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Master Thesis

Effect of pasteurization and pH on the formation of microbial metabolites during enzymatic pre-treatment of organic waste

Submitted by

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Statutory declaration

I hereby declare that I am the sole author of this work; no assistance other than that permitted has been used and all quotes and concepts taken from unpublished sources, published literature or the internet in wording or in basic content have been identified by footnotes or with precise source citations.

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Abstract

Products that contribute to a sustainable economy can be generated with microbial fermentation. The carbon required for this process is usually obtained from plant crops, which leads to competition with food production. Organic waste needs anyway a special treatment to avoid CH_4 emissions from landfills and contains unexploited carbon sources. Pre-treatment, e.g. with industrial enzymes, can enhance degradation to simple molecules. Hence, garden waste, brewer's spent grain, and organic municipal solid waste were enzymatically hydrolysed with and without previous pasteurization for inhibition of indigenous microorganisms and the results were compared. The highest possible glucose concentrations were aimed because this sugar is used as an energy and carbon source in many fermentations. Furthermore, metabolic products like lactic acid were measured because of their potential for applications and to estimate microbial growth. Production of lactic acid (11 g/L), as well as glucose (2 g/L), was the lowest within garden waste. The greatest raise of glucose (11 g/L) was measured within brewer's spent grain, however, within organic municipal solid waste lactic acid rose by 21 g/L and achieved a total concentration of 44 g/L. Hence, it was tried to inhibit the lactic acid production within organic municipal solid waste by lowering the starting pH with lactic and sulfuric acid to pH 4 or by an elevated incubation temperature (50 °C). The combination of pH reduction with lactic acid and pasteurization achieved almost the same increase of glucose (12 g/L) within 42 h as the enzymatic hydrolysis of autoclaved samples within 72 h (14 g/L). Thus, optimizing lactic acid production is more practical because a higher increase and total amount are achievable without energy-intensive pasteurization.

Kurzfassung

Produkte, die zu einer nachhaltigen Wirtschaft beitragen, können mittels mikrobieller Fermentation hergestellt werden. Der dafür benötigte Kohlenstoff wird in der Regel aus Nutzpflanzen gewonnen, was zu einer Konkurrenz mit der Lebensmittelproduktion führt. Organische Abfälle müssen hingegen ohnehin im Zuge der Entsorgung speziell behandelt werden, damit keine CH_4-Emissionen auf Mülldeponien entstehen, und enthalten nicht ausgeschöpfte Kohlenstoffquellen. Eine Vorbehandlung, z.B. durch industrielle Enzyme, kann den Abbau zu einfachen Molekülen erleichtern. Dazu wurden Gartenabfälle, Biertreber und Biomüll mit und ohne vorherige Pasteurisation zu Hemmung der indigenen Mikroorganismen enzymatisch hydrolysiert und die Ergebnisse verglichen. Eine möglichst hohe Glukosekonzentration wurde angestrebt, weil dieser Zucker als Energie- und Kohlenstoffquelle für viele Fermentationsprozesse dient. Metabolite wie Milchsäure wurden ebenfalls wegen ihres wirtschaftlichen Nutzens gemessen, und um das mikrobielle Wachstum abschätzen zu können. Bei den Gartenabfällen war die Produktion der Milchsäure (11 g/L) als auch die der Glukose (2 g/L) am niedrigsten. Biertreber hatte die höchste freigesetzte Menge an Glukose (11 g/L), aber der Milchsäuregehalt von Biomüll stieg um 21 g/L und erreichte eine Gesamtkonzentration von 44 g/L. Es wurde versucht die Milchsäureproduktion beim Biomüll durch Verringern des Anfangs-pH-Wertes mit Milch- und Schwefelsäure oder durch Erhöhung der Inkubationstemperatur auf 50 °C zu inhibieren. Mit der Reduktion des pH-Wertes durch Milchsäure und Pasteurisierung des Substrats wurde nach 42 h fast dieselbe Glukosekonzentration (12 g/L) freigesetzt wie bei der enzymatischen Hydrolyse von autoklavierten Proben nach 72 h (14 g/L). Sinnvoller ist es daher die Milchsäureproduktion zu optimieren, weil dadurch sowohl eine höhere Steigerung der Umsetzung als auch eine höhere Gesamtkonzentration des Produktes ohne energieintensives Pasteurisieren erreicht wurde.

Contents

Abbreviations

AD	Anaerobic digestion
BSG	Brewer’s spent grain
DM	Dry matter
FW	Food waste
GHG	Greenhouse gases
GW	Garden waste
MO	Microorganism
OMSW	Organic municipal solid waste
OW	Organic waste
PHA	Polyhydroxyalkanoate
PHB	Poly-3-hydroxybutyrate
PLA	Polylactide or polylactic acid
RO-water	Reverse osmose water
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
TKN	Total Kjeldahl Nitrogen
VFA	Volatile fatty acids

1. Introduction

1.1. Definition of organic waste

The European Waste Framework Directive 2008/98/EC describes biodegradable wastes or biowastes as wastes from gardens and parks, food and kitchen wastes, and waste generated from food processing plants (European Council, 2008). A simpler definition for organic waste (OW) is that it includes all organic matter or unused by-products from the food processing industry that are biodegradable and have a biological origin like animals or plants (Yazid et al., 2017). The main types defined by these descriptions are food (FW), garden (GW), and organic municipal solid waste (OMSW) (López-Gómez *et al.*, 2020).

FW includes edible products, which cannot be sold due to alterations of their quality, discarded food from households, or food leftovers from canteens or restaurants. Waste derived from these origins is composed of 30 % w/w dry basis starch as their main carbohydrate, lipids (12-37.7 %), and proteins (10–20 %). However, the composition varies a lot between different seasons, menus, and countries (López-Gómez *et al.*, 2020). Besides households, restaurants, and canteens, big amounts of FW are generated by food processing plants like from the sugar, fruit juice, or beer industry. The quantity and composition of these food residues are more predictable. For example, brewer's spent grain is a by-product of the brewing industry and consists throughout the whole year of about 15 to 27% lignin, 12 to 25% cellulose, 19.2 to 41.9% hemicellulose, and 14 to 31% protein (Mussatto *et al.*, 2008; Pinheiro *et al.*, 2019).

In addition to food also non-edible material is defined as OW. GW consists of lignocellulosic material like garden cuttings i.e., grass, pieces of bushes, or fallen leaves (López-Gómez *et al.*, 2020). The composition of this woody waste is about 40 % cellulose, 20–30 % hemicelluloses, and 25–30 % lignin (Shi *et al.*, 2013). The amounts of GW highly depend on seasons and do not occur on such a large scale as FW but the trend for more green areas in cities could raise the quantities of GW in the next years (Cubas-Cano *et al.*, 2020; López-Gómez *et al.*, 2020).

The third class of waste is OMSW which arises, if FW and GW are collected together. Thus, it can contain food leftovers as well as grass cuttings and is, therefore, the most heterogenous type of OW (López-Gómez *et al.*, 2020). Despite OMSW's local and seasonal variations López-Gómez *et al.* (2020) compared data of OMSW's composition from cities all over the world. This mixture of GW and FW consists of 6.1 to 35.0 % fat, 12.5 to 26.6 % proteins, 12 to 21.1 % cellulose, 5.1 to 5.5 % hemicellulose, and 16.3 to 20.7 % starch (López-Gómez *et al.*, 2020). Apart from these biopolymers OMSW already contains approximately 15 g/L lactic acid in a racemic mixture of L- and D-enantiomers produced from the naturally occurring lactobacillus (Probst *et al.*, 2013).

1.2. Commonly used organic waste treatment methods

The described OW is annually produced in huge amounts and in addition to wasting food the conversion of organic matter into greenhouse gases (GHG) such as CO_2 and CH_4 in landfills is a big problem (Gao *et al.*, 2017). FW alone contributes after the USA and China to the third-highest CO_2 emissions, if it is imagined as a country (López-Gómez *et al.*, 2020). Accordingly, adequate waste management and treatment are necessary. The most common ones are incineration, landfilling, composting, and anaerobic digestion. Incineration is an easy way to reduce the size of the discarded matter, but OW has a high water content, hence a low heating value and the combustion process causes air pollution as well (Eco.cycle, 2011; Girotto *et al.*, 2015). However, the method with the highest ecological impact by spoiling the environment for animals and humans is landfilling (Gao *et al.*, 2017). Many of these waste treatment sites have no suitable drainage system. Hence, toxic compounds are leaching into the groundwater and polluting big drinking water reservoirs. Additionally, the biodegradable waste is buried under anaerobic conditions that lead to the formation and release of mostly methane and a smaller extend carbon dioxide. Modern landfills containing only stabilized waste and capturing the leachate and methane, but it still should be the last option (Vaverková, 2019). The member states of the EU are encouraged to treat organic waste in an eco-friendly way i.e. anaerobic digestion (AD) and composting (European Council, 2008). The latter can be operated on an industrial scale or even at home. Composting in the own garden saves energy and land for a big plant and transport costs to the widely distributed waste bins. Nevertheless, not everybody is able to compost his own OW and so industrial sites are necessary. In these plants, organic material is decomposed by generating optimal aerobic conditions for the degrading microorganisms (MO). The composting process contains several reactions including hydrolysis, proteolysis, ammonification, nitrification, carbon mineralization, and humification (Cáceres *et al.*, 2018). This waste treatment method leads like landfilling to the emission of GHG but mostly CO_2 and less methane. Reduction of the latter pollutant is important because it has a 26 times higher influence on climate change than carbon dioxide (Cao *et al.*, 2019; Gao *et al.*, 2017).

Another solution for treating OW is AD, which is the biological conversion of biomass to biogas (Figure 1) by a consortium of different MOs. It starts with the hydrolysis of polysaccharides, lipids, and proteins into simple compounds like sugars, fatty or amino acids. The MOs excrete enzymes that are able to degrade these macromolecules into their constituents. In the next step called acidogenesis, these substances are metabolized into volatile fatty acids (VFA) and alcohols. During the acetogenesis, acetic acid, CO_2, and H_2 are produced, which are utilized by methanogenic Archaea to form CH_4 (Kasinath *et al.*, 2021). Depending on the substrate the biogas contains 50-75 % methane, 25-50 % CO_2, and small amounts of H_2S (Herout *et al.*,

2011; Kasinath *et al.*, 2021). Apart from the generation of electricity and heat the residues are used to fertilize agricultural land and make AD one of the most environmentally friendly treatments of OW (Kasinath *et al.*, 2021).

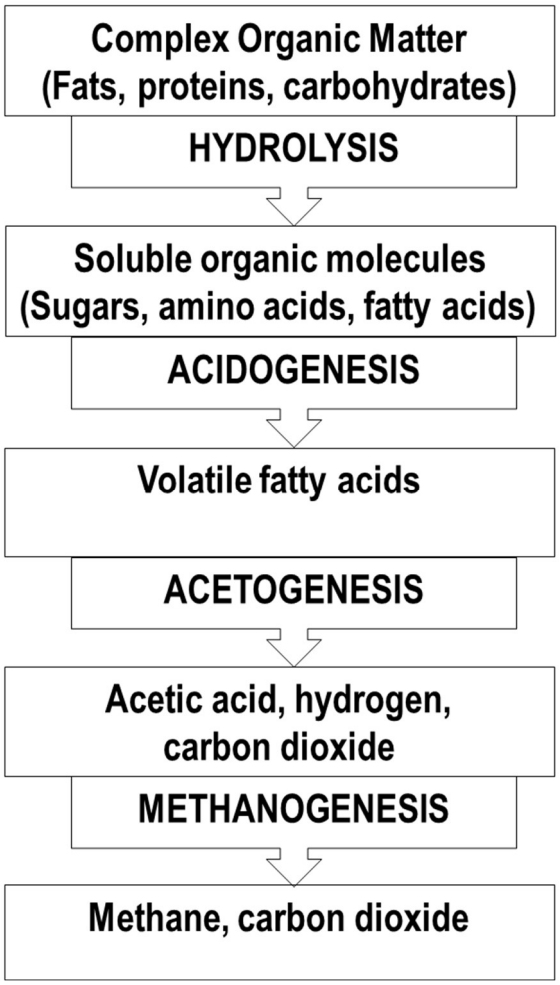

Figure 1: The AD process (Kasinath et al., 2021)

1.3. Fermentation of organic waste

1.3.1. Organic waste as alternative substrate for industrial fermentation

Since the oil crisis in 1973, the production of bio-based substances has been investigated to become more independent from fossil fuels (Akermann *et al.*, 2021). The first biomass which was used to generate first-generation biofuels like bioethanol were food crops. However, that means land, water, and other resources are used to produce fuels instead of food, while starvation is still a severe problem in many countries over the world. A solution for this problem is second-generation substrates like lignocellulosic material because they are inedible for humans and composed of carbohydrates mainly in form of cellulose and hemicellulose.

Nevertheless, this kind of biomass is covered with lignin that requires harsh pre-treatment methods to detach or degrade it (Tan *et al.*, 2014).

Due to the recalcitrance of second-generation biomass using waste for fermentation of third-generation biofuels and biobased chemicals like lactic acid, PHB or citric acid has become a well-investigated research topic (Sirohi *et al.*, 2021). Organic waste is an inexpensive material, that is not in competition with food production. In addition, using OW as biomass can contribute even to a zero-waste economy. Apart from these reasons, OW still contains sugars, organic acids, minerals, and proteins and has a high water content (Yazid *et al.*, 2017; López-Gómez *et al.*, 2020; Sirohi *et al.*, 2021). Therefore, it was already successfully used to generate different biotechnological products like polyhydroxyalkanoates (PHAs), lactic acid, and ethanol (Reis *et al.*, 2003; Kalogo *et al.*, 2007; López-Gómez *et al.*, 2020).

The production of biofuels still cannot compete with fossil fuels because prices for petroleum and natural gas are comparably low. In addition, sun, wind, and water can be used to generate renewable energy instead of biomass (Panwar *et al.*, 2011). The production of carboxylic acid platform chemicals like lactic, propionic, and succinic acid could be more attractive from an economical point of view (Tan *et al.*, 2014; López-Gómez *et al.*, 2020). Yet, many chemical building blocks are derived from processes where toxic chemicals and heavy metals catalyze the reactions and fossil fuels are used as raw material (Li *et al.*, 2020). However, these biobased chemicals, which can be used to generate a variety of products have higher market values compared to biofuels, which are needed in bulk amounts and low prices. Hence, biobased building blocks have applications in many industries such as textile, pharmacy, and cosmetics and can be generated from OW and thereby replace petrochemicals (Agler *et al.*, 2011; Tan *et al.*, 2014; López-Gómez *et al.*, 2020).

