
  [ 5.9301]    [    4.9623]    [ 12.6963]    [  4.0145]

Columns 8 through 10

  'Caproate'    'Pediococcus'    'Megasphaera'
  [  0.3685]    [     2.7004]    [     1.7435]
```

Listing 2. The simulation input (a) and output (b) values with increased amount of *M. cerevisiae,* so that the initial OD in the reactor (V=1500) corresponds to 0.5.

a) input

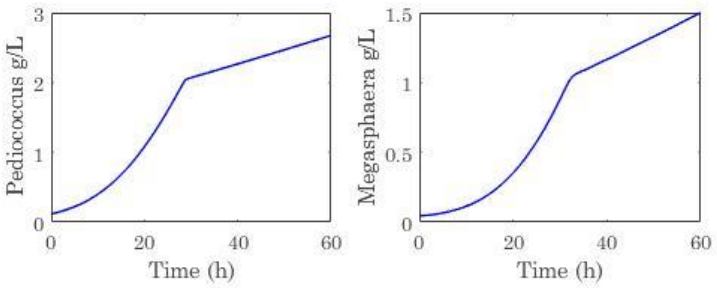
```
%% INPUTS OF THE MODEL

% Set reactor parameters
V = 1;                       % Volume of the reactor (L)
pH = 6.5;                    % Reactor pH

% Set the initial concentration in the reactor

C_ini(1) = 23.16;               % Glucose initial concentration (g/L)
C_ini(2) = 24.18;              % Fructose initial concentration (g/L)
C_ini(3) = 15;               % Acetate initial concentration (g/L)
C_ini(4) = 0;                % Propionate initial concentration (g/L)
C_ini(5) = 0.117;             % Sugar degrader initial concentration (g/L)
C_ini(6) = 0.043;             % Lactate degrader initial concentration (g/L)
```

```
% Set the simulation time
t = 60;                    % Reaction time (h)

% Select graphs at the end of the simulation
flag_graph = 1;            % 1= graphs are activated, 0= graphs are deac-
tivated
```

b) output

```
The concentration of the compounds at the end of the batch are (g/L):
  Columns 1 through 4

    'Glucose'         'Fructose'     'Lactate'     'Acetate'
    [1.3907e-197]     [  8.7374]     [ 0.0516]     [ 9.3723]

  Columns 5 through 8

    'Propionate'     'Butyrate'     'Valerate'     'Caproate'
    [    4.9642]     [ 14.0942]     [  4.0176]     [  0.3313]

