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Hydrogen Production from Enzymatic Pretreated Organic Waste with *Thermotoga neapolitana*

Julian Tix [1], Fabian Moll [1], Simone Krafft [1], Matthias Betsch [2] and Nils Tippkötter [1,*]

[1] Bioprocess Engineering, FH Aachen University of Applied Sciences, Campus Juelich, 52428 Juelich, Germany; tix@fh-aachen.de (J.T.); fabian.moll@fh-aachen.de (F.M.); krafft@fh-aachen.de (S.K.)
[2] Elsa-Brandström-Straße 27, 52134 Herzogenrath, Germany; matthias_betsch@web.de
* Correspondence: tippkoetter@fh-aachen.de

Abstract: Biomass from various types of organic waste was tested for possible use in hydrogen production. The composition consisted of lignified samples, green waste, and kitchen scraps such as fruit and vegetable peels and leftover food. For this purpose, the enzymatic pretreatment of organic waste with a combination of five different hydrolytic enzymes (cellulase, amylase, glucoamylase, pectinase and xylase) was investigated to determine its ability to produce hydrogen (H_2) with the hydrolyzate produced here. In course, the anaerobic rod-shaped bacterium *T. neapolitana* was used for H_2 production. First, the enzymes were investigated using different substrates in preliminary experiments. Subsequently, hydrolyses were carried out using different types of organic waste. In the hydrolysis carried out here for 48 h, an increase in glucose concentration of 481% was measured for waste loads containing starch, corresponding to a glucose concentration at the end of hydrolysis of 7.5 $g \cdot L^{-1}$. In the subsequent set fermentation in serum bottles, a H_2 yield of 1.26 mmol H_2 was obtained in the overhead space when Terrific Broth Medium with glucose and yeast extract (TBGY medium) was used. When hydrolyzed organic waste was used, even a H_2 yield of 1.37 mmol could be achieved in the overhead space. In addition, a dedicated reactor system for the anaerobic fermentation of *T. neapolitana* to produce H_2 was developed. The bioreactor developed here can ferment anaerobically with a very low loss of produced gas. Here, after 24 h, a hydrogen concentration of 83% could be measured in the overhead space.

Keywords: biological hydrogen; organic waste; dark fermentation; hydrolysis; pretreatment

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1. Introduction

A viable substitute for the reliance on fossil fuels for energy and material requirements is the utilization of renewable energy sources and biomass. These can be employed for the generation of biofuels and bioproducts [1]. The idea of extracting energy and raw materials from organic substances is encapsulated in the term biorefinery [2]. Organic wastes from municipalities, food waste, and agroindustrial residues are ideal feedstocks for use in biological conversion processes in biorefinery chains and represent a promising resource [1]. The ability of some hyperthermophilic bacteria, such as *Thermotoga*, to anaerobically ferment organic waste to form bioproducts has attracted considerable interest in the waste management industry [3]. *T. neapolitana* is able to form H_2 from glucose via dark fermentation, with acetate as the primary end product of fermentation [4]. In order for *T. neapolitana* to metabolize this organic waste, it must first be pretreated to break down complex sugar compounds such as cellulose into simple sugars, primarily glucose. For this purpose, the organic waste is first crushed and the non-digestible materials are separated [5]. Then, these digestible products are pasteurized at a minimum of 70 °C for one hour [6]. With the help of enzymes, the complex organic waste can be hydrolyzed, thus releasing sugars from the complex biomass [7]. Hydrolases, in particular, are capable of breaking down various polysaccharides or complex organic molecules [8,9]. Initial studies

have already investigated the use of multi-enzymatic pretreatment for biogas production. Strategies for enzyme dosing have been explored to improve the anaerobic digestion of various complex organic materials, and this review further outlines the challenges and opportunities in enhancing the enzymatic hydrolysis of these substances for biogas generation [10]. Pilot-scale experiments were conducted at the wastewater treatment plant in Ostróda, where a fixed combination of three enzymes—amylase, lipase, and protease—was continuously applied. The study aimed to investigate the effects of enzyme dosing on the sludge digestion process and the amount of biogas generated [11]. In a follow-up study, defined sets of mixed cultures were used to ferment both pure and mixed bio-wastes for hydrolysis and hydrogen (H_2) production. Various bacterial strains were employed for hydrolysis and subsequent hydrogen formation [12]. To date, no extensive research has been conducted on the multi-enzymatic pretreatment of organic waste for the microbial production of hydrogen through anaerobic microorganisms via dark fermentation.

1.1. Organic Waste

Organic waste can be used as feedstock in biological conversion processes in biorefinery chains. Due to its abundance and low cost, organic biomass has become increasingly important in recent years [3]. Organic municipal waste is a reliable resource and accounts for 44% of the world's waste composition [13]. Private households in Germany composted around 2.6 million tons of organic waste in 2020, which corresponds to an average of 31 kg per inhabitant. In addition to self-composted household waste, this includes a further 11.8 million tons of organic waste, collected by waste disposal companies. This brings the total amount of organic waste from the residential sector, not including organic waste from agriculture and the manufacturing industry, to 14.4 million tons [14]. The composition of organic waste is subject to regional, seasonal and temporal fluctuations [15]. Biodegradable materials encompass a variety of substances categorized into the three primary groups of organic matter: carbohydrates, proteins, and lipids. These groups collectively offer a balanced supply of carbon, nitrogen, sulfur, minerals, vitamins, and other small molecules. However, the most important parameter in biogenic biogas and hydrogen production is the carbohydrate content of the substrates. This correlates linearly with the respective gas yields, while the protein and lipid content does not make any significant contributions. The content of biodegradable carbohydrates is therefore a key parameter when identifying the most suitable organic waste [1]. The pretreatment of organic waste for use in biological gas-producing processes includes the separation of non-digestible substances and shredding [5]. For biogas plants processing slaughterhouse and food waste, EU legislation mandates sanitation measures [16]. The standard procedure involves pasteurization at 70 °C for one hour [6]. In this process, the waste is first mixed and then crushed to achieve a particle size smaller than 12 mm before pasteurization. Only then may it enter the anaerobic digestion process [17]. In addition to pasteurization, there are other methods for hygienization in the biogas process. During pretreatment by enzymatic hydrolysis, the complex, insoluble organic materials are hydrolyzed by extracellular enzymes and thus converted into fermentable structures. Thus, an improvement in biogas production has been reported when crude and commercial enzymes are used in the pretreatment of complex organic matter. Consequently, enhancements in biogas production have been observed when crude and commercial enzymes are employed in the pretreatment of complex organic matter. However, the high cost of producing commercial enzymes restricts their use in large-scale biogas plants, thereby impeding the overall process due to these additional expenses [10].

1.2. Enzymes

Cellulase, amylase, glucoamylase, pectinase, and xylanase are key enzymes in biotechnology, each performing specific functions in the hydrolysis of biomass [18]. Cellulose is the most abundant biomaterial on Earth. In the biosphere reserve, plants are significant contributors to the cellulose pool. Thus, cellulose is a crucial component of plant biomass, alongside lignin and hemicelluloses [19,20]. Cellulase, comprising endoglucanase, exoglu-

canase, and β-glucosidase, converts cellulose to glucose, although its efficiency is limited by hydrolysis product accumulation [21–23]. Amylase cleaves glycosidic bonds in starch to produce dextrins and oligosaccharides, with α-amylase being particularly important in industry [24]. Amylases include exo-amylases, which hydrolyze the non-reducing end of starch, and endo-amylases, which hydrolyze internal bonds [25]. They are categorized into α, β, and γ subtypes, with α and β being most studied [8]. Glucoamylases hydrolyze α-1,4 glycosidic bonds from non-reducing ends, releasing glucose [26,27]. Their activity can be inhibited by various compounds [28]. Fruits, vegetables, and mushrooms are significant sources of α-amylase and α-glucosidase inhibitors, including anthocyanins, flavonoids, phenolic acids, and more [29]. Pectinases degrade pectin, an acidic heteropolymer abundant in plants and fruits [30,31]. Pectin's main component, galacturonic acid, constitutes about 70% of the cell wall, along with other sugars [32]. Pectinase includes polygalacturonase, pectin lyase, and pectin esterase. Polygalacturonase hydrolyzes pectin to galacturonic acid monomers [33]. Pectin lyase cleaves α-1,4 glycosidic bonds by trans-elimination, forming glucuronide with an unsaturated bond [33,34]. Pectin esterase breaks pectin into pectic acid and methanol [33]. Xylanases degrade the β-1,4-glycosidic backbone of xylan, a major carbohydrate in the hemicellulosic fraction of plant tissue [35–37]. Their activity can be inhibited by the compounds formed during hydrolysis and fermentation [38].