1.3.2. Enzymatic hydrolysis of OW as a pre-treatment method

OW has no economic value but it still consists of carbohydrates as biopolymers. To promote microbial degradation the biological waste is treated before being used as a nutrient source. Hence, optimizing pre-treatment of the third-generation substrates is a necessity to raise the overall efficiency of the fermentation process. Physical, chemical, and biological pre-treatment methods can be found in the literature (Cesaro *et al.*, 2014). Besides steam explosion and ultrasound as an example for physical pre-treatment, also chemical methods, such as alkaline or acid hydrolysis, are applied. Nevertheless, biological options like the usage of enzymes are probably the most promising ones. Apart from their ability to higher conversion rates these biocatalysts do not generate as many toxic hydrolysates as acid-driven degradation and are not hazardous for the environment (Tan *et al.*, 2014). However, the added enzymes should be adapted to the treated biomass to obtain optimal yields. Jordan *et al.* (2007) for example

determined the lipid, carbohydrate, protein, and lignin content of their substrate and added industrial lipases, cellulases, hemicellulases, proteinases, and laccases in the same ratio as the biopolymers. This enzyme composition is suitable for lignocellulosic material. In contrast, starchy biomass such as FW needs amylases to hydrolyse the polysaccharides (López-Gómez *et al.*, 2020). The catalytical biomolecules are very effective in the degradation of biopolymers but they also can be inhibited in several ways. Enzymes have all different optimal temperatures and pH ranges, which influence their hydrolytic activity. Furthermore, in some cases the products of the hydrolysis can inhibit the biocatalysts as feedback inhibition (Öhgren *et al.*, 2007).

Two methods can be applied in order to minimize the throwbacks of industrial enzyme addition. The hydrolysis and fermentation can be operated either separately (SHF) in time and location, or simultaneously, which is called simultaneously saccharification and fermentation (SSF). There are several aspects to consider before choosing an option. One disadvantage of simultaneous employment of enzymes and MOs is their different optimal working conditions. Regarding SHF, the proper temperature, pH, time, etc. can be selected for both steps and lead to a higher reaction rate of the enzymes (Öhgren *et al.*, 2007). The final product yield of SSF, however, is in most cases higher because the hydrolysates are immediately metabolized. Thus, feedback inhibition of the enzymes is prevented. Furthermore, toxic compounds from the hydrolysis of lignin or other substances are neutralized during fermentation and have the ability to increase productivity by stressing the microbes. Apart from hydrolysates fermentation products like ethanol can be beneficial for SSF by preventing microbial contamination (Öhgren *et al.*, 2007, Tan *et al.*, 2014). Nevertheless, if OW is supposed to be converted into feed for many different fermentation processes SHF is the better choice (Paz *et al.*, 2019).

1.3.3. Mixed or pure cultures

In addition to choosing the best-suited pre-treatment strategy, the decision about applying a pure or mixed culture is also important. Waste can be sterilized with chemicals or most commonly with wet heat and used as a substrate for only one strain. This process is relatively simple, because the medium is sterile, and the aimed fermentation product depends just on the added MO. The production of L-lactic acid fits this strategy well as the formation of the right enantiomer is critical for many applications like the PLA synthesis. For instance, López-Gómez *et al.* (2020) generated L-lactic acid with high purity using *Bacillus coagulans* and sterile OW.

Nevertheless, one reason to use OW as a substrate is its low price, which makes biobased products economical more competitive compared to fossil-derived ones. However, the sterilization process and measurements to avoid microbial contamination are cost-intensive, raise the price for eco-friendly substances, and require energy, which is often derived from

fossil fuels (Gahlawat *et al.*, 2020). Therefore, mixed cultures, which have been used for centuries in environmental biotechnology to treat complex matter are an alternative for the fermentation of biological waste (Liang *et al.*, 2014). The disadvantage of this method is that the outcome from a mixed consortium is a mix of compounds, which needs a difficult downstream process to separate the substances (Agler *et al.*, 2011). Apart from subsequent separation, selective pressure to obtain pure compounds can be applied. In the biogas production, external energy sources (e.g. light) and electron acceptors (e.g. oxygen, nitrate, sulfate) are excluded to allow only the formation of CO_2 and methane as the final products, because they have the lowest free energy content per electron (Kleerebezem *et al.*, 2007). Another example is the enrichment of PHA forming MOs by using sequential feeding and famine phases due to the better adaptation to the periods with no added nutrients (Reis *et al.*, 2003; Kleerebezem *et al.*, 2007).

1.4. Applications

There are already some examples that show how to produce biotechnological products from OW with pure or mixed cultures. Since the 1970s, bioethanol was meant to make us more independent from fossil fuels by replacing gasoline and it is still the most important biofuel by covering around 65 % of the global biofuel market (Sarbishei *et al.*, 2020). To date, most of the bioethanol production competes with the food industry by using edible crops. Nevertheless, the conversion of OW into bio-based ethanol has already been accomplished (Öhgren *et al.*, 2007; Tan *et al.*, 2014; Sarbishei *et al.*, 2020; Slathia *et al.*, 2020).

In addition to the generation of biofuels also biopolymer production from waste has already been achieved (Reis *et al.*, 2003; Costello *et al.*, 2014; Izaguirre *et al.*, 2019; Sirohi *et al.*, 2021). These are polymers that are bio-based, biodegradable, or in the best case both. As an example, poly-3-hydroxybutyrate (PHB) has similar properties to fossil-derived polymers, which makes it a promising biological alternative. These short-chain PHAs are produced from MOs and naturally used for energy storage. The biosynthesis starts with a condensation reaction of two acetyl-CoA molecules to form acetoacetyl-CoA, which is reduced and forms 3-hydroxybutyryl-CoA. In the end, PHB synthase produces PHB from its C4-monomers. This biopolymer remains stable until high temperatures, has resistance to UV radiation, and has a low oxygen permeability. Thus, it is a potential alternative for food packaging. Now, researchers are looking for MOs with higher productivity and also to improve the properties of PHB and make fossil-derived plastics obsolete (Sirohi *et al.*, 2021).

Another very prominent bio-based substance with an annual production of $472*10^3$ kg is lactic acid (Li *et al.*, 2020). This carboxylate platform molecule is used for several applications like in the food, textile, and cosmetic industry (Bastidas-Oyanedel *et al.*, 2015; Liang *et al.*, 2014).

Furthermore, lactic acid is converted into lactide to generate another biopolymer called polylactic acid (PLA). It is crucial for this process to have a pure enantiomer or a defined ratio of D- and L- lactic acid. The chemical synthesis process generates a racemic mix of the two forms, but certain MOs produce L-lactic acid with high optical purity. Therefore, 90 % of the worldwide production is already bio-based and even OW as biomass was described in several scientific publications (Liang *et al.*, 2014; López-Gómez *et al.*, 2020; López-Gómez *et al.*, 2020). Apart from lactic acid's value as a product and chemical building block, this substance can be used as a substrate for MOs. Thus, the applications of bio-based lactic acid are even enlarged (Mansour *et al.*, 2008; Sonomoto *et al.*, 2010; Bertin *et al.*, 2014; Moens *et al.*, 2017).

2. Objectives

The goal of this master thesis is to inhibit microbial growth during enzymatic hydrolysis of OW. This should result in hydrolysates with a high sugar content, which implies low substrate losses through aspiration or anaerobic fermentation. The obtained hydrolysates can be employed in diverse applications.

As a prerequisite, the inhibition process has to avoid temperatures above 100 °C, the usage of toxic chemicals, high amounts of water, and high pressure to keep the energy consumption and environmental pollution low. Therefore, pasteurization is used to prevent microbial activity during enzymatic hydrolysis. First, three OW types, GW, BSG, and OMSW are enzymatically pre-treated and the results are compared. In addition to released sugars, produced metabolites like lactic acid, acetic acid, and ethanol are measured to monitor microbial growth.

OMSW is considered as the most promising substrate, and therefore further inhibition treatments are applied to assess the efficiency of enzymatic hydrolysis. An elevated incubation temperature (50 °C) and addition of lactic or sulfuric acid to lower the starting pH (pH 4) are combined with pasteurization. Finally, autoclaved samples are treated with the enzyme mix to compare enzymatic hydrolysis with sterilized and unsterilized substrates.

In addition, the composition of GW, BSG, and OMSW is determined and compared with each other and results from the literature. For example, the protein, hydrolysed carbohydrates, and water-soluble substances content are measured.

3. Materials and Methods

3.1. Chemicals

Table 1: Used chemicals and degree of purity

Chemical	Company	Degree of purity
D (+) Glucose	Roth	ACS water-free
Glucose Monohydrate	Roth	99.50 %
D-Xylose	Duchefa	>99 %
D (-) Fructose	Sigma	99 %
L (+) Arabinose	Roth	99 %
D (+) Cellobiose	Fluka	99 %
Sodium hydroxide (50 %)	Donau Chem	-
Sodium hydroxide (1.0 mol/L)	Fluka	-
Hydrochloric acid (1 mol/L)	VWR	-
Boric acid	Merck	-
DL-Lactic acid	Roth	90 %
Methanol	Fischer Chemical	99.8 %

3.2. Organic waste and slurry preparation

For the present experiments, three types of waste were used. Organic municipal solid waste (OMSW), brewer's spent grain (BSG), and garden cuttings called garden waste (GW) in the following text were received from a waste management company in the CAFIPLA-consortium (Figure 2).

Figure 2: Pictures of untreated OMSW (A), BSG (B), and GW (C).

Impurities like plastic or stones, which would have damaged the cutting device, were removed before further treatment. Subsequently, the wet substrates were mixed with the required amount of water to obtain 15 % dry matter (DM) slurries by using a professional blender (Emerio, PBL-108642, 2L, 1500 W),

3.3. Enzymatic hydrolysis of organic waste

Figure 3: Enzymatic hydrolysis of OMSW in a conical flask.

An industrial enzyme mix that contained non-starch-polysaccharide hydrolysing enzymes (glucanases, cellulases, and xylanases) was provided by a project partner and used for all experiments in a 16.7 % (w/w) ratio of g enzyme solution per g DM. The enzymatic hydrolysis was carried out in 250 mL conical flasks (Figure 3). Starting volume of the enzyme-substrate mixture was around 250 mL. The samples were incubated at 39 °C (except for some inhibition experiment samples) and mixed with 180 rpm (Infors HT, Multitron). Samples were taken with serological pipettes, centrifuged at 3428 rcf for 10 min (Eppendorf, 5920R) and just the supernatant was brought in for pH measurement and HPLC analysis.

3.4. Incubation in laboratory-scale bioreactors

Before using the parallel bioreactor system DASGIP® (Eppendorf), the pH sensors were calibrated with a pH 7.0 and pH 4.0 buffer solution. Furthermore, stirrers, temperature sensors, and off-gas condensers with filters were implemented. For controlling agitation and temperature and recording them together with the change of pH, a control unit and the DASware control software were used. Each bioreactor was filled with 400 g 15 % DM slurry and 10 g enzyme solution. This enzyme-OMSW-solution was incubated for 30 min at 39 °C to lower the viscosity of the slurry by enzymatic hydrolysis. Then, the bioreactors were put into the DASGIP bioblock, the lid with the stirrer, sensors, off-gas filter, and sampling site was added and the system was ready to start.

3.5. OMSW slurry titration

Three 15 % DM slurries were prepared as described in “3.2 Organic waste and slurry preparation”, incubated at room temperature for 1 hour, and centrifuged at 3428 rcf for 10 minutes (Eppendorf, 5920R). The pellet was removed, and the supernatant was used for the measurements. To carry out and record the titrations a titration device (Metrohm, 721 NET Titrino) and suitable software (Tinet 2.5) were used. First, the starting pH of the duplicates was measured and then 1 M HCl or 0.5 M NaOH was added to reach the target pH of 2.0 or 10.0. The titration settings are shown in Table 2.

Table 2: Titration settings for measuring the buffer capacity of OMSW

Parameters	Settings
Waiting time before first pH measurement	50 s
Max. rate	2.5 mL/min
Min. rate	40 µL/min

3.6. Dry mass and ash determination

DM was measured by weighing the sample in a crucible with an analytical balance (ENTRIS, 224I-1S) and incubating it for at least 24 hours at 105 °C in an oven with subsequently cooling in a desiccator. After weighing the crucibles with the dried samples, the crucibles were incinerated in a muffle oven (Heraeus, M110) at 550 °C to remove all compounds except the ash.

3.7. Water and methanol extraction

3.7.1. Water extraction

4 to 5 g of the lyophilized (Christ, Gamma 1-16 LSCplus, and Alpha 2-4 LSCplus) and crushed (Emerio, PBL-108642, 2L, 1500 W) samples were transferred into extraction tubes (33 x 80 mm, cellulosic fiber) until one-third of the tube was covered. The filled extraction tubes were placed into the Soxtherm®-tubes and approximately 100 mL MilliQ-water was added until the samples were completely submerged. Subsequently, the extraction procedure (the program can be seen in Table 3) was carried out by an automated Soxhlet device (Soxtherm®, Gerhardt). 10 mL of the water containing the extractives were taken for HPLC and nitrogen analysis. The remaining liquid was poured into glass beakers to determine the weight of the water-soluble substances after drying at 105 °C. The extraction temperature was extended due to the cooling effect of the hood.

Table 3: Soxtherm® program for water and methanol extraction

Parameters	Water extraction	Methanol extraction
Temperature	280 °C	240 °C
Cooking phase	1h 30min	1h 30min
1st solvent removing	4*15 mL	5*15 mL
Extraction	1h 50min	1h 50min
2nd solvent removing	6 min	6 min
Interval	2 min	2 min
Impulse	6 sec	6 sec
Duration	3h 33min	3h 29min

3.7.2. Methanol extraction

After the water extraction, the samples stayed in the Soxtherm®-tubes, were covered with methanol (Fischer Chemical) and the appropriate program for the alcohol extraction was selected (Table 3). Then, the remaining methanol in the Soxtherm®-containers was poured into glass beakers for weight determination and the treated samples were dried on plates. Most of the solvent evaporated under the hood, drying was completed with an oven at 60 °C.

3.8. Analysis of Klason-Lignin and acid-soluble substances

The following procedure is an adapted version of the “Determination of Structural Carbohydrates and Lignin in Biomass” (Sluiter *et al.*, 2004). One of the requirements for this protocol is a previous water and methanol extraction. Therefore, the dried samples after the extractions were used for this analysis. To obtain an equal size, the waste samples were treated with an ultra-centrifugal mill (Retsch, Z1000, 1mm sieve). This device has two different settings 10 000 and 15 000 rpm, 10 000 rpm were sufficient for our experiments. Approximately 1 g of milled material was used to determine the DM and ash and 0.3 g for the Klason-Lignin analysis in flasks. The first hydrolysis step was initiated by adding 3 mL of 72 % sulfuric acid and incubating this mixture for 1 hour at 30 °C and 200 rpm (Infors HT, Multitron). Next, 84 mL RO-water were added to the flasks to get a 4 % acid concentration, and 3 mL 72 % sulfuric acid were pipetted to 84 mL of the standards STD1 and Cellobiose STD (Table ***4***) to monitor possible losses. For the second hydrolysis step, these solutions were heated up in an autoclave (Systec, VE-120) to 120 °C for 20 min. To account for any losses during

Table 4: Composition of the standards used for the Klason-Lignin and acid-soluble substances determination

STD1	Cellobiose STD
D (+) Glucose	D (+) Cellobiose
D-Xylose	D (-) Fructose
L (+) Arabinose	

autoclaving additional flasks were filled with 84 mL standard solution (STD1 and Cellobiose STD) with 3 mL 72 % sulphuric acid. Sugar concentration was measured before and after autoclaving. The cellobiose STD was used to prove the acidic hydrolysis. To separate the acid-soluble from the insoluble substances, the mixture was filtered through micro-glass fiber paper (Ahlstrom Munksjö). The filtrate was used for HPLC analysis and the retentate was washed two times and then dried at 105 °C to determine the weight of the acid-insoluble solids.