  Columns 9 through 10

    'Pediococcus'     'Megasphaera'
    [     2.8585]     [     1.5813]
```

Listing 3. The simulation input (a) and output (b) values with addition of 10g/l fructose to reference concentration of 14.18 g/l. Initial media acetate concentration of 15 g/l.



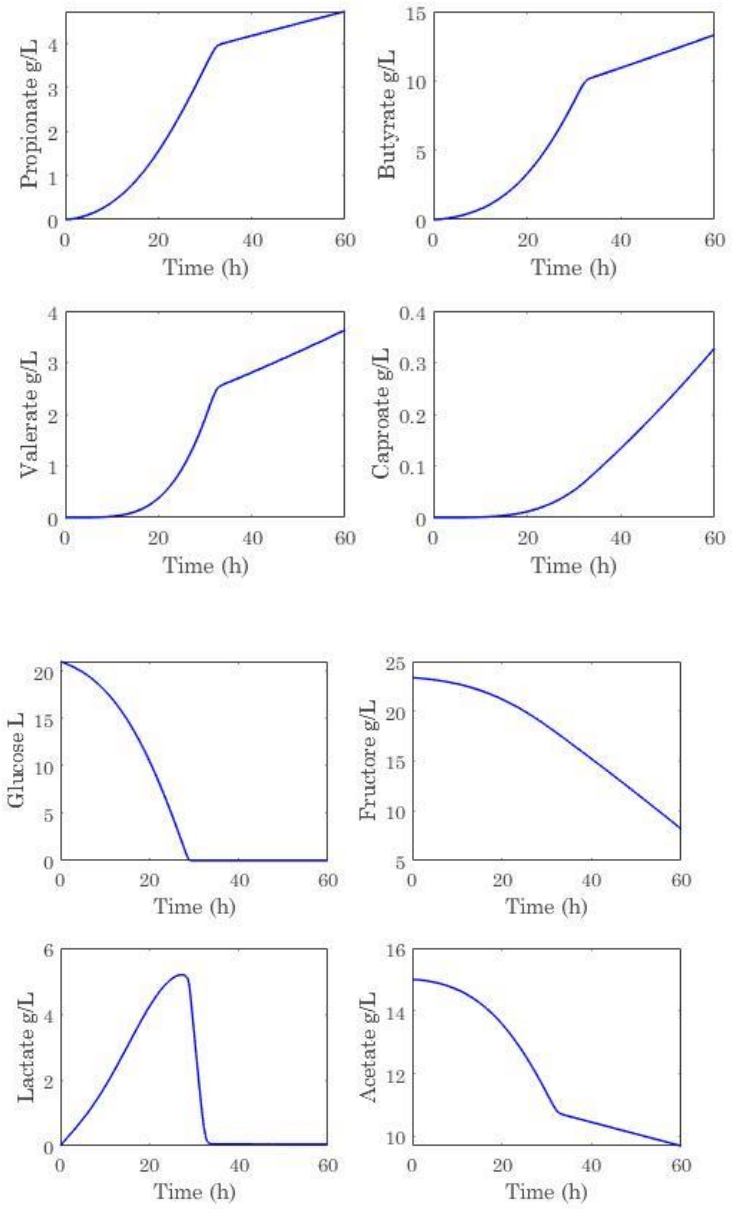



Figure 1. Examples of simulation output graphs.

a) input

```
% Set reactor parameters
V = 1;                      % Volume of the reactor (L)
pH = 6.5;                   % Reactor pH

% Set the initial concentration in the reactor

C_ini(1) = 33.16;               % Glucose initial concentration (g/L)
C_ini(2) = 14.18;              % Fructose initial concentration (g/L)
C_ini(3) = 15;              % Acetate initial concentration (g/L)
C_ini(4) = 0;                % Propionate initial concentration (g/L)
C_ini(5) = 0.117;             % Sugar degrader initial concentration (g/L)
C_ini(6) = 0.046;             % Lactate degrader initial concentration (g/L)

% Set the simulation time
t = 60;                    % Reaction time (h)

% Select graphs at the end of the simulation
flag_graph = 1;             % 1= graphs are activated, 0= graphs are deac-
tivated
```

b) output

```
The concentration of the compounds at the end of the batch are (g/L):
  Columns 1 through 3

    'Glucose'        'Fructose'      'Lactate'
    [3.6385e-172]    [1.2725e-09]    [2.3208e-09]

  Columns 4 through 7

    'Acetate'    'Propionate'    'Butyrate'    'Valerate'
    [ 8.1462]    [    5.8307]    [ 16.9461]    [  5.6150]

  Columns 8 through 10

    'Caproate'    'Pediococcus'    'Megasphaera'
    [  0.2948]    [     3.5725]    [     1.8778]
```

Listing 4. The addition glucose (10g/l) to reference concentration of 23.16 g/l. Initial media acetate concentration of 15 g/l

a) input

```
%% INPUTS OF THE MODEL

% Set reactor parameters
V = 1;                      % Volume of the reactor (L)
pH = 6.5;                   % Reactor pH

% Set the initial concentration in the reactor

C_ini(1) = 23.16;               % Glucose initial concentration (g/L)
C_ini(2) = 24.18;              % Fructose initial concentration (g/L)
C_ini(3) = 10;              % Acetate initial concentration (g/L)
```

```
C_ini(4) = 0;                % Propionate initial concentration (g/L)
C_ini(5) = 0.117;             % Sugar degrader initial concentration (g/L)
C_ini(6) = 0.046;             % Lactate degrader initial concentration (g/L)