1.3. Hydorgen Production from Household Biowaste

Hydrogen production from biowaste has emerged as a sustainable approach to addressing the dual challenges of waste management and energy production. Various methods have been developed to convert biowaste into hydrogen, each utilizing different substrates and conversion techniques, which in turn influence the efficiency and yield of hydrogen production. Thermal and microbial degradation methods are a cornerstone in biowaste-to-hydrogen conversion. The microbial route leverages the metabolic pathways of specific microbes. Techniques such as photofermentation, dark fermentation, and two-stage fermentation have demonstrated efficiency in hydrogen production. These processes utilize a range of biowaste materials, converting them into hydrogen through complex biochemical reactions facilitated by microbial activity. Asri et al. reported that dark fermentation achieved hydrogen yields of 2.83 mol $H_2 \cdot mol^{-1}$ glucose hexose, photofermentation produced up to 1.82 mol $H_2 \cdot mol^{-1}$ glucose substrate, and two-stage fermentation integrated both processes to enhance overall hydrogen yields to 3.50 mol $H_2 \cdot mol^{-1}$ glucose [39]. The anaerobic fermentation of food residue biomass has been shown to produce hydrogen at a concentration of up to 48.2% in biogas [40]. Dark fermentation is widely recognized for its potential to produce hydrogen from various organic wastes, including household biowaste. This method involves the microbial or enzymatic breakdown of organic matter, leading to the production of hydrogen. Studies by Dahiya et al. have shown that dark fermentation can achieve hydrogen yields of up to 2.83 mol $H_2 \cdot mol^{-1}$ hexose when optimized with specific microbial consortia and conditions [41]. Baeyens et al. highlighted the importance of process optimization and integration to enhance the efficiency of hydrogen production from biowaste. They identified critical challenges, such as the need for improved microbial strains, better process control, and economic assessments to ensure viability at a commercial scale. They emphasized the necessity of scaling up laboratory successes to industrial applications, which involves addressing both technical and economic hurdles [42]. Talapko et al. further explored biological hydrogen production methods, including dark fermentation, focusing on optimizing microbial consortia and operational conditions. They found that using defined microbial consortia and adjusting factors such as pH, temperature, and substrate concentration could significantly enhance hydrogen yields. Their research demonstrated that optimized conditions could lead to a more robust and scalable process, achieving higher hydrogen production efficiencies [43]. Finally, anaerobic digestion is an established method for methane production, which can be coupled with hydrogen production. Moretti et al. investigated the pretreatment and digestion of household biowaste to improve the yield of both methane and hydrogen. They reported a substantial increase in

biogas yield, with methane production and hydrogen production significantly improved through optimized pretreatment processes, showcasing the effective use of biowaste as a sustainable feedstock [44].

1.4. Thermotoga Neapolitana

The phylum Thermotogae encompasses more than 52 species across four orders: Thermotogales, Kosmotogales, Petrotogales, and Mesoacidotogales. The order Thermotogales includes two families, Thermotogaceae and Fervidobacteriaceae, characterized by the presence of conserved sequences [3]. Within the Thermotogaceae family, there are two genera: *Thermotoga* and *Pseudothermotoga*. These anaerobic, rod-shaped bacteria are encased in an envelope-like structure called the "toga", which creates large periplasmic spaces at the poles of the cells. The genus Thermotoga includes species such as *T. neapolitana* [3,45]. Members of the Thermotogaceae family have been isolated from geothermal environments worldwide, including oil reservoirs, submarine hot springs, and continental solfataric springs, with an optimal growth temperature range of 77–80 °C [46]. *Thermotoga* is capable of producing H_2 near the Thauer limit in anaerobic fermentation; this is 4 mol $H_2 \cdot mol^{-1}$ glucose and is reached when the primary end product of fermentation is acetate [4]. In addition to the H_2 that is formed, CO_2, acetate, and, in smaller amounts, lactate, ethanol, and alanine, are produced [3,46]. *T. neapolitana* produces H_2 primarily via the fermentation of organic matter that has previously been hydrolyzed [47]. The characteristics regarding the utilization and conversion of biodegradable organic waste with H_2 production and the sequestration of CO_2 for energy recovery and the production of other value-added products make *T. neapolitana* a promising candidate in the biotechnological field [3]. Through the fermentation of organic matter, *T. neapolitana* produces H_2. This process is dark fermentation [47]. In this process, energy is predominantly produced through glycolysis using the Embden–Meyerhof–Parnas (EMP) pathway. Besides the EMP, approximately 15% of the Entner–Doudoroff (ED) pathway is also active. The EMP pathway is the most prevalent route for glucose and other hexoses' oxidation, generating energy (ATP), reducing equivalents (NADH), and producing pyruvate, which can either be fully oxidized to acetate or utilized in biosynthesis as acetyl-CoA [3]. Additionally, *T. neapolitana* utilizes a capnophilic lactic fermentation (CLF) mechanism. This mechanism is triggered during glucose fermentation under CO_2 fumigation and involves combining acetate and CO_2 from glycolysis to produce lactate without compromising H_2 yield. This process offers advantages over traditional dark fermentation for H_2 production, enhancing the degradation of carbon substrates [3,47]. Under CLF conditions, *T. neapolitana* shifts its glucose utilization by downregulating the EMP and activating the ED and/or the oxidative pentose phosphate pathway. The most important feature of CO_2-induced molecular adaptation in *T. neapolitana* is related to the additional production of reducing agents through an EMP/ED-OPP switch; thus, the overexpression of flavin-based complexes such as NFN and RFN may occur [48]. Due to the capnophilic lactic acid fermentation, a reduction in lactate production has no influence on the H_2 yield, as was initially thought; however, the lactate production comes from the end product of glycolysis, i.e., acetate and additional CO_2 gassing [49]. The hydrogenase in *T. neapolitana* shows a very similar homology to that in *T. maritima*, which has been well researched [47,50]. Two moles of acetate, two moles of CO_2, and four moles of H_2 can be fermented from one mole of glucose. Hydrogen synthesis depends on a hydrogenase that synergistically transfers electrons from ferredoxin and NADH to protons. The synthesis of four moles of hydrogen consumes two moles of NADH generated by classical glycolysis and four moles of reduced ferredoxin [47]. Starting from glucose, pyruvate is synthesized in the EMP and ED. The pyruvate is then metabolized to lactate or acetate and CO_2. Lactate is formed here in the event of NAD^+ deficiency. NAD^+ is regenerated here through the formation of lactate. No hydrogen is formed in this process. This is only synthesized when acetate and CO_2 are formed, in which case the oxidized ferredoxin is reduced by the hydrogenase, releasing H^+. Ferredoxin oxidoreductase converts pyruvate with coenzyme A to acetyl-CoA, while ferredoxin is reduced with the release of H^+ ions [47,50].

2. Materials and Methods

2.1. Examination of the Enzymes on Different Substrates

To predict the individual hydrolysis efficiencies of various enzymes, a comprehensive experimental setup was designed. For the hydrolysis process, industrial enzymes were used and supplied by ASA Spezialenzyme GmbH, Wolfenbüttel, Germany, considering the potential for future scale-up and cost constraints. As detailed in Table 1, five distinct enzymes were employed in the enzyme mix. These enzymes were prediluted in an acetic acid acetate buffer, maintaining a pH of 4.7, to facilitate optimal enzymatic activity. The concentrations of the added enzymes were based on the manufacturer's specifications. This approach aimed to generate predictive data on the hydrolysis yields, enabling an estimation of hydrolysis efficiency based on the material being analyzed. The substrates were prepared at a final concentration of 40 $g \cdot L^{-1}$ dry mass (DM) in each batch. An initial experiment was conducted using a self-composed organic waste sample, which was formulated based on various household waste fractions (UH16.1). Subsequently, for further investigations, experiments were carried out using different compositions of bulk chemicals available in the laboratory. These laboratory-based compositions were designed to simulate various types of organic waste, allowing for a controlled and systematic analysis of enzymatic hydrolysis processes. When using moist substrates, the additional water content was accounted for by adjusting the volume of the buffer solution accordingly. This step ensured a consistent substrate concentration across all experimental conditions.