3.9. Total Kjeldahl Nitrogen (TKN) and unbound NH_4-N determination

An automated Kjeldahl unit (BÜCHI, Autokjeldahl Unit K-370) was used for the analysis of TKN and the unbound ammonium content. For measurement of TKN, samples had to be digested first to convert the bound nitrogen into solved ammonia ions. Therefore, between 0.1 and 1 g liquid or solid material was weighed into BÜCHI glass tubes. After the sample addition, the glass tubes were rinsed with water, one Kjeldahl tablet (Thompson&Copper LTD, CT-AA20) per tube, and 20 mL of 96% H_2SO_4 were added. In the digestor (BÜCHI, K-438), the solutions were heated up to 370 °C and after 4 hours the device was turned off. The measurement was conducted on the next day by putting the BÜCHI tubes into the Kjeldahl titration unit and choosing the TKN program. Then, the device pumped 30 % NaOH to the sample and heated it to convert the ammonium ions into ammonia, which evaporated, condensed again, and dropped into 2 % boric acid. 1 mol NH_3 forms 1 mol $B(OH_4)^-$, which is titrated with 0.05 M HCl. The amount of nitrogen in the sample can be calculated from the required volume of HCl.

Ammonium was measured directly without digestion and the program for ammonium of the automated Kjeldahl unit was selected.

3.10. Phosphate analysis

The phosphate concentration of the supernatant described in “3.5 OMSW slurry titration” was obtained with a kit (HACH, LCK 350). The instructions of its manual (HACH Lange GmbH, 2020) for ortho-phosphate were followed, and the samples were diluted 1:10 to reach the possible measuring range of 2.0–20.0 mg/L PO_4-P. The blue dye of the formed phosphate complex was analyzed by a photometer (HACH, DR 3900) at 880 nm. The device already showed the concentration in mg/L PO_4-P.

3.11. Determination of sugars, organic acids and ethanol with HPLC

This method was used for the HPLC analysis of acid-soluble substances, water extractives, substances in the liquid fraction of a 15 % slurry without and with enzymatic hydrolysis. The substances that can be analyzed with the HPLC system (Table 5) were externally calibrated in a range of concentrations comprised between 10 and 1000 mg/L. The concentrations were determined by the peak’s area.

For sample preparation proteins had to be removed with a Carrez-precipitation, which only works properly between pH 4 and pH 6. For that purpose, the samples were centrifuged (Beckman, GS-15) for 10 min at 14 324 g and either H_2SO_4 (pH 4) or NaOH (pH 13) was added to obtain the desired dilution and to adjust the pH of the samples accordingly. Then, a 2% (v/v) $K_4[Fe(CN)_6]\cdot 3\ H_2O$ solution and a 2% (v/v) $ZnSO_4\cdot 7\ H_2O$ solution were added to precipitate the proteins. After centrifugation (Beckman, GS-15) for 30 min at 14 324 g, the supernatant was filtered through 0.20 µm filter membranes into glass vials.

The retention times of xylose, galactose, rhamnose, and fructose are too close together to quantify them separately with this system. Therefore, the detected peak or double-peak was integrated as xylose, and results were given as xylose-SUM concentrations.

Table 5: Description of the used HPLC system

Column type	ION 300 (Transgenomic, Omaha USA)
Detector	Refractive index detector (Agilent 1100)
Temperature	45°C
Flow rate	0.325 mL/min
Solvent	0.01 N H_2SO_4

3.12. Calculations

Recalculation factor:

(adapted from A. Sluiter, R. Ruiz, C. Scarlata, J. Sluiter and Templeton, 2008)

Some values of the substrate analysis are determined from OW material after water and methanol extraction. The recalculation factor is used to convert results after the extraction (g/100 g extracted DM) to values applying to the starting material (g/100 g DM). Hence, all results are referring to the original substrate.

$$E_W = \frac{SW_{WE}}{IW_S * \frac{DM_{BE}}{100}} * \frac{V_{St}}{V_{Sa}} * 100$$

$$E_M = \frac{SW_{ME}}{IW_S * \frac{DM_{BE}}{100}} * 100$$

$$F_E = \frac{100 - (E_W + E_M)}{100}$$

E_W [g/100g DM]	Content of water extractives
E_M [g/100g DM]	Content of methanol extractives
F_E	Extraction recalculation factor
SW_{WE} [g]	Sample weight of the water extractives after drying at 105 °C
SW_{ME} [g]	Sample weight of the methanol extractives after drying at 60 °C
IW_S [g]	Initial sample weight
DM_{BE}	Sample's dry matter before extraction
V_{St} [g]	Total volume of extractives before sampling for further analysis
V_{Sa} [g]	Actual volume of extractives after sampling for further analysis

Crude Protein:

Crude Protein is calculated from the subtraction of the unbound ammonium concentration from the total amount of nitrogen and multiplied with the factor 6.25. There is no conversion factor defined specifically for OW. Hence, crude protein content is estimated from the measured nitrogen using 6.25, which is generally applied for food and feed (Casal *et al.*, 2000).

$$\mathrm{NH4} = \frac{\mathrm{M}_N * \mathrm{Titer} * \frac{(\mathrm{V}_{\mathrm{NH4}} - \mathrm{V}_{\mathrm{B}})}{1000}}{\mathrm{IW}_{\mathrm{S}} * \frac{\mathrm{DM}_{\mathrm{AE}}}{100}} * 100 * \mathrm{F}_{\mathrm{E}}$$

$$\mathrm{TKN} = \frac{\mathrm{M}_N * \mathrm{Titer} * \frac{(\mathrm{V}_{\mathrm{TKN}} - \mathrm{V}_{\mathrm{B}})}{1000}}{\mathrm{IW}_{\mathrm{S}} * \frac{\mathrm{DM}_{\mathrm{AE}}}{100}} * 100 * \mathrm{F}_{\mathrm{E}}$$

$$\mathrm{CP} = (\mathrm{TKN} - \mathrm{NH4}_4) * 6.25$$

NH_4 [g/100g DM]	Unbound ammonium content
TKN [g/100g DM]	TKN content
CP [g/100g DM]	Crude protein content
M_N [g/mol]	Molar mass of 1 nitrogen atom (14.007 g/mol)
Titer [mol/L]	Molar concentration of the titration solution (HCl, 0.05 mol/L)
V_{NH4} (mL)	Titration volume of the sample with the NH_4-program
V_{TKN} (mL)	Titration volume of the pre-digested sample with the TKN-program
V_B (mL)	Titration volume blank
DM_{AE}	Sample's dry matter after extraction

Klason-Lignin:

(adapted from Sluiter *et al.*, 2004)

Klason-Lignin is defined as the part of lignin, which is insoluble after hydrolysis with concentrated acids.

$$LIG_{AIL} = \frac{\left(R - \left(\frac{CP * R}{F_E * 100}\right) - \left(\frac{Ash_{Ex} * R}{100}\right)\right)}{IW_S * \frac{DM}{100}} * 100 * F_E$$

LIG_{AIL} [g/100g DM] Acid insoluble Klason-Lignin content

R [g] Retentate after acid hydrolysis

Ash_{Ex} [g/100g extracted DM] Ash content of DM after water and methanol extraction

4. Results and Discussion

4.1. Substrate analysis

Table 6: Chemical composition of OMSW, BSG, and GW in g/100g DM.

	OMSW	BSG	GW
Dry Matter (g/100g)	42.7*	26.9 ± 0.17	62.8 ± 1.2
Ash	26.2 ± 0.9	3.4 ± 0.1	23.8 ± 0.7
Total Crude Protein (calculated)	9.5	16.0	8.2
Water Extractives	15.7 ± 0.3	19.7 ± 1.0	8.8 ± 0.4
Water Soluble Ash (calculated)	1.8	1.5	4.9
Water Soluble Crude Protein	2.1 ± 0.1	1.1 ± 0.2	0.9 ± 0.2
Water Soluble Substances by HPLC	4.7 ± 1.6	8.8 ± 0.4	4.0 ± 0.5
Lactic Acid	3.1 ± 1.0	1.1 ± 0.1	1.0 ± 0.1
Mono- and Disaccharides	0.5 ± 0.1	7.1 ± 0.3	0.9 ± 0.1
Undetermined Water Extractives (calculated)	7.1	8.3	(-1.0)
Methanol Extractives	8.1 ± 0.1	10.1 ± 0.8	3.8 ± 0.7
Insoluble Compounds (calculated)	76.2	70.2	87.4
Insoluble Ash	24.4 ± 1.3	1.9 ± 0.0	18.9 ± 0.2
Insoluble Crude Protein	7.4 ± 0.4	14.9 ± 1.5	7.3 ± 0.9
Lignocellulose (calculated)	43.7	31.5	55.1
Klason-Lignin	14.8 ± 0.9	8.1 ± 0.1	24.9 ± 0.1
Glucan (after hydrolysis)	16.5 ± 1.0	6.5 ± 0.7	16.9 ± 0.2
Xylan (after hydrolysis)	5.7 ± 0.1	6.8 ± 0.6	7.9 ± 0.1
Arabinan (after hydrolysis)	6.7 ± 0.0	10.1± 0.3	2.7 ± 0.5
Undetermined Insoluble Compounds (calculated)	0.7	21.9	6.1

**single measurement*

Within this master thesis three different substrates, OMSW, BSG as an example for industrial FW, and GW, were compared. Several analytical methods were used to find the most promising biomass for the enzymatic hydrolysis experiments. The dry matter values were

obtained by lyophilisation instead of the usual method of drying by 105 °C in an oven. The rest water content of the lyophilised material is still between 1 and 4% (w/w) (Grossmann *et al.*, 2018). However, bigger quantities can be dried with this method which is necessary to lower the bias caused by the waste's high heterogeneity. Table 6 depicts that the ash content of OMSW was the highest with 26.2 g/100 g DM but closely followed by GW with 23.8 g/100 g DM. BSG, however, contains less ash. In addition to ash, the crude protein content that could not be extracted was determined. In BSG even 14.9 g/100 g DM were found, twice as much as in OMSW and GW. However, the water extractable crude protein content was very low in all three substrates. Within all analysed wastes the total amount of water extractives was twice as high as the amount of methanol extractives. However, less than half of the water-extractable substances could be quantified by HPLC analysis. Water extractives from OMSW mainly consist of lactic acid (3.1 g/100 g DM) and less of mono- and disaccharides (cellobiose, maltose, glucose, arabinose, xylose). In contrast to OMSW, BSG contains less lactic acid and more mono- and disaccharides. The chromatographically detected sugars made 7.1 g/100 g DM compared to 1.1 g/100 g DM of lactic acid. Within GW, both values are equally low with around 1.0 and 0.9 g/100 g DM, respectively. The negative value of the calculated undetermined water extractives might be the result of the high uncertainties of the total amount of water extractives and the water-soluble substances measured by HPLC. The combination of GW's heterogeneity and the different methods used for substrate analysis could be another explanation for the negative result. Regarding the insoluble compounds, GW's Klason-Lignin content was, as expected, the highest with 24.9 g/100 g DM. OMSW followed with 14.8 g/100 g DM and BSG had the lowest content with 8.1 g/100 g DM. The acid hydrolysed glucan values of GW and OMSW are markedly higher than the corresponding values for the hydrolysable hemicellulose. In BSG, more hydrolysed arabinan and xylan are found than glucan. Furthermore, the sugars that are derived from acid hydrolysis exceed by far the water-extractable carbohydrates from OMSW and GW, but also BSG. The high ash content in OMSW and GW is a potential disruptive factor for the analytical methods but also for enzymatic hydrolysis. Considering just the ash content, BSG is a much better choice than the other two substrates. The protein content is another advantage of BSG because the amino acid chains provide MOs with energy and nitrogen and are not protected by lignin. However, most of the present proteins were not dissolved in water and alcohol. Table 6 also depicts that the total amount of HPLC detected substances are divergent from the gravimetrically measured water extractives. Thus, not all soluble compounds were identified. Nevertheless, the chromatographically measured high amount of lactic acid in the water extractives from OMSW implies that most of the simply accessible carbohydrates are already metabolized from MOs. However, in BSG the amount of water-extractable sugars is even a third of the acid-hydrolysed glucans and hemicellulose. This means that the accessible pentoses and hexoses

can be quadrupled by degrading the remaining polysaccharides. The data demonstrates that for GW and OMSW a hydrolysis step is necessary because of the low amount of water-extractable sugars and the higher values of carbohydrates that can be hydrolysed by concentrated acid. However, the enzymatic breakdown of GW's and OMSW's polymers will be more challenging due to the higher lignin content compared to BSG's. Especially GW contains much of this recalcitrant material because mainly lignocellulosic matter belongs to this type of waste. The determined Klason-Lignin values though should just be compared within each other and not taken as total values because during analysis filter paper was used instead of filter crucibles to separate the acid-insoluble substances. Hence, the acid-insoluble protein and ash content could not be measured. Instead, the values were calculated with the protein and ash determination before the acid hydrolysis. Furthermore, the ash should not be more than 10 % of the DM to obtain accurate results according to the protocol (Sluiter *et al.*, 2004).

The low water-soluble mono- and disaccharide and the high hydrolysable carbohydrate content of OMSW depict that a hydrolysis step is necessary to access the sugars. The hydrolysable glucans are probably celluloses from plant material in OMSW. The amount of hydrolysable xylan and arabinan can be considered as hemicellulosic components. Hence, degradation of these carbohydrates with cellulases and xylanases from the enzyme mix during the following enzymatic hydrolysis experiments is expected. The included glucanases are supposed to hydrolyse glucans, which do not have the beta-1-4-glucosidic linkages from cellulose. Measured hydrolysable carbohydrates of BSG suggest that more xylose-SUM and arabinose instead of glucose will be found during enzymatic hydrolysis. However, the amount of glucose can be doubled with the water-soluble mono- and disaccharides. The high amount of Klason-Lignin in GW will hamper the enzymatic degradation of present carbohydrates. Therefore, the released amount of sugars will not be as high as the enzymatic hydrolysis of OMSW despite the similar amounts of hydrolysable glucans, xylans, and arabinans. Comparing the analyzed substrates with results from the literature is difficult because the composition differs at different seasons, local areas, or generally the definition of the waste type. Nevertheless, the amount of cellulose mentioned by López-Gómez et al. (2020) is similar to hydrolysable glucan at this substrate analysis (Table 7). The hemicellulose content from the literature is comparable with just hydrolysable xylan or arabinan and the protein content is twice as high even if the extracted and insoluble protein amount are summed up. Cellulose, hemicellulose as well as Klason-Lignin of BSG from Paz et al. (2019) are much higher than determined in my thesis. Only the ash content is similar to the results in the literature. These differences can occur due to diverse degrees of degradation of the carbohydrates during the production process in different brewing plants. The composition of GW depends the most on seasonal variations but the results from the literature and this work are comparable (Cubas-

Cano *et al.*, 2020). The values of the ash content vary considerably, which could be due to more intense impurity removal.