% Set the simulation time
t = 60;                    % Reaction time (h)
```

b) output:

```
The concentration of the compounds at the end of the batch are (g/L):
  Columns 1 through 4

    'Glucose'        'Fructose'    'Lactate'    'Acetate'
    [3.6793e-253]    [  5.4925]    [ 0.0544]    [ 5.1927]

  Columns 5 through 8

    'Propionate'    'Butyrate'    'Valerate'    'Caproate'
    [    5.4474]    [ 13.8003]    [  4.8729]    [  0.4051]

  Columns 9 through 10

    'Pediococcus'    'Megasphaera'
    [     3.0528]    [     1.7317]
```

Listing 5. The addition of fructose (10g/l), compared to reference concentration of 14.18 g/l with initial acetate concentration of 10 g/l. Simulation input (a) and output (b) values.

a) input

```
%% INPUTS OF THE MODEL

% Set reactor parameters
V = 1;                      % Volume of the reactor (L)
pH = 6.5;                   % Reactor pH

% Set the initial concentration in the reactor

C_ini(1) = 33.16;              % Glucose initial concentration (g/L)
C_ini(2) = 14.18;             % Fructose initial concentration (g/L)
C_ini(3) = 10;              % Acetate initial concentration (g/L)
C_ini(4) = 0;                % Propionate initial concentration (g/L)
C_ini(5) = 0.117;             % Sugar degrader initial concentration (g/L)
C_ini(6) = 0.046;             % Lactate degrader initial concentration (g/L)

% Set the simulation time
t = 60;                    % Reaction time (h)
```

b) output

```
The concentration of the compounds at the end of the batch are (g/L):
  Columns 1 through 3

    'Glucose'        'Fructose'      'Lactate'
    [4.3455e-226]    [9.3669e-24]    [2.4795e-23]

  Columns 4 through 7

    'Acetate'    'Propionate'    'Butyrate'    'Valerate'
    [ 4.5716]    [    6.1179]    [ 15.2445]    [  6.2087]