Table 1. Final concentrations of the industrial special enzymes in the respective hydrolysis batch. The enzymes are from ASA Spezialenzyme GmbH, Wolfenbüttel, Germany. Predilutions were prepared in acetic acid–acetate buffer (pH 4.7).

Enzyme	Concentration
Cellulase TXL	10 $\mu L \cdot g^{-1}$
Amylase FL	0.2 $\mu L \cdot g^{-1}$
Glucoamylase	0.2 $\mu L \cdot g^{-1}$
Pectinase	0.33 $\mu L \cdot g^{-1}$
Xylanase 2	50 $\mu g \cdot g^{-1}$

2.2. Pretreatment and Cultivation of Organic Waste

In this work, the substrates listed in Table 2 were examined for hydrogen yields. The substrates used were organic waste materials taken from private and municipal waste loads. These had to be prepared and processed for further tests. The substrates were first crushed using a knife mill (Grindomix GM 300 from Retsch GmbH, Haan, Germany). The particles were reduced to a minimum size of 7 mm depending on the lignification level. The lower the lignin content, the smaller the particle size. For enzymatic hydrolysis, it is also important to determine the moisture content of the substrate beforehand. For this reason, the moisture content of the respective substrates was determined using a moisture analyzer (DBS 60-3 from Kern & Sohn GmbH, Balingen-Frommern, Germany). The shredded organic waste was enzymatically hydrolyzed using a self-compiled enzyme mix. As can be seen in Table 1, five different enzymes were used for the enzyme mix. The additional water present in the batch due to the use of the moist substrate was subtracted from the volume of the buffer. Due to possible contaminations in the respective biogenic residues, the entire preparation was pasteurized for 2 h at the later cultivation conditions of 77 °C on a magnetic stirring plate with temperature control (Premiere Stirring Hot Plate 88-1 from C & A Scientific Co. Inc., Manassas, VA, USA). After pasteurization, the respective buffered enzyme mixture was added. After successful hydrolysis, the Schott bottle containing the hydrolyzate was heated to 90 °C for 15 min on a magnetic stirring plate with temperature control. The hydrolyzate was then distributed into 50 mL plastic reaction tubes and centrifuged at 10.001 RCF for 15 min using a centrifuge (MPW-380 R from MPW, Warsaw, Poland). The supernatant was then pooled in a beaker and 20 $g \cdot L^{-1}$

NaCl and 6 g·L^{-1} PIPES (Carl Roth) were added while stirring with a magnetic stirring plate (IKAMAG RED from IKA-Werke GmbH & Co KG, Staufen, Germany). The pH was then adjusted to pH 7.5 by adding 1 M NaOH, which corresponds to the conditions of the TBGY medium. PIPES was added for buffering at pH 7.5, as the acetic acid acetate buffer could no longer buffer in this range. The finished hydrolyzate can then be used for other tests, as well as the cultivation in the other parts of the experiment.

Table 2. Used substrates. The substrates used here were primarily organic municipal waste, which were tested in different compositions. Samples 8 to 13 came from a municipal waste disposal company (Stadtwerke Bergheim GmbH). In addition to the samples listed here, sample No. 16 was added in further course, which was self-assembled organic waste. The various samples were stored at −18 °C.

Sample	Substrate
1	Compost from garden and household waste—collection summer/fall 2021 (2–6 months old)
2	Organic waste (1 week old)
3	Garden cuttings, spring cuttings—winter grasses and hedge cuttings (1 month old)
4	Garden cuttings, autumn—grass clippings and leaves (5 months old)
5	Mashed potatoes
6	Vegetable waste—carrots and asparagus
7	Vegetable waste—lettuce, peppers, carrots, cucumber
8	Organic waste from municipal waste management, sample 1.1—food residues mainly cabbage
9	Organic waste from municipal waste management, sample 1.2—food residues esp. spring onion, potato peel, cabbage
10	Organic waste from municipal waste management, sample 1.3—food residues, mainly spring onion, potato peel, cabbage
11	Organic waste from municipal waste management, sample 2.1—bread rolls, cucumber, potato peels, Thuja hedge trimmings
12	Organic waste from municipal waste management, sample 2.3—Thuja hedge trimmings
13	Organic waste from municipal waste management, sample 3.1—branch cuttings
14	Lawn cutting
15	Press cake lawn cut—Honroso (low) late cut
16	Defined organic waste 50% starch (potato + bread) + 25% lignocellulose (hedge trimmings + grass) + 25% vegetable/fruit peelings (apple + cucumber)

2.3. Cultivation and Strain Maintenance of *T. neapolitana*

Cultivation, serial testing of the various substrates, and strain maintenance were carried out in serum bottles with a nominal volume of 120 mL. The working volume was 25 mL in each case. All work was carried out under sterile conditions in the microbiological safety cabinet. First, precultures were prepared. For this purpose, modified TBGY media, according to Childers [51], were placed in the autoclaved serum bottles. The bottles were then gassed with nitrogen for 20 min. After successful oxygen removal via gassing with sterile nitrogen, the cultures were inoculated with 10% of inoculum (*T. neapolitana*) under anaerobic conditions (Anaerobic Vinyl Chamber Type C from Coy Laboratory Products Inc., Grass Lake, MI, USA). Then, the precultures were incubated for 16 h in the incubator (KS 4000 i control from IKA from IKA-Werke GmbH & Co KG, Staufen, Germany) at 77 °C. This was then used for strain maintenance and as an inoculum for the main cultures. The main cultures were prepared in the same way as the precultures described above and inoculated

with a fresh preculture. Before inoculation, the optical density of the precultures was measured at wavelengths of 540 nm using a photometer (Cary 3500 UV-Vis Engine from Agilent Technologies Inc., Santa Clara, CA, USA). This should be approx. 0.200 at 540 nm to achieve optimum starting conditions. Subsequently, 250 µL L-cysteine was added to reach the final concentration of c = 1 $g \cdot L^{-1}$. These serum bottles were then also sealed and gassed with nitrogen for 20 min. After 10% *T. neapolitana* preculture was inoculated under anaerobic conditions, the cultures were incubated at 77 °C and 150 rpm in the incubator for 48 h. As an alternative to the main cultures, which were prepared with TBGY medium and the additional stock solutions, hydrolyzate of the various biogenic substrates (see Table 2) was used to investigate the hydrogen yields using *T. neapolitana*. For this purpose, 22.25 mL hydrolyzate (with 6 $g \cdot L^{-1}$ PIPES and 20 $g \cdot L^{-1}$ NaCl, at pH 7.5) was added to the autoclaved serum bottles (covered with aluminum foil).

2.4. Fermentation in the Anaerobic Bioreactor

The gaseous diffusion of H_2 and pH control problems under hyperthermophilic conditions necessitated the development of a new anaerobic bioreactor system, as conventional laboratory reactors had reached their limits. A Schott bottle, in this case a 1 l Schott bottle, serves as the reaction vessel. This was placed on a magnetic stirring plate with temperature control. The pH value was monitored using a specially developed pH measuring cell. A bioprocess controller (BioFlo 120 from Eppendorf SE, Hamburg, Germany) served as the control unit for the fermentation, which supplies the exhaust gas cooler with cooling water and regulates the pH value with 1 M NaOH. The gas obtained, primarily hydrogen, was collected in the overhead space of the Schott bottle and in the gasbag (5 l aluminum gasbag from Westfalen AG, Münster, Germany). Since the rapidly decreasing pH value during cultivation has a negative influence on the hydrogen yields and the success of the cultivation, constant pH control was necessary, in addition to the addition of PIPES for buffering. For this purpose, a new anaerobic reactor system was developed that was highly impermeable to hydrogen, which thus enabled the final balancing of the hydrogen yield with the added substrate. The Schott bottle was sealed with a black butyl rubber stopper, which was fixed in place with a perforated cap. A stainless steel tube was then inserted through the rubber stopper. This tube was used to transfer gas from the reactor into a gasbag. The tube also passed through a water cooler to a separate condensed medium. A pH electrode (pH combination electrode 405-DPAS-SC-K8S from Mettler Toledo, Columbus, OH, USA) was then placed in the measuring cell. The medium was then pumped through the measuring cell using a stand cassette pump (ISM851 from Ismatec SA, Glattbrugg, Switzerland). The medium was pumped out of the reactor via a cannula and then pumped back through a second cannula. The reactor vessel used here was a 1 L Schott bottle with a working volume of 700 mL. In Figure 1, the anaerobic bioreactor is shown. At the start of fermentation, 623 mL of the respective hydrolyzate was filled into a Schott bottle, and this was then connected to the system. Then, 7 mL of L-cysteine was added via a cannula, which corresponded to a concentration of 1 $g \cdot L^{-1}$, analogous to the work in the serum bottles. After adding the hydrolyzate, the system was pasteurized at 77 °C for two hours. During these two hours, the system was gassed with nitrogen. The reactor was then inoculated with 70 mL preculture. The hydrogen measurement took place in the overhead space of the Schott bottle, as it can be assumed that the gases are distributed equally in the Schott bottle and gas bag. After cultivation, the gas bag was sealed and separated from the system. The gas volume in the gasbag was then determined using water displacement.