Table 7: Chemical composition results from the literature in g/100g DM

	OMSW	BSG	GW
Cellulose	16.5	32.84 ± 0.08	24.8
Hemicellulose	5.3	25.85 ± 1.55	15.7
Klason-Lignin	-	17.57 ± 0.36	21.6
Proteins	17.7	-	18.2
Ash	-	3.26 ± 0.06	7.2
Reference	(López-Gómez *et al.*, 2020)	(Paz *et al.*, 2019)	(Cubas-Cano *et al.*, 2020)

4.2. Enzymatic hydrolysis of different substrates

In addition to the substrate analysis of the three waste types, enzymatic hydrolysis was carried out with GW, BSG, and OMSW to find the most promising substrate for further optimizations. The influence of pasteurization and enzyme mix addition were investigated at these experiments (Table 8). For pasteurization of the samples, 15 % DM slurry was heated until its core reached a temperature of 70 °C and then incubated for 1 hour at that temperature. This pre-treatment step was included to inhibit microbial growth and gain hydrolysates with high sugar content.

Table 8: Experimental settings for enzymatic hydrolysis experiments of different OWs. The cells with "X" are the chosen settings for the approach.

Abbreviation	Pasteurized	Enzymes
U		
U-E		X
P	X	
P-E	X	X

4.2.1. Garden waste

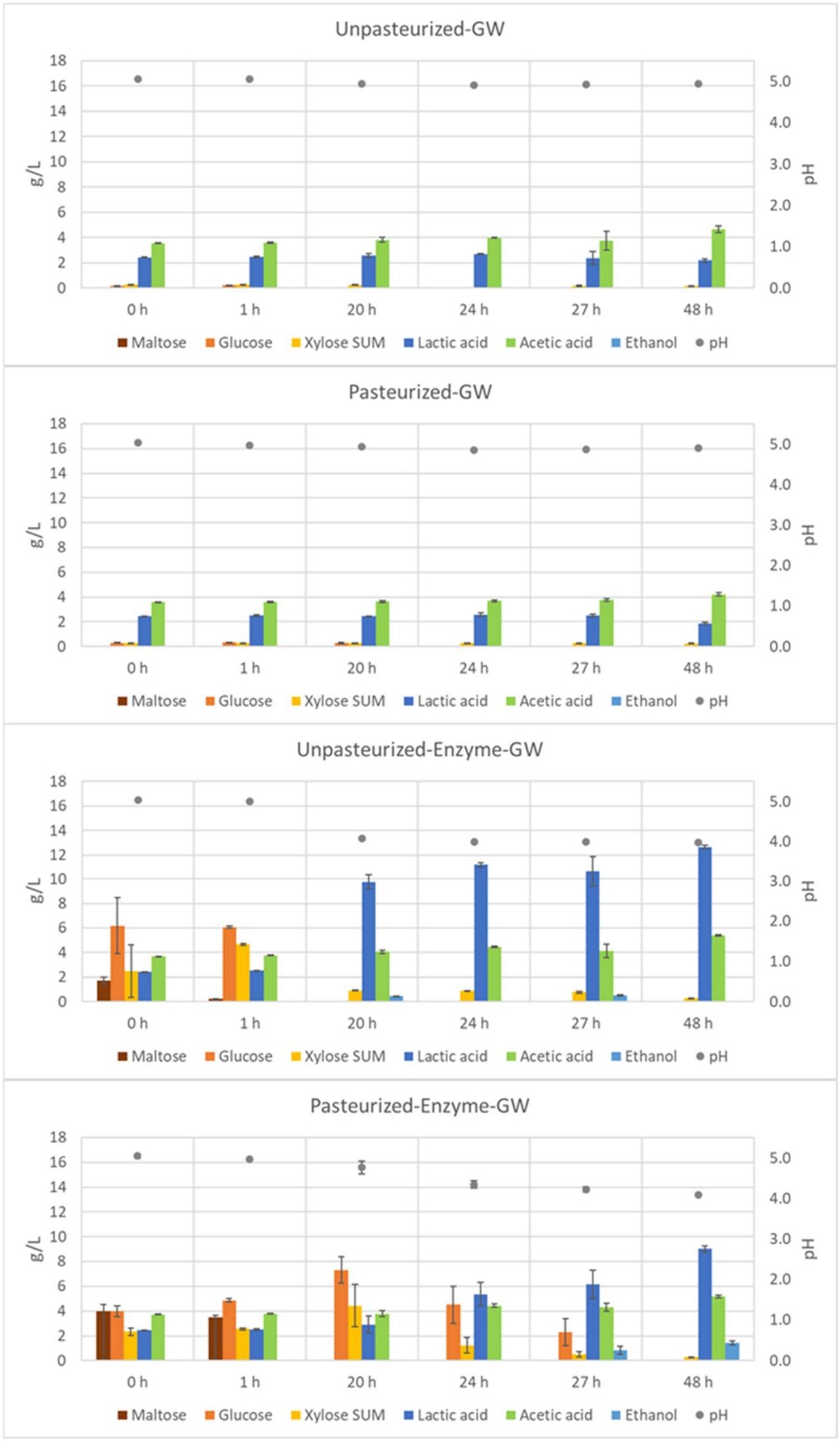

Figure 4: pH and concentration of maltose, glucose, xylose-SUM, lactic acid, acetic acid, and ethanol at certain timepoints of enzymatic hydrolysis of garden waste. The error bars are the SD values of triplicates.

In the pasteurized and unpasteurized reference samples of GW, no change of organic acid concentrations was observed (Figure 4). Lactic and acetic acid were at around 2 and 4 g/L respectively. Additionally, the pH remained constantly at pH 5.0 and the sugar concentration was around the detection limit (U-GW and P-GW). However, by adding enzyme solution to the unpasteurized GW, lactic acid rose sharply within 24 h from 2 to 11 g/L and elevated even to 13 g/L after 48 h. Additionally, the pH dropped during the accumulation of lactic acid from pH 5.0 to 4.0. Unlike lactic acid, the acetic acid concentration raised only by 1 g/L from the starting point of 4 g/L, and glucose was even decreasing until 19 h from 6 g/L below the detection limit. The xylose-SUM concentration fell as well from 5 to 1 g/L within the first 19 h but the residual amount was conserved until the end. Regarding the enzymatic treated and pasteurized samples, an accumulation of glucose from 5 to 7 g/L and xylose-SUM from 3 to 4 g/L was achieved after the 2 h equilibration phase until 19 h. Afterwards, the concentration of these sugars however fell to below the detection limit until 48 h. During the increase of the carbohydrates, the organic acid content remained the same. Nevertheless, simultaneously to the following decrease of the sugars lactic acid raised from 3 to 9 g/L and acetic acid from 4 to 5 g/L.

As expected from the substrate analysis results, the high degree of lignocellulosic material in this kind of waste hampered degradation in such a short time. Therefore, no change in the detectable substances was observed at U-GW and P-GW. However, MOs are definitely active in GW because lactic and also in smaller amounts acetic acid was immediately produced with the addition of enzyme solution which already contains carbohydrates. Unlike organic acids, sugars were not able to accumulate in the supernatant of U-E-GW due to the microbial activity. The residual xylose-SUM concentration just remained constant because the MOs probably prefer hexoses instead of pentoses. Another explanation could be that cellulose was not as quickly degraded as hemicellulose, which is easier to access, and xylose-SUM is just as quickly consumed by microbes as released by enzymatic hydrolysis. P-E-GW demonstrated that carbohydrate accumulation can be achieved by pasteurization. However, the increase was just 2 g/L and the microbial growth was inhibited only for the first 19 h. Comparing all four approaches, adding enzymes is effective for GW but lactic acid should be the target substance instead of mono- and disaccharides. In this case, pasteurization is not necessary because the increase of lactic acid by 11 g/L at U-E-GW is almost twice as high as in P-E-GW. Cubas-Cano *et al.* (2020) achieved a concentration of 103 g/L glucose with enzymatic hydrolysis. However, steam-explosion was used as pre-treatment, which needs high temperatures and much energy. Both are elevating the CO_2 emission for the enzymatic hydrolysis process. Additionally, inhibitors like 5-hydroxymethyl furfural are often formed during steam-explosion and chemical pre-treatment (Yu *et al.*, 2018). These compounds have to be removed before using the hydrolysate as a medium for fermentations.

4.2.2. Brewer's spent grain

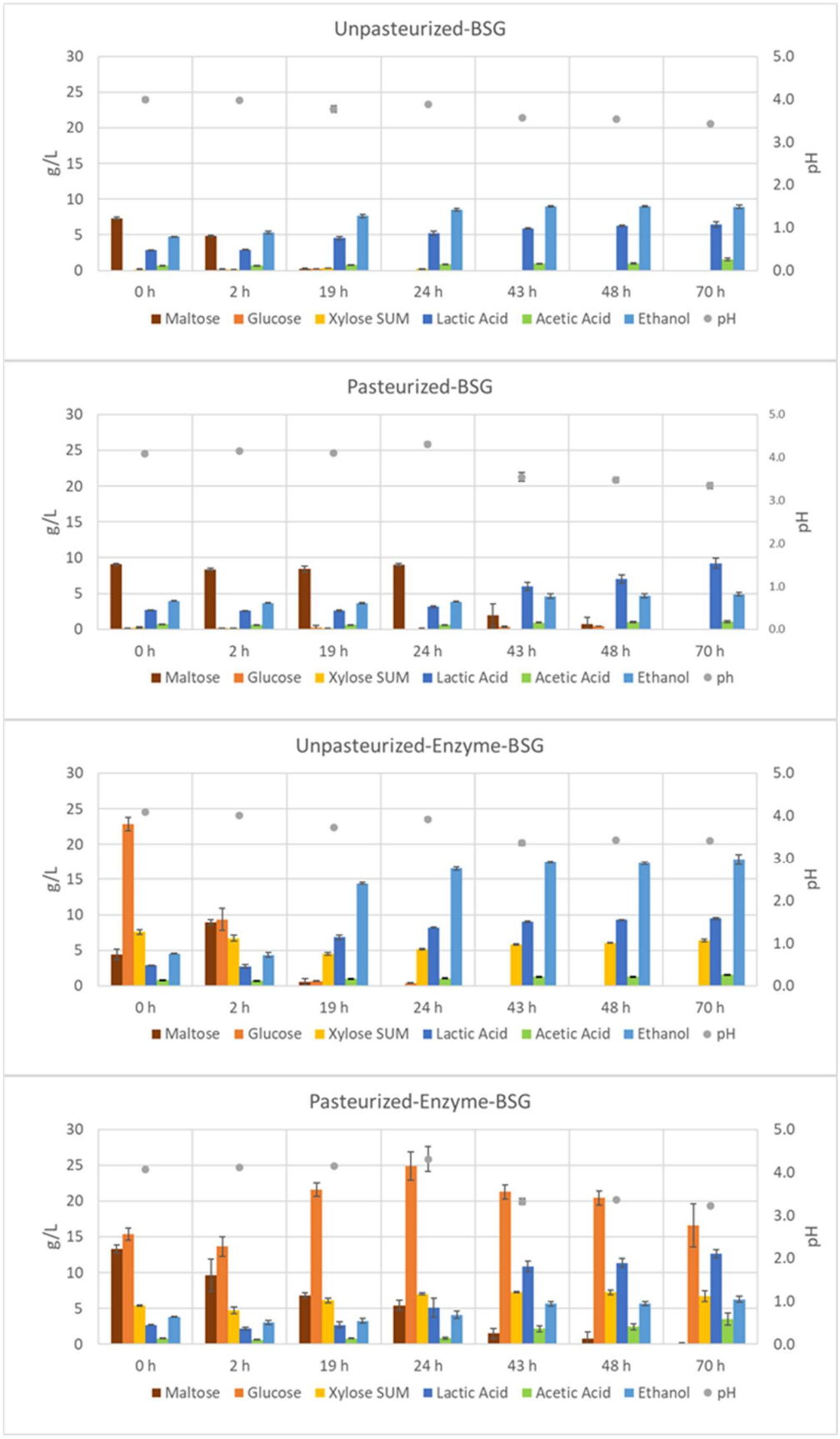

Figure 5: pH and concentration of maltose, glucose, xylose-SUM, lactic acid, acetic acid, and ethanol at certain timepoints of enzymatic hydrolysis of brewer's spent grain. The error bars are the SD values of triplicates.

In U-BSG, ethanol and lactic acid rose during the initial 24 h from 5 to 9 g/L and 3 to 5 g/L respectively (Figure 5). Afterwards, the concentration of these two substances elevated only by around 1 g/L until the end. During the increase of the microbial products, the pH decreased from 4.0 to 3.8. At the same time, the maltose level fell from 7 g/L below the detection limit and remained there until 70 h. The compounds in P-BSG had approximately the same starting concentrations as at U-BSG and stayed constant for 24 h. After this initial phase, the maltose concentration fell close to the detection limit within another 24 h and lactic acid exceeded the ethanol concentration and rose to 9 g/L until 70 h. The ethanol level however increased only by 1 g/L. Regarding U-E-BSG and P-E-BSG glucose and xylose were added together with the enzyme solution as already seen at the GW hydrolysis samples (4.2.1. Garden waste). At U-E-BSG, a glucose concentration of 23 g/L was measured at 0 h but after 2 hours of equilibration, the values were at 9 g/L. After 2 h incubation maltose had the same concentration as glucose but after 24 h both substances decreased below the detection limit. While the level of the carbohydrates fell ethanol rose from 5 to 17 g/L and lactic acid from 3 to 8 g/L. Like in U-BSG, the concentrations of the microbial product increased after 24 h only about 1 g/L. In P-E-BSG, the glucose concentration elevated after the equilibration phase from 14 g/L to 25 g/L at 24 h and slightly fell afterwards to 17 g/L. Despite pasteurization, the maltose level decreased from 10 g/L below the detection limit but the lowest point was only reached after 70 instead of 24 hours in U-E-BSG. The lactic acid and ethanol concentrations stayed constantly at respectively 2 and 3 g/L until 19 h. Afterwards, the organic acid rose to 13 g/L and the alcohol to 6 g/L. Although, their concentrations started to increase 4 hours before the highest glucose concentration was reached. Acetic acid marginally increased from 1 to 4 g/L during the whole process. Additionally, the pH values decreased with increasing acid concentrations at all approaches and the level of xylose-SUM did not change significantly during hydrolysis.

BSG is the only substrate with detected carbohydrates already at the beginning of the incubation. MOs are able to metabolize them and therefore, the lactic acid and ethanol concentrations were increasing without enzyme addition. However, the changes of the microbial products were different depending on whether the substrate was pasteurized or not. At U-BSG microbial production of lactic acid and ethanol increase almost in parallel but P-BSG depicts a shift in the metabolite pattern after pasteurization. That could be caused by the enrichment of spore-forming bacteria that predominately form lactic acid. This effect was also observed in the enzymatically hydrolysed samples. However, the constant xylose-SUM level indicates that either the present MOs are not able to metabolize pentoses or the xylose-SUM substances are as quickly consumed as they were generated. Nevertheless, pasteurization inhibited apart from ethanol also the lactic acid generation for 19 hours and led to an increase of glucose concentration by 11 g/L at P-E-BSG. Enzymatic hydrolysis of dissolved maltose contributed to this glucose peak because maltose concentration decreased while the microbial

product's level remained the same within P-E-BSG. P-BSG samples showed that after the inhibition phase MOs were consuming dissolved maltose. Unlike in the enzymatic hydrolysis of GW, in P-E-BSG were even after 70 h 17 g/L glucose and 7 g/L xylose-SUM left. Nevertheless, the carbohydrate addition from the enzyme solution has to be considered. Additionally, the biggest increase of ethanol was achieved without an energy-consuming pasteurization procedure and was higher than the highest glucose increase (11 g/L in P-E-BSG). Ethanol concentration increased by 14 g/L at U-E-BSG and achieved a total amount of 18 g/L. Paz *et al.* (2019) received similar glucose and xylose final concentrations with 18 and 6 g/L, respectively. The substrate from the literature was even autoclaved before enzymatic hydrolysis. The high input of sugars from the enzyme solution used in my thesis has to be considered. However, the comparable amounts of carbohydrates indicate that pasteurization could be sufficient to release as much hydrolysate as from a sterile substrate.

