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The potential for lactic acid production in brown juice

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In this thesis the potential for utilizing brown juice as a fermentation media for lactic acid production was examined. Green juice was initially utilized as fermentation media because it contains a Carbon source. Both juices are both characterized chemically, determining VFA, TS, VS, free sugars, Total Kjeldahl Nitrogen and soluble sugars. Bacteria were isolated from both the green juice and the brown juice. These bacteria were screened for lactic acid production together with a positive control *L. salivarius.* During this screening several fermentation conditions were also tested. The conditions chosen after completion were sterile aerobic conditions. Following the screening two isolated bacteria and *L. salivarius*, were tested for lactic acid production at different pH. The highest concentration, 21.5 g/L, was observed at pH 6.5 which is the value the future fermentation media were adjusted to. Finally, different Carbon sources (glucose, xylose and press cake) were tested in the brown juice yielding very little lactic acid production, but there was butyrate production. It was hypothesized that the switch to nonsterile conditions, were the primary reason for this. It was ultimately discovered that while a concentration of 21.5 g/L lactic acid could be obtained by fermentation in brown juice, there was a much bigger potential for butyrate production. The highest butyrate concentration obtained was 45 g/L.

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1 TABLE OF CONTENT

2 INTRODUCTION

2.1 GENERAL INTRODUCTION

The energy and chemical sectors are constantly growing and the current heavy dependency on fossil fuels in these industries are not considered sustainable (Rivas et al. 2016). Thus prompting the search alternatives. Through the recent years it has been recognized that there is potential in substituting products made from fossil fuels and petrochemicals with products produced from renewable biomass (Cherubini 2010). This has led to the development of biorefineries. Biorefineries are essentially a production plant where multiple products are manufactured from renewable bio based materials (Ecker et al. 2012). The products from a biorefinery can both be final products or platform products that are used in other production processes. One of the most important factors for a biorefinery is sustainability aspect, a successful should biorefinery is environmentally and economically sustainable (Pandey et al. 2015).

Biorefineries can be divided into different types depending on what type of biomass the biorefinery utilizes (Ecker et al. 2012). One type of biorefinery is what is referred to as a Green biorefinery which utilizes green biomasses as raw materials for the synthesis of products (Kamm et al. 2016).Grass is considered a green biomass and it is one that exists in abundance. While the biomass is seasonable (April – October), it can be harvested 3-4 from the same field during the growing season. It is estimated that one hectare of land can yield 7 tons drymatter or more every year (German Federal government, 2012).

One product that can be produced in a Green is animal feed. Europe does not produce enough proteins of a sufficient quality to be used for animal feed. Only around 45% of the protein used for animal feed in the European Union countries are produced in Europe (Scholey et al. 2016). To produce organic animal feed the biomass utilized would of course need to be organic as well.

Organic animal feed is limited due to the lack of supply of organic proteins. Which is in conflict with the expanding organic animal farming sector due to increased demand for organic products (Santamaria 2015). Which has led to the development of the OrganoFinery (see section 2.2). Currently the only products of the OrganoFinery is organic protein for animal feed, biogas and organic fertilizer. The option of integrating more products into the process is available and lactic acid production is a definite possibility. The lactic acid market is currently expanding due the applications in food and pharmaceutical industry as well as bioplastics (Abdel-Rahman et al. 2016). Lactic acid is already produced by microbial fermentation, but the traditional biomasses for these productions are feedstocks that are also used for food. In a biorefinery the production of lactic acid would be from a renewable source not used in food. Some biomasses like corn stover or algal biomass are currently under investigation for suitability for lactic acid production (Djukić-Vuković et al. 2013).

2.2 THE ORGANOFINERY PROJECT

The Organofinery project is focussed on producing organic protein feed from green biomass. It is based on a biorefinery concept so the waste products from the primary organic protein feed production are being utilized for other purposes like energy production and organic fertilizer.

A green crop, different type of grass preferably, is harvested and divided into two fraction using a screwpress. A liquid fraction called green juice and a solid fraction called presscake (Santamaria 2015). The presscake consists of insoluble compounds and other particles and is utilized for biogas. The green juice contains soluble compounds, enzymes among others (Thomsen et al. 2004). The green juice also contains a lot of proteins which is extracted through a fermentation. Lactobacillus Salivarius is inoculated into the green juice starting a fermentation producing lactic acid. This production causes juice to be acidified and the proteins to precipitate (Santamaria 2015). The protein paste are separated from the juice by centrifugation and used for animal feed. The remaining juice is referred to as brown juice and has so far been utilized for biogas with the presscake.

Brown juice stil contains a lot of nutrient (Thomsen et al. 2004) which means it could be utilized as a fermentation media for further lactic acid production if a new Carbon source was added to the juice. The leftover juice after a lactic acid fermentation could still be used for biogas production. A model of the OrganoFinery as it is intended to be in this thesis is seen in figure 1.

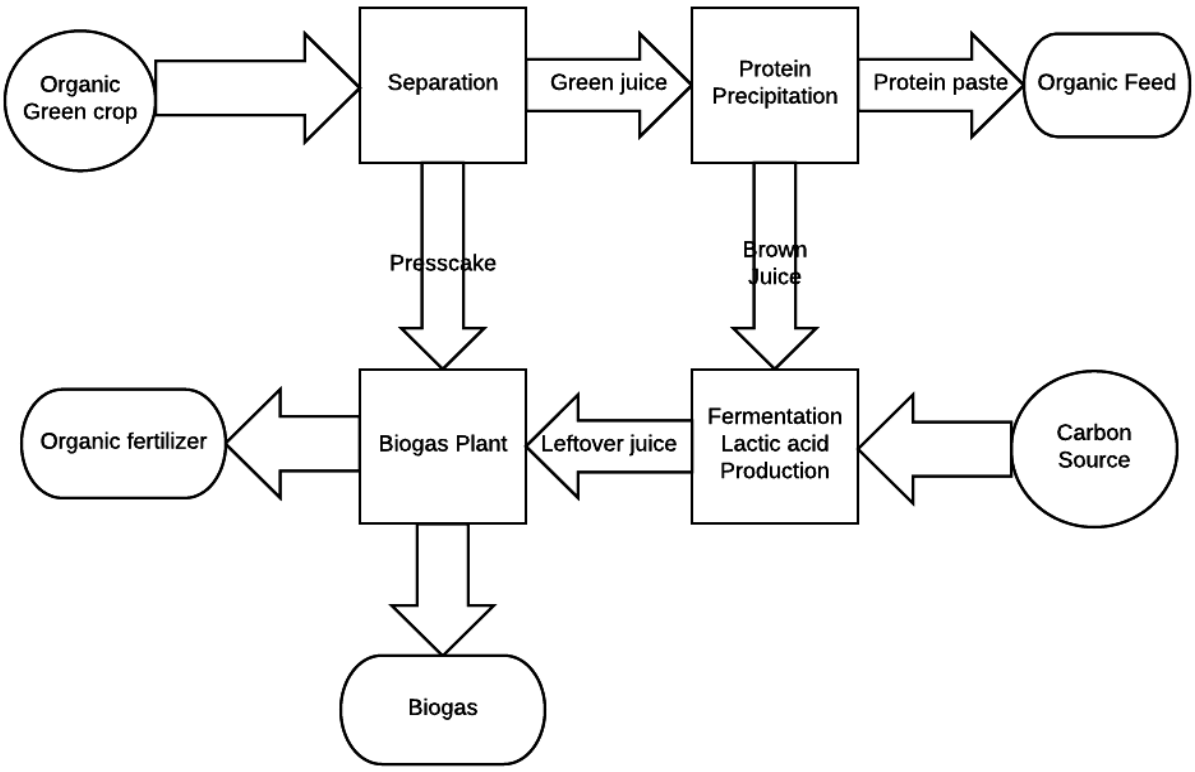

Figure 1: A model of the intended OrganoFinery

2.3 Scope of Project

In this master thesis an alternative use for the brown juice produced in the OrganoFinery is evaluated for its potential for utilization as fermentation media for lactic acid production. If the lactic acid production is successful, the process is intended to be integrated into the OrganoFinery. The potential is determined by screening for lactic acid production in the brown juice using different bacteria, determining the optimal fermentation conditions and most efficient Carbon sources.

Chapter 3 contains descriptions of lactic acid. The uses that lactic acid have, how it is produced and what the predicted future uses are for lactic acid. Chapter 3 also contains information about lactic acid bacteria in general and an overview over the metabolic pathways utilized for lactic acid production. The chapter also contains the theoretical mass balances expected from homo- and heterofermentative bacteria.

In chapter 4 the potential of brown juice and green juice as a fermentation media is determined. Both biomasses are chemically characterized in terms of TS, VS, free sugars, soluble sugars, Nitrogen content and Volatile Fatty Acids.

In chapter 5 bacteria are isolated from both the brown juice and the green juice and screened for lactic acid production in green juice. While fermentation conditions such as sterility and anaerobic conditions are also investigated, the main focus remains on the ability to produce lactic acid in the isolated bacteria when compared known lactic acid producers.

The bacteria selected from the initial screening in chapter 5 are in chapter 6 exposed to different pH values with the intention of determining if less acidic conditions could improve the output of lactic acid.

The isolated bacteria are identified in chapter 7. DNA is extracted from the isolated bacteria and sequenced with the purpose of identifying the bacteria. A description of the identified bacteria is included in the chapter making it possible to determine if the theoretical behavior of the identified bacteria is consistent with the observed.

The identified optimal conditions for the bacteria are tested with brown juice as fermentation media in chapter 8. Two different Carbon sources are also tested for suitability for lactic acid production in brown juice.

Another waste product from the OrganoFinery, the press cake, is considered as a Carbon source in chapter 9. It is considered if a more complex Carbon source can yield a satisfactory amount of product, avoiding having to use the more expensive simple sugars. Different ratios between the brown juice and Carbon source are also investigated.

Finally, the conclusive remarks and future prospects are presented in chapter 10.

3 LACTIC ACID

3.1.1 Properties & Applications of Lactic Acid

Lactic acid is an organic acid that has a wide range of uses in different industries like foods, pharmaceutical and chemical intermediates (Abdel-Rahman et al. 2010). In the Pharmaceutical industry lactic acid is among others used against osteoporosis, for the synthesis of certain drugs (Castillo Martinez et al. 2013). Within the food industry lactic acid is used for preservation of food due to the fact that lactic acid is safe to consume and the lactic acid bacteria dominates the microflora on several food products (Ghanbari et al. 2013)(Stiles 1996). Lactic acid is also used as an additive for foods as a flavor or buffering agent or as an acidulant (John et al. 2009). Lactic acid can also be converted into other chemicals such as pyruvic acid, lactate ester and acrylic acid. This conversion is possible because lactic acid is a hydroxycarboxylic acid, which means that lactic acid contains both hydroxyl groups and carboxyl groups (Gao et al. 2011).

Lactic acid exists in two different isomers D-lactate and L-lactate. These two isomeric structures can also bind themselves together and create a complex of DL-lactic acid, which is rather uncommon in compounds

that are chemically identical (Ikada et al. 1987). The isomeric structure of the lactic acid produced is very important because the application of the lactic acid can be very dependent on it. When using lactic acid for medicinal drugs or for the food industry the isomeric structure has to be solely comprised of L-lactic acid. This is because L-lactic acid is the only one of the two structures that can be metabolized by the human body (Castillo Martinez et al. 2013). When producing lactic acid for PLA products, the structural strength and degradability of PLA depends on which lactic acid structure is used and the ratio between them (Zhao et al. 2010)(Nair & Laurencin 2007). Thus PLA can be divided into poly L-lactic acid, poly D-lactic acid and poly DL-lactic acid (PLLA, PDLA and PDLLA) (Nair & Laurencin 2007).

3.1.2 Lactic Acid Production

Lactic acid can be produced either by chemical synthesis or by microbial fermentation. The primary chemical synthesis of lactic acid is based on strong acid hydrolysis of the petrochemical derivative lactonitrile (John et al. 2009). It is however considered unfeasible to produce lactic acid through chemical synthesis (John et al. 2009), which is why the majority of the lactic acid produced stems from microbial fermentation (Budhavaram & Fan 2009).

When producing lactic acid through microbial fermentation the efficiency of the process mainly depends on three factors. 1) The substrate utilized for the fermentation. 2) The organism utilized as lactic acid producer and 3) the production method (Abdel-Rahman et al. 2013). The substrates used for lactic acid production has traditionally been substrates containing starch that also been used for food production. Shifting to cheap waste products are currently under investigation (Djukić-Vuković et al. 2013).

Lactic acid bacteria are the organisms that usually carries out a lactic acid fermentation. Lactic acid bacteria are different genera of bacteria that can have lactic acid as either the only end product (homofermetative) or as a major final product (heterofermentative). Lactic acid bacteria are also known to be very demanding regarding nutrient content (Thomsen et al. 2007). The process of producing lactic acid is an oxygen free process (Thomsen et al. 2007), however most lactic acid bacteria are facultative anaerobes which means that lactic acid can be produced under aerobic conditions. This is an advantage in large industry scale production where it can be a problem keeping the fermenter oxygen free (Thomsen et al. 2004). Lactic acid bacteria are a general description of one out of four bacterial groups that can produce lactic acid. Lactic acid bacteria covers a group of Gram-positive bacteria with very different set of optimal growth conditions (Abdel-Rahman et al. 2013). Lactic acid bacteria species include, but are not limited to, *Carnobacterium, Lactococcus, Lactobacillus* and *Pediococcus* (Leroy & De Vuyst 2004). Most lactic acid bacteria are generally regarded as safe (GRAS), can tolerate very acidic conditions (pH lower than 5) and are generally mesophilic (Hofvendahl & Hahn–Hägerdal 2000). Lactic acid bacteria can grow in a pH range of 3.5-10 (Abdel-Rahman et al. 2013). The other bacteria groups capable of producing are some *Bacillus* strains (Ohara & Yahata 1996), *Escherichia coli. Corynebacterium glutamicum* (Abdel-Rahman et al. 2013). Some fungi like *Rhizophus oryzae* (Sun et al. 2012), yeast and microalgae are also capable of lactic acid production (Abdel-Rahman et al. 2013). Bacterial fermentations, which produces lactic acid falls into three categories the bacteria which solely produces L-lactic acid, bacteria that solely

produces D-lactic acid and the bacteria that produces the mixture between the two DL-lactic acid (Garlotta 2002). Because of the isomeric specificity of some of the product (Castillo Martinez et al. 2013), the choice of bacteria defines which buyers there are for the produced lactic acid.