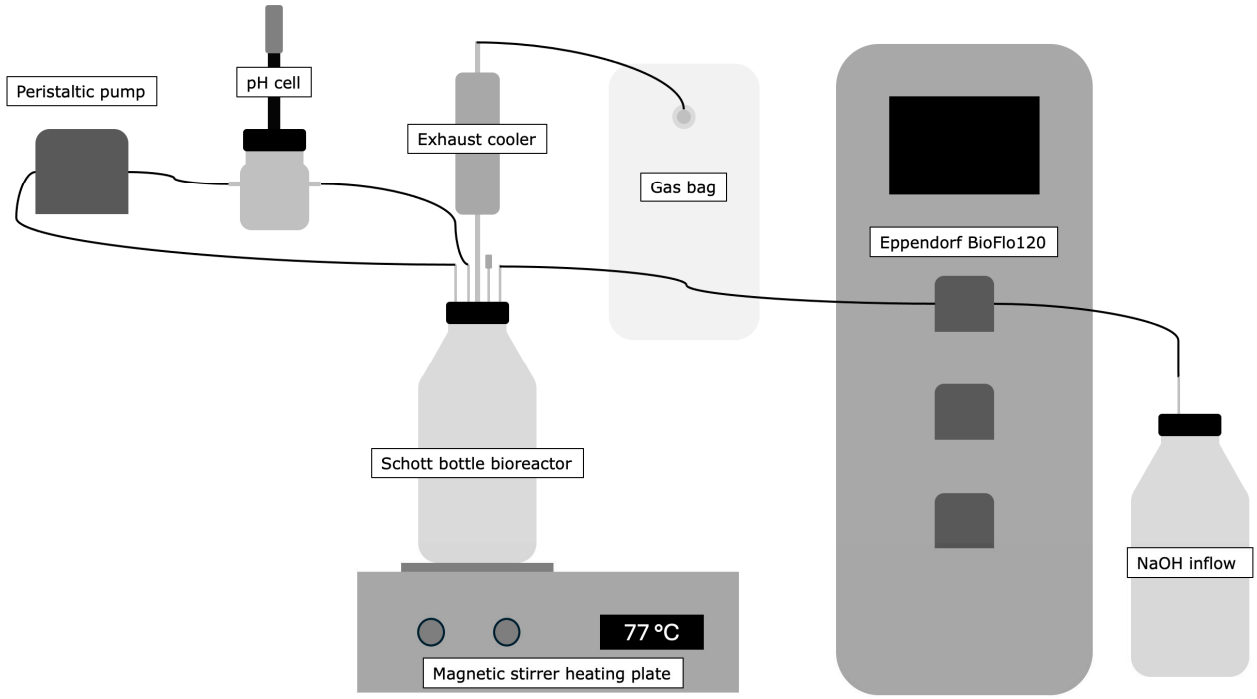

Figure 1. Schematic representation of the anaerobic bioreactor used here.

2.5. Analysis of the Fluid Products

The high-pressure liquid chromatography (HPLC) analysis of the liquid samples that underwent hydrolysis and cultivation was carried out using the HPLC system (Infinity II 1100/1260 Series from Agilent Technologies Inc., Santa Clara, CA, USA). The analysis was conducted using a Repromer H^+ column from Dr. Maisch GmbH, measuring 300 × 8 mm. The column temperature was meticulously maintained at 30 °C throughout the analysis. A sample injection volume of 5 µL was used for each analysis. The mobile phase consisted of 5 mM sulfuric acid, a choice made to enhance the separation efficiency of the analyts under investigation. The detection of analyts was carried out using an Infinity II Refractive Index Detector from Agilent Technologies Inc., Santa Clara, CA, USA. The total run time of each chromatographic analysis was set at 25 min, a duration determined to be sufficient for the complete resolution of all components. The flow rate of the mobile phase was set at 0.6 mL·min^{-1}.

2.6. Analysis of the Gas

The evaluation of gas samples collected from the headspace of serum bottles and Schott bottles was performed using the Mini GC AK LCGC 15, developed by "Arbeitskreis Kappenberg". Air was used as the carrier gas in the GC system. The column used was Chromosorb 102, with a length of 0.8 m. A Thermal Conductivity Detector (WLD) was employed. The analysis time ranged from 90 to 120 s, a duration determined to be sufficient for the complete resolution of the gas components. The temperature of the column was maintained at 20 °C, optimizing the separation efficiency. The injection volume for the GC analysis was set at 1 mL for measuring the gas product (H_2) of the fermentation.

2.7. Calculation of the Yields

With the results of the GC analysis and the measured overpressure, it is possible to calculate the amount of substance using the ideal gas equation:

$$n_{total} = \frac{p_{absolute} * V_{gas}}{R * T} \quad (1)$$

Using a peak area calibration with H_2, the respective concentration of the samples could then be determined and used in the gas equation.

3. Results and Discussion

3.1. Hydrolysis Efficiency of the Enzymes

Different compositions of organic wastes' efficiency in hydrolysis to glucose were tested. Since the main metabolic substrate of *T. neapolitana* is glucose, the analysis focused primarily on this. In addition, the main end product of hydrolysis is also glucose. Organic waste was composed in the laboratory on the basis of bulk chemicals. The samples were abbreviated as "BW" for organic waste. The compositions of the different samples are listed in Table 3; the results of this investigation are shown in Figure 2. The focus was on the enzymatic roles of cellulase TXL, amylase FL, glucoamylase AN, xylanase 2, and pectinase L-40, each with distinct functionalities in the hydrolysis process. The results over a 48 h period provided insights into the interaction between enzyme types and waste composition.

Table 3. Compositions of various organic waste were tested to analyze the efficiency of their hydrolysis to glucose. The organic waste was composed in the laboratory using bulk chemicals.

Sample	Cellulose	Lignin	Xylan	Pectin	Starch
BW1	50%	25%	25%	-	-
BW2	50%	-	25%	-	25%
BW3	25%	-	25%	-	50%
BW4	10%	50%	40%	-	-
BW5	-	50%	40%	10%	-
BW6	40%	-	20%	40%	-

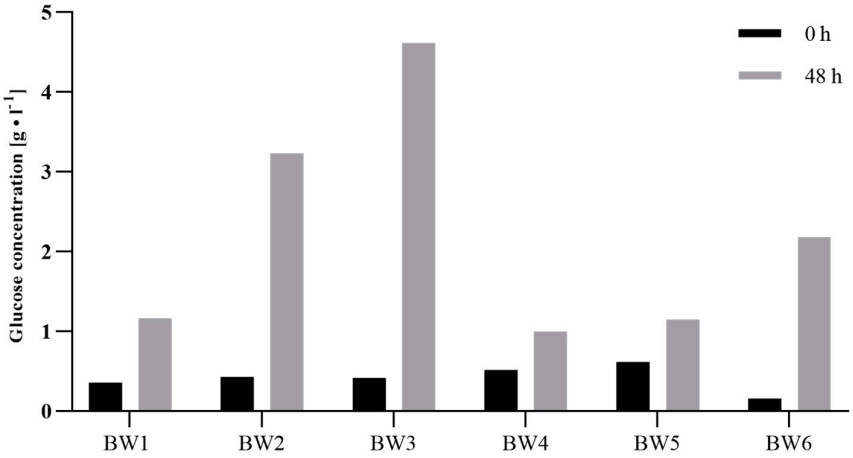

Figure 2. Results regarding the efficiency of hydrolysis for glucose production using laboratory-simulated organic waste compositions with the enzyme mix. This is a single determination. Samples were pasteurized for 2 h at 77 °C and incubated for 48 h at 150 rpm and 55 °C after the addition of the respective enzyme. The working volume here was 200 mL. Results were measured using Repromer H^+ column; 5 mM H_2SO_4; t = 25 min; flow rate 0.6 mL·min^{-1} at 30 °C.