4.2.3. Organic municipal solid waste

Previously collected data

The following results are based on unpublished data that were collected by Annika Putz before I joined the working group. Since my further experiments follow up on these findings, they are included and discussed here.

As already seen in the substrate analysis, water-soluble sugar concentration is low in OMSW. At U-OMSW and P-OMSW it is even below the calibration range. However, U-OMSW samples contained 24 g/L lactic acid, 5 g/L acetic acid, and 2 g/L ethanol at the beginning of enzymatic hydrolysis (Figure 6). Lactic acid remained around the same concentration during the whole process and ethanol stayed for the first 48 h constant and then decreased below the detection limits. In contrast, the acetic acid level raised to 13 g/L after 72 h. Despite the increase of the acid concentration, the pH was always at approximately 4.8. Regarding P-OMSW, the pH decreased from pH 5.0 to 4.5 within the first 24 h and did not change markedly afterwards. Additionally, acetic acid and ethanol had the same initial concentrations as within U-OMSW but the lactic acid reached lower concentrations (23 g/L). Within P-OMSW, lactic acid constantly increased to 34 g/L after 72 h. The acetic acid concentration rose also to 8 g/L after 72 h although it stayed the same until 24 h. Ethanol content did not change significantly during the process. From the two approaches with the enzyme addition, the first measured values were from 2 h. However, they were similar to the ones from U-OMSW and P-OMSW, and glucose and xylose-SUM were detected as well. The former had an initial concentration of 15 g/L and the latter around 5 g/L within U-E-OMSW and P-E-OMSW. Glucose was immediately consumed within 24 h and lactic acid simultaneously increased to 44 g/L within U-E-OMSW. Afterwards, the monosaccharide level stayed below the detection limit, but the

lactic acid concentration fell to 24 g/L after 72 h. The amount of xylose-SUM did not change as quickly as glucose at this approach. The pentose decreased however below the detection limit after 72 h like the hexose. Apart from lactic acid also acetic acid increased at U-E-OMSW constantly to a final concentration of 13 g/L. The ethanol concentration was not that consistent. It remained the same until 24 h, rose by 5 g/L, and declined below the detection limit after 72 h. The lactic acid values from P-E-OMSW varied a lot but a constant increase to the final 41 g/L can be noted. Acetic acid had similar high standard deviations. However, this organic acid achieved just a total concentration of 10 g/L.

Unlike at U-E-OMSW, within the pasteurized samples the sugar levels remained approximately the same until 24 h but the glucose content dropped markedly within the next 24 h and reached almost 0 g/L after 72 h. The xylose-SUM concentrations were maintained at around 2 g/L after 48 h and ethanol has not changed during the fermentation process.

OMSW does not contain such high concentrations of carbohydrates as BSG did, but the rising acetic acid level at U-OMSW indicates that the naturally occurring MOs can utilize available nutrients to grow. They either hydrolyse the remaining organic material and consume it immediately or they are using the produced lactic acid and ethanol for their metabolism. However, the spore-forming bacteria enriched through pasteurization seemed to preferably produce lactic acid, which was already observed at P-BSG and P-E-BSG and now at P-OMSW. Additionally, the rise of organic acids within samples without enzyme addition proves that the MOs are able to degrade OMSW without external enzymes, unlike GW. Apart from the higher heat resistance, the lactic acid-producing microbes metabolize the glucose faster than the other organisms. Total glucose concentration within U-E-OMSW derived from the enzyme mix and enzymatic hydrolysis was converted to lactic acid within 24 h. Afterwards, the four-carbon organic acid is used as a substrate to generate acetic acid and ethanol. This could indicate that most of the potential mono- and disaccharides are already hydrolysed at this point. The rising lactic acid concentration during the presence of glucose at P-E-OMSW supports this assumption. Glucose concentration remained constant at the beginning (P-E-OMSW), probably due to the microbial growth inhibition by pasteurization, which was already demonstrated in GW and BSG. Additionally, the slow decline of xylose-SUM was comparable to the experiments with BSG, probably for the same reasons (see 4.2.2 Brewer's spent grain). Another common ground to the previous substrates was the inability to accumulate high amounts of carbohydrates. Instead, the highest raise (21 g/L lactic acid) was again a microbial product and achieved at conditions without pasteurization i.e. less energy consumption. Furthermore, comparing all four OMSW experiments, it has to be noted that the pH mostly correlated with the lactic acid concentration. That makes the pH a good indicator for lactic acid monitoring.

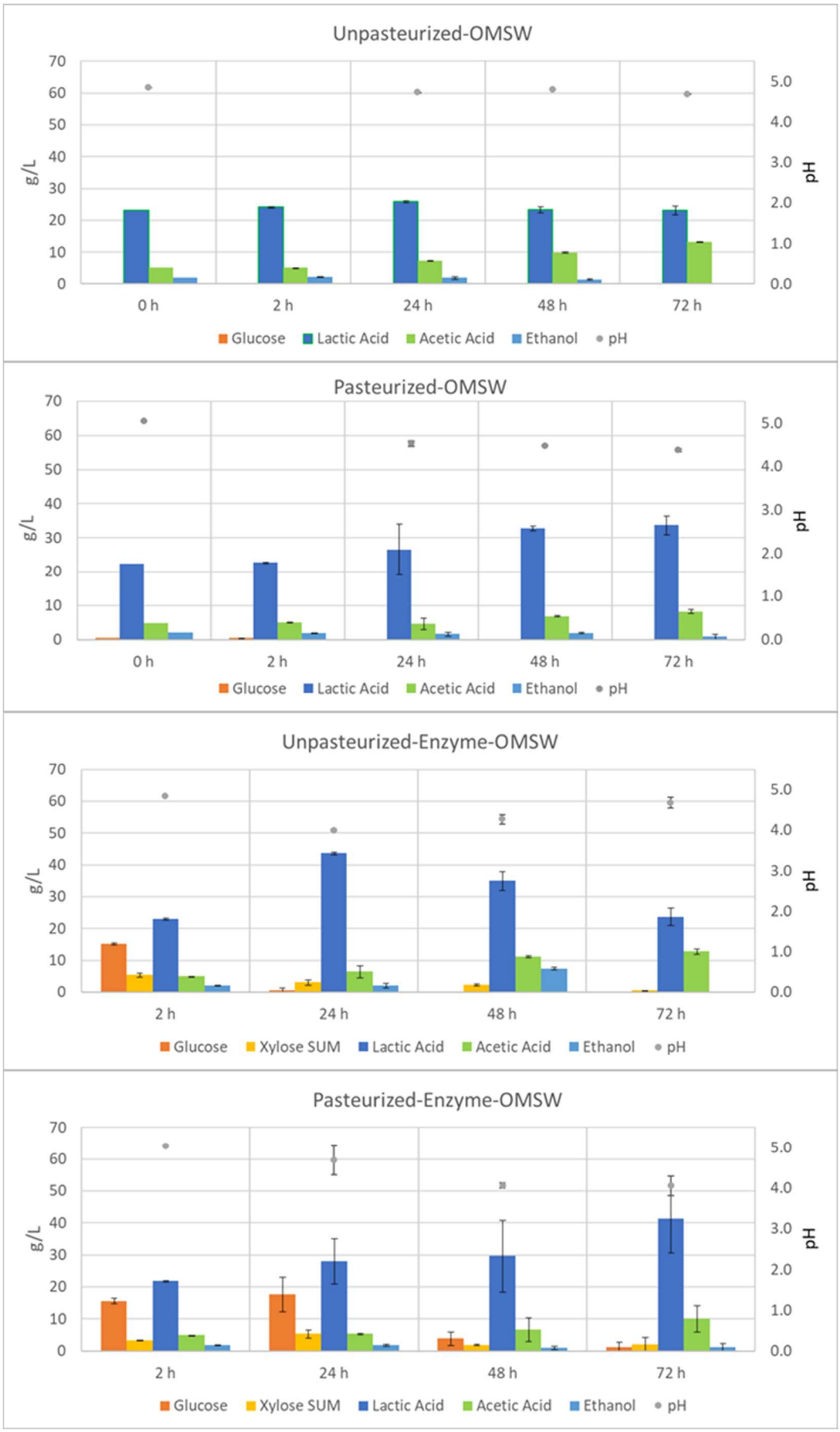

Figure 6: pH and concentration of maltose, glucose, xylose-SUM, lactic acid, acetic acid, and ethanol at certain timepoints of enzymatic hydrolysis of organic municipal solid waste. The error bars are the SD values of triplicates.

Replication of U-E-OMSW in a parallel bioreactor system:

During enzymatic hydrolysis in Pyrex flasks, the highest lactic acid concentration was reached with unpasteurised slurry after enzyme addition. By continuously monitoring the pH with the DASGIP reactor system, lactic acid production can be estimated from pH changes. This should help to identify the timeframe of lactic acid production during enzymatic hydrolysis. Additionally, samples were more often drawn for manual pH measurement and determination of the soluble substances.

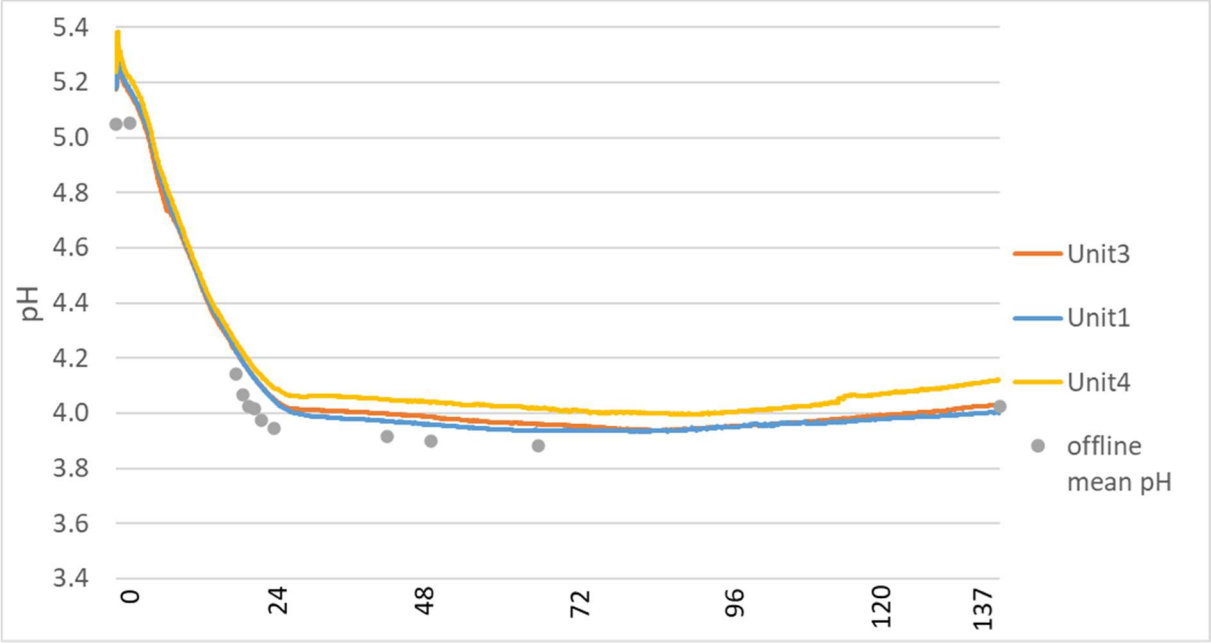

Figure 7: Online pH-values of U-E-OMSW replication in Unit 1, 3, and 4 of the parallel bioreactor system and the mean value of the offline pH measurement.

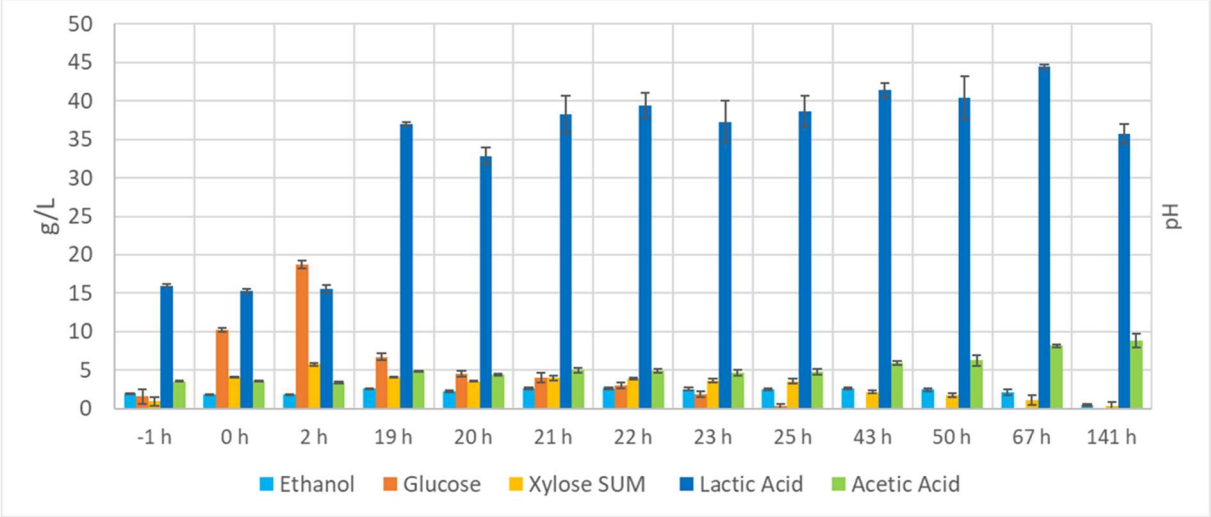

Figure 8: Concentration of glucose, xylose-SUM, lactic acid, acetic acid, and ethanol at certain timepoints at U-E-OMSW replication in the parallel bioreactor system. Timepoint "-1 h" depicts the measured values before the pre-incubation. Error bars show SD values of triplicates.

The continuous pH measurement exhibited an almost linear decrease of the pH from 5.3 to 4.0 within the first 24 h (Figure 7). A stationary phase followed the pH drop until approximately 96 h and at the end, the pH is slightly rising by around 0.1 pH units. Furthermore, the results of the three bioreactors differ at the most around 0.1 pH units and are consistent with the manually derived ones.