  Columns 8 through 10

    'Caproate'    'Pediococcus'    'Megasphaera'
    [  0.2691]    [     3.5840]    [     1.8707]
```

Listing 6. The addition of gluose (10g/l), compared to reference concentration of 23.16 g/l with initial acetate concentration of 10 g/l. Simulation input (a) and output (b) values.

# Appendix 4. **Summary of the Hydrolysis Experiments 1–4**

Table 1. The operational conditions and results from different hydrolysis (1–4) experiments after 42–48 hours.

| Experiment | Cabbage part | Enzyme product | Dosage % (w/w) | buffer % [ DW (g)/buffer (ml)] | Glucose (g/l) | Fructose (g/l) | Liquidity observations (1-5)* | pH | Time (h) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | leaf | Ctec2 | 1.5 | 25 | 6.3 | 3.0 | 2.8 | 4.40 | 42 |
| | mass | Ctec2 | 1.5 | 25 | 11.6 | 10.2 | 2.5 | 4.29 | 42 |
| | leaf | Ctec2 | 3 | 25 | 14.5 | 10.4 | 1.7 | 4.32 | 42 |
| | mass | Ctec2 | 3 | 25 | 13.9 | 11.7 | 1.7 | 4.81 | 42 |
| | stem | Ctec2 | 3 | 25 | 12.4 | 6.8 | 1.5 | 4.40 | 42 |
| 2 | leaf | Ctec2 | 6 | 2.5 | 6.4 | 3.9 | 1.7 | 4.99 | 48 |
| | mass | Ctec2 | 6 | 2.5 | 7.8 | 5.8 | 1.4 | 5.02 | 48 |
| | leaf | Ctec2 ; Htec2 | 2.7 ;0.3 | 2.5 | 6.1 | 3.8 | 1.7 | 5.00 | 48 |
| | mass | Ctec2 ; Htec2 | 2.7 ; 0.3 | 2.5 | 7.4 | 5.4 | 2.2 | 5.02 | 48 |
| | leaf | Ctec2 ; Htec2 | 5.4 ; 0.6 | 2.5 | 6.6 | 3.8 | 1.7 | 4.94 | 48 |
| | mass | Ctec2 ; Htec2 | 5.4 ; 0.6 | 2.5 | 8.2 | 5.2 | 1.6 | 4.96 | 48 |
| 3 | mass | Ctec2 | 6 | 20 | 15.2 | 10.5 | 1.5 | 4.24 | 48 |
