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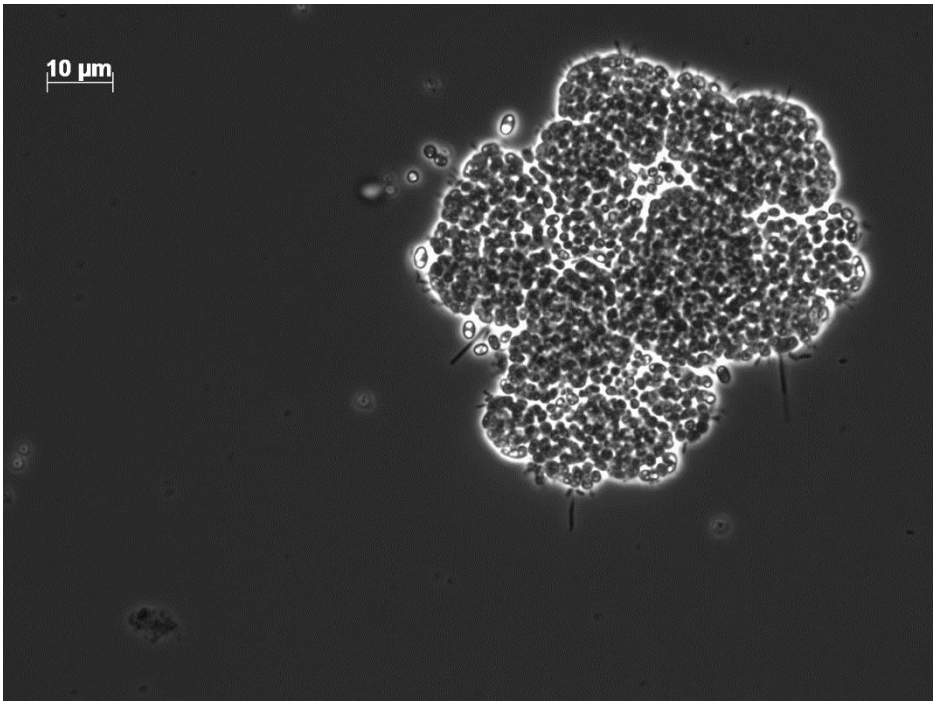

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Abstract

Pyrolysis is the thermal conversion of biomass in the absence of oxygen. In this process a biofuel called bio-oil is created. Bio-oil consists of many oxygenated compounds resulting in a bio-oil that is immiscible with other liquid fuels. To improve the characteristics of bio-oil, a water extraction can be performed. After the extraction, the water used for extraction (pyrolytic sugar) contains many different organic carbon compounds. Current research is focused on isolating and fermenting the large amount of sugars present in pyrolytic sugar. However, a broader spectrum of carbon substrates can be reached, if not only the sugars but also the organic acids and other more complex fermentable organic compounds are used for fermentation. Many of these complex organic compounds can be used for the fermentation towards organic acids and subsequently, fermented towards intercellular polyhydroxybutyrate (PHB). The produced PHB's can be used as bioplastics or converted to methyl-crotonate, which is an important building block for chemical industries. However, several of the organic carbon compounds present in pyrolytic sugar are toxic for micro-organisms. To achieve a successful fermentation, a solution for this toxicity must be found.

Several different detoxification methods have been tested for their inhibiter removal capabilities. From these results a detoxification strategy has been compiled. This strategy included an acid hydrolysis, XAD-4 resin treatment and a vacuum evaporation. These three successive steps were required for the conversion of anhydro-sugars to fermentable sugars and to remove several inhibitory compounds.

What followed were two fermentation processes. The first process was a subsequently fermentation of detoxified pyrolytic sugar by an anaerobic mixed microbial culture (acidogenic fermentation). The goal of this fermentation was to convert glucose and other complex organic compounds into small volatile fatty acids, which are the preferred substrates of PHB producing bacteria. The second process is with the detoxified and fermented pyrolytic sugar to serve as only carbon source for PHB fermentation.

From the results it can be concluded that it is possible to detoxify pyrolytic sugar sufficiently with the chosen detoxification strategy to remove most of the inhibitory compounds. Furthermore, the complexity of the carbon source can be reduced by acidogenic fermentation. In addition, it is possible to increase the concentration of small volatile fatty acids, the preferred substrate for PHB fermentations. Based on microscopy results it can be concluded that small PHB granules have been formed inside the bacterial cells. Thus, it can be concluded that a broad range of organic compounds in pyrolytic sugar can be used as carbon substrate for PHB production.

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Introduction

The depletion of fossil fuels and global warming caused by an increasing greenhouse gas emission leads to a search for alternatives to fossil fuels. These alternatives should be renewable and sustainable in order to decrease the dependence on fossil fuels. Some of the new alternatives are based on the conversion of biomass to liquid biofuel. In this biomass conversion, several main approaches can be used: chemical, biochemical and thermochemical processes. An example of a biochemical process is the production of bioethanol. Chemical processes lead to the formation of biodiesel as liquid bio-fuel. For the thermochemical processes, pyrolysis has been marked as one of the most promising options [1].

Pyrolysis

The thermal conversion of biomass in the absence of oxygen is called pyrolysis. Pyrolysis is executed at high temperatures which can range between 400-600 ˚C. In this process charcoal and gaseous products are formed. By condensing the gaseous products a liquid called bio-oil can be obtained. The charcoal and leftover gaseous products can be burned to provide the energy that is necessary for the pyrolysis. Pyrolysis can be classified into slow-pyrolysis and fast-pyrolysis. The type of pyrolysis depends on the heating rate and biomass residence time in the pyrolysis reactor. Slow pyrolysis is characterized by the slow heating rate and lower temperatures (400 ˚C). The main products of slow pyrolysis are charcoal and non-condensable gasses. To the contrary, fast pyrolysis is characterized by its high heating rate and high temperature (500 ˚C) and the main product is bio-oil. Nowadays, most research is focused on fast-pyrolysis as the bio-oil yield is significantly higher compared to slow-pyrolysis [2].

A disadvantage of the use of pyrolysis is that the produced bio-oil is not directly usable as liquid fuel. This is due to the high water content, pH, viscosity, density, elemental composition and thermal instability. These properties cause that bio-oil is immiscible in other liquid fuels [1, 2, 3]. Therefore, the bio-oil should be upgraded. The first step can be a water extraction to extract all water soluble compounds. An overview of obtaining pyrolytic sugar is shown in Figure 1. The water used in the extraction (pyrolytic sugar) contains large amounts of different organic carbon compounds from which several are toxic for micro-organisms. Furthermore, the obtained pyrolytic sugar can be seen as a bulk waste stream due to complexity of its mixture [1]. Due to this complex composition difficulties arise when isolating specific compounds and pyrolytic sugar could lead to negative environmental impact if not disposed correctly.

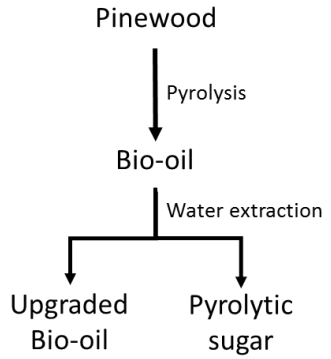

Figure 1. Schematic overview of obtaining pyrolytic sugar.

Pyrolytic sugar

The pyrolytic sugar is supplied by the company BTG (Biomass Technology Group). This is a company that focusses on the production of bio-fuels and bio-energy. The compounds and their corresponding concentrations in the pyrolytic sugar are dependent on different variables. These variables include: the organic substrate used for pyrolysis, temperature and substrate residence time. The pyrolytic sugar from BTG is originated from the pyrolysis of pine wood [4]. Therefore, the composition is for a large extent based on the thermal degradation products of (hemi-) cellulose and lignin (Figure 2). These compounds can be classified into 5 main categories [2, 4, 5]:

- Anhydro-sugars and (oligo-) saccharides
- Organic acids, including small volatile fatty acids
- Hydroxyaldehydes
- Hydroxyketones
- Phenolic compounds

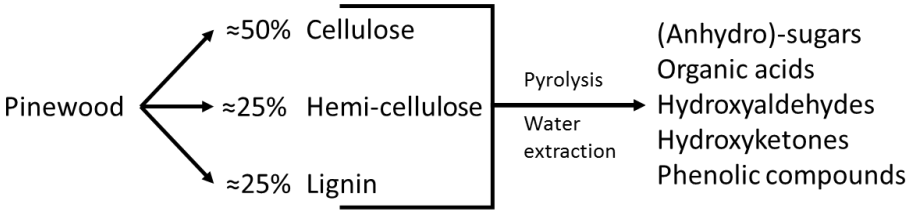

Figure 2. Overview of the organic composition change during the pyrolysis and water extraction.

The ratio between these five categories differs between the different distillation fractions and subsequent extraction steps. The toxic effect for micro-organisms is mainly derived from the phenolics, aldehydes and ketones present in pyrolytic sugar [5]. It has been reported that also the pre-treatment of the wood influences the thermal conversion and thereby the final composition of pyrolytic sugar [4]. For example, by pre-treating the wood, the concentration of levoglucosan (main anhydro-sugar) can be increased at expense of other compounds. The pre-treatment can be mechanical (i.e. shredding and drying) and chemical (i.e. nitric acid treatment).

Problem statement

As mentioned before, due to the complexity of the pyrolytic sugar mixture it is often economically unattractive to isolate compounds with high purity [6, 7]. A different way to valorise the pyrolytic sugar is to use microbial fermentation to reduce the complex mixture to simple products. The obtained pyrolytic sugar stream from BTG contains high concentrations of sugars, organic acids and other fermentable carbon compounds. The hypothesis is that the by-product of bio-fuel production can act as carbon source for fermentation to produce important biochemicals like ethanol, citric acid or polyhydroxybutyrate (PHB).

Current research is mainly focused on isolating and fermenting the sugars present in pyrolytic sugar. The sugars are fermented towards ethanol, which can be used as bio-fuel [8, 9, 10, 11, 12, 13]. The main disadvantage is that only the sugars of the pyrolytic sugar waste stream are used. To bypass this problem it is necessary to use more of the organic compounds present in pyrolytic sugar. A broader spectrum of substrates can be reached by using not only the sugars but also the organic acids and other more complex fermentable organic compounds.

However, not all organic compounds are easy biodegradable and can cause severe toxicity for micro-organisms. This toxicity can inhibit microbial growth and conversion and thereby preventing the production of the product of interest [9, 10]. The substrate toxicity can either be reduced by diluting pyrolytic sugar with water or by applying a detoxification strategy [10]. Dilution can be effective or economical unattractive, depending on the concentrations of toxic compounds. In the case of pyrolytic sugars supplied from BTG, dilution is insufficient due to reaching too low concentrations of sugars and organic acids. Therefore a detoxification strategy is necessary to be applied. This strategy should focus on removing toxic, with a minimal loss regarding to the sugars and organic acids present in pyrolytic sugar.

Polyhydroxybutyrate

PHB was chosen in this study as the fermentation product since the substrate precursors are present in high concentrations in the pyrolytic sugar [9]. Secondly, the anhydro-sugars can be hydrolysed to monomer sugars, which can act as carbon substrate for PHB production [14, 15, 16, 17] (Giin-Yu Amy Tan et all., 2014,. Lastly, PHB production is an aerobic process and therefore more complex organic molecules (i.e. aromatics) could be used as carbon substrate.

For the microbial PHB production, the focus will be on short chained length (SCL) polyhydroxy-butyrate and valerate (PHB/V). PHB/V can be produced from small volatile fatty acids (sVFA's), which include: formic, acetic, propionic, butyric and valeric acid [15]. PHB can be produced by a wide variety of bacteria as temporary cellular carbon storage [15].

The produced PHB's can be isolated and used as bioplastic or further thermally converted without prior cell lysis to (methyl-) crotonate. Crotonates are important intermediate building blocks used in several chemical industries like the textile, cosmetic or pharmaceutic industries [18]. In Figure 3 is depicted how PHB can be used as precursor for bulk chemicals required in many industries.

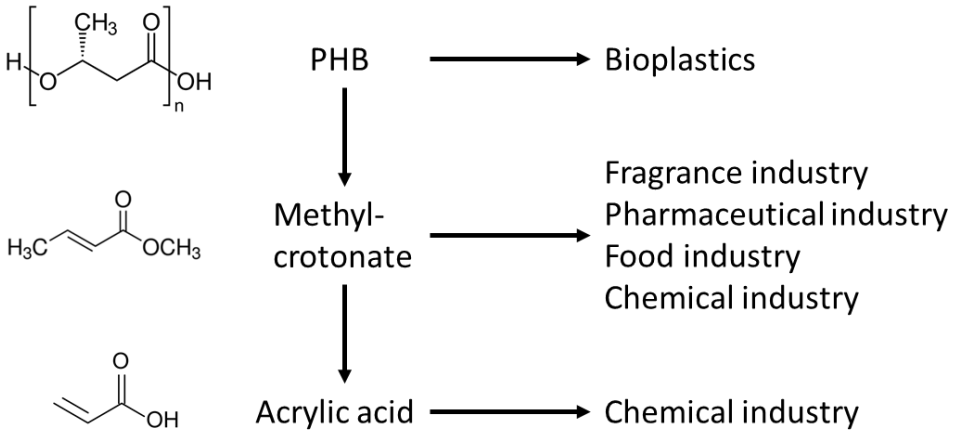

Figure 3. PHB as precursor for methyl-crotonate and acrylic acid used in large quantities in many different industries.

Research objectives

The aim of this project is to develop a fermentation process for PHB production using pyrolytic sugars as substrate.