Before pre-incubation measured glucose concentration was at 2 g/L (-1 h) and afterwards at 10 g/L (0 h) (Figure 8). The level was rising to 19 g/L after two hours of stirring. However, at 19 h only 7 g/L glucose was left and within the next 6 hours, the concentration fell below the detection limit where it remained until the end. Similar to glucose, xylose-SUM rose during the pre-incubation from 1 to 4 g/L and reached its peak of 6 g/L two hours after the DASGIP start. Xylose-SUM decreased not as fast as glucose. The pentoses, however, decreased afterwards consistently to almost 0 g/L at 141 h. Regarding the microbial products, ethanol stayed at around 2 g/L until 67 h and fell below the detection limit until the end and the acetic acid concentration rose from 4 to 9 g/L within the whole incubation. Like all the other substances produced by MOs, the lactic acid level was the same until 2 hours after the DASGIP started. Nevertheless, it increased from 16 g/L at 2 h to 37 g/L at 19 h. Lactic acid concentration varied between 19 to 25 h but a small raise to 39 g/L was achieved. Lactic acid concentration further elevated to 44 g/L at 67 h and declined afterwards to 36 g/L.

In contrast to previous hydrolysis experiments, at the beginning low glucose and xylose concentration were measured. Insufficient mixing before sampling could be the reason for the unusual carbohydrate concentrations. Furthermore, the lactic acid concentration decreased in this experiment only after 67 h and not already after 24 h as within U-E-OMSW in conical flasks (Figure 6). Different starting points for the decline of lactic acid are however recognized in the following hydrolysis experiments as well. Besides that, the measured microbial products were at similar levels as within flask experiments. Additionally, the correlation between lactic acid concentrations and manually and automatically determined pH could be confirmed. Hence, we can assume that the lactic acid concentration was increased almost linear until approximately 24 h and thus, also its highest raise ended at this point.

4.3. Buffer capacity of OMSW

Since GW had the lowest yield in obtained organic acids and carbohydrates and BSG has already other applications such as feed for cattle, it was decided to optimize the hydrolysis of OMSW. The major problem during previous experiments was the consumption of the hydrolysed sugars by MOs. Thus, one approach to inhibit microbial growth was lowering the starting pH to 4.0. Hence, the supernatant of the OMSW slurry was titrated to pH 2.0. Additionally, the titration curve to pH 10.0 was measured to investigate the buffer capacity of OMSW between pH 2.0 and 10.0. This facilitates interpretations of pH alterations during enzymatic hydrolysis.

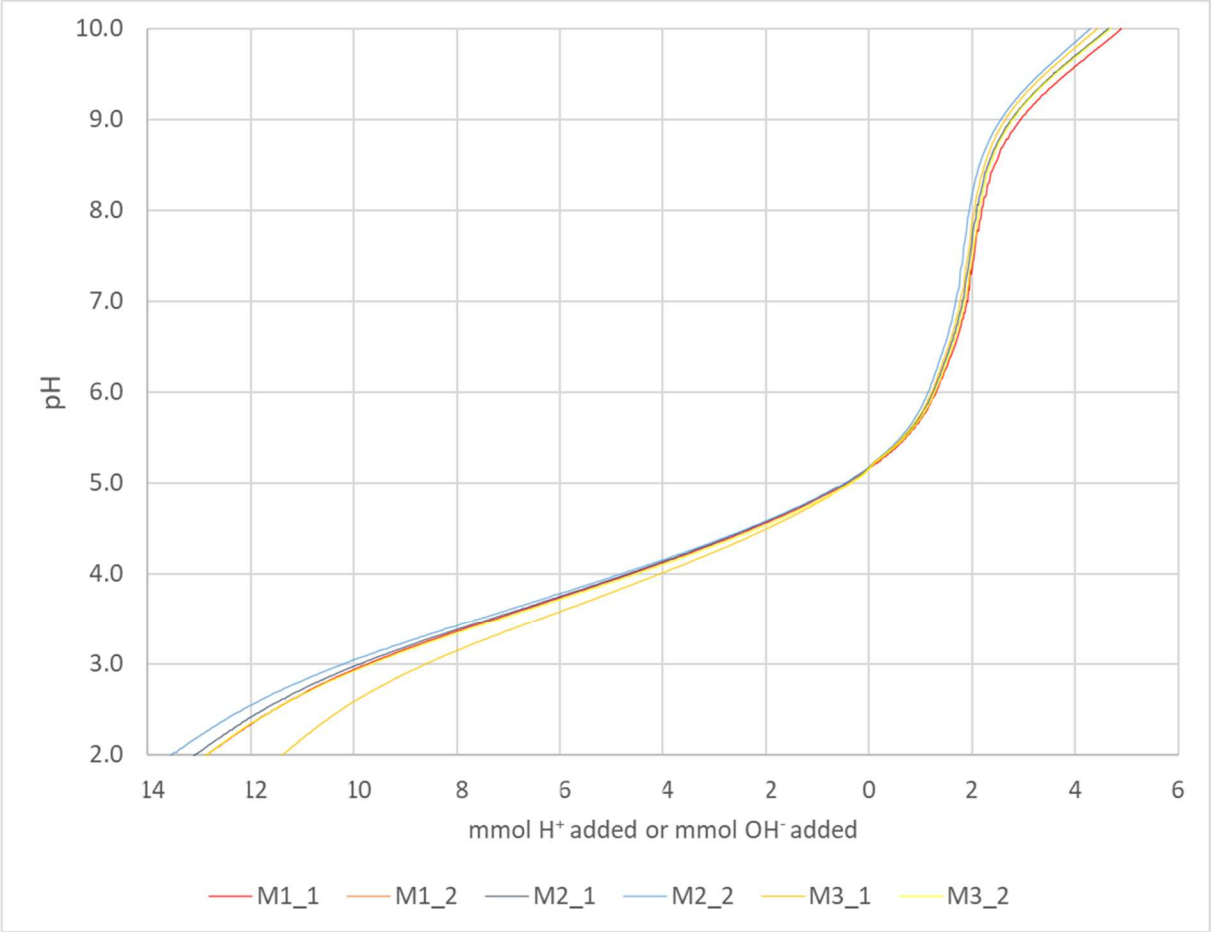

Figure 9: pH over mmol H^+ and OH^- added during titration of OMSW's supernatant to pH 2 and 10. "M1_1" and "M1_2" are the results from the first, "M2_1" and "M2_2" from the second, and "M3_1" and "M3_2" from the third slurry.

Table 9: R^2-values from the samples titrated to pH 2 calculated by Microsoft Excel.

Sample	**M1_1**	**M1_2**	**M2_1**	**M2_2**	**M3_1**	**M3_2**
R^2	0.9924	0.9922	0.9926	0.9928	0.9925	0.9927

The starting pH of all three slurries and their duplicates at titration to pH 2.0 was around pH 5.1 (Figure 9). The further development of the titration curve is linear until pH 3. Afterwards, the line slightly changed direction until pH 2.0. For the whole acid titration trend lines were set and their R^2 -values were calculated. They were at 0.99 (Table 9) for every titration to pH 2.0. Drawing all samples in one graph demonstrates that the starting pH of the different slurries was very similar just the ends were shifted from 11.4 to 13.6 added mmol HCl. According to the bias of the curves, titration to pH 10 was comparable to acid titration. The starting pH was around 5.2 and the course of the titration curve was similar in all samples (Figure 9). The longer the measurement took the more shifted were the different curves. Nevertheless, the required amount of NaOH to reach pH 10 varied only between 4.3 and 4.8 mmol. However, the course of the alkaline titration was different from the pH 2.0 titration. From the initial pH to approximately pH 6 the measured points are forming a plateau, followed by a sharp rise until pH 8 and the finishing titration went almost linear to pH 10.0.

R^2-values close to 1 approve that the titration curve from the starting pH 5.1 to pH 2.0 can be assumed as linear. This behaviour was caused by the mix of different organic acids in the supernatant. Lactic acid is present in the highest concentration with 17 g/L but due to the similar pK_a-value (Table 10) to the other containing substances, it is not possible to observe its influence. The concentration of phosphate is compared to the other acids very low and the contribution to the linear titration curve is marginal. The close starting pH results of the different slurries and their duplicates indicate the similar composition of present acids and bases which was confirmed by the HPLC, phosphate, and unbound NH_4 analysis (Table 10). The differences during the analysis could be caused by measurement errors of the pH meter or by the titration device that added not the exact amount of acid. Weak bases and acids have a buffering range from pK_a ± 1 pH unit. Hence, acetic and butyric acid with pK_a values of 4.75 and 4.82, respectively, are buffering between 3.75 to 5.82. This is the reason for the delayed increase of pH from the slurry compared to titration of pure lactic acid where the plateau stops at pH 5 (Ube *et al.*, 2017). The measured ammonium with pK_a 9.25 explains the plateau from around pH 8 until the end at pH 10. Konermann (2017) measured the buffer capacity of ammonium acetate and obtained a similar titration curve, which supports the above assumptions.

Table 10: Concentrations and pK_a-values of organic acids and ammonia from the supernatant of the three buffer capacity slurries measured by HPLC (Lactic, acetic, and butyric acid), phosphate determination kit (Phosphatic acid), and unbound NH_4 determination.

	pK_a-values	Slurry 1 [g/L]	Slurry 2 [g/L]	Slurry 3 [g/L]
Lactic acid	3.90	17	17	17
Acetic acid	4.76	4	4	4
Butyric acid	4.82	2	2	2
Phosphoric acid	2.14; 7.20; 12.37	0.16	0.15	0.15
Ammonia	9.25	0.97	0.99	1.01

4.4. Inhibition of microbial growth during enzymatic hydrolysis of OMSW

Slurry preparation, the subsequent pasteurization, and the enzyme addition are described in "4.2 Enzymatic hydrolysis of different substrates". Here, it was tried to inhibit or minimize microbial growth by a lowered pH or elevated incubation temperature at 50 °C. Lowered pH was reached by adding lactic acid as organic or sulfuric acid as mineral acid. These measures just slow microbial activity. To obtain results from enzymatic hydrolysis without interfering MOs, an additional experiment was implemented with autoclaved OMSW before the enzyme addition. The different settings of the inhibition experiments are shown in Table 11:

Table 11: Operating conditions for inhibition experiments with OMSW: Pasteurization as pre-treatment; Lactic acid addition for pH reduction to pH 4.0; Sulfuric acid addition for pH reduction to pH 4.0; 39 °C as incubation temperature; 50 °C as incubation temperature

Abbreviation	Pasteurization	Lactic acid	Sulfuric acid	39 °C	50 °C
U-E				X	
U-LA		X		X	
P-LA	X	X		X	
U-SA			X	X	
P-SA	X		X	X	
U-50					X
P-50	X				X

4.4.1. Lowering of the initial pH to pH 4.0

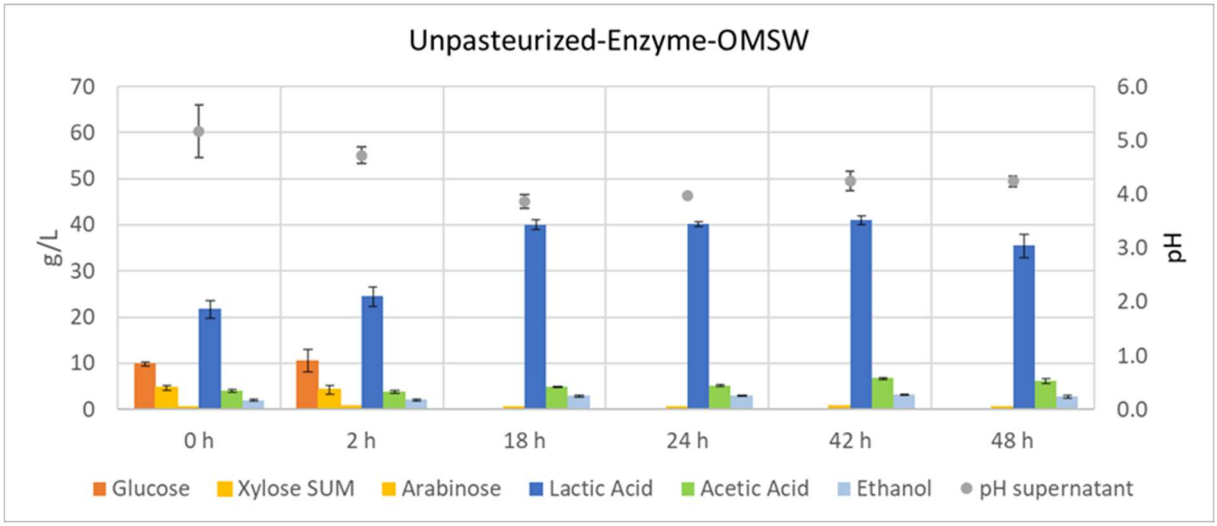

Figure 10: pH and concentration of glucose, xylose-SUM, lactic acid, acetic acid, and ethanol at certain timepoints. The error bars are the SD values of triplicates.

Addition of lactic acid:

The reference samples (U-E) for the three inhibition approaches were incubated under the same conditions as U-E-OMSW (4.2.3 Organic municipal solid waste). Within these samples, a marked rise from 25 to 40 g/L of lactic acid was achieved until 18 h (Figure 10). The acetic acid concentration increased as well but just about 2 g/L even after 48 h. Unlike the organic acids, glucose and xylose-SUM declined from 11 and 5 g/L, respectively, below the detection limit after 18 h. Additionally, the pH fell from above 5.0 to below 4.0 but increased afterwards slightly despite the rising organic acid concentration. In all inhibition samples between 2 and 3 g/L ethanol was found and the concentration remained constant during hydrolysis. Additionally, every sample except U-E-SA (Figure 12) and the autoclaved ones (Figure 14) had a drop of lactic acid concentration from 42 to 48 h by about 5 g/L.

Regarding the first inhibition experiment U-E-LA, the soluble substances stayed stable for 18 h (Figure 11). The pH remained the same until the end of the hydrolysis despite the accumulation of lactic acid after 18 h from 38 to 50 g/L within 24 h. However, the addition of lactic acid increased its starting concentration by about 10 g/L. During the rising of microbial products, the glucose concentration decreased from 14 g/L below the detection limit. The pasteurized P-E-LA samples showed a different development. The organic acids remained constant over the whole time and glucose was increasing from 15 g/L after equilibrium to 27 g/L after 42 h. Furthermore, the pH increased while the measured acids stayed the same. The xylose-SUM concentrations changed similar to glucose despite the smaller changes from maximal 3 g/L in both lactic acid-treated approaches.