Lactic acid is primarily produced through batch fermentation, since they give higher yields and final concentrations (Hofvendahl & Hahn–Hägerdal 2000). Some very high concentrations can be reached *Lactobacillus paracasei* has been reported to have a final concentration of 192 g/L lactic acid from a 200 g/L glucose concentration (Moon et al. 2012). The disadvantages of batch fermentation are the downtime between batches (Zhao et al. 2010), the limited substrate that can be added before the point of substrate inhibition is reached and the possibility of product inhibition since the lactic acid is not removed until the end of the fermentation. There also the possibility of running out of nutrients stunting cell growth (Abdel-Rahman et al. 2013). The issue of substrate inhibition can be countered by using fed-batch, which has also been reported to give higher yield (Hofvendahl & Hahn–Hägerdal 2000). While fed-batch solves the substrate inhibition issue the system is still closed for output so the issue with product inhibition still stands. There is also the possibility of running a continuous fermentation. These fermentations do not yield as much lactic acid as batch and fed-batch, but they have higher productivity. Because the system is open there no substrate or product inhibition, but the risk of contamination is bigger than it would be in a batch fermentation (Abdel-Rahman et al. 2013). A continuous fermentation also requires no downtime and the conditions will remain steady after they initially stabilize (Brethauer & Wyman 2010). The specific conditions under which the fermentation is started varies depending on which bacteria used.

Upon completed fermentation the lactic acid produced need to be separated from the broth and purified. The most traditional method entails the addition of Calcium carbonate raising the pH of the broth to 10. Afterwards the broth is heated and filtered. This results in the lactic acid being converted to Calcium lactate, all the cells are killed and the remaining proteins congeal. Any excess calcium carbonate is also removed and any remaining sugars are disposed of (Wang et al. 2015). Sulphuric is added acidifying the broth causing the Calcium lactate to convert to lactic acid and Calcium sulphate (Qin et al. 2010). Further lactic acid recovery can be obtained by distillation, esterification and hydrolysis (Wang et al. 2015) though these are utilized depending on what level of purity is needed (Datta & Henry 2006). There are two big disadvantages with this method, one is the high consumption of sulphuric acid and the other one is the waste product Calcium sulphate also known as gypsum. Approximate one ton of gypsum is produced for one ton of lactic acid produced, making the disposal of gypsum a major cost. Other methods for separation and purification are in development to avoid using sulphuric acid. Most of them are based on membrane-separation, particularly electrodialysis, microfiltration and ultrafiltration (Qin et al. 2010).

3.1.3 Pathways & Theoretical Mass Balances

When lactic acid bacteria produce lactic acid there are 3 primary pathways that can be utilized. These are the Embden-Meyerhof-Parnas pathway (EMP), the phosphoketolase pathway (PK) and finally the pentose phosphate pathway (PP) (Eiteman & Ramalingam 2015). The pathway utilized depends on whether the bacteria is homofermentative or heterofermentative and which sugar the bacteria are consuming (Abdel-Rahman et al. 2010). Figure 2 shows all the pathways utilized by lactic acid bacteria for lactic acid production.

A homofermentative bacteria fermenting glucose (a hexose sugar) is metabolized through the EMP pathway, also called the glycolysis. The EMP pathway metabolizes glucose to pyruvate in then steps. The EMP pathway can be divided into two stages; a preparation stage (stage 1) and an ATP production stage (stage 2) (Berg et al. 2012). Stage 1 coverts glucose to glucose 6-phosphate, then to fructose 6-phosphate and finally to fructose 1,6-biphosphate. In the final step of stage 1 fructose 1,6-biphosphate is divided to dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, so that one C-6 Carbon molecule becomes 2 C-3 molecules. Then stage 2 begins, with the conversion of the dihydroxyacetone phosphate to glyceraldehyde 3-phosphate. Glyceraldehyde 3-phosphate is converted to 1,3-bishophoglycerate which is converted to 3-phosphoglycerate. This is converted to 2-phosphoglycerate then phosphoenolpyruvate and finally to pyruvate (Berg et al. 2012). Finally, lactic acid is produced anaerobically from the pyruvate.

This gives a theoretical maximum yield of two mole of lactic acid from one mole of glucose (Eiteman & Ramalingam 2015), giving the mass balance seen below:

$C_6H_{12}O_6 \rightarrow 2\ C_3H_6O_3$

The PP pathway is an alternative metabolism to the glycolysis and begins after glucose has been converted to glucose 6-phosphate. The C-6 molecule is then cleaved to the C-5 molecule ribulose 5-phophate and CO_2 (Abdel-Rahman et al. 2010). From there the ribulose 5-phosphate is converted to either ribose 5-phospate or to xylulose 5-phosphate, the latter being generated depending on what the ribose 5-phosphate is converted to. From here there are two possibilities either the ribose 5-phosphate is utilized for nucleotides and nucleic acids or it is converted to molecules from the EMP pathway, which is where xylulose 5-phosphate is needed. The latter option is what eventually produces lactic acid. There are several metabolic steps before the PP pathway is linked to the EMP pathway. In the first step the ribose 5-phosphate and xylulose 5-phosphate is converted glyceraldehyde 3-phosphate and sedoheptulose 7-phosphate. Glyceraldehyde 3-phosphate and sedoheptulose 7-phosphate reacts with each other and produces Erythrose 4-phosphate and fructose 6- phosphate. The fructose 6-phosphate is then integrated into the corresponding step of the glycolysis. The erythrose 4-phosphate and xylulose 5-phosphate (from ribulose 5-phosphate), generates glyceraldehyde 3-phosphate and fructose 6-phosphate, both are integrated into the glycolysis (Berg et al. 2012).

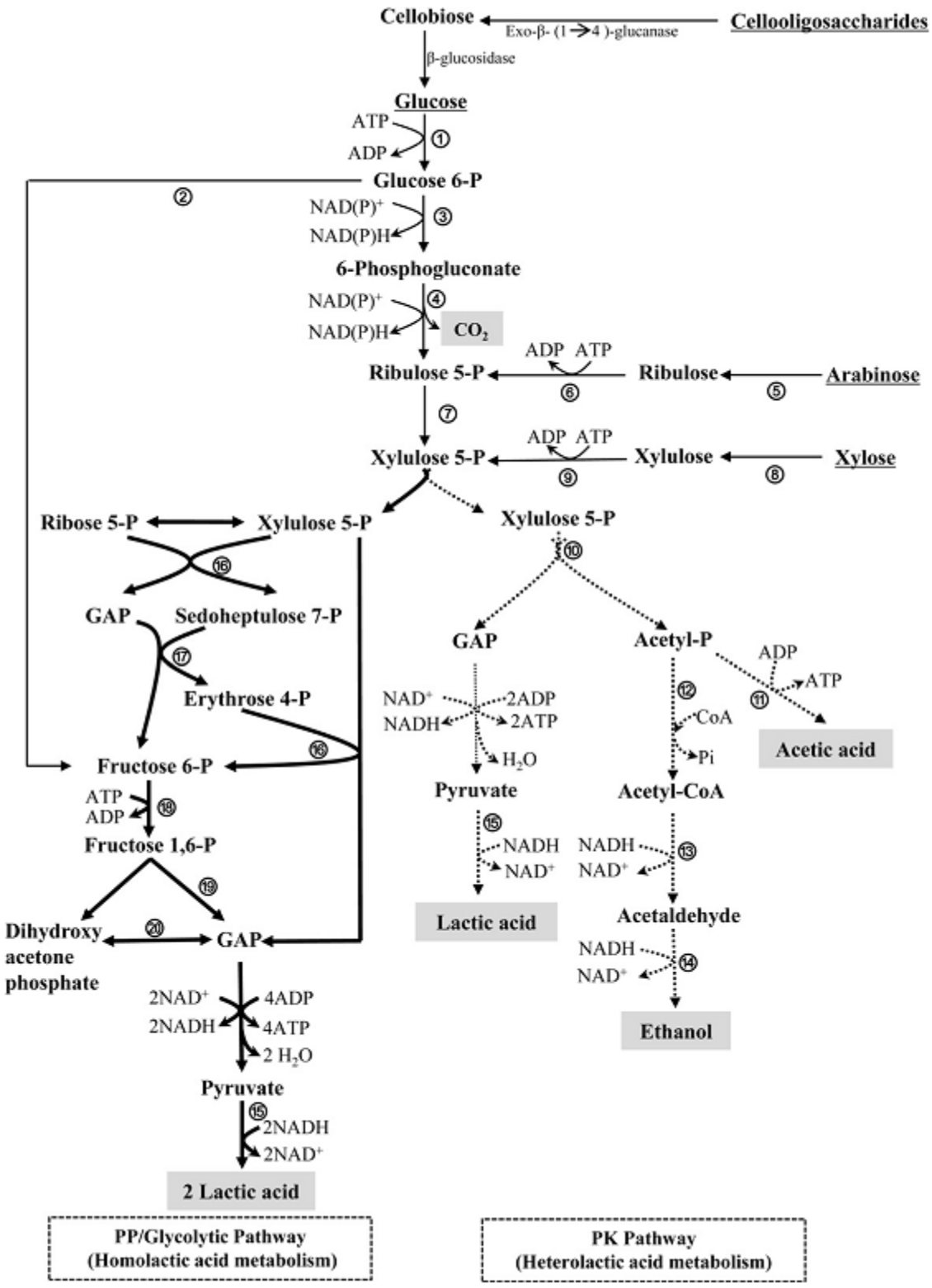

Figure 2: The pathways utilized by Homofermentative and heterofermentative lactic acid bacteria. The dotted lines indicates the PK pathway while the thick lines indicates the PP pathway (Abdel-Rahman et al. 2010).

PK pathway starts at xylulose 5-phosphate. This can have been generated either from the PP pathway or from the conversion of xylose to xylulose to xylulose 5-phosphate. Xylulose 5-phosphate is converted onto glyceraldehyde 3-phosphate and acetyl phosphate (Eiteman & Ramalingam 2015). The glyceraldehyde 3-phosphate is converted to pyruvate and then to lactic acid. The acetyl – phosphate can be converted to either acetic acid or to acetyl-CoA. Acetyl-CoA is the converted to acetaldehyde and then to ethanol (Abdel-Rahman et al. 2010).

Utilizing these two pathways a heterofermentative bacteria would if consuming glucose start using the PP pathway and then switch to the PK pathway after the glucose has been converted to xylulose 5-phosphate. If the substrate is xylose then the sugar enters the PK pathway if it is heterofermentative and the PP pathway if it is homofermentative (Eiteman & Ramalingam 2015)(Abdel-Rahman et al. 2010). This gives the following theoretical mass balances:

Homofermetative bacteria utilizing xylose:

$3\ C_5H_{10}O_5 \rightarrow 5\ C_3H_6O_3$

Heterofermentative bacteria utilizing xylose

$C_5H_{10}O_5 \rightarrow C_3H_6O_3 + C_2H_4O_2$

Heterofermentative bacteria utilizing glucose

$C_6H_{12}O_6 \rightarrow C_3H_6O_3 + C_2H_4O + CO_2$

3.1.4 Polylactic acid Production and Applications

In recent years that has been increasing focus on using lactic acid to create polylactic acid (PLA) polymers, which is used in bioplastics. The demands for lactic acid is estimated to increase with 5-8% a year, due to the interest in PLA production (Yadav et al. 2011). The term PLA does not refer to a specific compound but rather a series of related compounds (Saeidlou et al. 2012). PLA have applications in different areas, in the food industry used for packaging and labelling (Armentano et al. 2013). While in the medical industry PLA is also used for developing biodegradable medical appliances like sutures and scaffolds, polylactic acid is also used for orthopedics (Gupta & Kumar 2007).

The production of PLA consists of three steps: first is the production of lactic acid, second is the formation of lactide from the lactic acid, and third the generation of polylactic acid (Gupta et al. 2007). One of the most popular methods is ring opening polymerization method (Lasprilla et al. 2012). In this method lactide is formed by removing the water from lactic acid through evaporation under vacuum causing polymerization. Using a catalyst the polymer is depolymerized into the cyclic dimer lactide (Van Wouwe

et al. 2016). PLA are then formed by a ring-opening polymerization, this reaction is often catalysed by a stannous octoate (Madhavan Nampoothiri et al. 2010).

4 CHARACTERIZATION OF GREEN JUICE AND BROWN JUICE

4.1 OVERVIEW & PURPOSE

The purpose of this chapter was to gain an understanding of the potential of the green juice and brown juice as fermentation media. Hence the two juices were characterized to determine the pH, total solids (TS), volatile solids (VS), volatile fatty acids (VFA), free sugars and other chemical compounds. Total Kjeldahl Nitrogen (TKN) and weak acid hydrolysis to determine soluble sugars, were also determined.