To reach this aim, a proof of concept should be showed for all separate steps towards the production of PHB. To show the proof of concept the following research objectives should be reached:

1) Show that microorganisms are able to grow on pyrolytic sugars.
2) Develop a detoxifying strategy to promote the cell growth.
3) Implement an acidogenic fermentation for the production of volatile fatty acids.
4) Use of small volatile fatty acids (VFA's) as substrate for PHB production.

Background

Inhibitory compounds

Toxicity is indicated by cells that have an increased lag phase or are unable to grow. This toxicity can be caused by several reasons of which the most significant are: spontaneous reactions with molecules within the cell, ATP depletion, NAD(P)H depletion or loss of cell membrane integrity [19]. The major growth inhibitors present in pyrolytic sugars include: the organic acids, furfural, 5-hydroxymethylfurfural (5-HMF), glycolaldehyde, acetol and several phenolic compounds. These inhibitors should be effectively removed or diluted to low concentrations. This removal can be done chemically, biologically or a combination of both.

To solve these toxicity problems the following steps can be done.

- Dilute the pyrolytic sugar stream to dilute all the toxins present in this stream.
- Increase cell inoculum concentration to increase degradation rates.
- Using adapted cell strains which are more tolerant.
- Extract all inhibitory compounds by filtering (i.e. active carbon).
- Perform a fermentation that will degrade toxic compounds to organic acids.
- Oxidation of pyrolytic sugar fraction to reduce toxicity and increase the biodegradability.

Detoxification methods

To reduce the toxicity of pyrolytic sugar and thereby the inhibition of bacterial growth and conversion, several detoxification methods have been chosen. Some of these detoxification methods are already described in literature for pyrolytic sugar [8, 9, 10]. However, these methods focus often only into the recovery of the sugars. In this study not only are the sugars of importance but the organic acids as well. To detoxify regarding the minimal loss of glucose and acetic acid, the following five detoxification methods have been selected.

First of all, the activated carbon treatment was chosen due to the easy access as this can be made from the by-product of pyrolysis itself. The active carbon treatment has proven itself over the years for its good removal of organic compounds [8, 13, 20, 21]. After adsorption, the activated carbon can be re-activated by solvent extraction or heating.

Secondly, the calcium hydroxide has been selected due its good performance in detoxification of pyrolytic sugar [10]. Besides calcium hydroxide, sodium hydroxide can also be used for overliming. The calcium hydroxide is capable of precipitating different phenolics. Moreover, due to the increase in pH to alkaline conditions the reactive compounds (i.e. acetol, glycolaldehyde)) in pyrolytic sugar can react to less toxic compounds [10].

Furthermore, vacuum evaporation is an exceptional choice due to the relatively low boiling point of acetic acid (118.1 ˚C). However, it has been reported that if the pH of pyrolytic sugar is above 7, the acetic acid will not evaporate due to forcing it into the salt sodium acetate [22]. Therefore, this vacuum evaporation could be an effective method for evaporating the aldehydes and ketones from pyrolytic sugar without losing the acetic acid.

Moreover, the solvent extraction is a well-known method for detoxification of pyrolytic sugar [20]. For pyrolytic sugar choosing a suitable solvent is a difficult task due to the complexity of pyrolytic sugar. Especially, the difference in polarity of the inhibitors is inconvenient. In this study ethyl acetate is used. Ethyl acetate is capable to dissolve phenolics and other low polar compounds and will not dissolve the sugars present [20]. After identification of the inhibitors present in pyrolytic sugar another solvent can be chosen to remove that compound specifically.

Lastly, the XAD-4 resin is chosen for its high affinity to low polar organic compounds like the phenolics that are present in pyrolytic sugar. In literature, the XAD-4 resin is used more often to remove phenols from waste water streams [23, 24, 25, 26]. However, this resin treatment has not been studied intensely for detoxification of pyrolytic sugar.

Table 1. shows a summary of organic compounds that the detoxification methods should remove from pyrolytic sugar.

Table 1. List of detoxification methods and their associated function for pyrolytic sugar.

Detoxification method	Should remove all:
Activated carbon treatment	Aldehydes, ketones, aromatics, furans
Calcium hydroxide overliming	Aromatics, furans
Vacuum evaporation	Aldehydes, ketones, furans
Solvent extraction	Aldehydes, ketones, aromatics, furans
XAD-4 resin treatment	Aromatics, furans

Diluted acid hydrolysis

Most of the sugars present in pyrolytic sugar are not directly fermentable, but they consist in the anhydrous or oligo form. In order to use the anhydrous sugar fraction a hydrolysis step is necessary to perform. The hydrolysis reaction is catalysed by the presence of strong acids and it requires heating for optimal yields. During the hydrolysis the anhydrous sugars will react with water and be converted towards their hydrous form and oligo-saccharides will be cleaved towards the corresponding monomers. For pyrolytic sugar the strategy to gain the optimal yield has been researched. Using 0.1M of concentrated sulphuric acid, a temperature of 120 ˚C and a residence time of 1 hour most of the anhydrous sugars are converted with minimal by-products formed [28]. The most abundant anhydro-sugar in pyrolytic sugar is levoglucosan. After hydrolysis levoglucosan is converted towards the fermentable sugar D-glucose as showed in Figure 4. Other detected sugars are fructose, mannose, arabinose, xylose and sorbitol [9, 28].

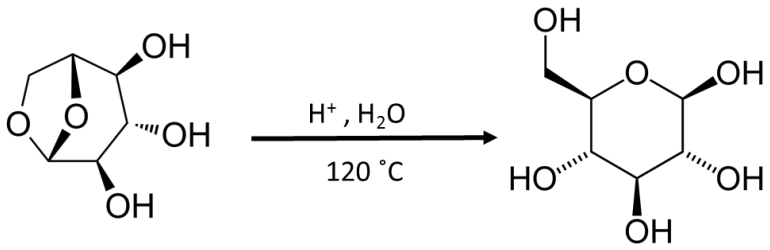

Figure 4. Acid hydrolysis of levoglucosan to D-glucose

Mixed microbial culture

Most research on the recovery of waste/by-products of biochemical processes are focused on using pure or genetically modified organisms [28]. However, waste bulk streams with a low value are often not valuable enough for aseptic recovery. In order to decrease the cost, a mixed microbial culture (MMC) can be used since sterility is not required/wanted for bulk streams. A second reason for the use of MMC is that, microorganisms can be selected by the operating conditions of the reactor. Especially, for toxic environments the operating conditions are important as it will select for tolerant and/or toxic compound degrading microorganisms, while still producing acids or PHB's.

PHB production

For the PHB production a MMC can be used that is selected for high cellular PHB storage capability. Therefore, it is known that this culture is able to produce up to 90% of its dry-weight in PHB [29 , 30]. However, this is under optimal conditions with a large amount of VFA's present and with known nitrogen concentrations [29, 30, 31, 32]. For pyrolytic sugar, the carbon source is more complex and therefore more difficult to ferment. Furthermore, the nitrogen content is unknown in the pyrolytic sugar. A different problem could be the inhibition caused by toxic compounds, which can limit the carbon uptake rate, inhibit growth or force growth instead of PHB production [33].

Materials and methods

Pyrolytic sugar

Pyrolytic sugar was obtained from BTG-group located in Hengelo. They are specialized in the thermal conversion (pyrolysis) of wood to bio-oil. After upgrading the bio-oil with a cold water extraction a waste stream is produced called pyrolytic sugar. This study uses the pyrolytic sugar as carbon source for fermentation.

Diluted acid hydrolysis

To reduce the polysaccharides and anhydro-sugars to their corresponding monomer sugars, pyrolytic sugar was treated with a diluted acid hydrolysis. In literature it has been reported that 0.1 M Sulphuric acid (H_2SO_4) at 120˚C for 1 hour results in the most optimal conversion towards glucose with minimal degradation products forming according to literature [8,9]. After hydrolysis, the sulphate ions were precipitated by addition of barium hydroxide. The precipitate was removed by centrifuge (5000 rcf, 15 min).

pH neutralisation

The raw pyrolytic sugar has a pH value of 2.3 due to the many organic acids present. PHB producing bacteria often require a pH value of around 7 [29]. Therefore, a neutralisation step was required. Neutralisation of pyrolytic sugar was performed by adding sodium hydroxide while stirring. The sodium hydroxide was added slowly to avoid rapid polymerization in alkaline conditions. The precipitate was either removed by centrifuge (5000 rcf, 15 min) or syringe filter dependent on the volume of the sample.

Detoxification methods

To reduce the toxicity of pyrolytic multiple detoxification methods were tried and analysed for their result. The toxicity is analysed with HPLC and microbial growth experiments. The obtained detoxified pyrolytic sugar should be less toxic with a minimal loss in sugars and carboxylic acids.

The following detoxification strategies were performed and analysed:

- Calcium hydroxide, $Ca(OH)_2$ overliming:
  The calcium overliming technique can be used to precipitate the phenolic compounds. A range of 50-100 g of $Ca(OH)_2$ per litre of pyrolytic sugar was used to neutralize all acids. The suspension was shaken for 4 hours at 60˚C. After incubation the suspension was centrifuged (15 min, 7000 RPM) to separate the formed precipitates from the detoxified pyrolytic sugar.
- Activated carbon (Norrit) treatment:
  The activated carbon treatment can be used to bind among other the phenolic compounds To detoxify pyrolytic sugar a 10-30% (w/v) activated carbon suspension was made. The pH of the pyrolytic sugar was either pH 2 or neutralised to pH 7 with sodium hydroxide. This suspension was incubated for 20 hours at room temperature and shaken with 150 RPM. After incubation the suspension was vacuum filtered to separate the activated carbon from the pyrolytic sugar.
- Vacuum evaporation:
  The evaporation method allows separating a selection of toxic compounds from pyrolytic sugar (i.e. acetol and furfural). For the vacuum evaporation a rotary evaporator (Buchi Rotavapor R-200) was used. The water bath temperature was set at 80˚C and the minimum pressure was

set at 45 mbar. This results in evaporating compounds with a boiling point up to 170˚C. To prevent acids from evaporating the pyrolytic sugar solution was neutralized to ensure that all carboxylic acids are in a dissociated form. The pressure was lowered stepwise to 45 mbar to prevent uncontrolled boiling of pyrolytic sugar. The vacuum evaporation was stopped when a thick brown viscous liquid was left over. Afterwards, the brown liquid was dissolved in distilled water to reach a volume equal to the starting volume.

- Hydrophobic polyaromatic resin (Amberlite XAD-4):
  XAD-4 resin is a polyaromatic adsorbent for small hydrophobic compounds Therefore, this resin in capable of binding phenolic compounds present in pyrolytic sugar. A 10-20% (w/v) XAD-4 suspension was made to detoxify pyrolytic sugar. The pH of the pyrolytic sugar was either pH 2 or neutralised to pH 7 with sodium hydroxide. This suspension was incubated for 20 hours at 30˚C and shaken with 150 RPM. After incubation the suspension was vacuum filtered to separate the resin from the pyrolytic sugar. The resin was subsequently washed with distilled water and eluted with methanol for analysis.
- Solvent extraction:
  Ethyl acetate was used to extract compounds like phenolics from pyrolytic sugar. Two different ratios of ethyl acetate were used for extraction. 1:1 and 4:1 for ethyl acetate to pyrolytic sugar respectively. These solutions were incubated for 20 hours at 25˚C and shaken with 150 RPM. After incubation the solution was rested for 6 hours to have a good separation of liquids. Left-overs of ethyl acetate were removed with the rotary evaporator. The water bath temperature was set at 40˚C and the pressure was set at 200 mbar.

After the most promising detoxification method was selected combinations of other methods were made to check for further decrease toxicity. For the successive acidogenic and PHB fermentations a combination of a XAD-4 resin treatment and vacuum evaporation was used.

Fermentation

Several fermentations have been performed each with their own goal. First, aerobic fermentations have been performed to test different detoxified pyrolytic sugar samples. Secondly, to reduce the complexity of carbon sources in pyrolytic sugar an acidogenic fermentation has been performed. Lastly, the detoxified and fermented pyrolytic sugar has been fermented again for PHB production with help of TU Delft.

Toxicity

To test the toxicity of pyrolytic sugar a pure culture of the model microorganism *Pseudomonas putida* KT2400 was used. This organism was obtained from System and synthetic biology (SSB) department of Wageningen University. *P. putida* is capable of growing on the hydrolysed sugars, acids and phenolics that are present in pyrolytic sugar [21]. Secondly *P. putida* is capable of producing polyhydroxyalkanoates [34]. Lastly, *P. putida* is relatively tolerant to the toxic compounds present in the pyrolytic sugar compared to other micro-organism like Escherichia coli and Saccharomyces cerevisiae. *P. putida* is able to withstand relatively high concentrations of phenols (0.7% v/v) [34].

P. putida is grown aerobically at 30˚C at pH 7 at either rich or minimal media. All experiments were conducted in sterile environment. All equipment and media components was either sterilized with an autoclave (121˚C, 20min) or filtered through a 0.2 µm syringe filter.

Toxicity characterization has been performed on both liquid and solid media. Petri dishes with solid media were inoculated with an inoculation loop from an overnight culture grown on rich media. This overnight culture is cultured from an adapted strain of *P. putida*. The adapted strain is selected by slowly increasing the pyrolytic sugar concentration in petri dishes and continues inoculating with the largest colony.