| | mush | Ctec2 | 6 | 20 | 16.5 | 10.5 | 1.25 | 4.19 | 48 |
| | mass | Ctec2 | 6 | 0 | 22.3 | 15.5 | 1.5 | 4.08 | 48 |
| | mush | Ctec2 | 6 | 0 | 27.1 | 17.1 | 1.25 | 4.17 | 48 |
| | mass | Ctec2 ; Htec2 | 5.4 ; 0.6 | 20 | 13.7 | 11.2 | 2.17 | 4.22 | 48 |
| | mush | Ctec2 ; Htec2 | 5.4 ; 0.6 | 20 | 14.7 | 12.0 | 1.25 | 4.19 | 48 |
| 4 | mass | Ctec2 | 6 | 0 | 23.2 | 13.7 | 2.50 | 4.25 | 48 |
| | Mush | Ctec2 | 6 | 0 | 24.0 | 16.4 | 2.33 | 4.08 | 48 |
| | mass | Ctec2 | 7.7 | 0 | 30.8 | 17.9 | 2.38 | 4.28 | 48 |
| | Mush | Ctec2 | 7.7 | 0 | 25.7 | 16.4 | 2.33 | 4.22 | 48 |
| | mass | Ctec2 | 12 | 0 | 27.1 | 14.0 | 2.17 | 4.17 | 48 |
| | Mush | Ctec2 | 12 | 0 | 29.4 | 17.0 | 2.25 | 4.12 | 48 |

# Appendix 5. **Data from Fermentations Investigating the Impact of pH: $CO_2$, Sugar, Acetate and Lactate Concentrations**

Table 1. Concentrations of glucose, fructose and acetate right after inoculation.

| | *pH6.0_1* | *pH6.0_2* | *pH6.5_1* | *pH6.5_2* | *pH7.0_1* | *pH7.0_2* | *Average (g/l)* | |
|---|---|---|---|---|---|---|---|---|
| *Glucose (g/l)* | 22.19 | 22.93 | 24.57 | 21.31 | 23.93 | 24.03 | 23.16 | 1.14 |
| *Fructose (g/l)* | 13.77 | 14.23 | 15.77 | 13.77 | 13.74 | 13.78 | 14.18 | 0.73 |
| *Acetate (g/l)* | 10.10 | 10.24 | 11.24 | 9.92 | 10.87 | 10.05 | 10.40 | 0.48 |

The averages from Table 1 were used as initial input values in the MATLAB® simulation runs.

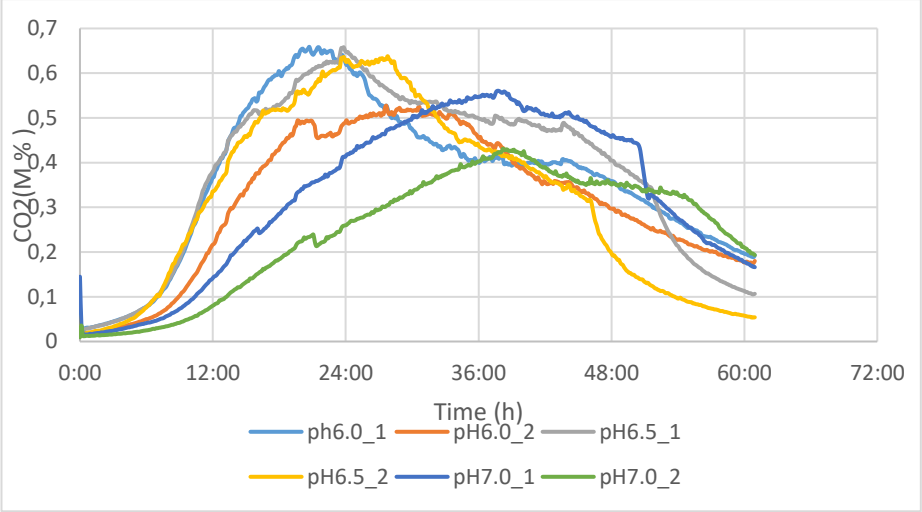



Figure 1. Molar $CO_2$ percentage obtained from the outlet gas during fermentations.

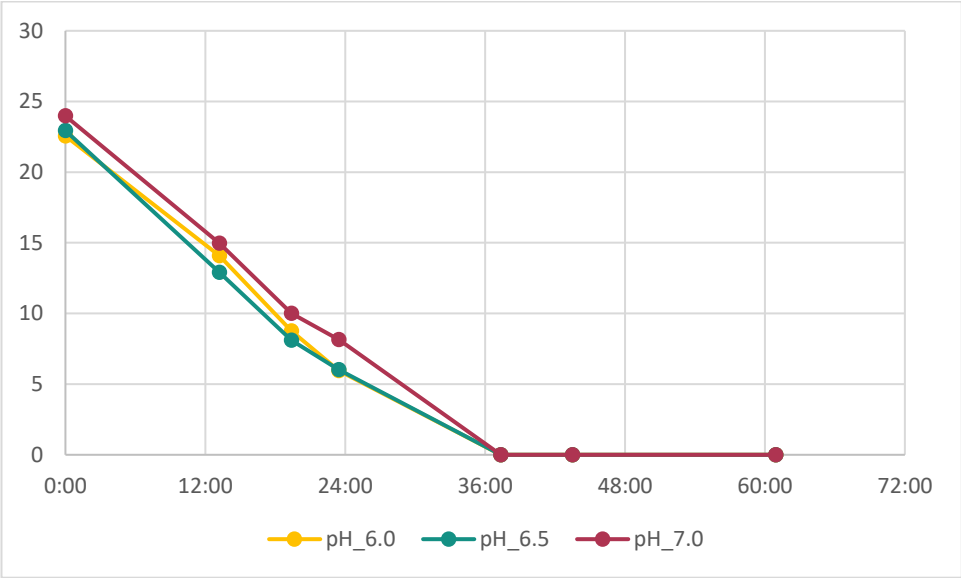