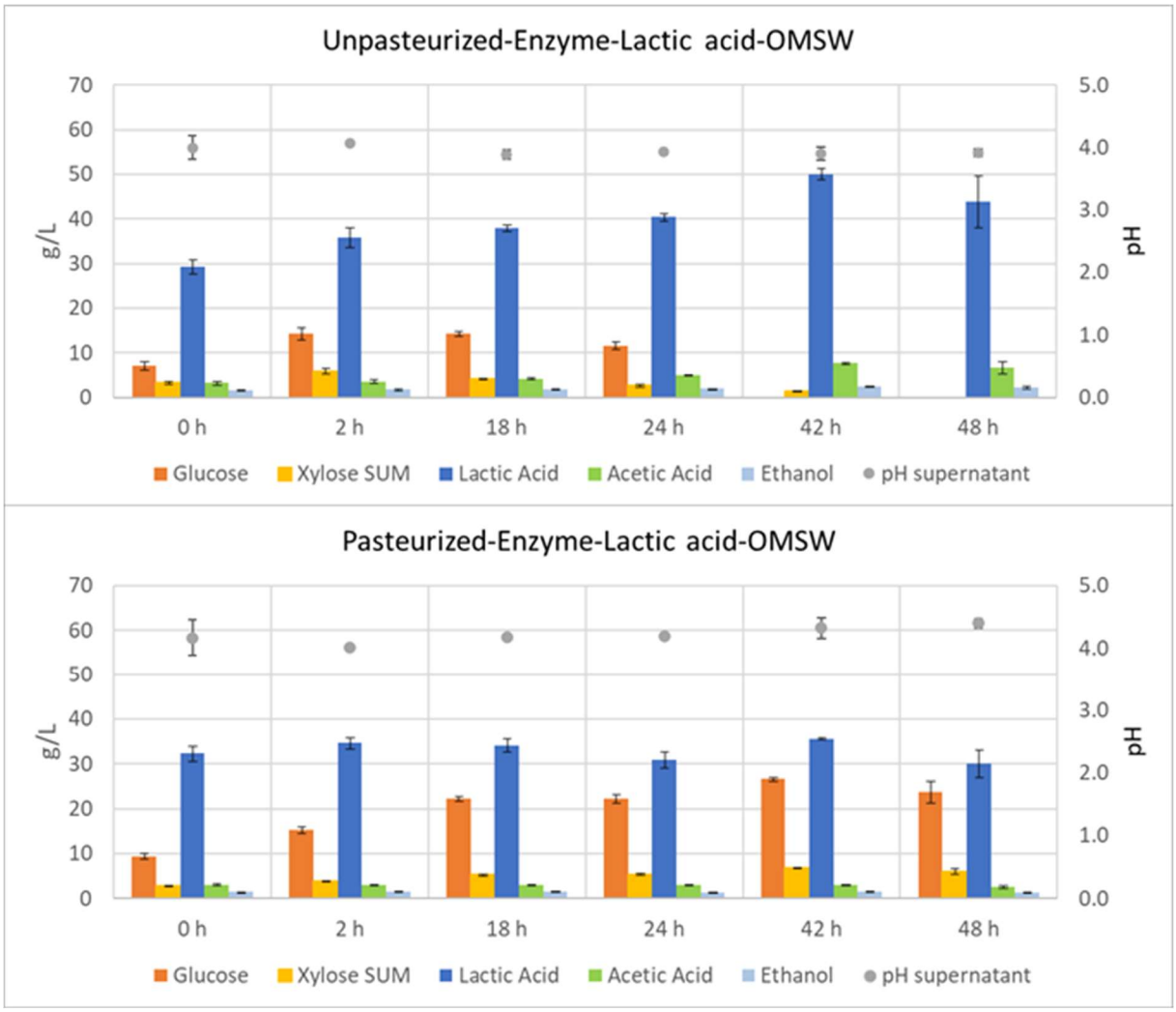

Figure 11: pH and concentration of glucose, xylose-SUM, lactic acid, acetic acid, and ethanol at certain timepoints. The error bars are the SD values triplicates.

The consumption of all sugars added with the enzyme mixture and released by enzymatic hydrolysis was again noted in the reference samples. This unwanted effect was slowed down by the lower starting pH but only for 24 h. The present MOs had to adapt during this first period to the lowered pH but afterwards, they were able to metabolize the carbohydrates. Mainly lactic but also acetic acid producers seemed to be the dominant microbes in the OMSW. Nevertheless, the constant concentrations of sugars together with constant products from microbial growth during the first 24 h at the unpasteurized samples suggest that the added enzymes cannot work properly under these conditions. However, the combination of lowered pH and pasteurization inhibits the microbial growth more efficiently and achieved a maximal increase of glucose and xylose-SUM of 12 and 3 g/L respectively. The sudden decrease of all measured substances from 42 to 48 h within the reference as well as the acid-treated samples could be caused by aerobic metabolism initiated by a declining amount of substrate from sampling. However, MOs could also have influenced the pH to reach their optimum. Afterwards, they started to digest all the present substances which would also explain the

constant pH despite decreasing acid concentrations from 42 to 48 h within U-E, U-E-LA, and P-E-LA.

Addition of sulfuric acid:

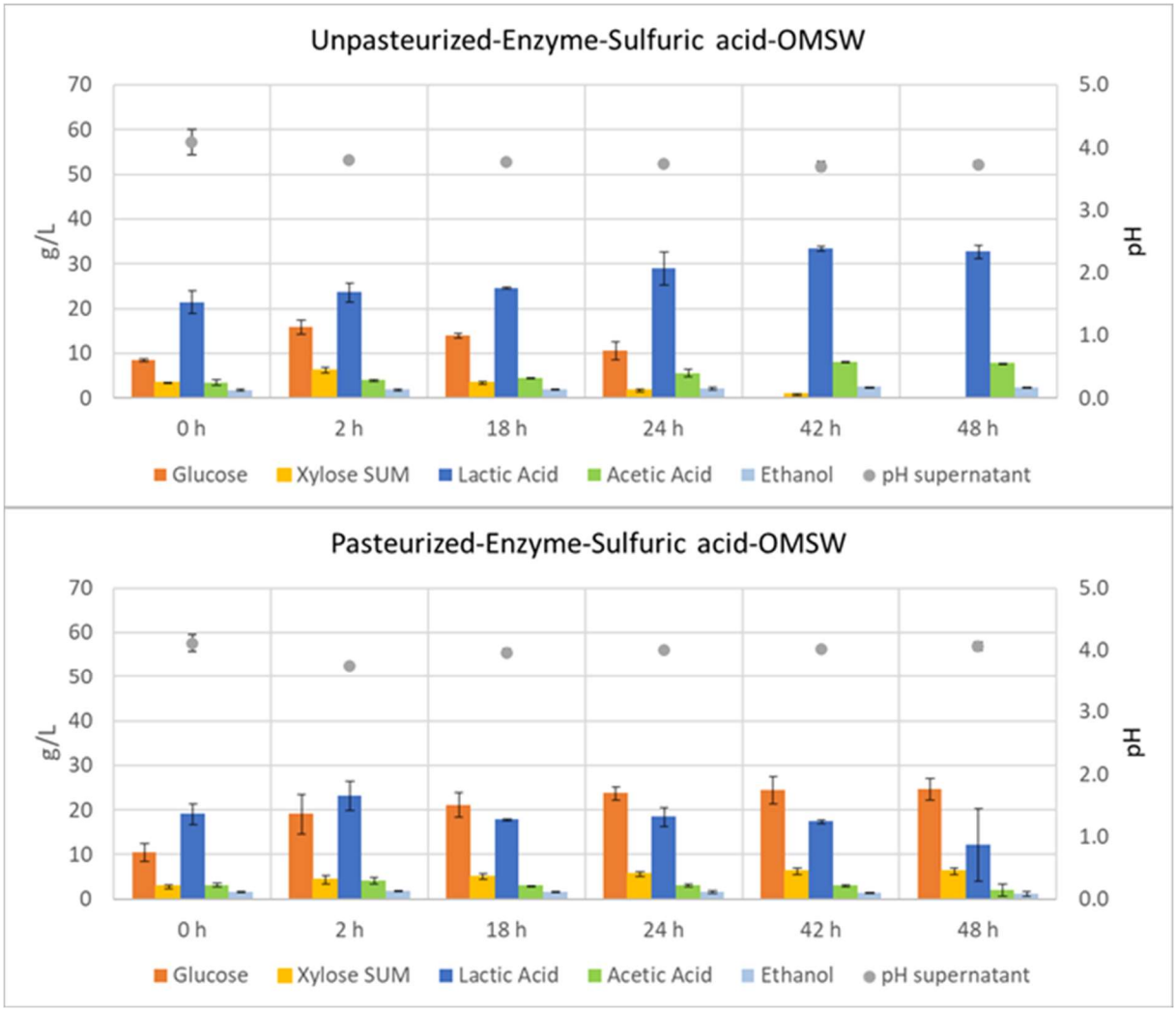

Figure 12: pH and concentration of glucose, xylose-SUM, lactic acid, acetic acid, and ethanol at certain timepoints. The error bars are the SD values of triplicates.

The inhibition experiment with sulfuric acid, but without pasteurization, showed almost the same results as U-E-LA. For the first 18 h, the production of lactic and acetic acid was inhibited, then both concentrations raised (Figure 12). The highest lactic acid concentration at 42 h was 33 g/L in contrast to 47 g/L at U-E-LA. The content of acetic acid was almost the same with 7 g/L in both approaches. Unlike in the lactic acid-treated samples, there was no measured decay of organic acids from 42 to 48 h. P-E-SA though behaved even more differently compared to P-E-LA. After 42 h, the glucose and xylose-SUM concentration reached their peaks with 24 g/L and 6 g/L, respectively, but the variability of the glucose values was higher than from P-E-LA. Furthermore, the lactic acid concentration decreased between 2 and 18 h from 23 to 18 g/L instead of staying constant like in the P-E-LA. In addition to lactic acid, acetic acid content was lowered during the same period. However, a reduction of only about 1 g/L

from the starting value of 4 g/L was measured. Afterwards, the concentration of both acids did not change until 42 h. Once again, a drop of lactic but also acetic acid level was observed from 42 to 48 h. Lactic acid decreased by about 5 g/L and acetic acid around 1 g/L. Unlike P-E-LA, the sugar concentrations remained constant in the last 6 h at P-E-SA. Furthermore, the pH of U-E-SA stayed at pH 3.7 after 2 h equilibration and within P-E-SA pH rose from 3.8 after 2 h to above pH 4 at 48 h.

Similar to lactic acid addition, sulfuric acid inhibits sugar consumption only for the first 18 h. Then, mostly lactic and also acetic acid producers were able to grow presumably by consumption of hexoses and pentoses. However, the constant carbohydrate concentration during the inhibition phase indicates that the mineral acid disturbed the added enzymes. The pH of U-E-SA was lower than the ones of the other acidified experiments. That could be the reason for the smaller decrease of organic acids between 42 and 48 h. It can be assumed that the growth of indigenous MOs at pH 3.7 was inhibited. Nevertheless, the pH stayed constant, while the organic acid concentrations rose. Either the present acids buffered at this pH or indigenous organisms might influence the concentration of organic acids in order to adjust the pH within U-E-SA. In contrast, the pH was as expected from the measured substances at P-E-SA, but it seems that MOs could also metabolize these organic acids at aerobic conditions to create a better growing environment. Since lactic and acetic acid are already to a large amount available in their undissociated form at pH 4, they are able to diffuse easier into cells and damage them. Another explanation could be that MOs survived the pasteurization, which prefer lactic and acetic acid instead of sugars as substrate. Thus, glucose and xylose-SUM concentrations almost as high as in P-E-LA were achieved. Although the increase of glucose between 2 and 42 h was smaller than in P-E-LA, the uncertainties of the P-E-SA's carbohydrate values are higher, which could explain the variations. The difference in lactic acid concentrations of U-E-LA and U-E-SA occurred because the particular organic acid was used to lower the starting pH of the slurry. However, the increase is with 14 and 10 g/L for U-E-LA and U-E-SA respectively almost the same. Therefore, the choice between lactic or sulfuric acid does not influence microbial growth.

4.4.2. Incubation at 50 °C

At the inhibition experiment with an elevated temperature but without pasteurization, the glucose concentration decreased from 13 to 8 g/L in the period of 2 to 48 h although the xylose-SUM values remained around 4 g/L during the whole experiment (Figure 13). Unlike the sugars, lactic acid increased from 23 g/L after 2 h to a maximum of 36 g/L at 42 h. However, there was no initial stable phase of this product of microbial metabolism. Instead, the concentration rose from the beginning. Additionally, the pH changed in correlation to lactic acid

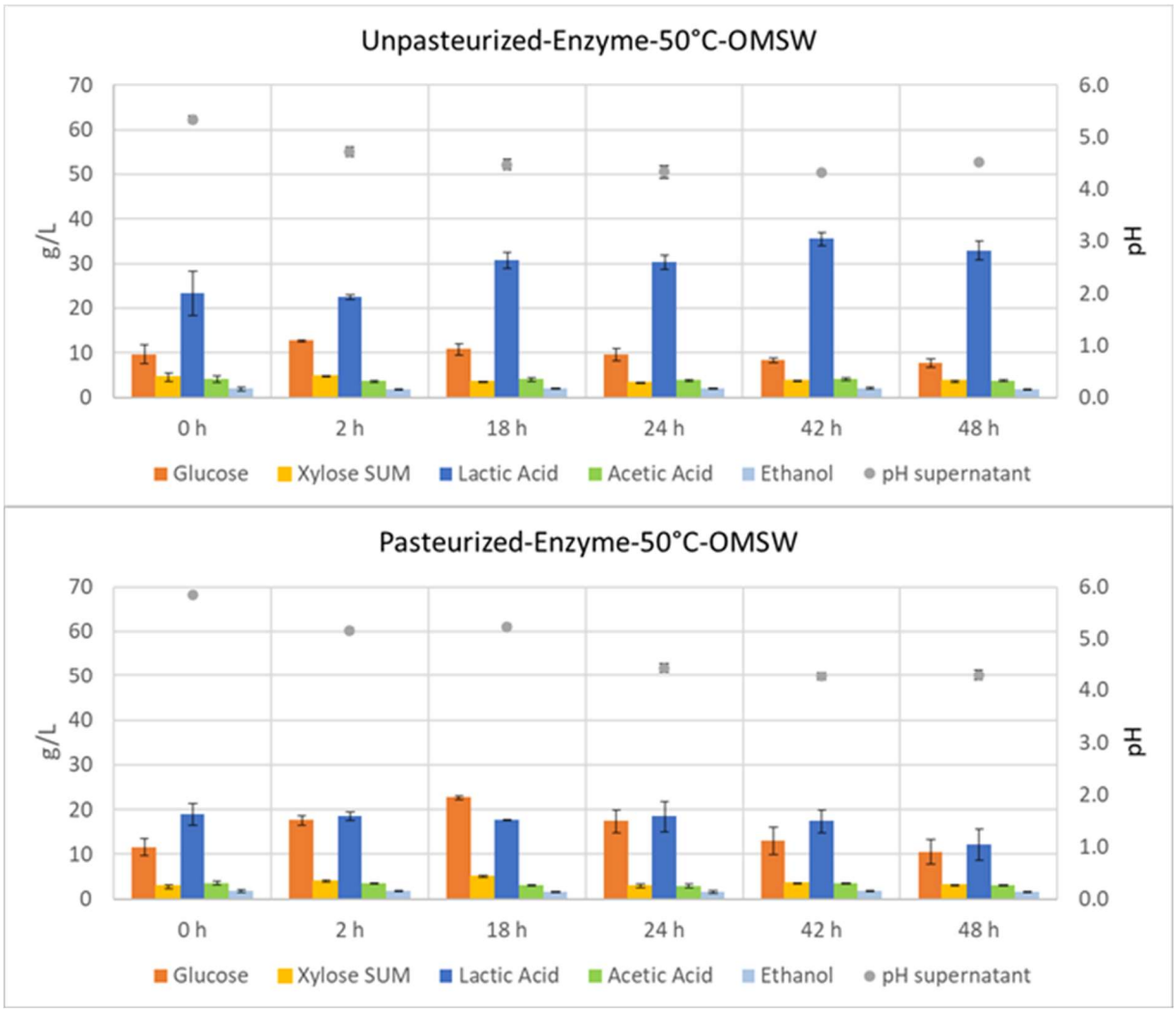

Figure 13: pH and concentration of glucose, xylose-SUM, lactic acid, acetic acid, and ethanol at certain timepoints. The error bars are the SD values of triplicates.

also during its drop from 42 to 48 h. At P-E-50, the lactic acid concentration remained stable at around 18 g/L except for the last 6 hours when the level dropped to 12 g/L. Acetic acid did not decrease from 42 to 48 h, but like lactic acid, its concentration remained the same during the whole process. Nonetheless, the glucose concentration descended from 23 g/L at 18 h to 11 g/L at 48 h. Xylose-SUM started to decline at the same time from 5 to 3 g/L but the decrease stopped at 24 h and the lower concentration lasted until 48 h. Regarding the course of pH in P-E-50, it fell from pH 5.2 to 4.4 within the 6 hours of 18 and 24 h without detection of any change in the acid composition only the sugar concentrations dropped at the same time.