4.2 MATERIALS AND METHOD

4.2.1 Chemicals & Solutions

MilliQ water, H_2SO_4 (2M, 4%), H_3PO_4 (17%), digestion reagent, Indicating H_3BO_3 (20 g/L), NaOH (300 g/L), $C_3H_7O_3$ solutions (alanine, Nitrogen conc. 8 g/L, 15 g/L, 25 g/L, 50 g/L, 80 g/L), NaOH-$Na_2S_2O_3$×5 H_2O (Sodium hydroxide sodium thiosulfate reagent), HCl (0,05 M), $Ba(OH)_2 \cdot H_2O$, clover grass green juice, red clover brown juice, stock solution.

4.2.2 Equipment

High Pressure Liquid Chromatograph Dionex Ultimate 3000, Centrifuge Hettich zentrifugation Mikro 200, Centrifuge Eppendorf MiniSpin plus, Centrifuge Thermo scientific Meraeus Multifuge X3R, pH meter Infolab WTW series pH 720, 105°C oven Memmert, 550°C oven Carbolite, Autoclave Certoclav Tisch-autoclave, Distillator Holm & Halby Büchi Distillation unit K-350, Digester Holm & Halby Büchi SpeedDigester K-438, Gas chromatograph PerkinElmer precisely Clarus 400 Gas Chromatograph, 0.0001 g balance, Büchi Scrubber B-414 Holm & Halby, MS2 Minishaker IKA®.

4.2.3 Materials

Automatic pipette, pipettes (10 ml, 25 ml, 50 ml, 1-5 ml, 30-300 µl), pipette tips, blue cap bottles (250 ml, 500 ml, 1 L), beakers, serum vials (117ml), rubber stoppers, clamps, chromatography vials, syringes (2 ml, 5 ml), 0,45 µm syringe filters, needles, Eppendorf tubes (1,5 ml), pH strips (pH 1-7), crucibles, tweezers, sample tubes, Erlenmeyer flasks (250 ml), burette, glass beads, Pyrex tubes, screw lids, falcon tubes (15 ml, 50 ml).

4.2.4 HPLC

A 1,5 ml sample was taken from each experiment sample and the pH was measured in all samples. The sample was acidified using 2M H_2SO_4 10% v/v, more 2M H_2SO_4 was added if the initial dose was insufficient to lower the pH. The samples were centrifuged at 10000 rpm for 10 minutes and the supernatant was removed and filtered through a 0.45 µm filter into a chromatography vial, that was subsequently sealed and analysed. The compounds tested for were glucose, xylose, arabinose, cellobiose, citric acid, succinic acid, glycerol, ethanol, 1,2-propanediol and 2,3-butanediol. The samples were analysed on a Dionex Ultimate 3000-LC system using an Aminex® HPX-87H column connected to a refractive index detector. H_2SO_4 with a 0.6 ml/min flowrate at 60°C was used as the mobile phase. The software used for integration was the Chromeleon from Dionex Corporation (Santamaria 2015).

4.2.5 VFA

A 1,5 ml sample was taken from each experiment sample and the pH was measured in all samples. The sample was using 17% H_3PO_4 (10% v/v), more 17% H_3PO_4 was added if the initial dose was insufficient to lower the pH. The samples were centrifuged at 10000 rpm for 10 minutes and the supernatant was removed and filtered through a 0.45 µm filter into a chromatography vial that was subsequently sealed and analysed. The VFAs tested were acetate, propionate, butyrate, iso-butyrate, valerate and iso-valerate. The VFAs are analysed with a gas chromatograph using an Agilent HP-FFAP capillary column that is 0.53 mm in diameter and 30 meters in length. The column is connected to a flame ionization detector (FID). The carrier gas utilized was Nitrogen gas with a flowrate at 13 ml/min. The temperature of the detector was 230°C and the temperature of the injector was 240°C (Santamaria 2015).

4.2.6 TS and VS

5 ml juice (green or brown) was added to pre-weighted, burned crucibles and weighted again. The crucibles were left overnight in an oven at 105°C and weighted again. Then, the crucibles were burned in an oven at 550°C and weighted again.

Equation for calculating TS:

$$TS\% = \frac{(A-B)}{(C-B)} \times 100\% \qquad \text{(eq. 1)}$$

Equation for calculating VS:

$$VS\% = \frac{(A-D)}{(C-B)} \times 100\% \qquad \text{(eq. 2)}$$

A: Weight of crucible + dry sample

B: Weight of crucible

C: Weight of crucible + wet sample

D: Weight of crucible + ash

All values for TS and VS calculations were in grams

4.2.7 Soluble sugars

A weak acid hydrolysis was performed to determine the soluble sugars. Unfortunately, there was not enough green juice left to perform this analysis, so the soluble sugar content was only determined for the brown juice. 5 ml sample was transferred to a screw lid Pyrex tube and 5 ml 4% H_2SO_4 was mixed in and the samples were shaken. After mixing 100 µl MilliQ water was added to the sample tubes and 100 µl a mixture with known sugar content called stock solution (D-xylose 8 g/L, L-arabinose 30 g/L, D-glucose 33 g/L) was added to the samples the tubes were again shaken. The lid was screwed on slightly loosened and the samples were autoclaved for 10 minutes at 121°C. The samples were filtered through 0.45 µm syringes filters and 5 ml filtrate was transferred to a falcon tube containing 0.5 g of $Ba(OH)_2 \cdot 8\ H_2O$. The samples were vortex mixed for 30 seconds and left at room temperature for 5 minutes. Samples were centrifuged at 4000 rpm for 5 minutes at 20°C. 2 ml supernatant was removed and mixed with 2 ml eluent, pH was adjusted to between 2 and 3. 1 ml of the mixture was prepared for HPLC. HPLC samples of the pure stock solution were also prepared.

4.2.8 Total Kjeldahl Nitrogen

The total Nitrogen was determined using the micro-Kjeldahl method. 1 ml sample was mixed with 24 ml milliQ water in a digester column. 25 ml of each of the 5 alanine solution were added to 5 separate columns (one column for each concentration), and 25 ml milliQ water was added to another column as a blank. 10 ml digestion reagent was added to each column together with five glass beads. The digester was connected to the scrubber and the boiling began starting the digestion. The digestion continued until the samples in the column turned green, the digestion was stopped and the materials were left for cooling. 20 ml water was added to the samples for dilution and 10 ml boric acid was added to an Erlenmeyer flask and inserted into the distiller. 10 ml Sodium hydroxide thiosulfate reagent was added to the tubes containing the samples and immediately afterwards inserted in the distiller and 2 minutes' distillation was started. After distillation the 0.05 M HCl was titrated into the distillate until the indicator turned pink.

Equation for calculating for Total Kjeldahl Nitrogen:

$$\frac{mg\ TKN}{L} = \frac{(VHCl_{sample} - VHCl_{blank}) \times N_{HCl} \times 14 \times 1000 \times X_{dilution}}{V_{sample}} \qquad \text{(eq. 3)}$$

$VHCl_{sample}$: Volume of HCl added to sample during titration

$VHCl_{blank}$: Volume of HCl added to the blank during titration

V_{sample}: Volume of the sample used for determination

N_{HCl}: Molarity of the HCl used for titration

$X_{dilution}$: Dilution of the sample

4.3 Results and Discussion

	Drymatter (TS) kg/ton	Organic matter (VS) kg/ton	Total Kjeldahl Nitrogen kg/ton	Free sugars* kg/ton	Lactic acid kg/ton	Acetate kg/ton	VFAs** kg/ton	Other compounds*** kg/ton	Soluble sugars Kg/ton	pH
Green juice	62.55	51.51	1.72	28.13	0.26	0.38	0.22	1.09	X	5.6
Brown juice	25.54	17.73	0.86	1.92	13.22	2.41	0.44	0.79	0.31	4.2

*Table 1: The chemical composition of green juice and brown juice shown in Kg/1000 Kg. It is assumed that the density of both juices is 1. No soluble sugars were measured for the green juice. *Free sugars is the sum of glucose, xylose, arabinose and cellobiose. **VFAs is the sum of propionate, iso-butyrate, butyrate, valerate and iso-valerate. *** Other compounds is the sum of citric acid, succinic acid, glycerol, ethanol, 1,2-propanediol and 2,3-butanediol*

Table 1 shows the results obtained from the characterization of brown juice and green juice. The green juice contains more solids and organic matter. This is to be expected since a large amount of solids is removed, when the protein paste is removed from the green juice (Santamaria 2015). In the green juice the organic solids consist primarily of free sugars, mainly glucose and xylose at around 15 kg/ton and 13 kg/ton respectively. The brown juice on the other hand contained less than 2 kg of free sugars. This is consistent with the fact that the free sugars should have been consumed from the green juice during the lactic acid fermentation (see OrganoFinery chapter 2.2). Other free sugars are arabinose and cellobiose which were only found in very small amounts or not at all. In the brown juice there are only a slight amount of soluble sugars, this is probably because most soluble sugars have already been fermented during the protein extraction.

The Nitrogen content of the biomasses is halved in the process from green juice to brown juice. This is expected since proteins are extracted from the green juice and proteins are constructed of amino acids which contains Nitrogen (Berg et al. 2012). The fraction of proteins removed from the brown juice depends on what type of grass has been used for making the green juice. From clover grass green juice

52% of the proteins are extracted while it is 67% when the green juice comes from red clover (not published data). Since the green juice and the brown juice used in this experiment are from different sources it is not possible to make completely reliable since the initial composition of the biomasses will differ. That being said the difference between the Nitrogen in green juice and brown juice appears somewhat consistent with the removal fractions.

In the same note the brown juice contained a lot more lactic acid than the green juice where there was almost no lactic acid to be found. Since the proteins are extracted from the green juice by producing lactic acid in the green juice. This is the expected result and also connects to the difference in pH between the two, brown juice contains more lactic acid and thus is more acidic with a pH of 4.2, while green juice has pH 5.6. The acetate in the brown juice is around 2.5 kg which is much higher than what is found in the green juice, this shows that the acetic acid is produced during the protein extraction fermentation. Any other compound or VFA found in the brown juice and green juice was only present in negligible amounts.

In the green juice approximately 20 kg of organic material has been left unidentified. This organic material is probably a mixture of soluble carbohydrates, lipids and other organic compounds. Where the main part is either consumed during fermentation or lost during the removal of the protein paste. In the brown juice the total amount of organic material is actually lower than the total amount of compounds found which is not possible. The most likely explanation is that some of the volatile organic compounds evaporate with the water during the drying for TS determination. This means that the dry matter content might also be underestimated. Most of the evaporated organic solids are likely to be VFAs (Vahlberg et al. 2013).

The characterization results will not be completely representative for all green juice and brown juice produced, especially not if the juices were to be used in industrial scale. There are several reasons for those future discrepancies. Firstly, the chemical composition of the brown juice and green juice is dependent on the biomass used in the production process. In this case the green juice was made from clover grass while the brown juice was from red clover. So not only are there more than one type of grass material used in the OrganoFinery, the fields containing mixed sorts will all so inevitably have different ratios between the species. Secondly, the growth conditions influence the chemical composition of the juices. Crops are grown of different types of soil, under different weather conditions and during different seasons. All these factors influence the crop composition and thus the composition of the juices. Thirdly, there is a difference between laboratory scale preparation and industrial scale preparation. For instance, the protein paste separation by centrifugation will be much more efficient when performed in the laboratory than it will be on an industrial scale. This means that the TS of the juices will be larger on an industrial scale. The difference mostly comes down to the fact that equipment efficiency decrease when dealing with larger amounts.

5 ISOLATION AND INITIAL SCREENING

5.1 PURPOSE & OVERVIEW

Different bacteria were isolated from the green juice and the brown juice samples in order to obtain bacteria capable of producing lactic acid and growing in the juices. The bacteria were isolated from the juice with the assumption that bacteria capable of growing in a specific environment could be found in said environment. A few different production conditions were also tested during the initial screening, these conditions were aerobic/anaerobic and sterile/nonsterile. Initial screening was performed using green juice as substrate instead of brown juice. This was to avoid experimentation with sugar addition before optimal fermentation conditions and the lactic acid production potential of the bacteria had been determined. For the screening experiments a blank control was added and later in the experiment *L. salivarius* was used for a positive control since it is a known lactic acid producer and already utilized in the OrganoFinery. The experiment was run for 48 hours since a lot of lactic acid bacteria have a very fast production rate (Thomsen & Kiel 2008).

5.2 MATERIALS AND METHOD

5.2.1 Chemicals & Solutions

Red Clover green juice, Clover Grass green juice, Alfalfa brown juice, Sodium carbonate (0.4 g/L), N_2 gas, glycerol, agar, MRS media, EtOH (70%).

5.2.2 Equipment

Centrifuge Thermo scientific Meraeus Multifuge X3R, Incubator Formascientific CO_2 water jacketed incubator, pH meter Infolab WTW series pH 720, Laminated flowbench Holm & Halby Holten LaminAir, Autoclave Certoclav Tisch-autoclave, 0.0001 g balance

5.2.3 Materials

Serum vials, rubber stoppers, clamps, pipettes (50 ml, 2-20 µl, 30-300 µl), syringes (2 ml, 5 ml), needles, Eppendorf tubes (1,5 ml), falcon tubes (15 ml, 50 ml), blue cap bottles, petridishes, small glass vials (approximately 10 ml), cryotubes, grafting needle.