BW1 (50% cellulose, 25% lignin, 25% xylan) showed a moderate increase in glucose concentration from an initial 0.36 g·L^{-1} to 1.17 g·L^{-1}. This increment can be attributed to the high cellulose content, which is a preferred substrate for cellulase TXL. However, the presence of lignin and xylan, known for their resistance to enzymatic breakdown, likely impeded a more substantial increase in glucose yield. In the case of BW2 (50% cellulose, 25% xylan, 25% starch), an increase in glucose concentration was observed, rising from 0.43 g·L^{-1} to 3.23 g·L^{-1}. This substantial enhancement is indicative of the effective hydrolysis of the starch component, primarily facilitated by amylase. Known for its ability

to break down starch into simpler sugars, amylase likely played a pivotal role in this process. BW3 (25% cellulose, 25% xylan, 50% starch) exhibited the highest increase in glucose concentration, from 0.42 $g \cdot L^{-1}$ to 4.62 $g \cdot L^{-1}$. The pronounced effect in this mixture can be attributed to the higher starch content, which underwent efficient enzymatic breakdown, possibly due to a synergistic effect between the enzymes. Conversely, BW4 (10% cellulose, 50% lignin, 40% xylan) showed only a modest increase in glucose concentration, from 0.52 $g \cdot L^{-1}$ to 1.00 $g \cdot L^{-1}$. The high contents of lignin and xylan, which are less susceptible to enzymatic hydrolysis, likely contributed to this limited increase. BW5 (50% lignin, 40% xylan, 10% pectin) followed a similar trend, with an increase from 0.62 $g \cdot L^{-1}$ to 1.15 $g \cdot L^{-1}$. Despite the presence of pectin, which is generally more amenable to enzymatic breakdown, the high lignin and xylan contents seemed to restrict the hydrolysis efficiency. Interestingly, BW6 (40% cellulose, 20% xylan, 40% pectin) demonstrated an increase in glucose concentration, from 0.16 $g \cdot L^{-1}$ to 2.18 $g \cdot L^{-1}$. This suggests that pectin, in combination with cellulose, enhances the hydrolysis process, likely due to the complementary action of pectinase and cellulase. These findings underscore the critical role of substrate composition in enzymatic hydrolysis for glucose production. The results highlight the importance of selecting appropriate enzyme combinations based on the specific composition of the waste material. Mixtures with higher proportions of components that are more amenable to enzymatic breakdown, such as starch and cellulose, yielded higher glucose concentrations. This study opens new avenues for future research, particularly in optimizing enzyme combinations and concentrations to enhance the hydrolysis of lignocellulosic materials for efficient bioconversion processes.

In addition, the enzymes were tested for their hydrolysis activity. The aim was to evaluate the respective glucose formation proportions of the enzymes used (Table 1). Table 4 shows the results of an analysis of self-assembled organic waste (sample 16). Here, the substrate was treated with individual enzymes and then with the various enzymes together, i.e., the enzyme mix. The components' self-assembled organic waste consisted of 50% starch (potato + bread), 25% lignocellulose (hedge cuttings + grass), and 25% vegetable/fruit peelings (apple + cucumber). Before the various enzymes were added, the substrate was also pasteurized for two hours at 77 °C on the magnetic stirring plate. The various enzymes were then added, and the different preparations were incubated for 48 h at 55 °C and 150 rpm. One batch each of cellulase TXL, amylase FL, glucoamylase AN, xylanase 2 and pectinase L-40 was prepared. Sample UH16.1, in which the five enzymes came together (enzyme mix), served as a reference. In fact, there was an increase in glucose concentration for the substrate hydrolyzing with cellulase TXL from 0.86 $g \cdot L^{-1}$ to 0.89 $g \cdot L^{-1}$, marking only a 3% increase. The cellulase TXL used here is an endoglucanase (EC 3.2.1.4), but the three different forms of cellulase act together in a cellulase complex [22]. The enzymatic components work sequentially in a synergistic system to degrade cellulose and convert it into the usable energy source, glucose. Endoglucanases randomly hydrolyze the β-1,4-glycosidic bonds within the cellulose molecule, creating new chain ends and producing oligosaccharides of varying lengths [52]. The exoglucanases release cellobiose units that show a recurring reaction from the chain end. With the help of β-glucosidase, the formed cellobiose is converted into glucose [21]. This enzyme, primarily used in the textile and paper industries, proved to be less effective in terms of glucose production from lignocellulosic material. The high lignin and lignocellulose contents in the tested samples, comprising hedge and grass clippings, further contributed to the lower glucose yield. In addition, the hedge cuttings in the sample formed a highly lignified sample with a high lignin and lignocellulose content, which means that the glucose yield was also lower. The composition of the agricultural lignocellulosic biomass consisted of approximately 10–25% lignin, 20–30% hemicellulose, and 40–50% cellulose [21]. The enzymatic hydrolysis of lignocellulose can be improved via various pretreatment strategies for material containing lignocellulose. These pretreatment strategies include, for example, pretreatment with acid, bases, or steam-explosin, as well as organosolv digestion [53]. It can be assumed that lignocellulose is broken down by the endoglucanase and the oligosaccharides are

broken down by other enzymes like xylanase. Xylose, for example, is a component of hemicellulose [21]. This can be hydrolyzed by Xylanase 2, which is contained in the enzyme mix. The highest glucose concentrations after 48 h were achieved by amylase FL and glucoamylase AN. Both enzymes showed remarkable efficiency in increasing glucose concentration, especially in starch-rich waste.

Table 4. Results of the hydrolysis efficiency of the individual enzymes. The five enzymes were added to a model substrate. In addition to the individual enzyme measurements, the enzyme mix was also analyzed. This is a single determination. Samples were pasteurized for 2 h at 77 °C and incubated for 48 h at 150 rpm and 55 °C after the addition of the respective enzyme. The working volume was 200 mL. Measured using Repromer H^+ column; 5 mM H_2SO_4; t = 25 min; flow rate 0.6 mL·min^{-1} at 30 °C.

Enzyme	Glucose Concentration g·L^{-1}			
	0 h	2 h	24 h	48 h
Cellulase TXL	0.86 ± 0.01	0.63 ± 0.01	0.77 ± 0.01	0.89 ± 0.01
Amylase FL	1.18 ± 0.01	1.49 ± 0.01	3.41 ± 0.03	4.19 ± 0.04
Glucoamylase AN	1.59 ± 0.02	3.74 ± 0.04	5.81 ± 0.06	6.51 ± 0.06
Xylanase 2	0.84 ± 0.01	0.71 ± 0.01	0.80 ± 0.01	0.86 ± 0.01
Pectinase L-40	0.82 ± 0.01	0.85 ± 0.01	1.80 ± 0.02	2.54 ± 0.02
Total				14.98 ± 0.15
Enzyme mix	1.29 ± 0.01	2.91 ± 0.03	5.27 ± 0.05	7.50 ± 0.07

Amylase FL increased the glucose concentration by 254% to 4.19 g·L^{-1}, while glucoamylase AN led to a 310% increase, reaching 6.51 g·L^{-1}. This enhancement is attributed to the high starch content in the organic waste, primarily composed of bread and potato peelings. The proportion of starchy substances in the defined organic waste was 50%, which was found in the form of bread and potato peelings. The amylase FL used here is an α-amylase (E.C.3.2.1.1), which catalyzes the hydrolysis of internal α-1,4-glycosidic bonds in starch to obtain products such as glucose and maltose [24]. The glucoamylase AN used here is an α-glucosidase (EC 3.2.1.20) that hydrolyzes the α-1,4-glycosidic bonds from the non-reducing ends of the polysaccharide substrate, releasing glucose [26]. A strong increase in the glucose concentration can be achieved by the two enzymes, particularly when starchy components are present in the waste loads. The application of xylanase, however, did not result in a notable increase in glucose concentration. The levels remained almost constant over a 48 h hydrolysis period, starting at 0.84 g·L^{-1} and marginally increasing to 0.86 g·L^{-1}. As mentioned above, it can be assumed that the endoglucanase used here breaks down the lignocellulose-containing substrate and thus releases xylose [21]. Pectinase L-40 was effective in hydrolyzing plant and fruit peels in the waste, leading to a 209% increase in glucose concentration from 0.82 g·L^{-1} to 2.54 g·L^{-1}. As mentioned above, it can be assumed that the endoglucanase used here breaks down the lignocellulose-containing substrate and thus releases xylose [21]. Pectinase was effective in hydrolyzing plant and fruit peels in the waste, leading to a 209% increase in glucose concentration from 0.82 g·L^{-1} to 2.54 g·L^{-1}. This result suggests the potential of pectinase in breaking down pectin, a natural acidic heteropolymer found in plants and fruits. This is one of the main components of the cell wall of all higher plants [31]. The study concluded that individual enzymes yielded higher glucose concentrations compared to their combined application. The highest glucose concentrations were reached when using glucoamylase, although this could potentially inhibit the activity of other enzymes. These findings underscore the importance of selecting and combining enzymes strategically for efficient glucose production from various biomass types. The study opens avenues for future research, particularly in optimizing enzyme combinations and concentrations for the enhanced hydrolysis of lignocellulosic material.