After four days of growth, the petri dishes were inspected to check for grown colonies and their corresponding size. Liquid media was used initially for characterization of growth in pyrolytic sugar samples but due to spontaneous precipitation and browning the growth measurement were inaccurate. However, liquid media was used for checking the biodegradability of known carbon sources and unidentified compounds present in pyrolytic sugar.

Acidogenic fermentation

For performing the acidogenic fermentation, anaerobic sludge was obtained from the wastewater treatment Bennekom. The slurry was acidified to pH 5 and heated to 70˚C for 30 min to select for acidogenic spore forming bacteria. The obtained sludge was used for selecting an undefined mixed microbial culture (MMC). The MMC was grown in anaerobic conditions at 36˚C on minimal media at different constant pH values (6-8.5). The MMC was selected for volatile fatty acids (VFA's) production while keeping ethanol concentrations low. Afterwards the MMC was adapted for growth on pyrolytic sugar by stepwise decreasing the dilution of pyrolytic sugar. Fermentations on pyrolytic sugar are based on minimal media enriched with vitamins and trace elements to reduce the growth lag phase caused by the toxic environment. All acidogenic fermentations have been performed in non-sterile conditions.

The acidogenic fermentations were performed in a 0.5 L batch lab scale reactor with internal water jacket (Figure 5). This water jacket is heated by an external water bath to 36˚C. The content of the reactor was continuously stirred with a magnetic bead and a magnetic stirrer underneath the reactor. The pH was measured and controlled at 6.5 with a Titrino plus from Metrohm. pH adjustments were made with 5M NaOH. For the anaerobic fermentations the reactor was flushed with N_2 gas for 5 minutes (200 ml/min). Accumulation of pressure inside the reactor was prevented with a water trap. Every day a 1mL sample was taken and frozen to be analysed later. Fed batch fermentations were performed by injecting an extra pulse (10% v/v) of pyrolytic sugar based on the volume present in the reactor.

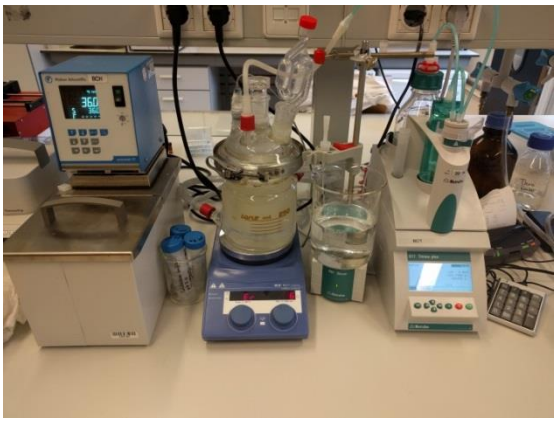

Figure 5. Set-up for the acidogenic fermentation

The nitrogen limited fermentations were performed in the lab of bioprocess technology (BPE, Wageningen University). Four mini bioreactors from Applikon were used and controlled with mycontrol from Applikon (Figure 6). The working volume was 200 mL, temperature 36˚C and the pH was maintained at 6.5 and anaerobic conditions were assured by a constant stream of N_2 gas (10 mL/min). pH adjustments were made with 2.5M NaOH. Off gas was first condensed before it was filtered in a 0.45µm gas filter and fed into the local off gas system. Every day a 1mL sample was taken for analysis.

Figure 6. Set-up for the nitrogen limited fermentations.

Media

For the growth experiments both minimal media and rich media was used. The composition of these media can be found in Table 2 and 3. Minimal media can be enriched by the addition of optional compounds. Agar plates were made by dissolving agar in the (enriched) media.

Table 2. Minimal and rich media composition

Defined minimal media	Concentration (mM)
Na_2HPO_4-$2H_2O$	33.7
KH_2PO_4	22
NaCl	8.55
NH_4Cl	9.35
$MgSO_4$-$7H_2O$	1
$CaCl_2$-$2H_2O$	0.3

Optional compounds	**Concentration (g/l)**
Carbon source : Glucose/ Acetic acid	0-20
Pyrolytic sugar	0-300
Trace elements 100x	1X
BME Vitamins 100x	1X
Yeast extract	1-10
Anti-Foam B	0.005
Agar	15
Undefined rich media	
Luria broth base (LB), Miller (L1900)	35

Table 3. Trace element composition

100X Trace element solution	**Concentration (µM)**
EDTA	13400
$FeCl_3$-$6H_2O$	3100
$ZnCl_2$	620
$CuCl_2$-$2H_2O$	76
$CoCl_2$-$2H_2O$	42
H_3BO_3	162
$MnCl_2$-$4H_2O$	50.5

PHB production

For polyhydroxybutyrate (PHB) production an aerobic mixed microbial culture was used in the Biotechnology department of TU Delft (Prof. Dr. ir. M. Loosdrecht). The selected MMC is capable of reaching 90% PHB (w/w) [29]. The culture fermented on the detoxified and fermented pyrolytic sugar substrate in accumulation experiment (Figure 7). In an accumulation experiment there are minimal nutrients present except for the carbon source. Therefore, the bacteria are able to store this excess carbon source in the form of PHB. The presence of PHB is visualized with an optical microscope (1000x) and afterwards analysed following their protocols in the lab of TU Delft.

The used mixed culture is selected for its high PHB storage capability by using the feast and famine method with nitrogen limited growth. In the feast period there is sufficient carbon source present but only limited growth due to low nitrogen availability. Therefore, the microorganisms are forced to store the carbon in the form of PHB. In the famine period there is no carbons source present and the microorganisms are required to use their stored PHB for cell maintenance and growth.

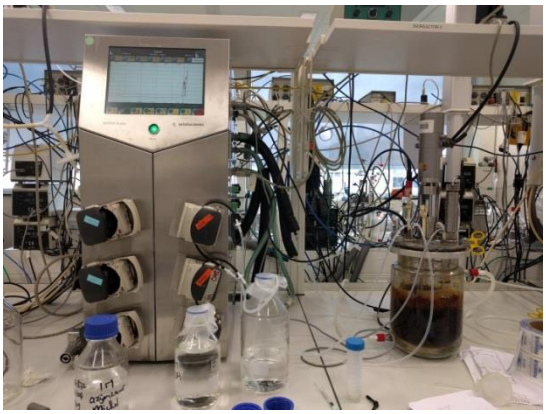

Figure 7. Reactor set-up for the PHB accumulation experiment in TU-Delft.

HPLC

High-Performance Liquid Chromatography (HPLC) was performed on a Ultimate 3000 with a Biorad aminex HPX-87H column (7.8x300mm, 50˚C) for organic acids. 5mM H_2SO_4 (aq, 0.5 mL/min) was used as eluent. The samples (injection 10 µl) were analysed using a UV-detector at 210 and 280 nm and a RI detector for 70 minutes. Quantification was performed by calibrations with pure compounds. All samples were at least ten times diluted to ensure that peak area's are within the calibration range.

Phenol analysis was performed on a Acquity ultra HPLC with a BEH C18 column (2.1x150mm, 40˚C). A gradient mixture of 0.02% formic acid (eluent A) and 70% acetonitrile was used as eluent (eluent B) at a flowrate of 0.35 mL/min as explained in Table 4. The samples (injection 5-50 µl) were analysed using a UV-detector at 210 and 280 nm.

Table 4, gradient used for phenol analysis (uHPLC)

Time (min)	Ratio solvent A to B
0-2	100:7
2-18	0:100
18-18.1	100:7
18.1-20	100:7

Chemical oxygen demand analysis

To analyse the amount of organic carbon in pyrolytic sugar, a chemical oxygen demand (COD) analysis was performed. In this analysis all organic compounds are oxidised toward CO_2. By using the COD analysis it is possible to quantify the removal of organic carbon during the detoxification. Secondly, by combining the COD and HPLC results, a balance can be made how much of the organic carbon is identified by HPLC.

The COD analysis is performed with a HachLange COD analysis kit (1000-10000 mg O2/L). Samples were diluted between 25-300 times in order to reach the sufficient oxidation range. After the addition of 0.5 mL of sample to the reaction mixture, the suspension was digested for 2 hours at 148˚C. After the digestion the absorption is measured with a spectrophotometer at 680 nm. This adsorption is proportional to the amount of oxidation and thus to the amount of organic carbon that is present in the sample.

Total phenolic content

To analyse the total phenolic content in pyrolytic sugar, a Folin-Ciocalteu (FC) analysis was performed. This analysis is often used in the wine industry to estimate the total phenolic content. Recently, the FC method is also tested for its reliability on pyrolytic sugar and bio-oil [5]. The FC method is based on the chemical reduction of tungsten and molybdenum oxides. The products of the chemical reduction have a blue colour with an absorption maximum at 765 nm. The light intensity is proportional to the concentration of phenolics.

A calibration curve was made with *p*-coumaric acid with a range of 0-500 mg/L. Pyrolytic sugar samples were diluted either 10 or 100 times dependent on the their expected phenolic content. 20 µL of sample was diluted in 1.58 mL distilled water, followed by 100 µL FC reagent (2N, Sigma Aldrich) and mixed by pipetting. After 1-8 minutes of incubation, 300 µL of sodium carbonate solution (200 g/L) was added, mixed with a vortex and incubated for 2 hours at room temperature. At last, the absorbance was measured in a spectrophotometer with 1 cm cells.

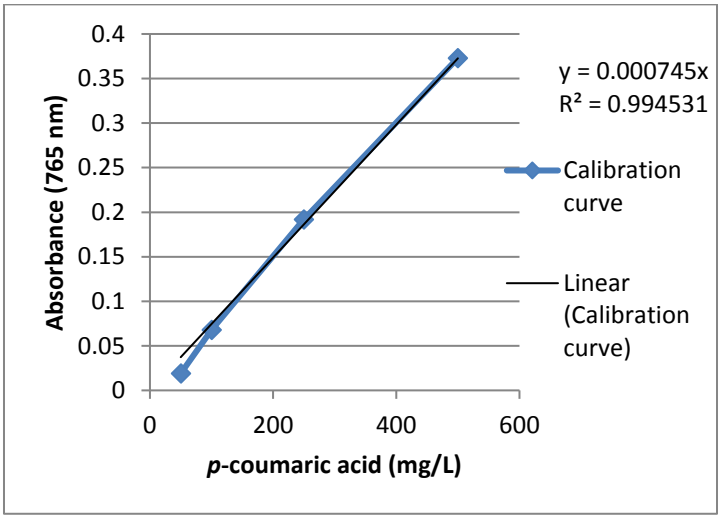

Figure 8. Calibration curve for the FC analysis. *p*-coumaric acid is representative for other phenolics.

Results and Discussion

In this chapter the results that are found are presented and discussed, evaluating if pyrolytic sugar is a suitable carbon substrate for PHB fermentation. Starting with the toxicity of pyrolytic sugar and how this can be effectively reduced. Followed by increasing the organic acid content by fermentation and reducing the carbon source complexity. Lastly, if micro-organisms are able to utilize pyrolytic sugar and convert the organic acids into intracellular PHB granules. A list with a rough quantification of important carbon compounds in raw pyrolytic sugar can be found in Appendix A.

Inhibitor identification

As mentioned before, the main problem of the fermentation of pyrolytic sugar is the microbial toxicity. The toxicity originates from numerous compounds present in pyrolytic sugar and they should be removed to gain an efficient fermentation.

An example of the toxicity and reactivity is the response of changing the pH of pyrolytic sugar. The dark brown/red solution changes to light brown if the pH is closer to 7. However, the solution turns dark brown again after increasing the pH further to alkaline conditions. This could be caused by oxidation of hydroxy-phenolics towards quinone's and pyrone's [35].

By comparing chromatographs of different detoxifications methods with their corresponding microbial toxicity gained by growth experiments, it was possible to indicate peaks that correlate with toxicity. Figure 9 shows two chromatograms from raw pyrolytic sugar and calcium overlimed pyrolytic sugar. From the observed peaks that are removed several are identified. This list includes: glycolaldehyde, ethylene glycol, glyoxal, glyoxalic acid, glycolic acid, acetol, 5-HMF, furfural, methanol, catechol and phenol. All of these compounds are known for their microbial toxicity [5].

Some peaks were not identified but classified as correlated to microbial toxicity. Interestingly, the major peak found was correlated to toxicity. This peak is indicated in red in Figure 9 with a retention time (RT) of 34 minutes. Based on the polarity, size and absorption at different wavelengths (210nm, 256nm, 280nm), it could be an oxygenated cyclic organic compound like 1,2-cyclopentanedione, 2(5H)-furanone, succindialdehyde or a nitrogenous cyclic compound like piperidine or pyrrolidine. Oxygenated polar phenolic compounds like pyrogallol can be excluded due to the lack of absorption at 256 and 280nm.

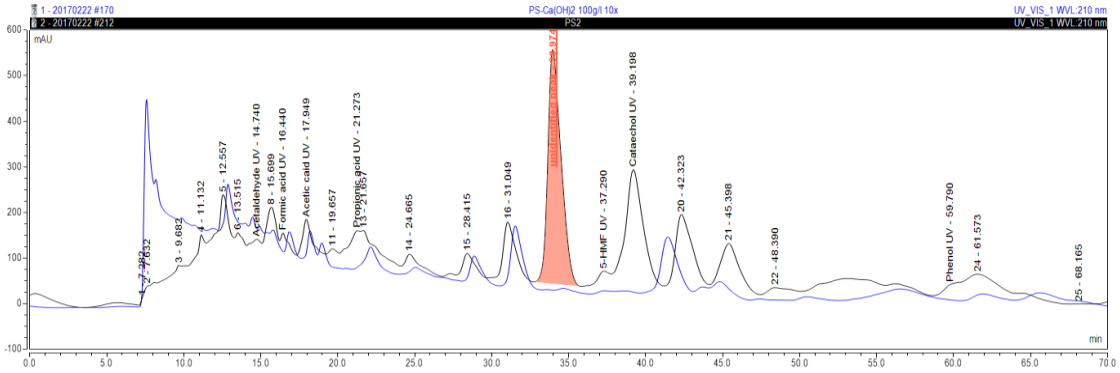

Figure 9. HPLC chromatogram comparison between raw pyrolytic sugar (black) and calcium overlimed pyrolytic sugar (blue). Detection is at 210 nm with a run time of 70 minutes.