5.2.4 Isolation of bacteria

MRS media was prepared and sterilized for 20 minutes at 121°C, prior to sterilization half the MRS broth was mixed with agar, 1,5 g agar to 100 ml MRS broth. The agar MRS media was distributed in petridishes, 20 ml in each. A dilution series was made by mixing 1 ml Red Clover green juice with 9 ml MRS media, and continuing the dilution up to 1:10000. 100 µl was plated on the petridishes from each dilution in duplicates. The plates were left in an incubator at 37°C overnight. 10 sterile glass vials were filled with 5 ml MRS broth

and a colony from the agar plates to the broth (one colony to one broth), the vials were sealed and incubated overnight at 37°C. All work was performed under a laminar flowbench. 5% inoculum v/v (40 ml) was transferred to a preculture for the isolated bacteria. The preculture was incubated overnight at 37°C. Samples were also prepared for storage. 900 µl of bacteria and 100 µl of sterile glycerol was pipetted to a cryotube under a laminar flowbench and stored in a -80°C freezer.

5.2.5 Screening for lactic acid production in bacteria isolated from Red Clover green juice

Four different conditions were tested during the screening: aerobic/sterile, anaerobic/sterile, aerobic/nonsterile and anaerobic/nonsterile. Clover grass green juice was added to the serum flasks and prepared accordingly. The inoculum was 5% of total volume (40 ml), blank control samples were also prepared. The samples were incubated at 37°C. Samples for HPLC and VFA were taken at time 0h, 24h and 48h, pH was measured at these time 24 and 48 hours. Anaerobic conditions were obtained by flushing the vials with N_2 prior to sealing and autoclaving. Sterility was obtained by autoclaving at 120°C for 20 minutes.

The experiment was repeated only using the aerobic/sterile conditions, for clearer results. *L. salivarius* a known lactic acid producer was used as a positive control.

5.2.6 Screening for lactic acid production in bacteria isolated from Alfalfa brown juice

Clover Grass green juice was added to the serum flasks and sterilized. The bacteria isolated from Alfalfa brown juice was inoculated using 5 % of total volume (40 ml) and the samples were incubated at 37°C for 48h. pH measurements, samples for VFA and HPLC were taken at 0h, 24h and 48h.

5.3 Results and Discussion

5.3.1 Isolation of bacteria

10 colonies were isolated from the plates and enriched. 4 were isolated from green juice and 6 were isolated from brown juice. The bacteria were given the working names: GJ A, GJ B, GJ C, GJ D, BJ A, BJ B, BJ C, BJ D, BJ E and BJ F.

5.3.2 Conditions: sterility and aerobic/anaerobic

The bacteria isolated in green juice was used for a screening for lactic acid production under different conditions. The results from this experiment (see appendix 12.1) were upon examination found unreliable due to several problems with the results.

When measuring initial conditions, the assumption was made that since the same biomass is used for all bacteria the initial control samples could be used for initial values in all of the samples which unfortunately created some slight discrepancies. One discrepancy was that some samples to have an increase in glucose and xylose concentration between initial samples and 24 hour samples. There are two possible explanations for this. One is that the soluble sugars are being degraded and glucose and xylose are released as a result. The other explanation is that for this experiment initial samples were not taken from the individual experiment samples, but rather directly from the green juice before distribution. This means that the individual variation of the samples is not taken into account. The inoculum also contains some sugar residue which enters the sample with the inoculum without being included in the initial sugar concentration.

The large difference in initial conditions between sterile and nonsterile vials is also an issue. The big difference in sugar content and lactic acid concentration between the nonsterile and sterile vials suggests that fermentation has already occurred in the nonsterile vials. The most likely cause for this is that the samples were improperly stored and thus was not inhibiting the bacteria already in the green juice from fermenting.

From the control sample it can be seen that lactic acid is produced from the bacteria already in the green juice. This means that it will not be possible to determine the lactic acid production potential of the different bacteria if they are grown in a mixed culture since it will be difficult to distinguish the production of the bacteria tested from the bacteria already in the non-sterile juice. Leading to the decision that further experimentation should be performed under sterile conditions.

For aerobic and anaerobic conditions there was overall a slight increase in concentration under anaerobic conditions compared to the aerobic conditions. This increase was however small enough that the added cost and effort of making the process anaerobic will most likely be larger than any extra output. Hence the decision was made to continue the experiments using aerobic conditions.

5.3.3 Screening for lactic acid production

The results from the new green juice screening with aerobic sterile conditions can be observed in figure 3 and figure 4. The results show that the bacteria isolated in green juice do not have good potential for lactic acid production. The samples GJ A and GJ D seems to have no production or sugar consumption at all, which most likely means that the bacteria have not grown at all. This is supported by the fact that there was almost no fall in pH during the 48 hours of fermentation. Bacteria GJ B and GJ C both had some lactic acid production, however neither was in an amount large enough to continue fermenting with either. Both of the bacteria isolated in green juice had a final lactic acid concentration well below 10 g/L. This could be because the other fermentation conditions are not optimal for the growth for these bacteria. For instance, the pH was not adjusted in any way so the fermentation started at around pH 5. The final pH was 4.5 for GJ B and 4 for GJ C. This could mean that the conditions were too acidic for the bacteria and it might be possible to have gotten better results if the samples had been pH controlled. Another matter

is that the green juice should have a pH at 5.6, however all of the samples had an initial pH at 5 or lower. This indicates that the pH gets lowered during the preparation, this was examined more closely in another experiment (see chapter 6).

The positive control *L. salivarius* was the best lactic acid producer observed in the screening, the sample had a final lactic acid concentration at approximately 13.3 g/L. That lactic acid concentration is actually quite a bit lower than what it is possible to obtain from this strain (Thomsen & Kiel 2008), showing that the fermentation conditions can be optimized. *L. salivarius* had no other production, which is consistent with the fact that *L. salivarius* is homofermentative (Garlotta 2002).

The only compounds produced in the samples in any significant amount was acetate and lactic acid. This was expected as since the metabolic pathways in lactic acid bacteria only should be able to produce these two compounds and ethanol. The other compounds and VFAs tested for was only present in negligent amounts, which is why they have not been included in the graphs.

None of the bacteria isolated in green juice seems to be able to consume xylose, in fact xylose seems to be produced in small amounts instead. This should not be possible, which means that either xylose is released from degradation of soluble sugars or it is a measurement variation. The latter is more likely since the negative control (GJ X) also has an increase in xylose despite having no bacterial activity.

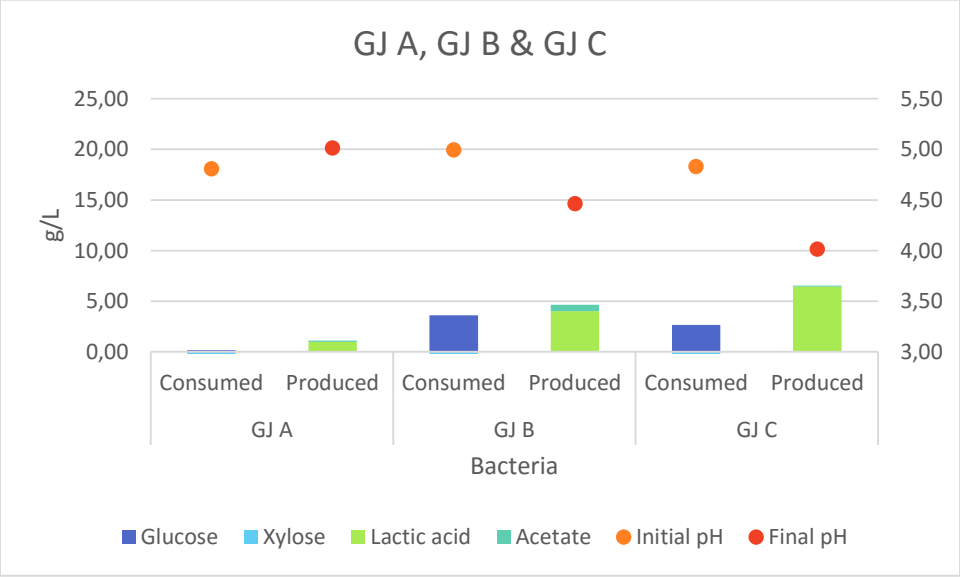

Figure 3: The consumed sugars and produced lactic acid and acetate seen in GJ A, GJ B and GJ C after 48 hours of fermentation

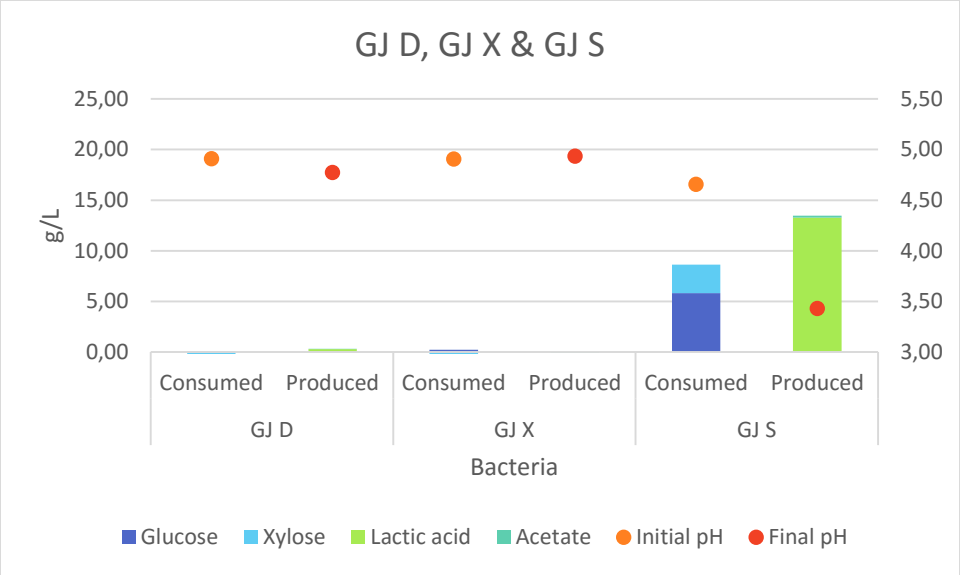

Figure 4: The consumed sugars and produced lactic acid and acetate seen in GJ D, L. salivarius and the control sample (GJ X) after 48 hours of fermentation

The bacteria isolated in brown juice seen in figure 5 and figure 6 appears to have higher production than the bacteria isolated from green juice. This could be explained by the brown juice being more acidic than the green juice, thus it should contain bacteria which are more tolerant to lower pH values and thus can ferment longer. This is supported in the final pH values of the bacteria. With the exception of GJ C all the bacteria isolated in green juice had a final between 5 and 4.5. Whereas the bacteria isolated from the brown juice had a final pH between 4 and 3.5.

BJ A, BJ B, BJ C and BJ D all had a similar lactic acid production, but it is only slightly higher than what was observed for GJ C. BJ E and BJ F however did manage to produce lactic acid enough to reach a final concentration of just over 10 g/L, which is worth looking into improving. This time the positive control *L. salivarius* reached a final lactic acid concentration of 15 g/L, which is a slight increase that might be explained by the increased xylose consumption observed. BJ E and BJ F also produced quite a bit of acetate, meaning they are both heterofermentative bacteria. This means that the acetate is a byproduct of the lactic acid production.

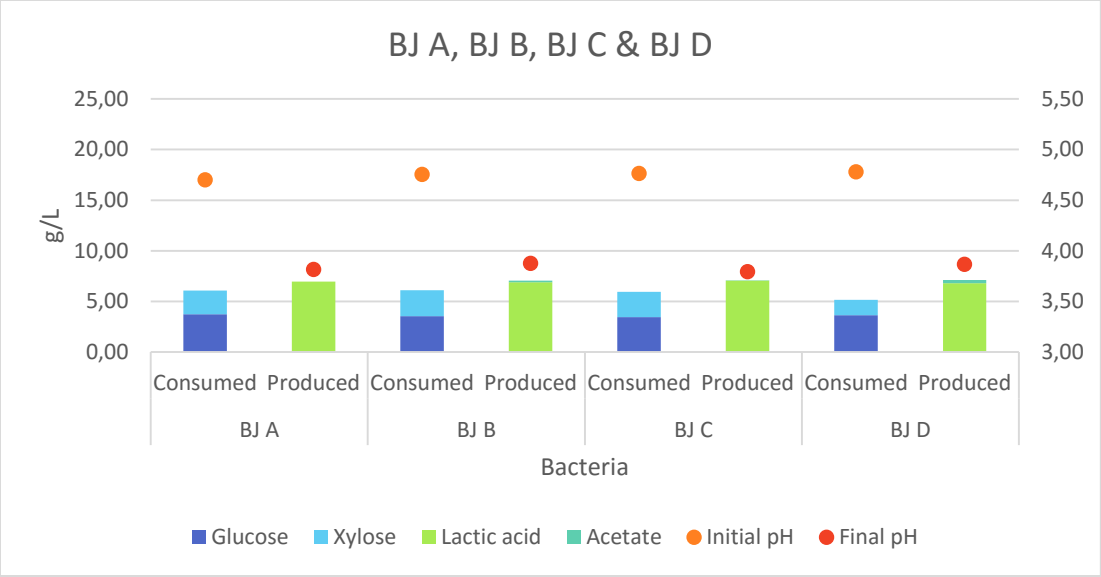

Figure 5: The consumed sugars and produced lactic acid and acetate seen in BJ A, BJ B, BJ C and BJ D after 48 hours of fermentation