3.2. Set Fermentation with Hydrolyzate

For the cultivation in serum bottles, a hydrolysis of various substrates was initially conducted. The numbering of the samples corresponds to that in Table 2. The nomenclature was abbreviated as 'H' for hydrolyzate and 'C' for cultivation. The amount of glucose that was converted after 48 h of hydrolysis into different substrates is depicted in Figure 3. It was observed that glucose yields varied depending on the substrate. Except for H1, all other substrates showed the presence of glucose at the onset of hydrolysis. For H1, the glucose concentration remained at 0% throughout the 48 h of hydrolysis, with no increase observed. The glucose content was consistently 0.07 $g \cdot L^{-1}$ at the beginning and end, dropping slightly to 0.05 $g \cdot L^{-1}$ after 2 h. This could be attributed to variations in high-performance liquid chromatography (HPLC) measurements. A possible explanation for the lack of increase in glucose in H1 could be the age of the compost (ranging between two and six months), leading to the degradation of easily digestible polysaccharides via microorganisms present in the compost. Another factor could be the pH value of the compost, which is typically slightly alkaline [54]. This might have resulted in a higher pH during hydrolysis, rendering the enzymes inactive. Sample H_2, approximately one week old, showed an increase in glucose concentration from 1.66 $g \cdot L^{-1}$ to 3.18 $g \cdot L^{-1}$ over 48 h, a 92% increase, making it the fourth highest in terms of glucose concentration among the 15 hydrolyzes that were conducted. H3, primarily consisting of winter grasses and hedge trimmings, exhibited a 54% decrease in initial glucose concentration from 0.26 $g \cdot L^{-1}$ to 0.12 $g \cdot L^{-1}$. The low glucose concentrations could indicate measurement deviations or potential residual contamination consuming glucose during hydrolysis. The high lignin content in these samples likely hindered enzymatic hydrolysis, suggesting the need for alternative methods like lignocellulose hydrolysis. H4 is similar to H3; this 5-month-old sample showed a modest 19% increase in glucose concentration, indicating the need for alternative hydrolysis methods due to the high lignin content. H5 exhibited the highest increase in glucose concentration; this starch-rich substrate showed a 344% increase from 1.46 $g \cdot L^{-1}$ to 6.50 $g \cdot L^{-1}$, which can mainly be attributed to the action of amylase and glucoamylase enzymes. H6 and H7 showed minimal glucose formation, possibly due to the insufficient concentrations of pectinase and xylanase enzymes, which are crucial for breaking down pectin and xylan in plants and fruits. H8 to H10, primarily consisting of vegetable waste, showed increases in glucose concentration, with H11 exhibiting a 152% increase. The glucose formation in these samples is likely due to cellulase, xylanase, and pectinase activity. H11, including bread and hedge trimmings, showed a 244% increase in glucose concentration, attributed to starch in the bread and the action of amylase and glucoamylase. H12 and H13, consisting of hedge trimmings and branch cuttings, showed increases in glucose concentration of 69% and 67%, respectively, likely due to the presence of other substrates and the action of cellulase, xylanase, and pectinase. H14 showed a 65% decrease in glucose concentration, possibly due to unsuccessful pasteurization leading to microbial contamination. In contrast, H15 exhibited a 140% increase in glucose concentration, likely due to the action of cellulase, xylanase, and pectinase. The hydrolysis process was generally successful, with variations in glucose yield depending on substrate composition and enzyme activity.

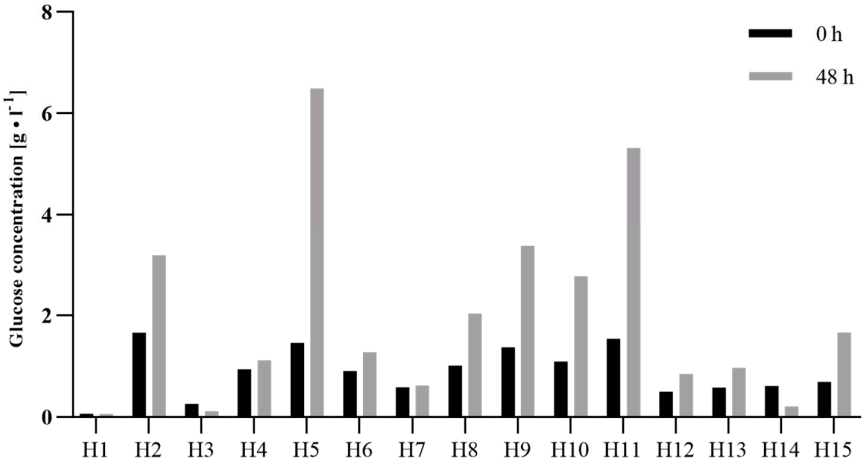

Figure 3. Results of hydrolysis of the various substrates, H1 to H15. This is a single determination. Samples were pasteurized for 2 h at 77 °C and incubated for 48 h at 150 rpm and 55 °C after the addition of the enzyme mix. The working volume was 200 ml. Results were measured using Repromer H^+ column; 5 mM H_2SO_4; t = 25 min; flow rate 0.6 mL·min^{-1} at 30 °C. The sample naming was analogous to the sample numbering and was supplemented with H for this part of the experiment.

The aim of this part was to analyze the hydrogen formation rates when using the produced hydrolyzates and cultivated in serum bottles using *T. neapolitana.* The samples were numbered analogously and given the abbreviation "C" to distinguish them. First, a cultivation with TBGY medium was carried out. This served as a reference value for the cultivations using hydrolyzate. For this purpose, the concentration of hydrogen formed after 48 h and the glucose concentration measured at the beginning are plotted in Figure 4. All samples were determined in triplicate, from which the mean values were then calculated. For C7, only two samples were included in the analysis, as leaks occurred in one sample and no H_2 concentration could be measured here. A yield coefficient of 3.92 mol H_2·mol^{-1} glucose was determined for sample C0. This is close to the Thauer limit, which is 4 mol H_2·mol^{-1} glucose [4]. Figure 4 shows that high levels of H_2 were measured in the headspace of the Schott bottles, particularly in samples C2, C8, C9, C10, C11, and C15. Samples C2 and C10 exceeded the amount of H_2 in the reference sample C0. A H_2 amount of 1.31 mmol was achieved for C2 and 1.37 mmol for C10. These were, therefore, higher than the reference sample C0, where the amount of H_2 was 1.26 mmol. However, the standard deviation for C0 is higher and the highest value achieved was 1.35 mmol. The glucose concentration at the start of fermentation is plotted next to the amount of H_2. Here, it can be seen that the amount of H_2 is in proportion to the glucose concentration previously formed by hydrolysis. C1, C3, C12, and C14 also showed a low amount of H_2 in the overhead space, in addition to the low glucose concentrations at the start of fermentation. As mentioned above, the sugar that was introduced may also be due to the precultures. The glucose concentration in H1 was 0.07 g·L^{-1} compared to 1.08 g·L^{-1} in C1. It can therefore be assumed that the glucose concentration increased here due to the glucose from the preculture that had not yet been consumed. Nevertheless, the glucose concentration in the samples is still too low. The concentration was 1.08 g·L^{-1} for C1, 1.95 g·L^{-1} for C4, 1.66 g·L^{-1} for C12, and 1.27 g·L^{-1} for C14. For C5, it is noticeable that the H_2 concentration is rather low compared to the high glucose concentration found here. With a glucose concentration of 6.86 g·L^{-1} at the start of fermentation, only 1.00 mmol of H_2 could be achieved after 48 h. The reason for this is the shaking incubator. As there were many sample bottles in the incubator during the batch fermentation, the heat input was slightly lower in the front bottles than in the back bottles. In addition, the mashed potato could also contain substances such as salts or lipids, which could have an inhibitory effect on *T. neapolitana*. In C12, no hydrogen formation could be observed. This was the hydrolyzate H12, which mainly contained hedge cuttings and had