Detoxification methods

In chapter 2 several detoxification methods are mentioned, which can remove the inhibitors from pyrolytic sugar. These methods were tested for their inhibitor removal and the corresponding influence on the toxicity of pyrolytic sugar. The toxicity analysis was tested by growth experiments with *P. putida*. In Table 5, the dilution is shown for different samples at the point that *P. putida* is unable to form colonies within 4 days. As reference, the toxicity for pyrolytic sugar has been determined. It was found that *P. putida* was unable to handle concentrations higher than 1% (v/v) pyrolytic sugar in rich media and 0.4% (v/v) in minimal media. The higher tolerance in rich media could be caused by the presence of amino acids and vitamins, which reduces the growth lag time. Secondly, it could be that glycolaldehyde is binding to amino acids forming a Schiff's base [36]. Thereby, a natural buffer arises that reduces the toxicity of pyrolytic sugar.

From the results of Table 5 can be concluded that calcium overliming and vacuum evaporation are the most effective detoxification methods to decrease the microbial toxicity. The least effective method was the solvent extraction with ethyl acetate.

Table 5. Comparison of toxicity removal by different detoxification methods.

Detoxification method	Toxic concentration of pyrolytic sugar (v/v)
Raw pyrolytic sugar, minimal media	0.4%
Raw pyrolytic sugar	1.0%
10% (w/v) activated carbon	3.0%
30% (w/v) activated carbon	5.0%
10% (w/v) activated carbon, pH 7	2.0%
1:1 diluted and 10% (w/v) activated carbon	2.0%
Ethyl acetate extraction 1:1	1.0%
Ethyl acetate extraction 4:1	2.5%
Calcium overliming 100 g/L	**10.0%**
10% (w/v) XAD-4 resin, pH 7	3.0%
20% (w/v) XAD-4 resin, pH 7	3.0%
Vacuum evaporation, boiling point up to 165 ˚C	**7.5%**

The following step was linking the determined microbial toxicity's to the results of the HPLC analysis. To compare the detoxification methods, three peaks have been selected. Namely, acetic acid, acetol and the unknown peak (RT=34 min, Figure 5). The glucose peak could not be integrated correctly due to small overlapping with another unknown peak. The selected peaks can be integrated without noise from other overlapping peaks and therefore, give a more reliable quantification. Table 6 shows the recovery (%) of the integrated peaks found before and after the detoxification method. The best method should have a high recovery of acetic acid and have a low recovery for acetol and peak (RT=34min).

Based on the recoveries found in Table 6, the vacuum evaporation and calcium overliming methods show the best inhibitory removal. For both methods there was no indication of acetic acid loss and both acetol and glycolaldehyde are completely removed while this was not the case with other methods. This suggests that the high decrease in toxicity is caused by the removal of acetol and glycolaldehyde. For the calcium overliming it is interesting that these compounds are removed because these compounds should not precipitate. Probably, due to the alkaline conditions reactive compounds like acetol have reacted to less toxic molecules. Note that, the calcium overliming and the vacuum evaporation reached an acetic acid recovery of over 100%. This is probably due to better integration caused by lowered background noise. It could be that by increasing the amount of calcium hydroxide, the detoxification becomes more effective. However, if the concentration exceeds the solubility of calcium acetate, a loss of acetic acid is expected.

The activated carbon treatment results showed removal of inhibitors, but at low pH is also showed removal of acetic loss, which is undesirable. This loss can be prevented by neutralising the pH of pyrolytic sugar before treatment. However, this seems to result in a less effective inhibitor removal.

Ethyl acetate extraction showed unwanted results for both acetic acid and inhibitory compounds removal. Together with the microbial toxicity analysis this solvent seems unsuitable for the chosen application. Solvent extraction with a different solvent is still an option. However, finding a solvent that can remove both non-polar and low polar compounds while, not removing acetic acid and sugars, is a difficult and time consuming process.

The XAD-4 treatment showed no loss in acetic acid but also a minimal removal of acetol and peak (RT=34 min). However, the toxicity analysis showed a visible improvement (Table 6). This can be explained by the HPLC chromatogram, which showed a clear reduction in the peaks of furans and phenolics. The hydrophobic groups on the resin have a low affinity for the more polar compounds like acetol. However, the XAD-4 resin is capable of binding the low polar aldehydes such glycolaldehyde (Appendix B).

The last detoxification method that is analysed is vacuum evaporation. By increasing the pH to be higher than 7, the evaporation of acetic acid can be prevented. The evaporation is prevented due to acetic acid being forced into the sodium acetate salt. Furthermore, any nitrogenous compounds like pyrimidine (boiling point of 123 ˚C) will be removed during the vacuum evaporation and thereby not influence the PHB fermentation. In Table 6 can be seen that the recovered acetic acid concentration for vacuum evaporation is higher than 100%. This is probably due to a better peak integration caused by lowered background noise. However, it is possible that acetic acid is formed during the heating of pyrolytic sugar. Furthermore, the vacuum evaporation method showed good removal of acetol and peak (RT=34min). The main disadvantage from this method is the evaporation of water. The evaporation of water causes a large energy requirement and a water diluted side-product.

Table 6. Comparison of polar compound removal by the different detoxification methods

Detoxification method, recovery (%)	Acetic acid (%)*a	Acetol (%)*b	Peak (RT=34min) (%)*b
10% (w/v) activated carbon	87.1	73.8	39.8
30% (w/v) activated carbon	38.0	36.0	14.3
10% (w/v) activated carbon, pH 7	102.6	110.4	56.1
1:1 diluted and 10% (w/v) activated carbon	40.9	48.9	55.6
Calcium overliming 100 g/L	115.9	7.4	N.D.
Ethyl acetate extraction 1:1	60.5	71.7	39.8
Ethyl acetate extraction 4:1	27.8	54.1	23.4
Vacuum evaporation, boiling point up to 165 ˚C	114.5	17.4	8.9
10% (w/v) XAD-4 resin	101.0	101.8	95.6
20% (w/v) XAD-4 resin	92.2	97.0	81.4

* The percentages are based on the concentrations found in raw pyrolytic sugar
a High recovery of acetic acid is desired
b Low recovery of acetol and peak (34min) is desired

From these previous results can be concluded that calcium overliming is the most effective detoxification method. However, calcium overliming requires a high weight per volume percentage of calcium hydroxide (at least 100 g/L). This could limit its economic potential. Furthermore, the re-use potential of the precipitated slurry is limited. Therefore, if previous conclusions and the re-use of the removed organic compounds are considered, a combination of XAD-4 resin together with a vacuum evaporation step is a promising detoxification strategy.

During the XAD-4 experiments it was found that the pH had a significant influence on the removal of inhibitors, shown in Table 7. This pH effect was only expected for phenolics due to conversion to the phenolate state if the pH reaches alkaline conditions. This effect can be seen at multiple compounds including the furans and the unknown peak (RT=34 min) (Table 7). However, this effect is already noticeable after neutralising the pyrolytic sugar. Therefore, it could be that while neutralising pyrolytic sugar some of these compound are reacting or precipitating. Because of the reduction in peak area's while neutralising, it cannot be concluded that furans are more easily removed at pH 7 compared to the pH of raw pyrolytic sugar. But it can be concluded that neutralising pyrolytic sugar has a positive effect on the inhibitor removal.

Table 7. Comparison of inhibitor removal by XAD-4 resin at different pH conditions

Detoxification method, Recovery (%)	Furfural (%)*	5-HMF (%)*	Peak (RT=34 min) (%)*
Pyrolytic sugar, pH 7	66.4	65.3	74.8
10% (w/v) XAD-4 resin	55.1	92.8	95.6
10% (w/v) XAD-4 resin, pH 7	20.1	50.5	56.4
20% (w/v) XAD-4 resin	27.6	79.2	81.4
20% (w/v) XAD-4 resin, pH 7	18.2	38.8	40.1

* The percentages are based on the concentrations found in raw pyrolytic sugar, low recovery is desired

Pyrolytic sugar detoxification

In this chapter the previous mentioned promising detoxification strategy is further researched. For the final detoxification strategy also the acid hydrolysis should be considered for the conversion of the anhydro-sugar. The new detoxification strategy is divided into 3 main parts:

1. Acid hydrolysis; converting the anhydro-sugars to fermentable monomers.
2. Phenol removal; using XAD-4 resin to remove phenolics and furans.
3. Removing of aldehydes and ketones; by evaporating all compounds with a boiling point up to 165C can be removed.

To quantify the organic compounds that have been removed during these detoxification steps, the chemical oxygen demand (COD) of pyrolytic sugar has been measured. In figure 10 the measured COD values are shown during the different steps in the detoxification strategy. Interestingly, the COD value after the hydrolysis seems to be higher than in raw pyrolytic sugar. Especially, because during the hydrolysis a black viscous gum has formed. The higher COD value could be explained by that the COD analysis has difficulties completely oxidizing polyphenols. After the hydrolysis, some of these polyphenols can be hydrolysed to smaller phenolics and thereby, more easily measurable by the COD analysis. Furthermore Figure 6 shows a clear reduction in the COD values during the detoxification strategy, indicating the removal of several inhibitors.

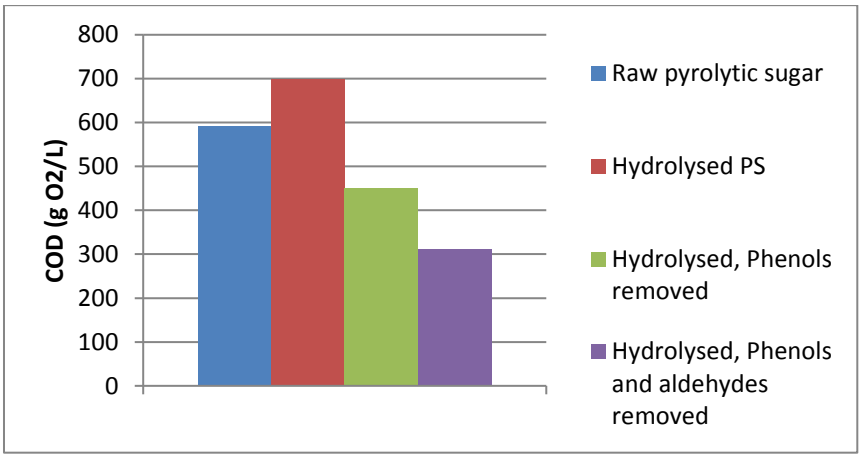

Figure 10. COD comparison between the successive detoxification steps.

To compare these different compounds with each other the value found from the phenolic and HPLC analysis have been normalized to the value found in raw pyrolytic sugar. The first detoxification step is the hydrolysis. In Figure 11 the relative concentrations are shown for the selected compounds. As can be seen, there is a significant reduction in the concentration of furfural and 5-HMF. This reduction is probably caused by polymerization reactions with degradation products or glycolaldehyde which was not detected anymore after the hydrolysis. The most known polymerization reaction is the production of humins during hydrolysis [37]. The same explanation can be used for the phenolics and acetol. Polymerization of phenolics has been noticed during hydrolysis in earlier studies [38].

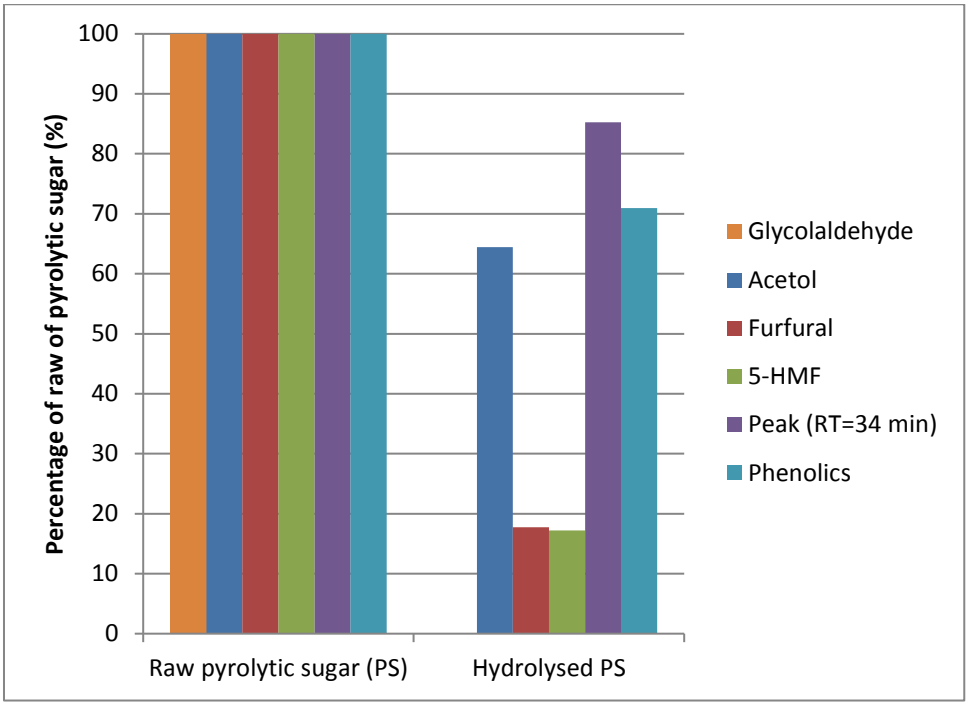

Figure 11. Inhibitory removal comparison between raw pyrolytic sugar and the hydrolysed sample. Hydrolysis time was 60 minutes at 120 ˚C

These polymerization reactions can be confirmed by the presence of a black viscous gum that was formed in the hydrolysis reactor. However, the viscous gum was not only observed on the bottom of the reactor but also above the liquid level what suggests that other volatile reactive compounds are polymerizing.