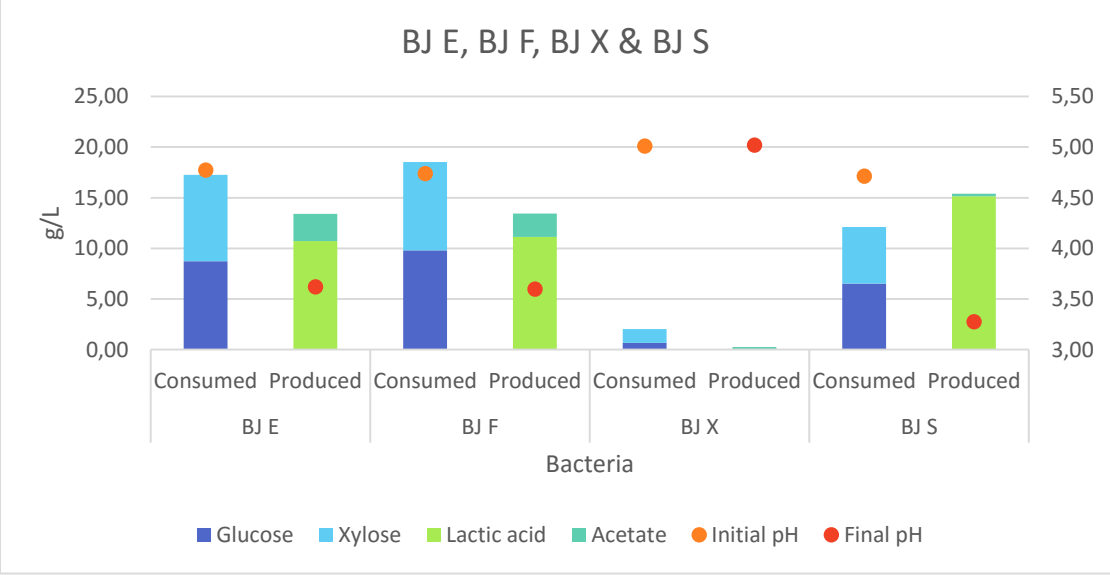

Figure 6: The consumed sugars and produced lactic acid and acetate seen in BJ E, BJ F, L. salivarius and the control sample (BJ X) after 48 hours of fermentation

Green juice has a total free sugar concentration of approximately 28 g/L, which means that all the samples still have plenty of free sugars left. Therefore, it is possible that the fermentation could continue for longer with a higher lactic acid gain. It is however also possible that the conditions will have become too acidic for the fermentation to continue as GJ C had the lowest final pH of the green juice bacteria at 4. While all the others as previously stated 4.5 or higher.

BJ E and BJ F has a higher sugar consumption than any other bacteria despite producing less than *L. salivarius*, making them quite inefficient.

6 LACTATE SCREENING WITH VARYING PH

6.1 PURPOSE & OVERVIEW

When starting the experiment, the pH should be 5.6 and from then on decreases as the fermentation continues. When the juice becomes too acidic the bacteria should stop fermenting which is assumed to be due to the bacteria being inhibited thus causing the production of lactic acid to stop. How acidic the fermentation media can become before fermentation stop depends on the bacteria used in the fermentation.

The If the initial pH was to be increased, then the fermentation should be able to continue longer thus increasing the lactic acid produced by the bacteria. Hence for the following experiment the green juice has been adjusted to different pH values and fermentation was prolonged to 72 hours.

Initial experimentation uncovered a problem with keeping the adjusted pH value, before the experiment even started. This means that the pH fall was due to something that happened during the preparation or inoculation. The primary cause for the fall in pH was suspected to be the sterilization method, autoclaving. An experiment was devised to help determine the cause for the decreased pH so that it could be countered for the experiment with the pH adjustment and if necessary further experiments.

6.2 MATERIAL AND METHOD

6.2.1 Chemicals and Solutions

Clover grass green juice, NaOH (1,57 M), HCl (1 M), MilliQ water, EtOH (70%).

6.2.2 Equipment

Incubator Formascientific CO_2 water jacketed incubator, pH meter Infolab WTW series pH 720, Autoclave Certoclav Tisch-autoclave, Laminated flowbench Holm & Halby Holten LaminAir.

6.2.3 Materials

Serum vials (117 ml), pipettes (30-300 µl, 50 ml), automatic pipette, pipette tips, rubber stoppers, cramps, magnet, magnetic stirrer, blue cap bottles, syringe, autoclave bags, falcon tubes (15 ml), needles, Eppendorf tubes (1,5 ml)

6.2.4 Fall in pH determination

Green juice was distributed between 8 serum vials, 40 ml in each. Each serum vials were adjusted to a specific pH (4.5, 5, 5.5, 6, 6.5, 7, 7.5 and 8) using 1,57 M NaOH when raising the pH value and 1 M HCl when lowering the pH value. The vials were autoclaved at 121°C for 20 minutes, afterwards pH was measured again.

6.2.5 pH adjustment and fermentation prolongation

Green juice was divided between five blue cap bottles, 450 ml each, and autoclaved at 120°C for 20 minutes. pH was adjusted in a laminar flowbench using 1 M HCl or 1,57 M NaOH, the buffers were filtered into the bottles using filter sterilization. The pH adjusted to was 7, 6.5, 6, 5.5 and 4,5. After adjustment green juice added to sterilized 117 ml serum vials and sealed with sterilized rubber stoppers, 8 vials for each pH value. A separate set of vials was prepared using the method described in chapter 5, this set had no pH adjustment. Three different bacteria were inoculated for each pH value, BJ E, BJ F and *L. Salivarius* at 5% v/v (total volume of 40 ml). The bacteria were tested in duplicates and for each pH value, a duplicate set of blanks were also prepared. The vials were incubated at 37°C for 72 hours and samples for VFA and HPLC were taken at 0h, 24h, 48h and 72h. pH was also measured at those time points.

The theoretical max yield and actual were calculated as following:

Theoretical lactic acid yield from glucose:

$$Theo.\ yield = \frac{2\ mol \times 90 \frac{g}{mol}\ lactic\ acid}{1\ mol \times 180 \frac{g}{mol}} \quad \text{(eq. 4)}$$

Theoretical lactic acid yield from xylose:

$$Theoretical\ xylose\ yield = \frac{5\ mol \times 90 \frac{g}{mol}\ lactic\ acid}{3\ mol \times 150 \frac{g}{mol}} \quad \text{(eq. 5)}$$

Theoretical lactic acid yield in the experiment:

$$Theoretical\ \ yield = \frac{Total\ consumed\ sugars\ (\frac{g}{L}) \times 1L}{Theoretical\ yield\ (glucose) + Theoretical\ yield\ (xylose)} \quad \text{(eq. 6)}$$

The actual yield seen in the experiment:

$$Actual\ yield = \frac{\frac{g}{L}\ Produced\ Lactic\ acid}{\frac{g}{L}\ Consumed\ sugars} \times 1\ L \quad \text{(eq. 7)}$$

6.3 Result and Discussion

6.3.1 Preliminary pH determination

In table 3 the varying pH before and after autoclaving can be observed. The results clearly show a fall in the pH, especially in the samples were the pH has been adjusted upwards, the higher the pH the bigger the decrease. These results prove that the large pH drop can be attributed to autoclaving.

Adjusted pH before autoclaving	pH after autoclaving	Difference
4.54	4.60	0.06
5.06	5.02	0.04
5.48	5.00	0.48
5.97	5.27	0.70
6.50	5.57	0.93
7.04	5.87	1.17
7.49	6.10	1.39
7.97	6.65	1.32

Table 2: The pH before and after autoclaving

Generally autoclaving usually causes some decrease in the pH in the media being sterilized, in this case green juice. This decrease is normally quite small if the media is without sugars. However if the media being autoclaved contains sugar, then the severity of the pH decrease is dependent on which sugars is found in the media (Thorpe et al. 2008). Green juice contains approximately 30 g/L of free sugars, around half of this is glucose which has a significant pH reducing effect on the media when autoclaved (Thorpe et al. 2008). The remaining free sugar in the green juice is xylose which also has been observed to have a pH reducing effect on the media which contains it when it is autoclaved (Hill et al. 1993).

It should be noted that while the samples adjusted to high pH values falls drastically, the lower pH values have a much smaller decrease, with the pH 5 and 4.5 samples having no decrease at all. This suggest that something other than the free sugar content and the autoclave is affecting the green juice. It could be a

matter of the buffer capacity of the proteins. Green juice contains a lot of protein which acts as a buffer counteracting decreases in the pH (Karow et al. 2013). This is supported by the fact that a lot of NaOH (1.57M) had to be used to adjust the pH up any higher than the standard 5.6 of the green juice. Also when higher pH values were reached the buffer used shifted from having minimal effect even in large amounts, to large effect in very small amounts. The adjustment to lower pH was however much easier. This suggest that the lower pH values are within the buffering area of the proteins while the higher pH values were not and thus being much more susceptible to factors affecting the pH values. In this case the autoclaving.

That the fall in pH can be contributed to autoclaving means that the pH cannot be adjusted before sterilization if autoclaving is the method of sterilization. No other sterilization method was considered feasible for the lab work, so the pH has to be adjusted under sterile conditions after the green juice has been autoclaved (see section 6.2.5).

6.3.2 pH value and fermentation duration

In figure 7 and figure 8 it can be observed that for BJ E the change of pH seems to have very little effect on the overall lactic acid production. The lactic acid produced in the uncontrolled sample is approximately 11.2 g/L and that concentration raises slightly from pH 5.5 up to pH 7, which has a lactic acid concentration of around 12.5 g/L. This is a difference of 1.3 g/L so the gain from increasing the pH is quite small. The acetate production actually seems to decrease at the higher pH values, which means that higher pH leads to a purer lactic acid production. The highest sugar consumption is seen in the pH 6 sample, with an approximate total of 19.7 g/L consumed sugar. The consumption of glucose and xylose is around 8.5 g/L and 11 g/L respectively, which is the highest result for both categories. The uncontrolled pH sample had the lowest consumption with 6.2 g/L for glucose and 8.4 g/L xylose consumption. For glucose the remaining samples had a consumption between 7 and 8 g/L with the higher pH samples having better results. The xylose consumption was at approximately the same level as the uncontrolled for the lower pH samples, while the higher pH samples was between 9.9 g/L and 11 g/L. The final pH was between 3.6 and 3.9 for all the samples.

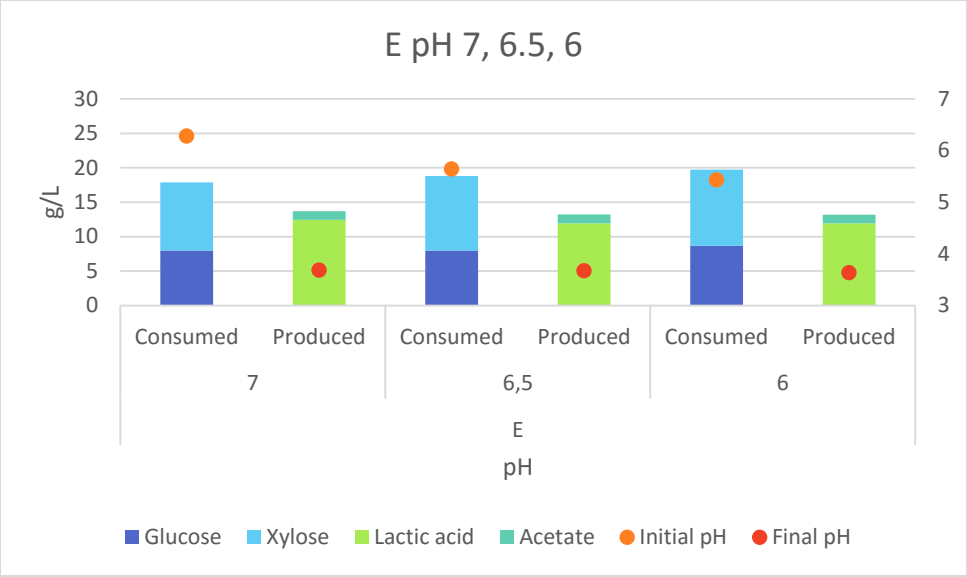

Figure 7: The sugar consumption, production of lactic acid and acetate and the change in pH for BJ E, at initial pH values 7, 6.5 and 6

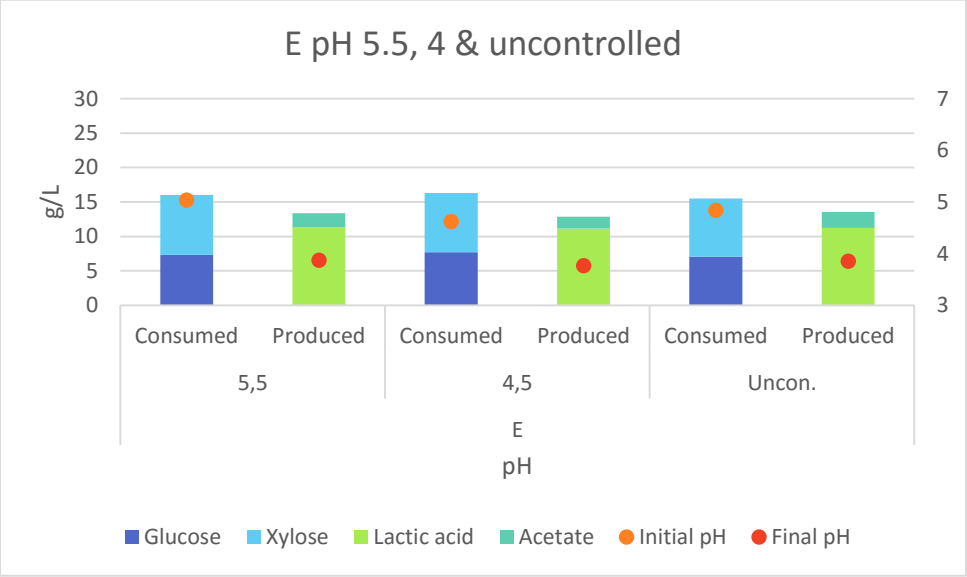

Figure 8: The sugar consumption, production of lactic acid and acetate and the change in pH for BJ E, at initial pH values 5.5, 4.5 and uncontrolled

In figure 9 and figure 10 it can be seen that BJ F has a very clear jump in consumption of sugars between the lower pH value and the higher pH value samples. The highest consumption for both is seen at pH 6.5, 11.8 g/L for glucose and 12.4 g/L for xylose, with a difference of 1-1.5 g/L when compared to the values of pH 7 and pH 6. The lactic acid production was varied over all the pH values. The highest production at

pH 7 with 13.6 g/L, and the second highest at pH 5,5 with 13.1 g/L. At uncontrolled pH the lactic acid production was at the lowest with 9.6 g/L. Acetate production was at the highest at lower pH, but never exceeding 2.2 g/L, so overall production was quite low as expected. The final pH was between 3.6 and 3.9. The samples with the lower pH samples were more similar to what was observed in BJ E.