The figures indicate that it is possible to inhibit the microbial digestion of glucose with an incubation temperature of 50 °C for 48 h even without pasteurization. But the carbohydrate consumption was only slowed down, and therefore no enrichment of the sugar concentrations was achieved. The lactic acid maximum was reached after 42 h. The lower hydrolyzation rate of the enzymes at 50 °C could slow lactic acid production. Further research should be done on finding the optimal temperature for the used enzyme mixture. Another reason could be the

inability of naturally occurring organisms to adapt to higher temperature. The sharper decrease of glucose and the entire consumption of the hexose after the adaption of MOs to the lowered pH in the previous examples support this hypothesis. As already noted at the acidified samples, pasteurization reduced the organic acid production more efficiently. However, the naturally occurring diverse microbial consortium recovered after 18 h and consumed the sugars. The decline of pH between 42 and 48 h indicates that acids were formed, which were not detected.

4.4.3. Enzymatic hydrolysis of autoclaved OMSW

Constant organic acid concentrations for at least 42 h were already achieved with the previous inhibition conditions but the possibility of microbial growth could not be excluded anyways. In P-E-SA, the lactic and acetic acid concentration decreased, in P-E-50 the sugar concentrations fell without detection of an increase of microbial products, and in almost all samples measured substances decreased from 42 to 48 h. Thus, the samples were autoclaved before enzyme addition and two different reference groups were used, U-E and AUT (Table 12).

U-E demonstrated the same results as the previous non-sterile hydrolysed samples. A marked increase of lactic acid and a decrease of sugars were measured within the first 19 h (Figure ***14***). Unlike U-E, none of the autoclaved samples showed relevant changes in the pH, organic acid, or alcohol concentrations until the end at 70 h. The pH was at 5.0, lactic acid concentrations were around 23 g/L and acetic acid remained at 4 g/L. Regarding the carbohydrates at AUT, xylose-SUM, and glucose were mostly below the detection limit or close to 0 g/L. Like in AUT, all measured values were the same in AUT-E, except for glucose and xylose-SUM. The pentose started with 3 g/L and achieved in the hydrolysed samples a maximal increase of 2 g/L within the first 24 h. The glucose concentration increased from 15 g/L to 26 g/L within 24 h and it even rose to 29 g/L after 70 h.

Table 12: Operating conditions for autoclaved OMSW.

Abbreviation	Enzymes	Autoclaved
U-E	X	
AUT		X
AUT-E	X	X

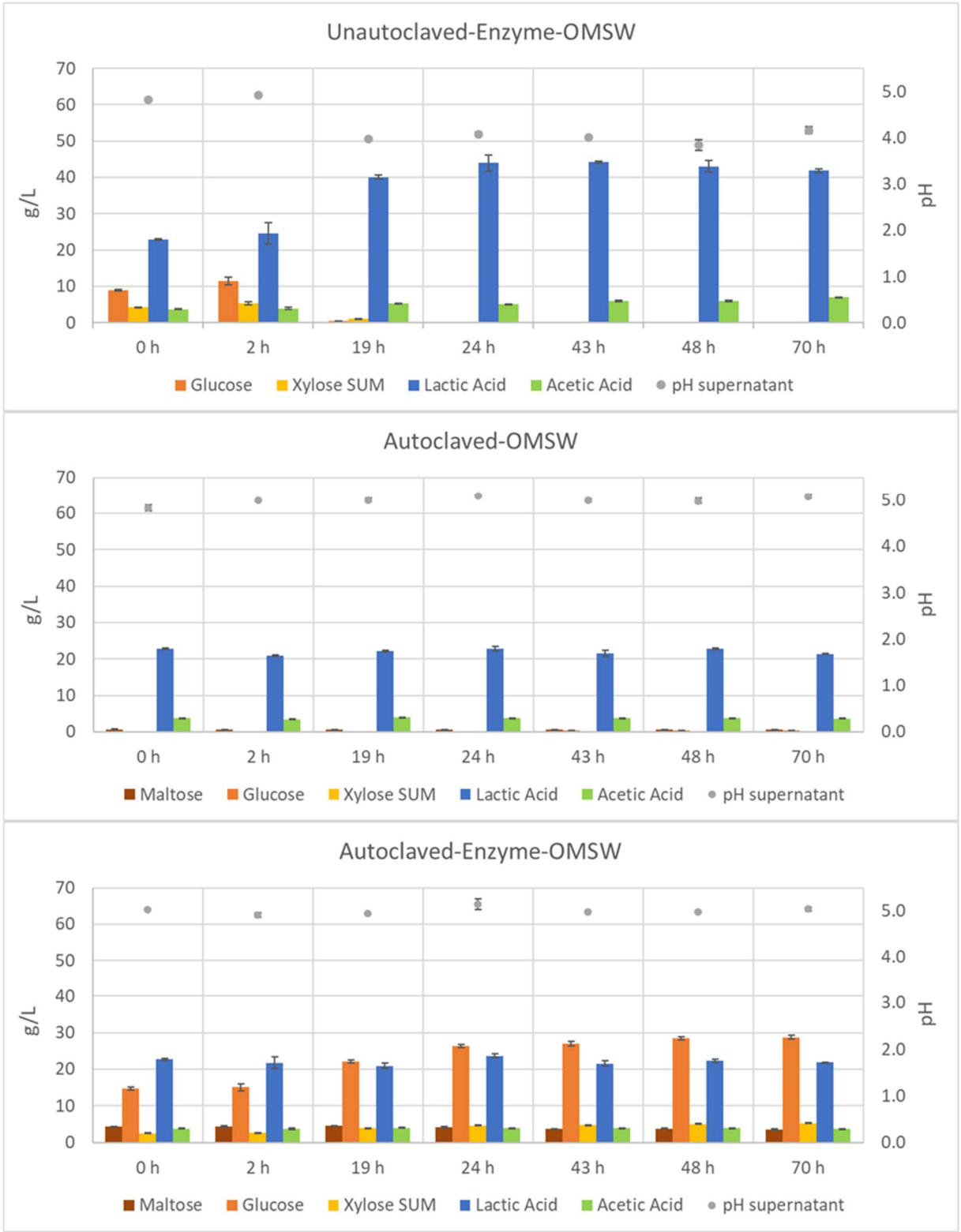

Figure 14: pH and concentration of glucose, xylose-SUM, lactic acid, acetic acid, and ethanol at certain timepoints. The error bars are the SD values triplicates.

The autoclaved slurries can be considered sterile during the whole experiment because the potential microbial products remained constant, and the stable pH supports this notion. However, the high temperature and pressure during autoclavation have to be seen as pre-treatment of the OW and the results of AUT-E and AUT are probably an overestimation of

the possible yield. Despite that, it can be assumed that the yield of xylose-SUM won't be more than 2 g/L with this enzymatic hydrolysis approach. Nevertheless, the experiment demonstrated that most of the sugars are already present after 24 h (26 g/L) which is possible due to the high enzyme activity. Furthermore, it was possible to detect for the first time the maximum glucose concentration through enzymatic hydrolysis of OMSW after 70 h (29 g/L). Even more relevant is the greatest increase of glucose (14 g/L) at AUT-E because around 15 g/L are introduced with the enzyme solution and are not released by the enzymes.

4.5. Comparison

The substrate analysis showed that OMSW (30.8 g/100g DM) and GW (30.2 g/100g DM) have a higher content of hydrolysable carbohydrates than BSG (22.7 g/100g DM) (Table 6). Nevertheless, BSG consist of more water-soluble di- and monosaccharides (7.1 g/100g DM) and proteins (16 g/100g DM). BSG has even the highest amount of all measured carbohydrates and proteins (45.8 g/100g DM) compared to OMSW (40.8 g/100g DM) and GW (36.6 g/100g DM). Additionally, OMSW and GW have higher lignin (14.8 and 24.9 g/100g DM) and ash content (26.2 and 23.8 g/100g DM). BSG contains comparable low amounts of these potential enzyme inhibitors (Klason-Lignin: 9.9 g/100g DM; Ash: 3.4 g/100g DM). Hence, the best choice for enzymatic hydrolysis would be BSG according to substrate analysis results.

The enzymatic hydrolysis experiments of all three waste types depict that around 15 g/L glucose and 5 g/L xylose-SUM are added with the enzyme solution. Therefore, the total amount of sugars is not as representative as the increase in carbohydrates. Furthermore, an equilibration period of approximately two hours is necessary for the homogeneous distribution of enzyme mix in the slurry. Hence, the change of measured substances is compared with the values after the equilibration period. In addition, the xylose-SUM concentrations were increased at some samples but not as much as glucose. Therefore, the focus of the experiments evaluating the efficiency of the enzymatic hydrolysis is on the glucose concentration changes. The highest carbohydrate concentrations were observed with pasteurization as pre-treatment for all types of waste investigated. However, enzymatic hydrolysis of GW confirmed that lignocellulose-rich waste is the most recalcitrant material, and in this case only an increase of 2 g/L of glucose was achieved. As expected from the substrate analysis, enzymatic hydrolysis of BSG led to the greatest raise of glucose (11 g/L). Paz *et al.* (2019) received even with autoclavation as pre-treatment similar glucose concentrations (18 g/L). The maximum glucose increase obtained during the enzymatic treatment of OMSW was in the same range as the value determined for the hydrolysis of GW. However, for the unpasteurized and enzymatically hydrolysed samples of OMSW, an increase of 22 g/L and a total amount of 44 g/L lactic acid concentration was measured. Hence, OMSW has a high potential for enzymatically releasing carbohydrates, but microbial growth has to be prevented.

Like in the experiments without pH adjustment, higher sugar concentrations were determined in the pasteurized samples than in the unpasteurized ones. Lowering the starting pH to pH 4.0 as inhibition treatment worked with lactic as well as sulfuric acid. The greatest raise of glucose in samples treated with lactic or sulfuric acid was 12 and 6 g/L, respectively. Additionally, 24 (P-E-LA) and 25 g/L (P-E-SA) glucose were still in the OMSW slurries after 48 h. The inhibition due to an elevated incubation temperature (50 °C) did not lead to such sugar concentrations as the acid treatment. Nevertheless, the highest increase (5 g/L) and end concentration (11 g/L) of glucose were observed in the pasteurized approaches. Hence, lowering the pH to 4.0 in combination with pasteurization is the better inhibition treatment. The increase and total amount of glucose in lactic acid-treated samples are even almost as high as in autoclaved ones (14 and 29 g/L, respectively).

Comparing the results with the literature it is clear that the achieved glucose concentrations are still too low. López-Gómez *et al.* (2019) used also autoclaving as pre-treatment, but it is mentioned that 47 g/L glucose are released by enzymatic hydrolysis within 72 h. However, the concentration of lactic acid (44 g/L) obtained without any inhibition treatments (U-E-OMSW) is considerably higher than the glucose concentration from sterile substrate (AUT-E). Fewer pre-treatment steps also result in lower costs and energy consumption. In addition, these reductions make this enzymatic hydrolysis more eco-friendly. The production of lactic acid from OMSW is also fast. Online pH measurement showed a sharp drop of pH until 24 h and constant values until 142 h. This implies that most lactic acid can be formed within 24 h, which lowers production time and costs. Lactic acid is a high-value product used in many industries, but the optical purity is very important for these applications. The ratio of L/D-lactic acid has yet to be determined, but a racemic mixture can be expected according to the work of Probst *et al.* (2013). López-Gómez *et al.* (2019) overcame this problem by inoculating the slurry with *B. coagulans*, which produces only L-lactic acid. In that manner, 83 % L-lactic acid purity was achieved with a racemic starting concentration of 15 g/L. However, lactic acid can be used as feedstock for fermentation as well. A big advantage of this application is that pure enantiomers are not required in the feed solution. Providing diverse MOs with lactic acid can be used for the production of different bio-based products like biobutanol or biodiesel (Mansour *et al.*, 2008; Sonomoto *et al.*, 2010; Bertin *et al.*, 2014; Darvishi *et al.*, 2017; Moens *et al.*, 2017).

5. Conclusions

In conclusion, all three OW types exhibit protein and carbohydrate content between 37 and 46 g/100 g DM and therefore could be used as a nutrient source for fermentation. However, BSG is favorable due to its lower ash and lignin content. The enzymatic hydrolysis of pasteurized BSG confirmed that the greatest rise of glucose (11 g/L) can be achieved with this substrate, compared to GW (2 g/L) and OMSW (2 g/L). However, the highest increase (22 g/L), as well as the highest total amount (44 g/L) of any measured substance, was achieved with lactic acid during enzymatic hydrolysis of OMSW without pasteurization as pre-treatment. That confirms that it should be possible to extract nutrients from OMSW if their consumption by indigenous MOs can be prevented.

Inhibition of microbial growth during the enzymatic hydrolysis of OMSW by lowering the pH to 4.0 or increasing the incubation temperature to 50 °C showed promising results. Lactic acid addition together with pasteurization managed to inhibit microbial growth and achieved almost the same sugar concentrations (27 g/L) as under sterile conditions. 29 g/L glucose from autoclaved OMSW seems low compared to results from other scientists, but even this value leads to a wrong conclusion because around 15 g/L were already added with the enzymatic mixture. 14 g/L are actually released by mechanical and enzymatic pre-treatment of OMSW considering the external glucose addition. Therefore, the focus should be set on lactic acid production. The repetition of enzymatic hydrolysis of OMSW without pasteurization and the online pH measurement even proved that most of the lactic acid generation can be achieved within 24 h. Apart from the higher total concentration after already 24 h (44 g/L), energy is saved because no heat pre-treatment is required. The ratio of L/D-lactic acid should be determined but a racemic mixture can be expected. To meet the aim of using OW for generating different products, lactic acid can be used as feed for other fermentations as well.

The production of lactic acid could be even improved. Inoculating OW already containing a racemic mix of lactic acid with L-lactic acid producers like *B. coagulans* can lead to high L-enantiomer purity. Additionally, lactic acid could be extracted during the fermentation to prevent feedback inhibition and raise the final yield.

Overall, the results show that there is still potential for optimization regarding the enzymatic hydrolysis; however, it could be demonstrated that it is possible to inhibit unwanted microbial growth without cost- and energy-intensive measures. Additionally, lactic acid as a high-valuable product as well as a potential feedstock for diverse fermentation processes was produced with even less energy consumption.

6. References

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