Figure 2. Glucose consumption for each reactor shown as g/l towards time (h)

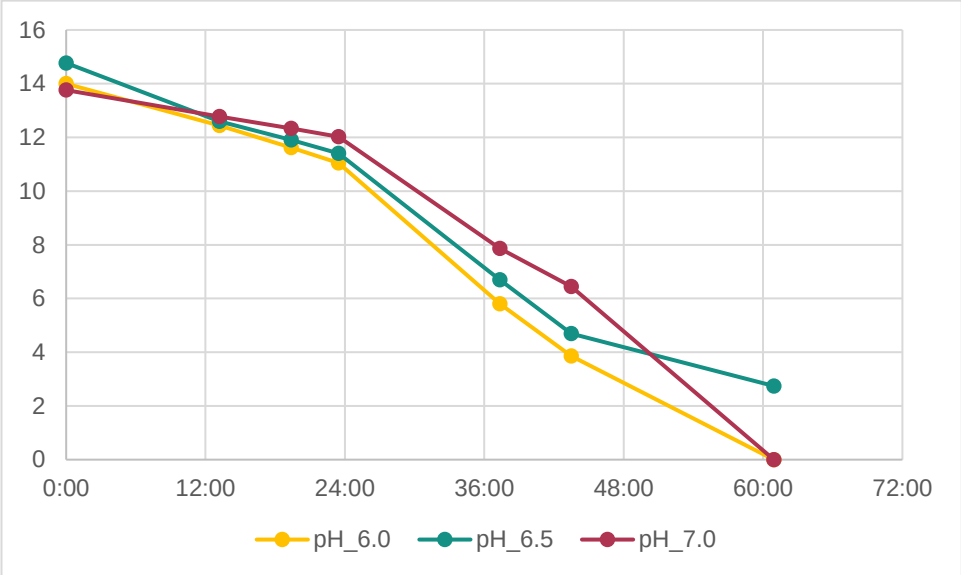



Figure 3. Fructose consumption for each reactor shown as g/l towards time (h).

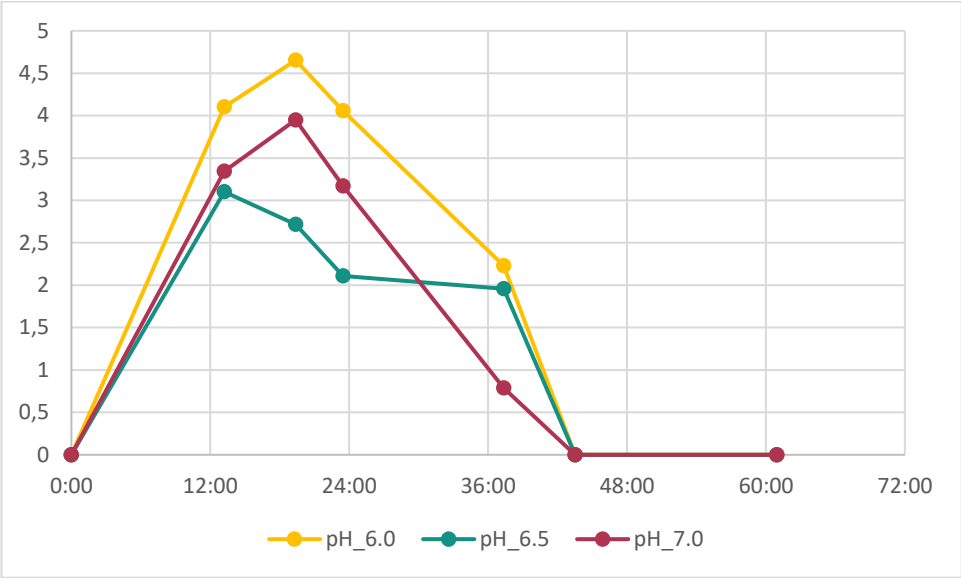



Figure 4. Lactate concentration during fermentation for each reactor shown as g/l towards time (h)

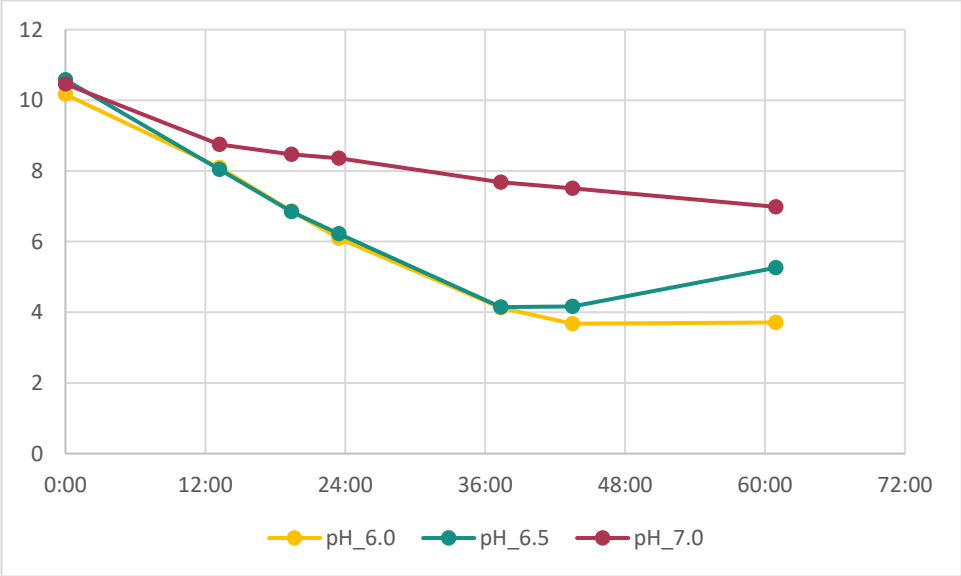



Figure 5. Acetate concentration during fermentation for each reactor shown as g/l towards time (h)

# Appendix 6. **Initial Inoculum Concentrations According to Dry Weight Measurements**

Table 1. Triplicate dry weight measurement results from inoculums incubated for fermentation experiments. Process explanations: F81–F86, fermentation investigating the influence of pH-values 6.0, 6.5 and 7.0; F87–F88, fermentations A &B investigating the impact of increased initial *M. cerevisiae* concentration; F89–F94, fermentations C-H, experimenting the influence of additional glucose, fructose, and acetate.

| **Process and working volume** | | **tube (dry)** | **tube +cells** | **diff.** | **Average (/1 ml)** | **g/l** | **Initial OD** | **Inoculum (ml)** | **calculated reactor OD** | **reactor concentration (g/l)** |
|---|---|---|---|---|---|---|---|---|---|---|
| pH | 1 ml | 1,0935 | 1,0944 | 0,0009 | 0,0017 | 1,7 | 5,222 | 7,66 | 0,1 | 0,0326 |
| 400 | | 1,0932 | 1,0956 | 0,0024 | | | | | | |
| | | 1,0995 | 1,1013 | 0,0018 | | | | | | |
| A-B | 1ml | 1,1016 | 1,1029 | 0,0013 | 0,0013 | 1,3 | 3,6 | | | |