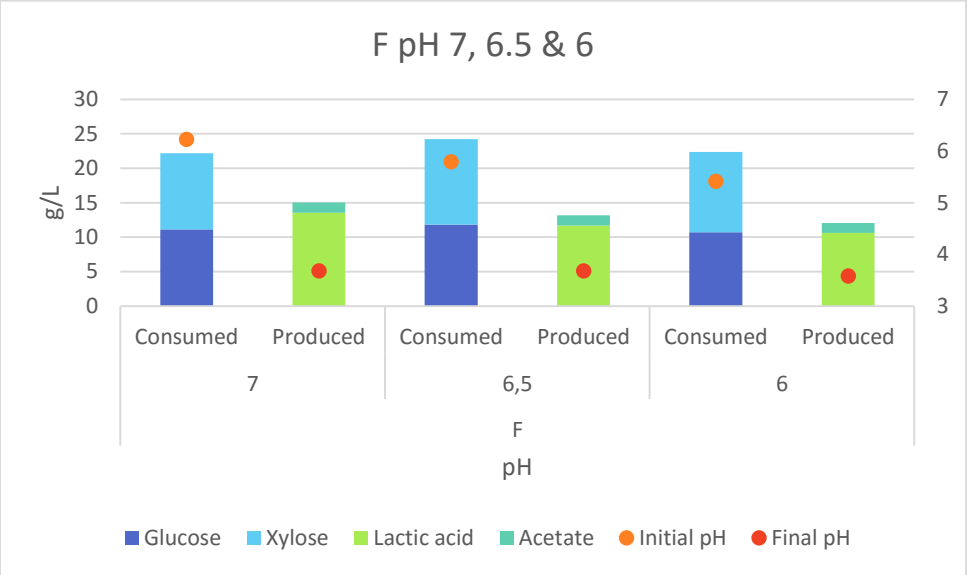

Figure 9: The sugar consumption, production of lactic acid and acetate and the change in pH for BJ F, at initial pH values 7, 6.5 and 6

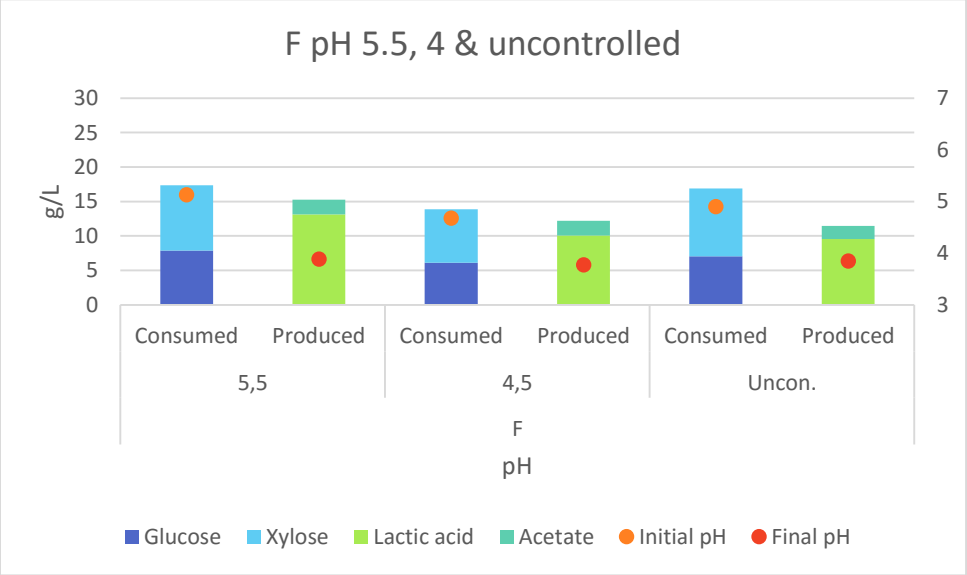

Figure 10: The sugar consumption, production of lactic acid and acetate and the change in pH for BJ F, at initial pH values 5.5, 4.5 and uncontrolled

Figure 11 and figure 12 shows that *L. salivarius* still has a much higher lactic acid production than bacteria E and F. The lowest concentration of lactic acid was obtained at pH 4,5 at 18.1 g/L, the highest was at pH 6.5 at 21.5 g/L which is 1.5 g/L higher than the closest competitor (which). At pH 6.5 the highest sugar consumption was also observed with a total consumption of around 17 g/L, with no significant difference between the glucose and xylose consumed. At lower pH there is less xylose consumption, which appears to be an overall general trend for all the bacteria. The final pH ranged from 3.2 to 3.4.

The initial pH measurements, was lower than they had been adjusted to despite the pH being adjusted after autoclaving. This is additional fall happens because the inoculum is very acidic, thus decreasing the pH. The fall in pH appears to be somewhere between 0.5-1. The decreasing effect of inoculum seems to fall at the lower pH values. Samples adjusted to pH 5,5 had a much smaller pH drop than the samples at higher pH, while the samples adjusted to 4,5 remained largely unaffected. This is consistent with what was previously observed, with the lower pH values being within the buffer capacity of the green juice while the higher pH values are much more sensitive.

For the control samples (see appendix 12.2) there were a few issues. For one thing the pH 7 sample had fall in pH that it should not have had, since there was no autoclaving after pH adjustment and no inoculum to decrease the pH. There was also some minor lactic acid production in the same sample, while the amount was only 0.3 g/L, it is almost ten times the amount seen in the other control samples. It is possible that the sample simply became contaminated with lactic acid thus the slight increase in lactic acid, which in turn affected the pH.

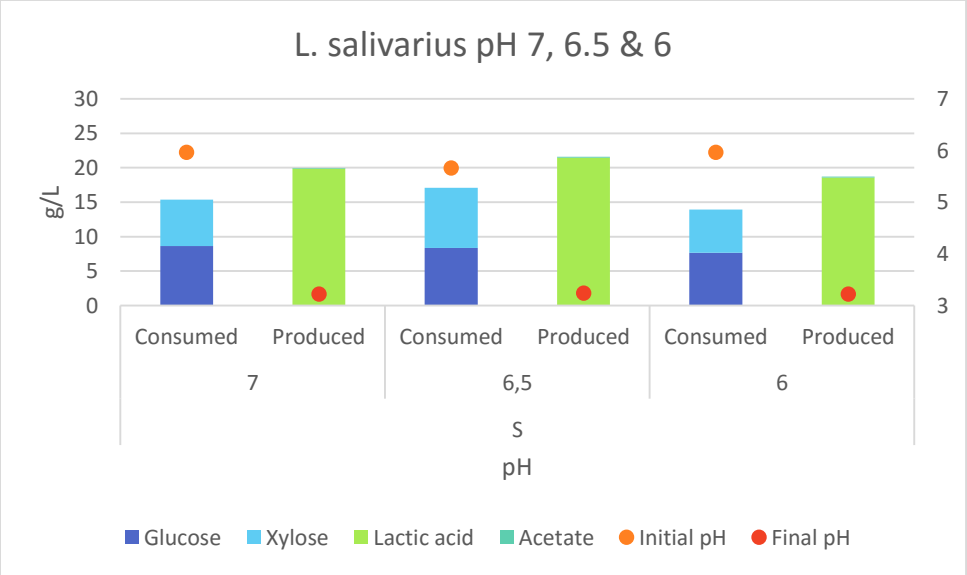

Figure 11: The sugar consumption, production of lactic acid and acetate and the change in pH for L. salivarius, at initial pH values 7, 6.5 and 6

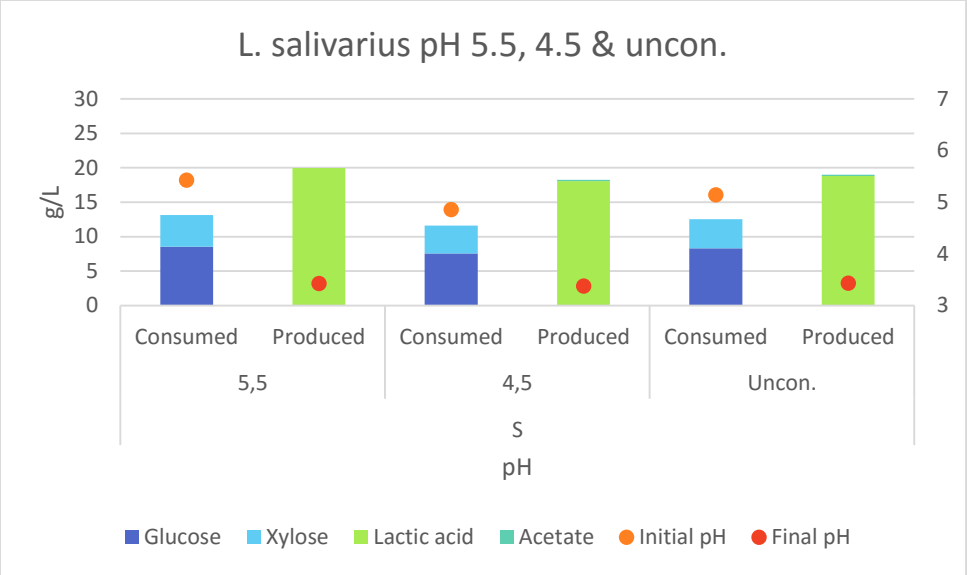

Figure 12: The sugar consumption, production of lactic acid and acetate and the change in pH for L. salivarius, at initial pH values 5.5, 4.5 and uncontrolled

In *L. salivarius* more lactic acid is produced than the amount of sugar consumed, this should not be theoretically possible. The most likely explanation is that soluble sugars are degraded and utilized for the lactic acid production. In BJ F pH 7, 6.5 and 6 there is a higher sugar consumption than lactic acid production. Meaning that BJ F are not very efficient or that the bacteria do not adapt well to higher pH values thus requiring more energy.

A general thing for all the bacteria is that there are still sugars left to consume in the green juice. From the green juice characterization, it is known that green juice contains 29 g/L free sugars, which is supported by the sugar concentrations observed in the initial HPLC samples. BJ F pH 6.5 had the highest total sugar consumption at 24 g/L. This means that it is possible for the bacteria to continue the fermentation. From the results seen table 4 it is very clear that an extra 24-hour fermentation did not yield a lot of extra lactic acid, but since all the bacteria had plenty of sugar left the decrease in lactic acid production was not due to lack of substrate. It is however possible that what is experienced is product inhibition, since lactic acid have an inhibitory effect on microbial activity (Iyer & Lee 1999). This is also evidenced by the low final pH, which mean that the conditions are acidic which could prevent fermentation. Lactic acid production leads to a decreasing pH.

The yield of the bacteria also falls between 48 hours and 72 hours. Looking at the results the explanation for the decrease in yield stems from the fact that very little lactic acid is being produced in the last 24 hours, some samples even has a decrease in lactic acid concentration. This can then be combined with the sugar consumption still happening albeit at a slower rate. One concern about the yield is that the calculated yield based on the data is occasionally a little bit higher than the theoretical maximum in BJ E and BJ F. For *L. salivarius* all the conditions had significantly higher yield than the theoretical maximum.

The explanation for the difference is most likely due to the unknown amount of soluble sugars in the green juice which are probably consumed by the bacteria along with free sugars. This means that the amount of sugars available for consumption during the fermentation is actually higher than what is presented during analysis. A higher sugar consumption would lower the actual yield and thus more realistic. However, some of the yield obtained from the *L. salivarius* are still very high even when the soluble sugars into account. The theoretical yield for BJ E and BJ F has probably been overvalued because the calculation was based on the assumption that the fermentation was homofermentative. BJ E and BJ F are heterofermentative bacteria, meaning that the theoretical yield should be smaller.