a glucose concentration at the end of hydrolysis of 0.85 g·L^{-1}. The glucose concentration of C12 was 1.66 g·L^{-1} at the beginning and dropped to 1.16 g·L^{-1}. However, the glucose concentration was still too low for *T. neapolitana* to show any growth and the quantities of hydrogen were too small to be detected. All in all, it can be concluded that C2, C8, C9, C10, C11, and C15 were able to produce high amounts of H_2 that were in the range of the TBGY medium. These were mainly organic waste, which consisted primarily of food waste. C15, as an exception, was the press cake from grass cuttings. This represents great potential for the use of organic municipal waste, as the hydrogen yields were in the range of, or even above, the TBGY medium. The prerequisite for successful fermentation, and thus the achievement of high hydrogen yields, is successful hydrolysis in which sufficient quantities of glucose are formed.

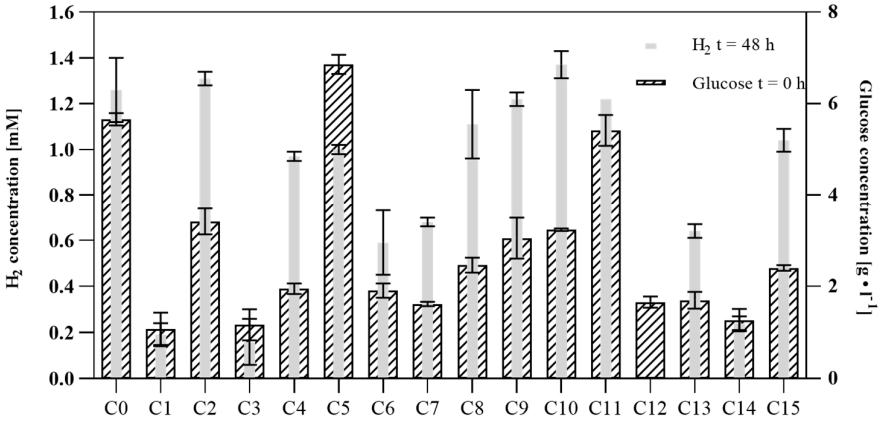

Figure 4. The amount of H_2 after 48 h in the various samples in relation to the glucose concentration at the start of the cultivation. Samples were studied in triplicate. Results were measured using Repromer H^+ column; 5 mM H_2SO_4; t = 25 min; flow rate 0.6 mL·min^{-1} at 30 °C. H_2 measured with Mini GC AK 15 using Chromosorb 105 column; WLD; injection volume 1 mL. Fermentation was carried out at 77 °C for 72 h at 150 rpm. Working volume was 25 mL in serum bottles. The results after 48 h are shown here. The sample naming was analogous to the sample numbering and was supplemented with C for this part of the experiment.

As previously described, *T. neapolitana* can produce H_2 in amounts close to the Thauer limit. This is 4 mol H_2·mol^{-1} glucose and is reached if the primary end product of the fermentation is acetate [4]. For this purpose, the hydrogen yield and the consumed glucose are plotted in Figure 4. Samples C2, C4, C5, C6, C8, C9, C10, and C11 exceed the Thauer limit. The reason for this is that *T. neapolitana* is able to metabolize not only glucose but also other hexoses and pentoses [23], or complex carbohydrates such as starch and cellulose to H_2 [55]. It can therefore be assumed that other sugars, or even more complex carbohydrates, are metabolized. However, these different carbohydrates are difficult to detect because a wide variety of different products are formed through hydrolysis. All in all, it can be said that this part of the experiment was successful and that H_2 could be formed via the use of the previously prepared hydrolysis from organic waste when cultivated using *T. neapolitana*. It is therefore possible to produce hydrogen from organic waste, especially food waste, which has great potential for the future. To achieve complete glucose consumption, and thus even higher H_2 concentrations, the pH value must be regulated, as this drops sharply in the first 24 h despite buffering using PIPES. It can be assumed that the CO_2 formed during cultivation is consumed by other metabolic pathways, such as capnophilic lactic acid fermentation, which can lead to an increase in the concentration of the hydrogen formed [47]. In their work, Nguyen et al. were able to detect 30% hydrogen in 120 mL serum bottles with a working volume of 50 mL [55]. In this study, the highest concentration of 23% was achieved with sample C10 after 48 h in a 25 mL preparation. Thus, the value achieved here was lower than that of Nguyen et al. However, the working volume was lower here, which led to a low concentration of hydrogen in the headspace [55].

3.3. Fermentation with Hydrolyzate in Anaerobic Bioreactor

As larger quantities of hydrolyzate were required in the bioreactor, work was carried out at a larger scale. For the fermentation in the anaerobic reactor, three different hydrolyses were carried out. The samples were labeled with the abbreviation "UH", which, in this case, stands for upscale hydrolysis. The numbering corresponds to that in Table 5. In contrast to the previous fermentation, substrate 16 was also used as a defined, self-assembled organic waste. The composition of sample 16 was 50% starch (potato + bread) + 25% lignocellulose (hedge cuttings + grass) + 25% vegetable/fruit peelings (apple + cucumber). The results of the various hydrolyses are shown in Table 5. With UH11.1, an increase in glucose concentration of 251% was achieved. The concentration increased from 1.29 $g \cdot L^{-1}$ glucose to 4.52 $g \cdot L^{-1}$. As sample H11 consisted of food residues, which, in addition to vegetable waste, also contained bread roll waste and hedge cuttings, it can be assumed that, in addition to cellulase, xylanase and pectinase, amylase, and glucoamylase were primarily responsible for the high glucose concentration at the end of hydrolysis. The highest glucose concentration was reached at UH16.1, with a glucose concentration of 7.50 $g \cdot L^{-1}$, which thus represents the highest value in this work. The initial glucose concentration of 1.29 $g \cdot L^{-1}$ was increased to 7.50 $g \cdot L^{-1}$ by the addition of the enzyme mix, which corresponds to an increase of 481%. Due to the presence of approx. 50% starchy components such as potato peelings and bread, amylase and glucoamylase are primarily responsible for the high glucose concentrations. The other components, such as 25% lignocellulose (hedge cuttings + grass) and 25% vegetable/fruit peelings (apple + cucumber), were then hydrolyzed by cellulase, xylanase, and pectinase. Here, it can be recognized that the glucose concentration was higher in sample UH16.1 than in UH16.2. In UH16.2, the substrate was first frozen before it was used. This was not the case with UH16.1, where the substrate was used directly. It can therefore be said that the differences in hydrolysis between the individual substrates are mainly due to the inhomogeneous composition and the freezing and subsequent thawing [56]. Furthermore, no increase in the acetate formed during hydrolysis was observed.

Table 5. Glucose concentration of hydrolysis UH. This is a single determination. Samples were pasteurized for 2 h at 77 °C and incubated for 48 h at 150 rpm and 55 °C after the addition of the enzyme mix. The working volume was 200 mL. Measured using Repromer H^+ column; 5 mM H_2SO_4; t = 25 min; flow rate 0.6 $mL \cdot min^{-1}$ at 30 °C. The sample naming was analogous to the sample numbering and was supplemented with UH for this part of the experiment.

Hydrolysis	0 h	2 h	24 h	48 h
		[$g \cdot L^{-1}$]		
UH11.1	1.29 ± 0.01	3.11 ± 0.03	5.10 ± 0.05	4.52 ± 0.04
UH16.1	1.29 ± 0.01	2.91 ± 0.03	5.27 ± 0.05	7.50 ± 0.07
UH16.2	1.50 ± 0.01	2.54 ± 0.02	5.47 ± 0.05	5.98 ± 0.06

The hydrolyzates that were produced were subsequently cultivated in an anaerobic reactor developed in-house. The experiments carried out in this section are abbreviated as "UC". The numbering of the samples can be seen in Table 1. Table 6 shows that the highest amount of H_2 substance was measured at UC11.1. A substance quantity of 27.07 mmol was measured here. The second highest amount of 12.63 mmol was achieved at UC16.2.