| 1700 | | 1,0958 | 1,0971 | 0,0013 | | | F87 | 210 | 0,5 | 0,161 |
| 1500 | | 1,0911 | 1,0924 | 0,0013 | | | F88 | 42 | 0,1 | 0,0364 |
| Same inoculum | 2 ml | 1,1011 | 1,1039 | 0,0028 | 0,0013 | 1,3 | | | | |
| | | 1,0995 | 1,1024 | 0,0029 | | | | | | |
| | | 1,0936 | 1,0958 | 0,0022 | | | | | | |
| C-H | 1 ml | 1,0923 | 1,0934 | 0,0011 | 0,0012 | 1,2 | 4,66 | 8,5 | 0,1 | 0,0248 |
| 400 | | 1,0989 | 1,1003 | 0,0014 | | | | | | |
| | | 1,0988 | 1,0998 | 0,0010 | | | | | | |
| | | | | | | | | | | |
| | | | | | | | | | | |
| | | | | | | | | | | |
| *Pediococcus* | | | | | | | | | | |
| *pentosaceus* | *E-153483* | | | Dry weights | | | Fermentations | | | |
| **Process and working volume** | | **tube (dry)** | **tube +cells** | **diff.** | **Average (/1 ml)** | **g/l** | **Initial OD** | **Inoculum (ml)** | **desired reactor OD** | **reactor concentration (g/l)** |
| F81 -F 86 | 1 ml | 1,0933 | 1,0976 | 0,0043 | 0,0033 | 3,27 | 8,916 | 4,5 | 0,1 | 0,0368 |
| 400 | | 1,0935 | 1,0962 | 0,0027 | | | | | | |
| | | 1,0904 | 1,0932 | 0,0028 | | | | | | |
| F87-F88 | | Calculated according to other inoculums | | | from F89-F94 | 3,4 | 9,8486 | | | |
| | | and measured OD | | | from F81-F86 | 3,61 | | | | |
| 1700 | | DW not determined | | | Average | 3,5 | | 15,3 | 0,1 | 0,031 |
| 1500 | | | | | | | | 15,3 | 0,1 | 0,0356 |
| F89-F94 | 1 ml | 1,0971 | 1,1013 | 0,0042 | 0,0038 | 3,83 | 11,2 | 3,6 | 0,1 | 0,0345 |
| 400 | | 1,0955 | 1,0986 | 0,0031 | | | | | | |
| | | 1,1002 | 1,1044 | 0,0042 | | | | | | |