The following step in the detoxification strategy is the XAD-4 resin treatment. This step was meant for the removal of furans and phenolics. The removal can be seen in Figure 12. Both the furfural and 5-HMF are almost completely removed and a large reduction in phenolics can be seen. There is also a reduction in the unknown peak (RT-34 min), which supports the hypothesis that it is a cyclic low-polar compound.

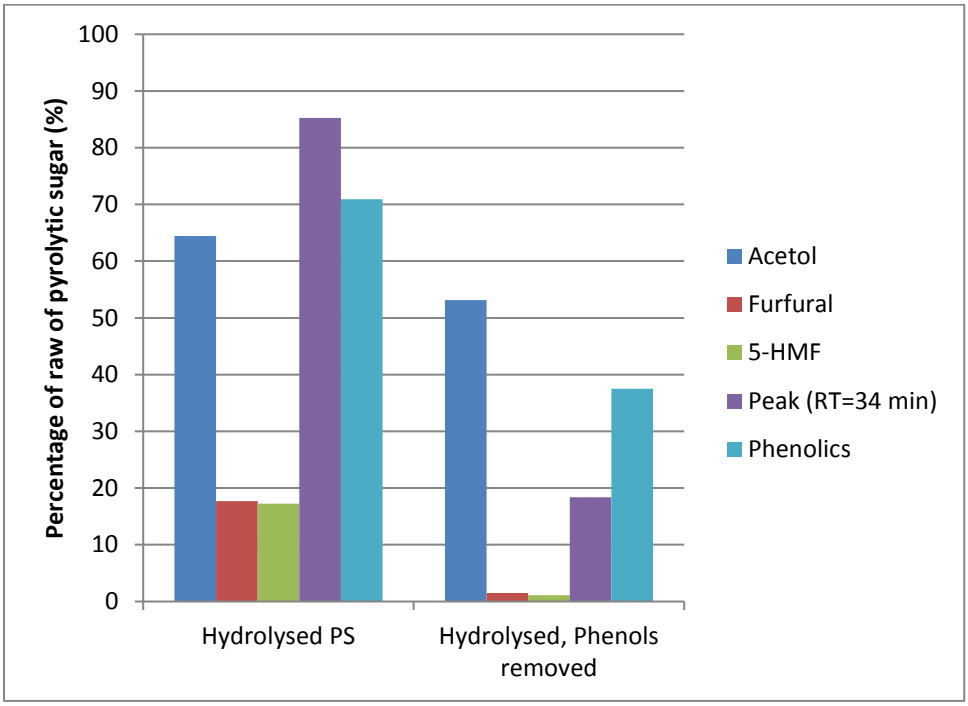

Figure 12. Inhibitory removal comparison between hydrolysed pyrolytic sugar and hydrolysed and XAD-4 treated pyrolytic sugar. Resin retention time was 20 hours at 25 ˚C

The last step of the detoxification strategy is the vacuum evaporation step. The vacuum evaporation was meant for evaporating aldehydes, ketones, furans, volatile phenols and water from pyrolytic sugar. In Figure 13 the remaining concentrations of the inhibitors are shown. Acetol is almost completely removed from pyrolytic sugar and the remaining furans are evaporated as well. However, there was only a small difference in the total phenolics visible. The small difference could be due to not all volatile phenolics (i.e. vanillin) being removed during the XAD-4 treatment. Interestingly, the unknown peak (RT=34 min) was completely evaporated from pyrolytic sugar. This suggests that the boiling point of the unknown compound is 165˚C or below.

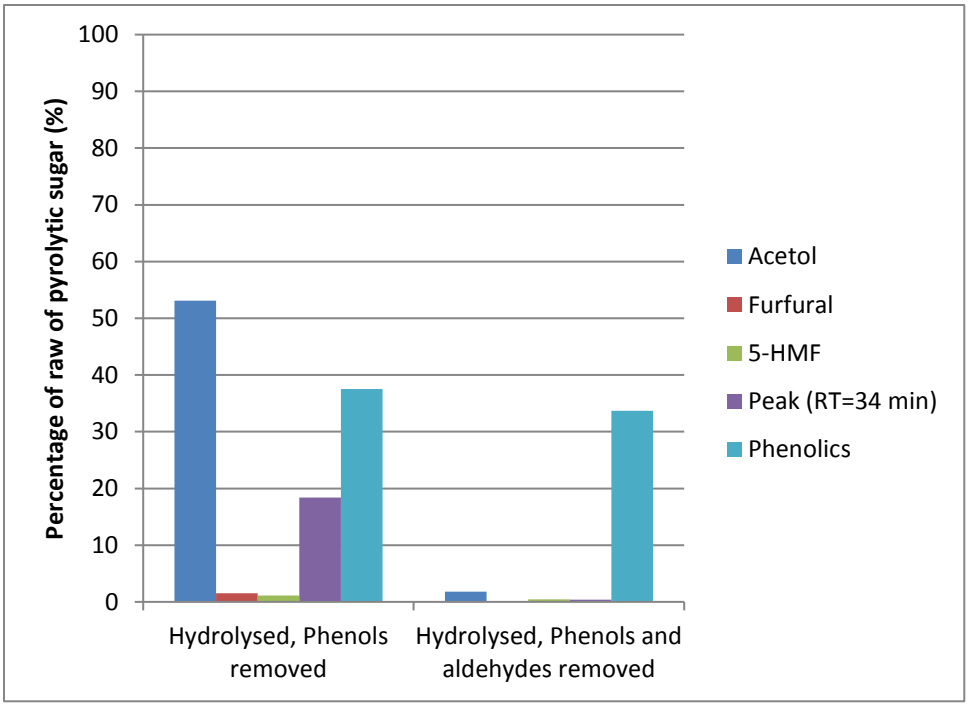

Figure 13. Inhibitory removal comparison between hydrolysed and XAD-4 treated pyrolytic sugar and hydrolysed, resin treated and vacuum evaporated pyrolytic sugar. Water bath temperate was 80 ˚C and pressure was reduced to 40mbar for 1 hour.

In Figure 14 an overview is given of the removal of inhibitors over the whole detoxification strategy. As can be seen, overall the detoxification is successful in removing inhibitors from pyrolytic sugar. Similar results can be seen in the HPLC chromatograms (Appendix C) where a comparison is made between raw and detoxified pyrolytic sugar.

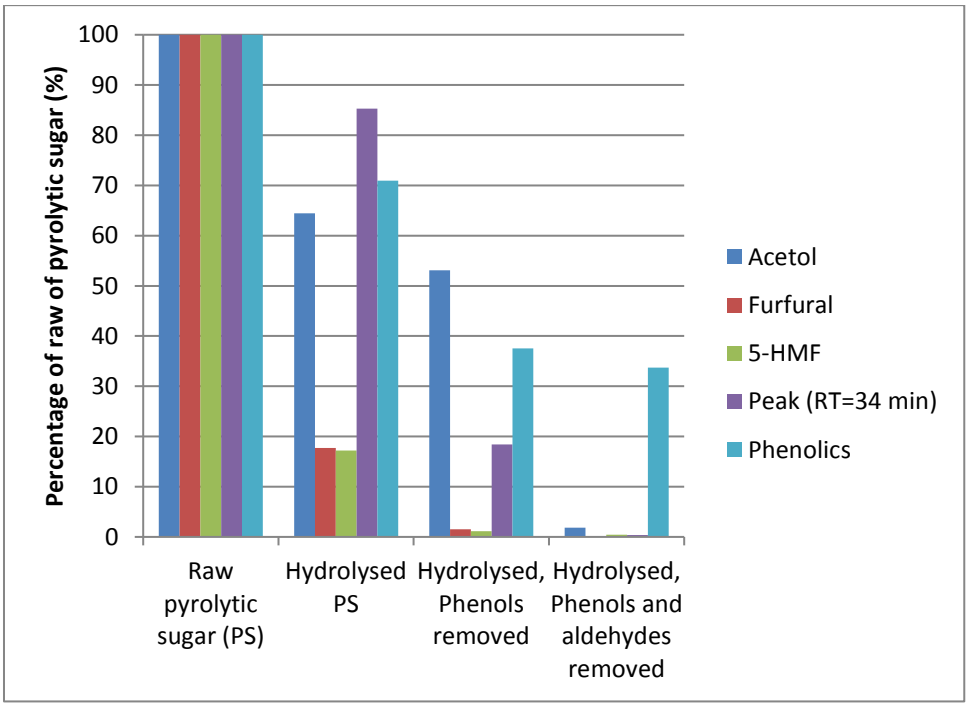

Figure 14. Overview of the inhibitory compound removal during the whole detoxification strategy.

To confirm that the current used detoxification strategy is sufficient for removing inhibitors, the losses of important carbon sources like glucose and acetic acid should be low. In Figure 15, the concentration profile of glucose and acetic acid is shown during the successive detoxification steps. As expected, the glucose concentration rises during the hydrolysis step due to the hydrolysis of levoglucosan. Glucose concentrations of average 38 g/L have been measured (Figure 11). Furthermore, there is an unexpected significant increase (unpaired t-test, p=0.0002) in the concentration of acetic acid after the hydrolysis. The increase of acetic acid could be due to acetic acid formation from degradation or better integration caused by a lowered background noise in the HPLC analysis.

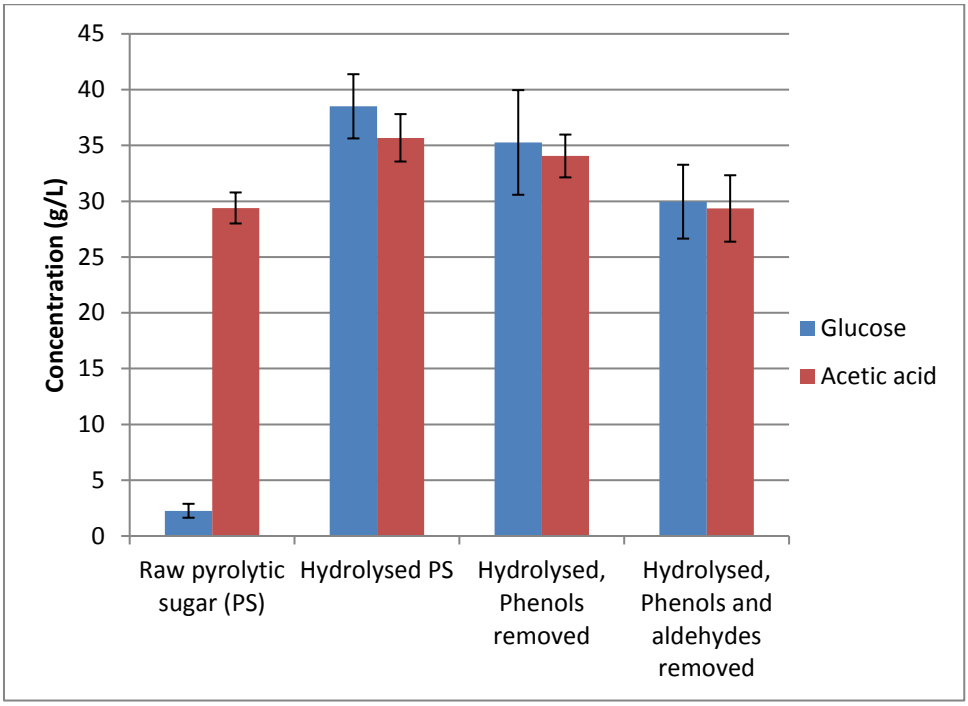

Figure 15. Overview of the concentration profile of glucose and acetic acid during the whole detoxification strategy.

In the following detoxification steps, both glucose and acetic acid show a reduction in concentration. Based on the mean values measured, the average loss over the XAD-4 resin and vacuum evaporation steps of glucose and acetic acid are 22 and 18% respectively. The loss has been calculated based on the average values measured in the hydrolysis step. Note that, the variations of the samples are noticeable (Figure 15). Therefore, it cannot be concluded which steps significantly causes the largest losses. However, from these results can be concluded that the loss of glucose and acetic acid is acceptable for this selected detoxification strategy. Note that, all detoxification steps are not optimized and therefore the loss of substrates could be lowered. The order of the detoxification methods was briefly studied but no significant results were found regarding the toxicity of pyrolytic sugar. However, the organic content and associate concentrations in the resin eluate and distillate can differ.

The total phenolic content was measured with the Folin-Ciocalteu (FC) analysis as mentioned in material and methods. This method was calibrated with *p*-coumaric acid. However, not all phenolics have an equal response towards this method [6]. Therefore, the total phenolic content is only a rough estimation of the phenolics present in pyrolytic sugar. In Figure 16, a summary of the total phenolic content is given over the different steps of the detoxification strategy. The FC analysis results in a starting concentration of almost 50 g/L of phenolics. After the detoxification this concentration is reduced to 17 g/L. The error bars show the differences between two independent measurements of a sample.