It is also noticeable that the amount of H_2 in UC16.1 was quite low, at 8.64 mmol, compared to the high glucose concentration at the beginning, which was 6.35 $g \cdot L^{-1}$. Due to the low density of hydrogen, it can be assumed that the hydrogen that is formed escapes from the system over the fermentation period [57]. Figure 5 shows the progression of the percentage H_2 content of the respective fermentation batches over the fermentation period of 72 h. Overall, in every sample, the percentage of H_2 content rises sharply within the first 24 h and, as mentioned above, reaches a peak between 24 h and 30 h, depending on the batch. The highest H_2 concentration was reached between 24 h and 30 h in each case.

For UC11.1, after 24 h, 83% H_2 was obtained; for U16.1, after 30 h, 70% H_2 was obtained; and for UC16.2, after 30 h, 72% H_2 was obtained. The H_2 concentrations then fell. For UC11.1, the concentration was 60%, for UC16.1, it was 41%, and for UC16.2, it was 45%. As can be seen here, the percentage H_2 content of the respective preparations fluctuates and decreases towards the end of fermentation compared to the values obtained between 24 h and 30 h. However, the substance concentration could only be calculated using the final volume after 72 h, leading to inaccuracies and deviations.

Table 6. Results of the fermentation in the anaerobic bioreactor after 72 h. It was fermented for 72 h at 77 °C on a magnetic stirring plate using temperature control. In addition, the pH value was actively controlled using a pH measuring cell. Working volume was 700 mL in an anaerobic reactor. The sample naming was analogous to the sample numbering and was supplemented with UK for this part of the experiment.

Fermentation	H_2 [%]	H_2 Volume [ml]	H_2 Substance [mmol]	H_2 [mol/kg DM Substrate]
UC11.1	60 ± 0.01	664.30 ± 16.30	27.07 ± 0.66	1.09 ± 0.03
UC16.1	41 ± 0.01	211.95 ± 5.20	6.00 ± 0.21	0.35 ± 0.01
UC16.2	45 ± 0.01	309.81 ± 7.60	12.63 ± 0.31	0.51 ± 0.01

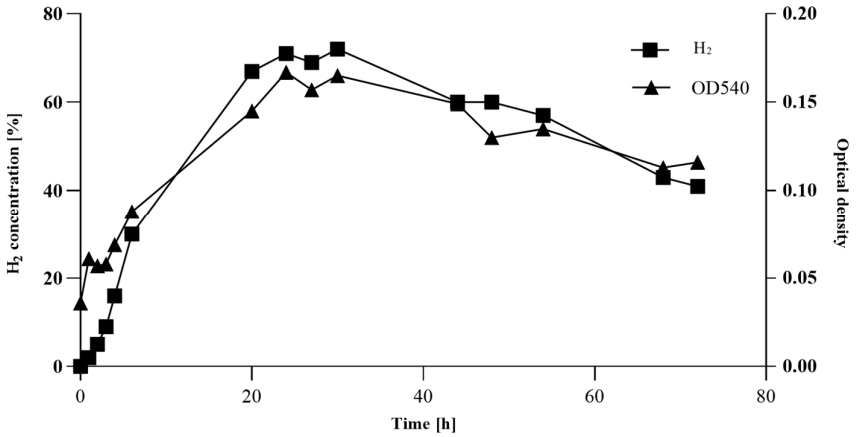

Figure 5. The dependence of H_2 content and OD_{540} at UC16.1 single determination. Results were measured using Repromer H^+ column; 5 mM H_2SO_4; t = 25 min; flow rate 0.6 mL·min 1^{-1} at 30 °C. OD was measured with Cary 3500 UV-Vis Engine from Agilent Technologies at 540 nm. Fermentation was carried out for 72 h at 77 °C on the magnetic stirring plate using temperature control. In addition, the pH value was actively controlled using a pH measuring cell. Working volume was 700 mL in an anaerobic reactor. The sample naming was analogous to the sample numbering and was supplemented with UH for this part of the experiment.

The course of H_2 and the OD_{540} are dependent on each other. In the first six hours, the OD_{540} rises from 0.036 to a value of 0.088. The H_2 content increases from 0% to 5% in the first two hours and continues to rise to 30% at time 6 h. After 24 h, both the H_2 and the OD_{540} reach their peak. A H_2 content of 71% could be measured. The OD_{540} reached a value of 0.167. Subsequently, both curves fell slightly and rose again slightly at 30 h. One explanation for this is that cell growth causes more sugar, especially glucose, to be metabolized, and thus H_2 is formed due to glycolysis [47]. Due to the amount of H_2 formed here in the gas and liquid phase, hydrogen acts as a strong inhibitor during dark fermentation [58]. Thus, cell growth is inhibited at a high H_2 concentration. If the hydrogen then escapes via slight leaks, and thus the H_2 concentration drops, the cells are less inhibited and cell growth, accompanied by increasing OD_{540}, is possible. This is particularly noticeable at 48 h. After the H_2 concentration drops to a value of 60% between

30 h and 48 h, the OD_{540} increases from 0.130 to 0.135. Subsequently, H_2 and OD_{540} decrease until 72 h. After 72 h, the percentage of H_2 is 41% and the percentage of OD_{540} is 0.116. One reason for this is the escape of hydrogen from the system. In addition, cell growth is restricted due to the high H_2 concentration and the limited glucose concentration. The glucose concentration at 48 h is 1.50 $g \cdot L^{-1}$ and drops to 0.88 $g \cdot L^{-1}$ at 72 h.

4. Conclusions

This study demonstrates the potential for producing hydrogen from biogenic municipal waste through enzymatic hydrolysis and subsequent fermentation using *T. neapolitana*. The hydrolysis efficiency varied significantly depending on the composition of the organic waste. Notably, substrates with a higher starch content, such as BW2 and BW3, showed the highest glucose yields, highlighting the effectiveness of amylase and glucoamylase in hydrolyzing starchy materials. Conversely, samples with a high lignin content, like BW4 and BW5, exhibited lower hydrolysis efficiency, indicating the challenge of breaking down lignocellulosic materials without additional pretreatment. The study highlights the potential of using food waste, particularly starch-rich substrates, for hydrogen production. However, for lignocellulosic materials, pretreatment strategies may be necessary to improve enzyme accessibility and hydrolysis efficiency. Current studies of our group are focusing on low-energy pretreatment strategies for cellulose-based waste materials, with promising first results. It has been shown that it is possible to produce hydrogen from biogenic municipal waste through enzymatic hydrolysis and anaerobic fermentation. The enzyme mix was particularly effective in enhancing glucose yields from composite substrates, as seen with UH16.1, which achieved the highest glucose concentration of 7.50 $g \cdot L^{-1}$, primarily due to the combined action of amylase, glucoamylase, cellulase, xylanase, and pectinase. This underscores the importance of selecting and combining enzymes based on substrate composition to maximize the efficiency of hydrolysis. Fermentation results revealed that *T. neapolitana* could achieve high hydrogen yields, reaching up to 83% H_2 in the reactor headspace after 24 h. This was organic waste consisting mainly of bread rolls, cucumber, potato peelings, and Thuja hedge cuttings. This shows the high potential of the strain to utilize a wider range of di- and monosaccharides occurring in a vast variety of household waste streams. The correlation between initial glucose concentration and hydrogen yield emphasizes the need for efficient hydrolysis to provide sufficient fermentable sugars. The mixture that was used improved the efficiency and robustness of hydrogen production. Future research should focus on optimizing enzyme combinations and concentrations, as well as exploring pretreatment methods for lignocellulosic waste. These may include tailored mixtures based on the substrate's composition. Additionally, investigating the scalability of this process and integrating it with existing biogas plants could enhance the economic viability and sustainability of hydrogen production from organic waste. In summary, the method developed in this study offers a promising approach for hydrogen production from organic waste, contributing to the establishment of a circular economy and providing a sustainable alternative to fossil fuels. The yield is significant, suggesting that the method developed can serve as a viable alternative to traditional hydrogen production technologies. The study highlighted the critical role of substrate composition and the effectiveness of specific enzyme combinations in optimizing glucose production, which is essential for efficient hydrogen fermentation. Converting existing biogas plants to incorporate this technology could further enhance the production of hydrogen, paving the way for a more sustainable energy future.

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