Lactic acid production	**Conc. 48 h g/L**	**Conc. 72 h g/L**	**Theo. Max yield g/g***	**Yield 48 h g/g**	**Yield 72 h g/g**
E 7	13.717	13.86	1	0.915	0.697
E 6,5	13.453	13.717	1	0.839	0.633
E 6	13.629	13.849	1	0.806	0.605
E 5,5	13.475	12.694	1	0.952	0.705
E 4,5	11.561	11.121	1	0.959	0.682
E uncon.	11.737	11.231	1	0.832	0.767
F 7	13.475	13.552	1	0.612	0.611
F 6,5	13.365	13.552	1	0.568	0.483
F 6	12.166	12.419	1	0.577	0.474
F 5,5	13.926	13.112	1	0.911	0.755
F 4,5	11.99	11.253	1	1.038	0.724
F uncon.	11.209	11.242	1	0.709	0.567
***L. salivarius* 7**	19.866	21.45	1	1.436	1.294
***L. salivarius* 6,5**	22.187	23.441	1	1.374	1.258
***L. salivarius* 6**	19.371	20.493	1	1.486	1.335
***L. salivarius* 5,5**	20.878	20.735	1	1.408	1.519
***L. salivarius* 4,5**	19.569	18.799	1	1.885	1.554
***L. salivarius* uncon.**	20.845	19.503	1	1.916	1.500

*Table 3: The lactic acid concentration, yield and productivity after 48 hours and72 hours for bacteria E, F and L. Salivarius. *Theoretical max is calculated for homofermentative bacteria*

Overall the pH adjustment seemed to have very little effect on BJ E since the final concentration of lactic acid only range between 11 g/L and 12.5 g/L. For BJ F the difference in lactic acid ranged from 9.5 g/L to 13.5 g/L which is a better improvement showing that pH changes have bigger effect here, however there was no real tendency to be spotted so it is hard to determine whether higher or lower pH is more effective as a general rule. The difference is probably due to the fact that different bacteria do not necessarily have

the same optimal conditions, meaning that the optimal conditions for the individual bacteria will vary (Abdel-Rahman et al. 2013). For L. salivarius the lactic acid concentrations were much higher than the other bacteria used. The range of the lactic acid concentration were between 18 g/L and 21.5 g/L, with the highest concentration being obtained at pH 6,5. Since the best lactic acid concentration were obtained at pH 6.5 this is the pH used for future experiments.

7 BACTERIAL IDENTIFICATION

7.1 PURPOSE & OVERVIEW

Since BJ E and BJ F were isolated from the brown juice it is not know which bacteria these two are. It would be an advantage to know which bacteria they are because than would be possible to seek additional information about optimal conditions. It will also clarify whether the bacteria are actually the same species, since the behaviour observed by the two bacteria was very similar during the screening, though not the pH control experiment. The bacteria chosen for identification were BJ A, BJ E and BJ F. BJ E and BJ F were chosen because these two are used in the experimentation. BJ A were chosen due to the behavioural similarities between BJ A, BJ B, BJ C and BJ D which led to the assumption that these bacteria are probably identical. To identify the bacteria genomic DNA was extracted from the bacteria and amplified using polymerase chain reaction (PCR) before it was purified and sent for sequencing.

7.2 MATERIALS AND METHODS

7.2.1 Solutions & Chemicals

Isopropanol, 70% ethanol, lysozyme (10 mg/ml), EDTA (ethylenediaminetetraacetic acid, pH 8, 50 mM), Sigma H_2O, 10x Buffer, dNTP Mix (10 mM), primers fD1 (10 µM) and rD1 (10 µM) (Weisburg et al. 1991), RUN polymerase, agarose, TAE buffer, ethidium bromide, distilled water, 6x loading dye, Thermo Scientific generuler 100 bp DNA ladder, mastermix (see appendix 12.3).

7.2.2 Equipment

Nanodrop 1000 spectrophotometer Thermo Scientific, AlphaImager® Mini Alpha Innotech, PowerPac ™ Basic Bio Rad, Techne Dri-block® DB-2D Buch & Holm A/S, TS-100 Thermo shaker Biosan, MS2 Minishaker IKA®, Sprout ™ (spinner), T3000 thermocycler Biometra, Centrifuge Eppendorf MiniSpin plus, T300 Thermocycler biometra, microwave Melissa.

7.2.3 Materials

Wizard® Genomic DNA Purification kit, Eppendorf tubes (1,5 ml), pipettes (100 – 1000 ml, 2 – 20 µl, 30 – 300 µl), pipette tips, PCR tubes, Erlenmeyer flask, GeneJet Gel Extraction and Clean Up Micro kit Thermo scientific.

7.2.4 DNA extraction and isolation

DNA was extracted from BJ A, BJ E and BJ F in duplicates. For the DNA extraction the protocol from Wizard® Genomic DNA Purification Kit was used, specifically the 3.G: Isolating Genomic DNA from Gram Positive and Gram Negative Bacteria (Promega 2014). Following the extraction and isolation, the DNA concentration and quality was tested using the Nanodrop spectrophotometer.

An 0,5% agarose gel was prepared using of 0,5 g agarose dissolved 50 ml 1x TAE buffer heated for 2 minutes in a microwave and cooled to around 60°C. Any evaporated buffer was replaced by distilled water before 1 drop of ethidium bromide was added and mixed into the solution. The gel was poured into the gel tray and left to solidify for approximately 20 minutes. 1 µl 6x loading dye was mixed with 5 µl DNA sample before being loaded in the gel, this was repeated for all the DNA samples, additionally 5 µl generuler was added in a lane in the gel. The gel was run at 80 V for 30 minutes and afterwards photographed using the AlphaImager to confirm the presence of DNA, indicating successful extraction.

The extracted DNA was amplified using PCR. PCR tubes were prepared with 48 µl mastermix and 2 µl DNA sample. The primers used were fD1* and rD1* and was a part of the mastermix (see appendix 12.3). After mixing the PCR was started the with the following program conditions: 94°C for 2 min., 30x (94°C for 30 sec., 59°C for 30 sec., 72°C for 90 sec), 72°C for 2 min., 4°C for hold. Following PCR another gel was prepared for evaluation of the PCR reaction. The PCR product was purified using GeneJet Gel Extraction and Clean Up Micro kit from Thermo scientific and the concentration of DNA was evaluated using the Nanodrop spectrophotometer. The DNA samples were then prepped for sequencing. 350 ng DNA was transferred to a PCR tube, 1 µl primer (rD1) and sigma water was added to a total volume of 7 µl. This was the concentration requested by the company doing performing the sequencing. The samples were then sent for sequencing at StarSEQ.

7.3 Results and Discussion

7.3.1 Purification

Sample	260/280	260/230	Ng/µl
A-1	1.80	2.30	393.0
A-2	1.84	1.84	84.6
E-1	1.87	2.16	359.8
E-2	1.89	2.08	200.8
F-1	1.89	2.19	427.9
F-2	1.69	1.84	349.0

Table 4: The purity levels of DNA and nucleic acid and the DNA concentration of the samples sent for sequencing

Table 5 shows the purity values of the DNA after purification and the concentrations of DNA in each sample. It is important that the values of the samples are within the recommended ranges, because it makes it more likely that sequencing is successful. 260/280 is a ratio that describes the purity of the DNA in the sample. The commonly accepted value for a sample to be regarded as pure is 1.8 (Thermo Fischer Scientific 2012). As seen in table 5 most of the samples contains pure DNA, the exception of this being F-2 which has a value that is slightly lower at 1.69, which could be indicative the sample being contaminated. Contaminants could be proteins or chemicals used during the extraction or purification process (NanoDrop Technologies 2007). 260/230 is a ratio that describes the purity of the nucleic acids. This is considered a secondary purity test and the accepted values for a pure sample are 1.8-2.2, lower values indicate contamination (Thermo Fischer Scientific 2012). As with the 260/280 measurements most of the samples are within these values, A-1 actually has a higher value, none of the samples have a score lower than required. The final value in the table is ng/µl which quite simply gives the concentration of DNA in the sample. The concentration of DNA in A-2 is a bit low but still usable, the rest of the samples had good high concentration.

7.3.2 Identification

Out of the six samples send for sequencing, five of the samples rendered usable sequence (see appendix 12.3). Sample F-1 had become too degraded to render any sequence. The five usable was run through a nucleotide BLAST (National Center for Biotechnology Information n.d.) for identifying the sequence, the results can be seen in table 6. All five of the sequences was identified to belong to the same species of bacteria, albeit different strains. The identified bacteria species was *Pediococcus pentosaceus*. The duplicates did not come up with the same strains, which is probably be due to the fact that the sequences are only partial and bacteria within the same species are bound to have similar sequences. Most of the samples had several hits in the database that had identical or similar scores to the ones seen in the table.

Sample	Strain	Identity
A-1	P. pentosaceus strain KID7	95%
A-2	P. pentosaceus strain KFF2	99%
E-1	P. pentosaceus strain FC6	96%
E-2	P. pentosaceus strain HS-2	89%
F-1	No sequence	No sequence
F-2	P. pentosaceus strain E24-168	95%

Table 5: The strains identified in the experiment

P. pentosaceus are gram-positive bacteria, which are facultative anaerobes (Tanasupawat 1993). *P. pentosaceus* is mesophilic and grows universally at 35°C. When exposed to higher temperatures the success of the growth depends on the specific strain. P. pentosaceus is capable of growing at pH 4.5 – 8 (Holzapfel & Wood 2014), though from previous experiments described in this report the pH from the samples containing these bacteria reached down 3.6. Lactic acid is the primary product obtained from a *P. pentosaceus* fermentation, however depending on the Carbon source the product can change, making the bacteria heterofermentative, consistent with the results obtained in the experiments. When glucose is fermented then lactic acid is always the product, however when xylose is fermented the product are acetate and lactic acid in equal molar amounts (Simpson & Taguchi 1995). This has not been observed in the experiments so far, however these samples also had access to glucose so it is possible that this behavior does fit. *P. pentosaceus* are not reported to produce gas when fermenting glucose (Tanasupawat 1993), however several of the samples from former experiments had overpressure at the end of the experiment which indicates gas production. It is of course possible that it is the consumption of other substrates that causes the gas production.

8 SUGAR ADDITION

8.1 PURPOSE & OVERVIEW

Brown juice does not contain a Carbon source which means that it is not possible for the bacteria to grow and produce lactic acid. Therefore, it is necessary to add a Carbon source to the brown juice before fermentation will occur. The bacteria have proven to be able to consume glucose and xylose in green juice, the precursor to brown juice. Hence these two sugars were the first ones to be tested out as Carbon sources added to the brown juice. The Carbon sources is added to separate vials and at different concentration to determine the most variable substrate concentration. Some of the samples from earlier experiments had overpressure so it was decided to measure gas production. The first set of vials were incubated for 5 days, however based on the results from that fermentation it was decided to continue

fermentation until it appeared that there was no more activity. The activity was determined by the gas production and the pH variation in the samples.

In the initial experiment performed the brown juice was autoclaved in accordance with the previous experiment, proved to lead to an unsuccessful fermentation. Upon investigating the reason for this lack of fermentation, it was discovered that autoclaving spoils the free amino acids and inactivates enzymes catalyzing the breakdown of polysaccharides. Furthermore products obtained from the Maillard-reaction increases in the brown juice (Thomsen et al. 2004), meaning a lessening of the substrates: sugars and free amino acids (Zumdahl & Zumdahl 2010) (Martins et al. 2001). While other sterilization methods were available, e.g. filtration, it was decided to do the fermentation under non sterile conditions.

8.2 Materials & Methods

8.2.1 Chemicals & Solutions

Red clover brown juice, NaOH (1,57 M), HCl (1 M), MilliQ water, EtOH (70%), $C_6H_{12}O_6$ solution (200 g/L), $C_5H_{10}O_5$ solution (200 g/L), H_2, N_2.

8.2.2 Equipment

Incubator Formascientific CO_2 water jacketed incubator, pH meter Infolab WTW series pH 720, Laminated flowbench Holm & Halby Holten LaminAir, SRI 310 gas chromatograph.

8.2.3 Material

Serum vials (117 ml), pipettes (1-5 ml, 30-300 µl, 50 ml), automatic pipette, pipette tips, rubber stoppers, cramps, needles, syringe, falcon tubes, Eppendorf tubes (1,5 ml).

8.2.4 Carbon sources

117 ml vials were prepared with brown juice in duplicates at pH 6,5 under non-sterile conditions. Half were prepared with glucose at 5 g/L, 10 g/L and 15 g/L respectively. The other half were prepared with xylose at the same concentration. 5% inoculum was added (*L. salivarius*, BJ E and BJ F respectively), making a total volume of 40 ml. The samples were incubated at 37°C and samples for HPLC and VFA was taken regularly and pH were measured at the same time intervals. After 48 hours Hydrogen was also measured. *L. salivarius* and the control samples were run for 5 days while the BJ E and BJ F samples were run for 19 days.

8.3 Results & Discussion

8.3.1 Carbon source addition

Due to the different fermentation time it is not possible to conclusively compare the results from all the bacteria. Furthermore, a preparation mistake appears to have been made for BJ E glucose samples, all of the samples had an initial glucose concentration of 5 g/L. The BJ E glucose samples (see appendix 12.4), seemed consume all glucose available to them with the exception of the 15 g/L sample. The lactic acid production was at around 10 g/L for BJ E glucose 10 g/L and BJ E 15 g/L, while BJ E 5 g/L had a final lactic acid concentration at approximately 5 g/L. the amount of produced lactic acid was almost nonexistent. For glucose 5 g/L the amount of consumed glucose and the amount of produced lactic acid appears to correspond with the theoretical yield possible. Also the final pH was higher than seen in the other glucose samples suggesting that the fermentation was not stopped due to product inhibition, as the other BJ E glucose samples ended up with a higher lactic acid concentration and a lower final pH. The other two samples had a much higher production of lactic acid then what should have been possible from the amount of glucose added. BJ E glucose 15 g/L actually had a slightly higher final glucose concentration than initial. This suggest that there was a release of soluble sugars, however the brown juice contains only very small amount of soluble sugars (0,31 g/L), which makes this explanation unlikely. Also the soluble sugars available should when broken down become xylose which is was not measured case. The acetate in the brown juice could work as a Carbon source but since there is very little change between initial and final acetate concentration that is unlikely. Since it is not possible for the bacteria to exceed the theoretical maximum yield, there must be some alternate Carbon source in the brown juice that is not accounted for. It could be that the protein left in the brown juice is degraded and utilized as a Carbon source.