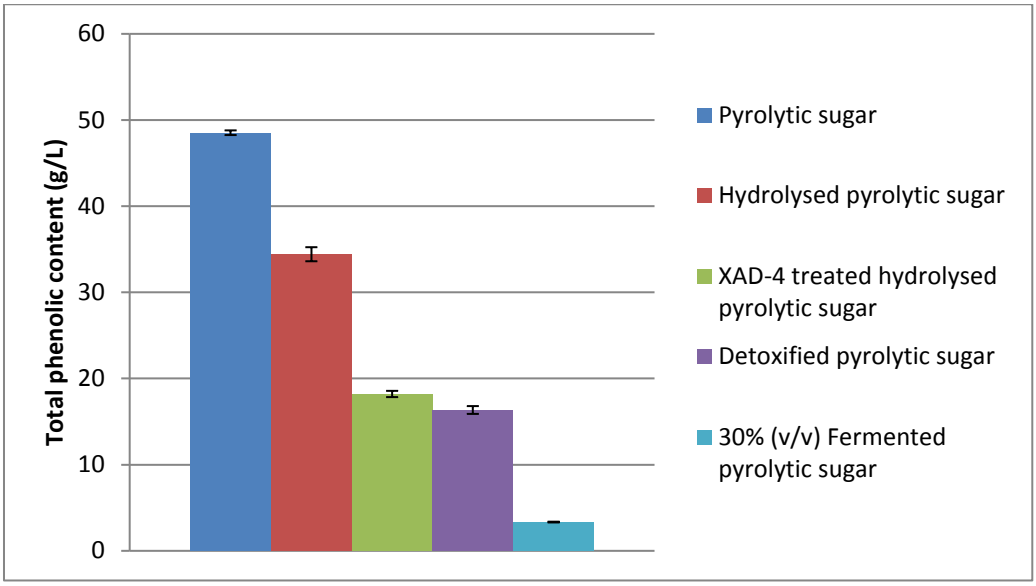

Figure 16. Overview of the total phenolic content during the detoxification of pyrolytic sugar. The phenolic content is measured with the FC analysis method.

Pyrolytic sugar fermentation

After detoxification of pyrolytic sugar it is possible to ferment the organic compounds in pyrolytic sugar towards PHB. However, as stated in the introduction most PHB producing micro-organisms have a carbon source preference. Secondly, the PHB production should be fast to have minimum carbon losses due to cell maintenance. Converting a complex range of carbon sources is more time consuming then a few simple carbon sources. Therefore, it is commonly accepted that small volatile fatty acids (VFA's) are the preferred substrate for PHB production [30, 31, 32]. These VFA's require only a few conversions to form PHB.

To summarize, the acidogenic fermentation is hypothesised to:

- Reduce carbon source complexity.
- Increase the VFA content in pyrolytic sugar
- Further reduce pyrolytic sugar toxicity.

Carbon source improvement

To reduce the carbon source complexity, an acidogenic fermentation was implemented. The fermentation should convert not only most of the sugars present into volatile fatty acids but also, more complex carbon sources. In Figure 17 a fed batch of the acidogenic fermentation profile is shown. The graph shows the concentrations of the wanted volatile fatty acids and ethanol during the fermentation. At 60 and 230 hour an extra pulse (10% v/v) of detoxified pyrolytic sugar was injected in the reactor, bringing the total concentration of pyrolytic sugar to 20 and 30% (v/v) respectively. In Figure 17 can be seen that small volatile fatty acids, ethanol and succinic acids are produced during the fermentation. The formation of ethanol and succinic acid should be prevented due to the increased energy requirement for the conversion towards PHB. By further adapting and selecting the MMC, the formation of unwanted side-products can be prevented. Note that, the ethanol concentration shown is a rough estimation due to overlapping peak in the HPLC chromatogram. From Figure 17 can be concluded that it is possible to increase the VFA content in pyrolytic sugar.

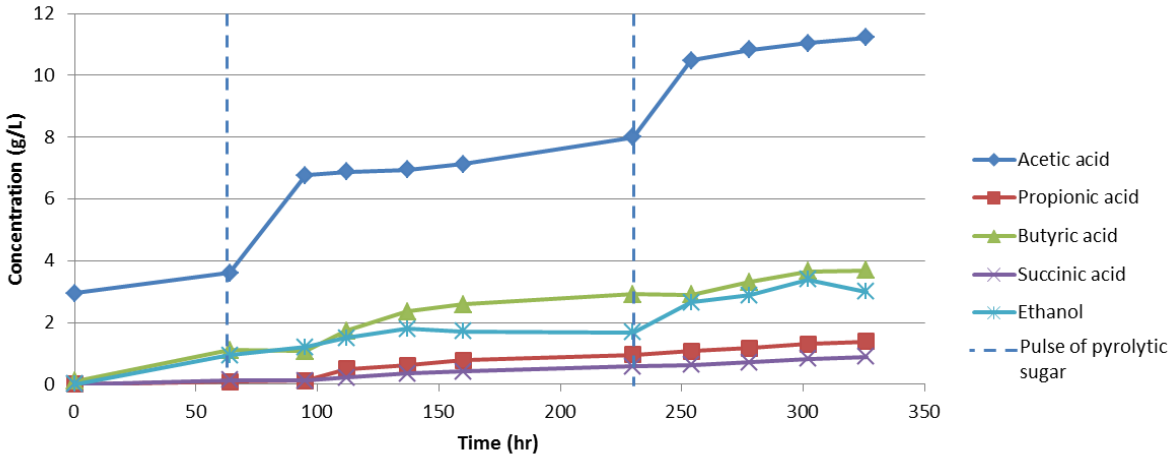

Figure 17. Product concentration profiles during the acidogenic fermentation. The starting concentration was 10% (v/v) detoxified pyrolytic sugar.

If the acidogenic fermentation reactor is operated in batch mode it is important to start with a low percentage of pyrolytic sugar to reduce the initial phase (data not shown). Higher concentrations of pyrolytic sugar should be possible if biomass is retained in the reactor. Thereby, reducing the lag phase and increasing the volumetric productivity, which will lower the total fermentation time.

At 30% (v/v) diluted pyrolytic sugar the MMC was still capable of producing volatile fatty acids. Increasing the pyrolytic sugar to 40% (v/v) was not performed due to time limitations. However, this can be tested in follow up research. Note that increasing the concentration of pyrolytic sugar further than 40% (v/v) will result in a significant increase in the total fermentation time caused by VFA toxicity. Increasing the pH of the medium can decrease the VFA toxicity due to acids being in their dissociated form.

The added volume of sodium hydroxide was monitored with the pH stat. The addition of sodium hydroxide is correlated to the production of organic acids in the reactor. Figure 18 shows the addition of sodium hydroxide over the total fermentation time. After 30 hours of growth, all glucose was converted into mainly organic acids, biomass and CO_2. The following 50 hours was spent to ferment other sugars like mannose and arabinose. Interestingly, Figure 18 also shows that after 80 hours when all monomer sugars are depleted, still organic acids were produced. This could be due to complex carbon compounds that were still converted towards VFA's. A second reason could be the conversion of ethylene glycol. Between 80 and 140 of the total fermentation time, all ethylene glycol present was degraded. The degradation productions of ethylene glycol are glyoxal, glycolic acid, glyoxylic acid and oxalic acid [39]. These acids are already present in pyrolytic sugar and a small increase has been observed (Appendix D). However, due to integration errors and overlapping peaks, the increase of concentrations of these acids could not be confirmed or quantified.

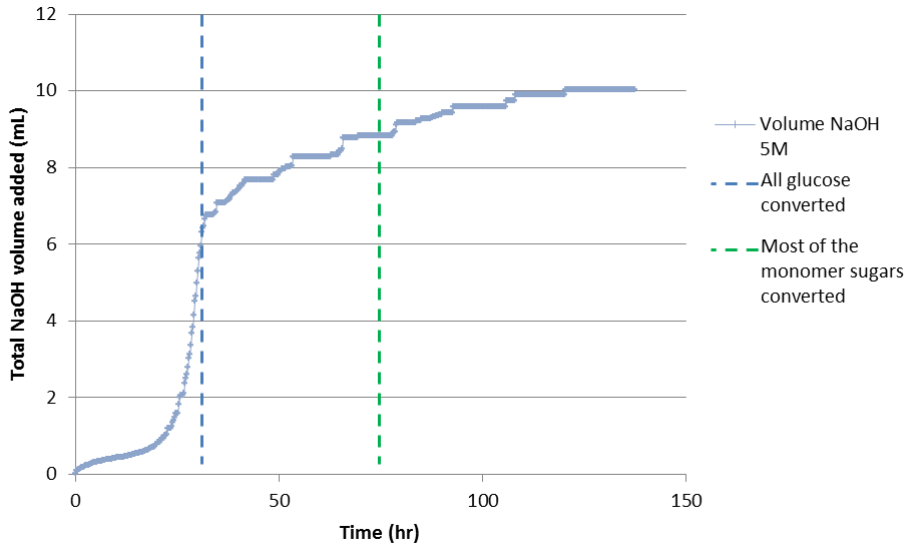

Figure 18. Sodium hydroxide addition during the acidogenic fermentation. Starting concentration pyrolytic sugar was 10% (v/v).

To confirm the hypothesis of converting complex carbon compounds to VFA's, a mass balance can be made. By quantifying the amount of glucose that is converted, the total Cmol that is converted can be calculated. This can be compared to the amount of Cmol that corresponds with the product spectrum. In theory, the products, CO_2 and biomass should result in 100% explained Cmol. In this study the biomass could not be quantified due to spontaneous precipitation of solids. If other sugars or complex carbon compounds are converted to VFA's, a percentage higher than 100% is expected. Figure 19 shows the total Cmol that is found in the products, which equals 237% compared to the conversion of glucose. The total Cmol calculation includes the theoretical CO_2 production for each product. Moreover, Figure 19 shows the Cmol distribution of the produced products. From this data can be concluded that, most of the conversion is towards VFA's but still a relatively large amount is converted towards ethanol. Furthermore, roughly >75% of the fermentable sugars present in hydrolysed pyrolytic sugar is glucose (Appendix E). Therefore, it is unlikely that other monomer sugars are the only cause of the high Cmol production per Cmol converted glucose.

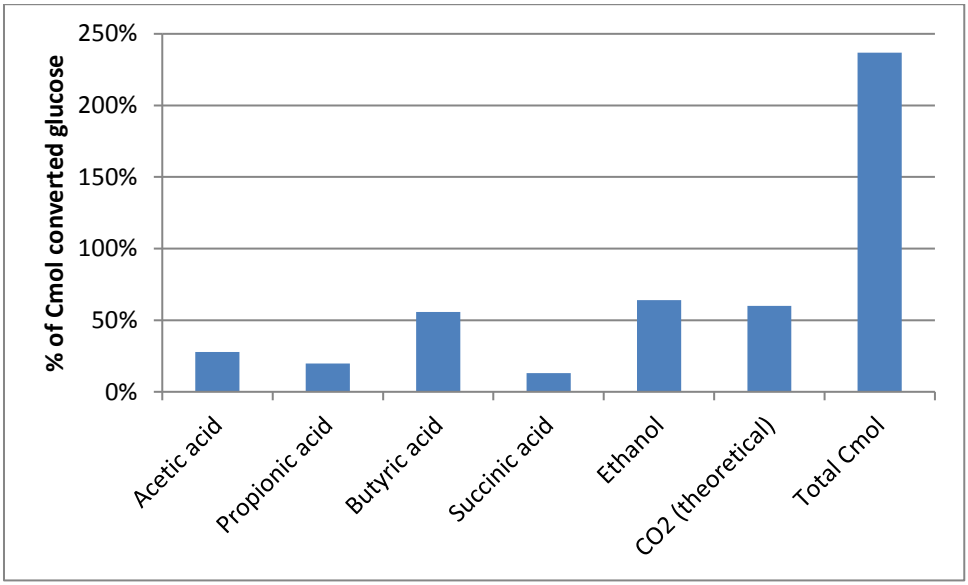

Figure 19. Cmol distribution at the end of the acidogenic fermentation. Percentages are calculated based on the amount of glucose converted.

Nitrogen limited fermentation

For PHB production it is important that the carbon source is in excess and other nutrients should be as limited as possible. To perform a PHB accumulation experiment the substrate should not contain any nitrogen compounds or ammonia, which can be used for biomass growth and prevent PHB formation. To reach the low amount of nitrogen it was important to perform the acidogenic fermentation under nitrogen limited conditions. With this limitation only traces of nitrogen should be present for the subsequent PHB fermentation. Moreover, any nitrogenous compounds present in pyrolytic sugar will be used during the fermentation. In Figure 20 two graphs are visible; the left graph (A) shows the product spectrum of the nitrogen limited fermentation, the right graph (B) shows the control with carbon limitation. Both graphs show a long lag phase of at least 50 hours and first lactic acid traces are found (Appendix F). Both graphs have similar pattern and therefore, it can be concluded that it is possible to have an acidogenic fermentation under nitrogen limited conditions. The product spectrums have a small differences, this is probably due to using a mixed microbial culture. With different nutrient limitations it could be that a different bacterial strain gains advantage and grows faster thereby, establishing microbial dominance in the reactor and influencing the product spectrum.

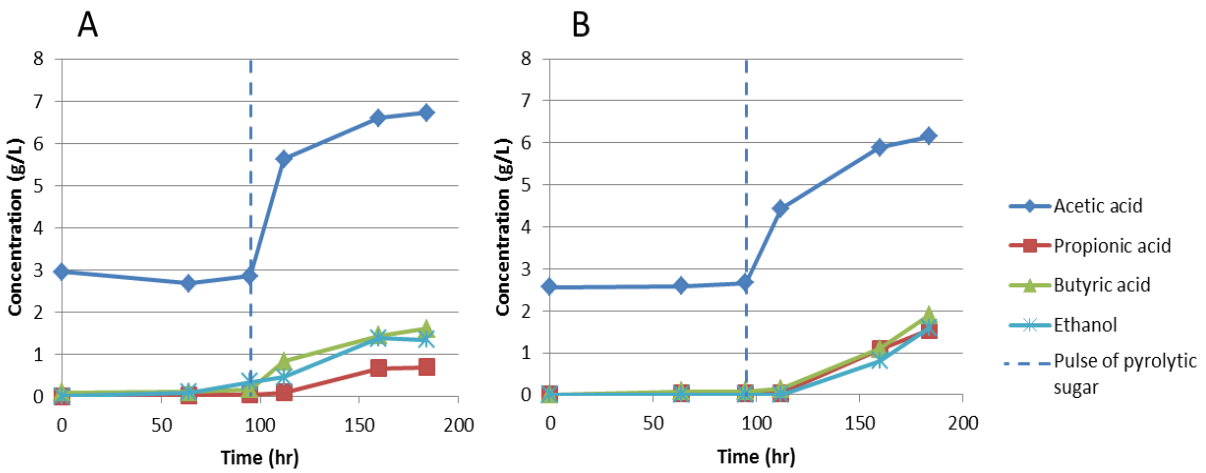

Figure 20. Product concentration profiles during the acidogenic fermentation. On the Left (A), fermentation with nitrogen limited conditions (100 mg NH4/L). On the Right (B), control fermentation with carbon limitation (500 mg NH4/L). Starting concentration was 10% (v/v) pyrolytic sugar.

Toxicity

It could be that in the acidogenic fermentation toxic compounds are fermented toward biomass or organic acids. After the detoxification, it is presumable that most of the toxicity is derived from the VFA's and the presence of phenolics.

Figure 21 shows the concentration profile of catechol during the acidogenic fermentation. It can be seen that slowly increases during the fermentation. This can be explained by that larger phenolics are degraded by cleaving off methoxy groups, replacing them for a hydroxy group [40]. In this conversion it is possible that a carboxylic acid is released. However, the release of acids from phenolics has not been researched in this study. The anaerobic phenolic conversion is shown in Figure 22 as found by Nakamura et all [40].

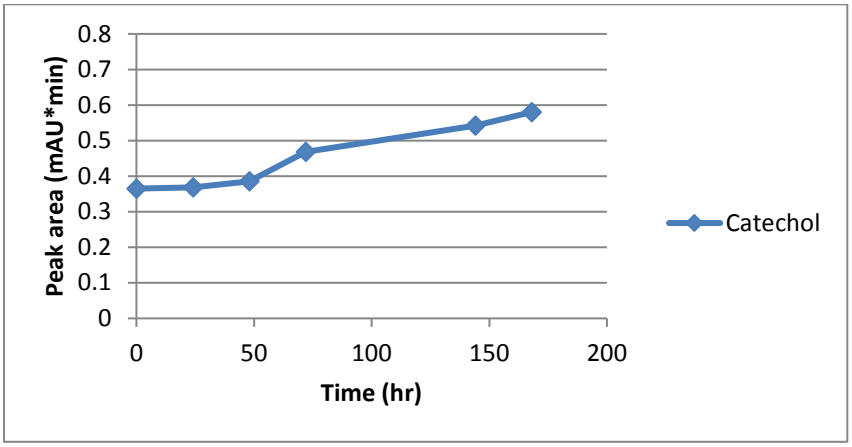

Figure 21. Catechol concentration profile during an acidogenic fermentation. Pyrolytic sugar concentration was 10% (v/v).

Anearobic degradation of small phenolics like catechol was not observed in this study. Anaerobic cleavage of the aromatic ring can be performed by several micro-organisms. However, they often require an external electron acceptor like nitrate, sulphate or Fe^{3+} [41], which were not present in high concentrations in the minimal media.

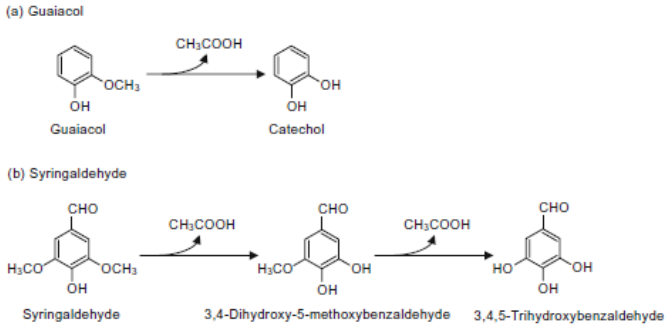

Figure 22. Possible anaerobic fermentation pathway to produce acetic acid from (a) guaiacol and (b) syringaldehyde. [40]

PHB production

The last step to show a proof of concept to produce PHB on pyrolytic sugar as carbon source is the PHB fermentation itself. To produce the PHB an accumulation experiment was performed. In Figure 23 (A) an optical microscopy picture is shown that shows the PHB producing bacteria at different magnifications. In the largest magnification picture the cells on the border of the aggregated cells show enlightened spheres within the cells. In Figure 23 (B) a clear example of the spheres in the cells is shown. These enlightened spheres represent the PHB granules in the cells. Note that, only the outer cells in the aggregate show the white PHB granules. Presumably, all cells are looking similar to the outer cells but due to lighting differences, this cannot be seen. The PHB granules are still small and represent only a small fraction of the cell mass. However, it can be concluded that PHB has been formed with pyrolytic sugar as carbon source.

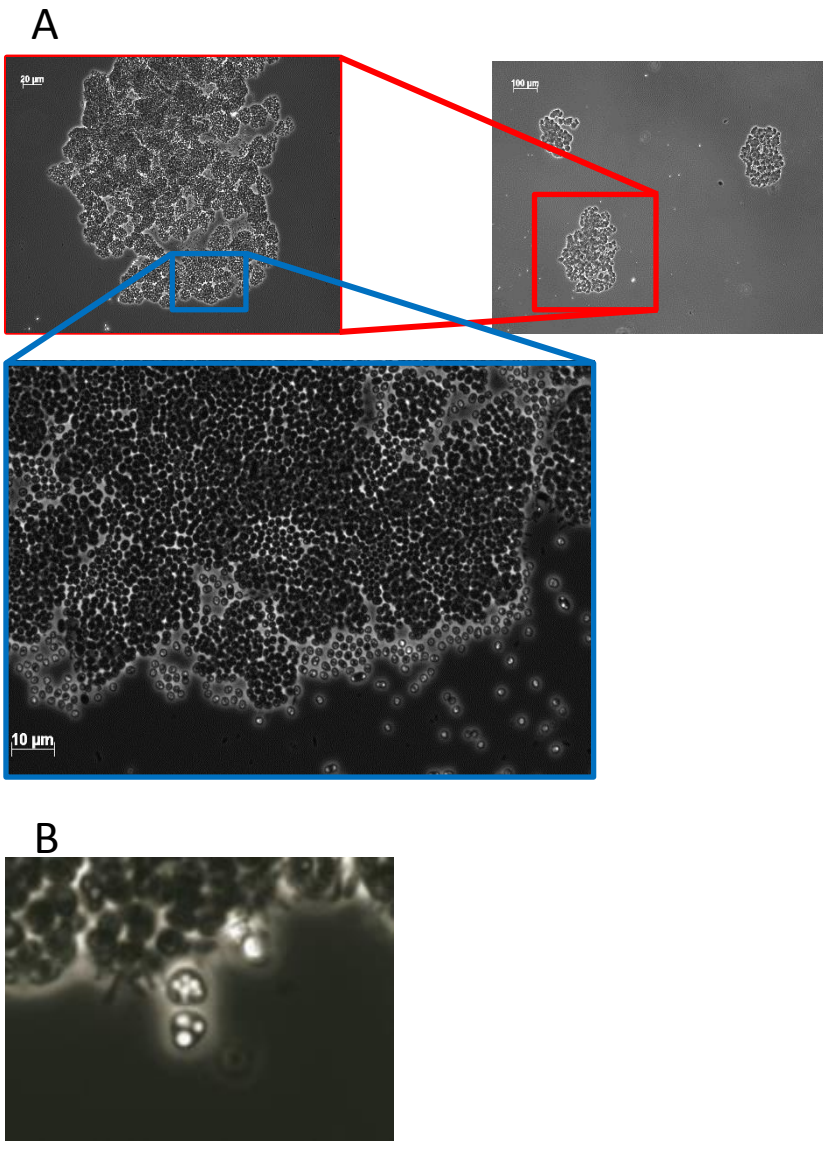

Figure 23. (A) Aggregated PHB producing bacteria at different magnifications (100x, 400x and 1000x). (B) Zoomed-in photo (1000x) of PHB granules within bacterial cells.

Conclusion

The objective of this study was to show a proof of concept of a fermentation process for PHB production using pyrolytic sugar as substrate. To show the feasibility of the concept it was important to show the possibility to detoxify pyrolytic sugar sufficiently for fermentation. Furthermore, growth experiments had to show the toxic concentration of pyrolytic sugar and the associated carbon source concentration. Lastly, to complete the proof of concept, the PHB fermentation had to show PHB granules within the cells.

From the results it can be concluded that it is possible to detoxify pyrolytic sugar sufficiently to remove most of the inhibitory compounds. Furthermore, the complexity of the carbon source is reduced by an acidogenic fermentation. With the acidogenic fermentation it is possible to increase the concentration of volatile fatty acids, the preferred substrate for PHB fermentations. Based on microscopy results it is concluded that small PHB granules have been formed inside the bacterial cells. Thus, it can be concluded that all three requirements to complete the proof of concept have been achieved.

Three different detoxification steps are necessary to remove most of the inhibitors present in pyrolytic sugar. These 3 steps are: hydrolysis, XAD-4 resin treatment and vacuum evaporation. With this strategy, the required dilution of pyrolytic sugar was reduced, which allows both the acidogenic and PHB fermentation on pyrolytic sugar.

Recommendations

Multiple recommendations can be made as this study was only to show a proof of concept. To achieve a feasible detoxification process, optimization is required to increase inhibitor removal and minimize the energy and material that is required for detoxification. The recommendations made, are the basis for further research with fermenting pyrolytic sugar and can be divided into three parts: analysis, detoxification and fermentation.

Analysis

Due to the complexity of pyrolytic sugar, there is a high background noise and multiple overlapping peaks in the HPLC chromatograms. Further studies should optimize the HPLC method to have better peak separation, meaning that the quantification of peaks is more accurate. Furthermore, many of the peaks are not identified (Figure 9). To further study the detoxification of pyrolytic sugar, it is interesting to identify more inhibitory compounds that should be removed before performing the fermentations. Especially, the identification of other furans and small aromatics could be interesting as these compounds inhibit microbial growth severely.

As previously mentioned, knowledge about the amount of nitrogen in pyrolytic sugar is crucial for the PHB fermentation. Therefore, an elemental, total nitrogen and ammonia analysis are necessary before and after detoxification. Knowing the amount of nitrogen, the ammonia addition before the acidogenic fermentation can be changed to ensure nitrogen limited conditions. Furthermore, the ammonia content after the fermentation can be analysed to check for left-over ammonia traces.

Further studies could implement the biological oxygen demand (BOD) analysis. This analysis should give information about the fermentation ability of the organic compounds present in pyrolytic sugar. This additional information can support the conclusions about the detoxification and give important information about the use of pyrolytic sugar as carbon source for PHB fermentation.

Detoxification strategy

From the results it was concluded that the acid hydrolysis causes reactive compounds to actively polymerize. Further studies could focus on the order of detoxification steps including the hydrolysis. This should give insights in the inhibitor removal and efficacy of the detoxification strategy. The eventual goal should be to prevent the polymerization during the hydrolysis.

Calcium overliming is well suited for removing inhibitors from pyrolytic sugar as mentioned in the chapter results (detoxification methods). In this study the removal of phenols and other inhibitors is not further researched. However, this method is a potentially good method for detoxifying pyrolytic sugar. More research could focus on the waste product of the calcium overliming and on reducing the amount of calcium hydroxide that is necessary to perform the overliming.

The XAD-4 treatment was performed in batch form. However, this could be improved if pyrolytic sugar is run over a column packed with XAD-4 resin. By using a column the treatment time can be shortened and possibly a better removal of phenols can be achieved while minimizing the losses of acetic acid and glucose. However, this requires more research in the binding capabilities of the resin.

A different way of optimizing the resin treatment could be the use of XAD-7 resin. This type of resin has more affinity for polar compounds compared to the XAD-4 resin [27]. Therefore, the XAD-7 resin should be more capable of removing the more polar compounds like glycolaldehyde and acetol. Note that, more polar groups on the resin can affect the affinity for acetic acid and higher losses should be expected.

It has been found that some of the acetic acid and glucose lost during the XAD-4 treatment can be recovered by washing the resin with water. This washing water contains the water soluble acetic acid and glucose but also small amounts of furans and catechol. Therefore, it could be studied if the column washing water can be used for the dilution of pyrolytic sugar.

The vacuum evaporation requires a significant amount of energy and the larger part of the evaporated compounds is water. In addition, it was found that the vacuum evaporation was mainly necessary for the removal of acetol. Therefore, this method could be replaced with a different detoxification step, which can remove or convert acetol. For example, the aldol condensation of acetol by increasing the pH to alkaline conditions.

Another recommendation is to study the phenols that are removed. The XAD-4 resin can be eluted with methanol, ethanol and acetone to achieve a solvent with high content in furans and phenols. It could be interesting to research the potentials of such a side-product.

Further studies could research other detoxification methods. For example, the oxidative catalysis of phenols can produce acetic acid. Therefore, pyrolytic sugar can be detoxified while increasing the fermentable carbon sources [42, 43]. The oxidative catalysis in pyrolytic sugar itself can be difficult due to the reactive compounds present, which can cause polymerization and deactivation of the catalyst. However, it might be interesting if the eluted XAD-4 phenolics can be catalysed and re-mixed with pyrolytic sugar.

Fermentation

For the acidogenic fermentation the culture selection can be continued. The MMC can be selected for better adaption to high concentrations of pyrolytic sugar. Furthermore, with the culture selection unwanted side-products can be minimized. For example, the production of ethanol and succinic acid is unwanted during the acidogenic fermentation due to the extra energy requirement for the conversion of these products to PHB in the successive fermentation.

Another recommendation is the optimization of the fermentation conditions. By changing the reactor conditions (i.e. the pH), the product spectrum can be influenced to improve the ratios between different products. This can be interesting if it is known which substrate is preferred by the PHB producing bacteria after adaptation to pyrolytic sugar.

An important recommendation is to change the reactor type. Currently, a batch type fermentation was used. A batch type reactor is not optimal for the use of toxic substrates due to the severe toxic inhibition which lowers biomass growth and conversion. Growth inhibition can be reduced by the use of a continuous reactor whereby the dilution rate is based on biomass growth and VFA's production. Furthermore, the amount of nitrogen flushing out of the reactor can be controlled and remained stable because of the steady state in these continuous reactors. For example, it is possible to use a granular continuous reactor. In this type of reactor the biomass will retain in the reactor. This will reduce the necessary addition of nitrogen that is required for biomass growth. Furthermore, due to higher concentrations and retention of biomass a higher dilution rate can be established. A second option, is the immobilisation of the acidogenic culture. By immobilizing the MMC, the biomass is mostly retained in the reactor and high biomass concentrations can be reached. In addition, an important advantage is the reduced local toxicity [44, 45]. Due to the reduced local toxicity, the volumetric production rate of VFA's could be higher even if the mass transfer limitations are considered.

Except for recommendations for the acidogenic fermentations, it is possible to give some recommendations for the PHB production as well. An important factor for the PHB producing culture is the selection and adaptation to the toxicity of pyrolytic sugar. It is important to select a strain, which has a minimal metabolic inhibition due to the presence of pyrolytic sugar. Secondly, the culture should convert most of the organic compounds present in pyrolytic sugar. The PHB producing culture can be selected by the known Feed and Famine strategy [16] and stepwise increases of the concentrations of pyrolytic sugar.

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Appendix

A

Table 8. Quantification of compounds found in raw pyrolytic sugar

Compound	Concentration (g/L)
Formic acid	8
Acetic acid	40
Propionic acid	1.2
Butyric acid	0.6
Methanol	0.5
Levoglucosan	45
Glucose	2
Acetol	11
Glycolaldehyde	45
Ethylene Glycol	12
5-HMF	5

B

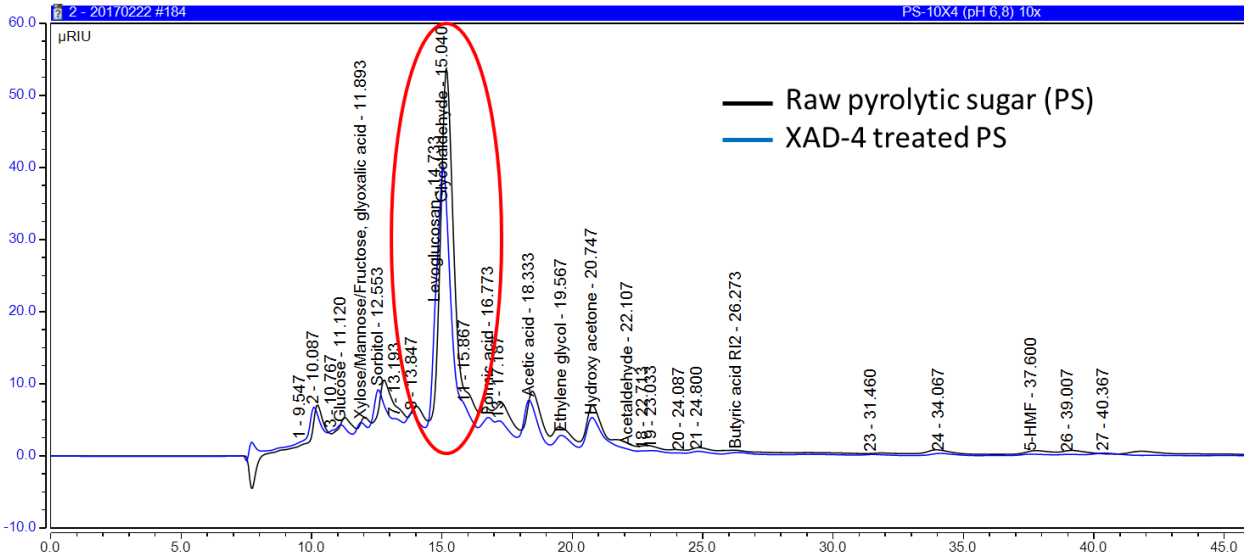

Figure 24. HPLC chromatograms of raw pyrolytic sugar (black) and XAD-4 resin treated pyrolytic sugar (blue). The red circle indicates the glycolaldehyde peak.

C

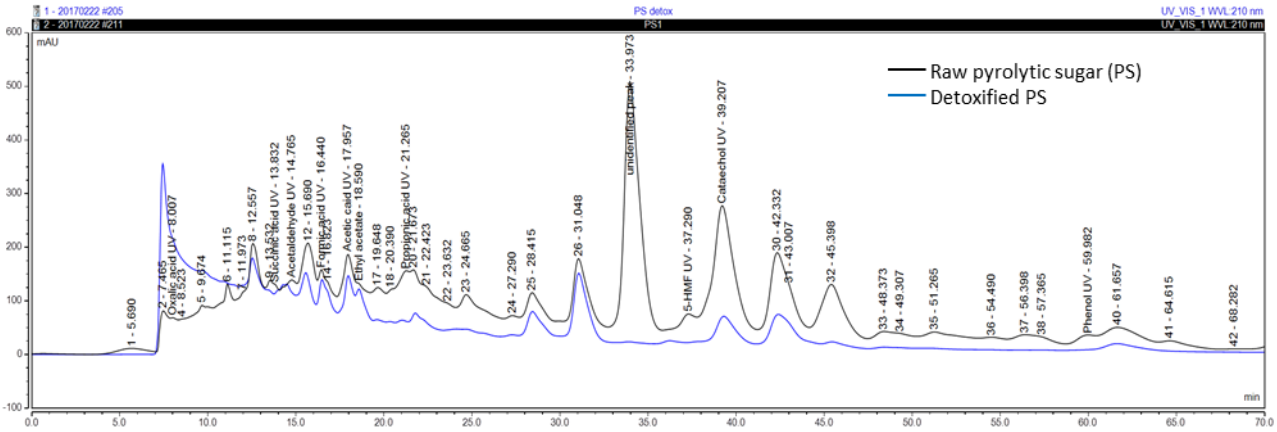

Figure 25. HPLC chromatograms of raw pyrolytic sugar (black) and detoxified pyrolytic sugar (blue) measured at 210 nm.

D

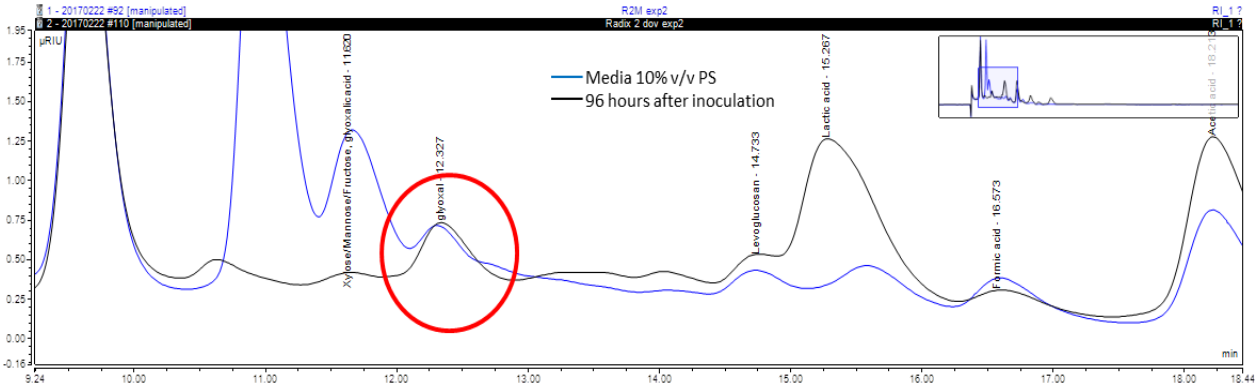

Figure 26. HPLC chromatograms of the fermentation media (blue) and 96 hours fermented pyrolytic sugar (black). The red circle indicates the glyoxal peak.

Sample	Peak area (µRIU*min), red circle
Media 10% v/v PS	0.0491
96 hours after inoculation	0.1393

E

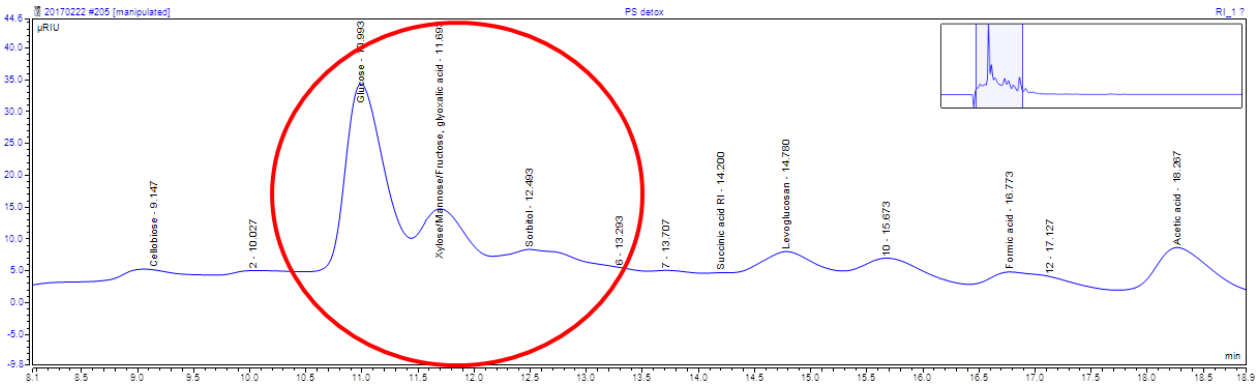

Figure 27. HPLC chromatogram of detoxified pyrolytic sugar. The red circle indicates several monomer sugar peaks, which include glucose, xylose, mannose, fructose and sorbitol.

F

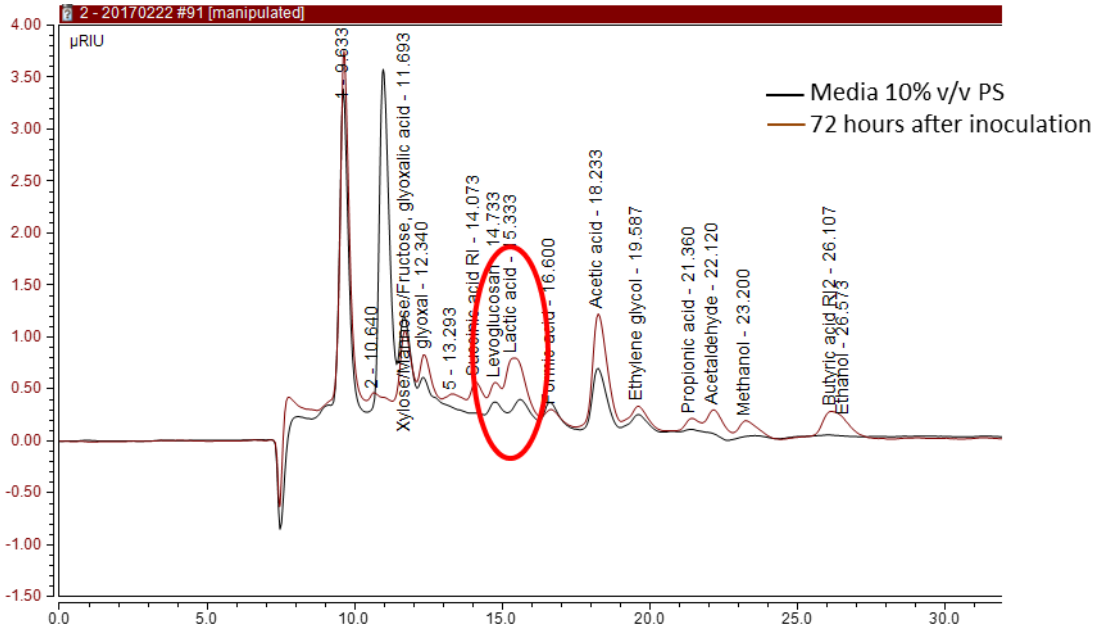

Figure 28. HPLC chromatograms of the fermentation media (black) and 72 hours fermented pyrolytic sugar (brown). The red circle indicates the increased lactic acid peak.