The BJ E xylose samples showed (see appendix 12.4) more cohesive results, the produced amount of lactic acid and acetate seems correspond with the amount of xylose that is consumed in each sample. The xylose 15 g/L had a xylose consumption of 10 g/L, which suggest that somehow further fermentation was stopped. It was probably inhibition from the low pH, though BJ E has earlier continued to ferment at lower pH values. Xylose 5 g/L and xylose 10 g/L have both consumed all the available sugar. The final pH for xylose 5 g/L are higher than the final pH of the other BJ E xylose samples indicating that the fermentation could have continued if more Carbon was available, which is consistent with the consumption of the other samples. The Hydrogen production in all the BJ E was negligent (see appendix 12.4).

Due to the different behavior of the duplicate all the BJ F samples were analyzed. In figure 13 a very large difference between the two 5 g/L samples can be observed. The 5 g/L-1 sample consumed all the glucose (5 g/L) and produced approximately the same amount of lactic acid. In 5 g/L-2 sample consumed all the glucose and then also consumed all the lactic acid in the sample, while producing almost 35 g/L butyrate. The lactic acid bacteria used should not be capable of producing butyrate, even a heterofermentative bacteria should only be capable of producing lactic acid, acetate or ethanol. This can be explained by the fact that the brown juice has not been sterilized due to the fact that the only sterilization method available

(autoclaving) rendered the brown juice useless as a fermentation media (Thomsen et al. 2004). In a non-sterilized media, bacteria already exist in a mixed culture in the brown juice. As previously seen in the screening experiment a pure culture cannot necessarily compete if inoculated in a mixed culture. The results seen in 5 g/L-2 is the result of the mixed culture metabolism instead of the pure culture. For the mixed culture it would seem that the glucose is first converted to lactic acid and then lactic acid is converted to butyrate, probably to increase the pH in fermentation broth. This theory is evidenced by the change in pH over time in this sample (see appendix 12.4), in the first 48 hours the pH falls to 4.7 and then begins to increase again reaching 6.4 after eight days, before starting to fall again. The fall in pH suggest that either there is an intermediate compound between lactic acid and butyrate with an even higher pH or the butyrate is being consumed again. A measurement from day 13 shows a butyrate concentration at 39 g/L which suggest that it is the latter option. 5 g/L-1 sample is probably the result of the pure BJ F culture and it is likely that the results observed with BJ E is also solely the result of the pure culture rather than the mixed culture. It is however odd that the same pure culture sometimes outcompetes the mixed culture and sometimes not so another explanation that has merit is that the metabolism of the mixed culture in its first step matches the behavior of a pure culture, and then the fermentation for some reason stops before the other metabolic steps can be started. BJ E and BJ were both isolated from the mixed culture in the brown juice so it is actually a likely explanation.

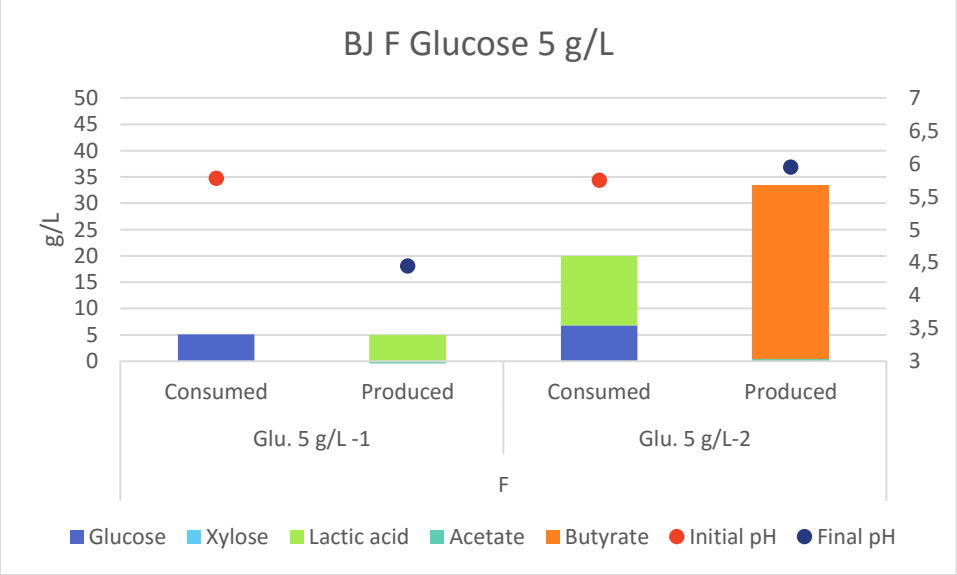

Figure 13: The consumption and production in BJ F glucose 5 g/L samples

In figure 14 the results from BJ F 10 g/L glucose can be observed. These results mimic the results seen in 5 g/L-2, except there is more glucose to consumed. Both samples have nearly identical behavior. 10 g/L glucose is consumed in both, corresponding with the amount added, approximately the same amount of lactic acid is also consumed in both samples. Both samples produce butyrate in large amounts both

reaching almost 40 g/L. There is a slight difference in the final pH values, but that probably does not really have any significant meaning.

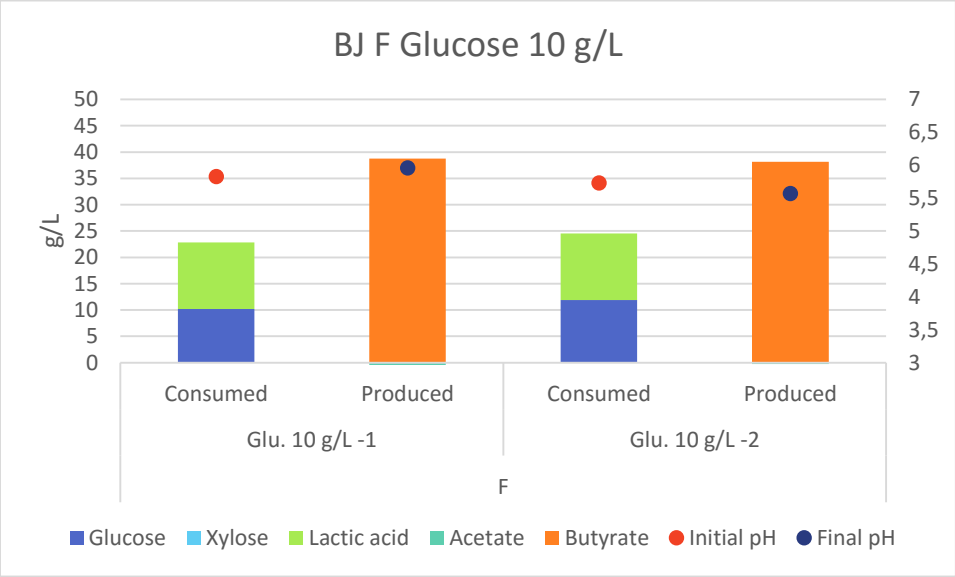

Figure 14: The production and consumption in BJ F glucose 10 g/L samples

In figure 15 the results from BJ F glucose 15 g/L can be observed and just like with glucose 5 g/L samples the two samples show different behavior for sample 15 g/L-1 around 10 g/L of the glucose is consumed and around 10 g/L of lactic acid is produced. This does indicate that it is the pure culture at work and not the mixed culture. However, a small amount butyrate is produced which means that it cannot be the pure culture. It is possible that the production of butyrate is simply delayed, and the slight production observed is simply the beginning of the production. Looking at the data it shows that there actually is a very slight consumption of lactic acid (around 2 g/L), which could explain the slight production of butyrate. The low pH further shows that there has been very little in terms of counter production to the lactic acid. 15 g/L-2 consumed all the glucose, 15 g/L, but only very little lactic acid. This does not quite fit with the amount of butyrate produced which is approximately 32 g/L. one explanation is that with way the graphs present the data the development in concentration over time is not represented. This means that anything that is produced and then consumed again does not show on the graphs. From the other results it can be seen that with sufficient glucose 10 g/L of lactic acid can be produced, brown juice also contains around 10 g/L lactic acid. This means that the total concentration of lactic acid in the samples when all the glucose is consumed is around 20 g/L. Due to the nature of the graphs and the available data the lactic acid will only show as a consumed product when the final concentration is lower than the initial concentration. Even then only the difference between the initial and the final concentration is shown. This means that while it might seem like only 2 g/L lactic acid has been consumed it could actually be much more, due to lactic acid production. The amount of lactic acid consumed and the final pH suggests that the butyrate

production was not finished when the experiment was ended, this is also supported by the higher butyrate concentrations obtained in some of the other samples.

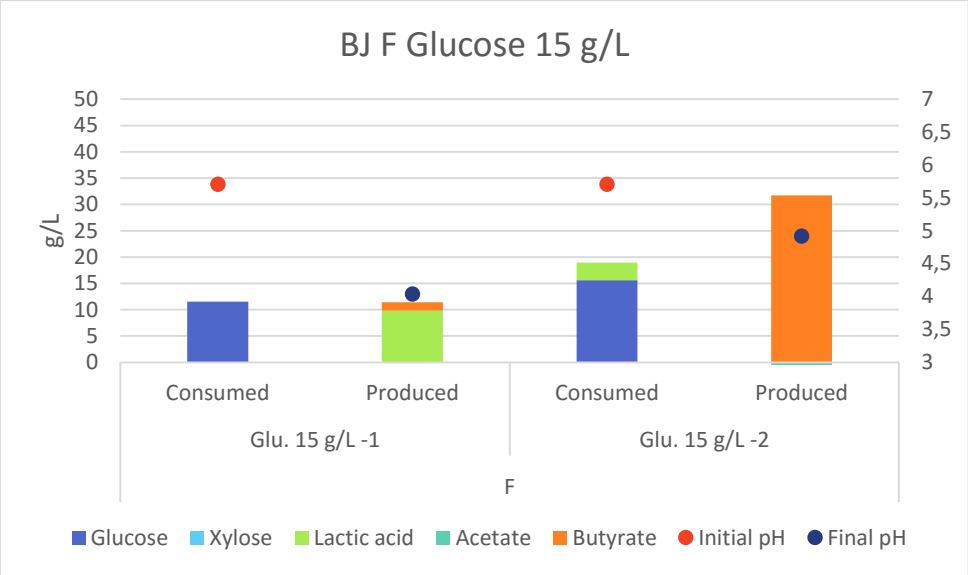

Figure 15: The production and consumption in BJ F glucose 15 g/L samples

In figure 16 the results from BJ F xylose con be observed. 5 g/L xylose have not been fully consumed, but there are still lactic acid and acetate production beyond what there should be with this amount consumed xylose, further supporting the theory that the brown juice contains an unknown substance that can function as a Carbon source. The 10 g/L xylose sample consumed the available xylose and produced a corresponding amount of lactic acid and acetate. A slight amount of butyrate is also seen suggesting that the butyrate production has begun. The 15 g/L xylose consumed the 15 g/L of xylose and final production was approximate 5 g/L lactic acid and 30 g/L butyric acid. The presence of lactic acid in the produced section suggests that the butyric acid production could have continued. It is slightly interesting that the final pH is similar for all the samples despite the difference in products, it indicates that not enough butyrate has been produce to provide a significant pH increase. The Hydrogen production seen in figure 17 was quite small and only four of the samples had any Hydrogen production of note and none of these samples exceeded 10 ml/gVS. Lactic acid bacteria should not be able to produce Hydrogen only CO_2 if they are heterofermentative so the Hydrogen production is likely the result of the mixed culture.

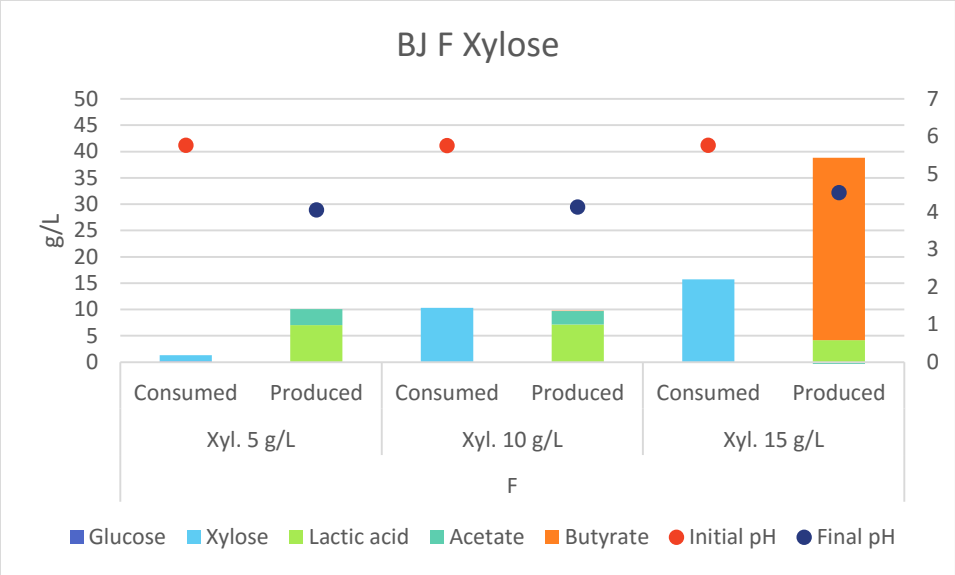

Figure 16: The production and consumption in BJ F xylose samples

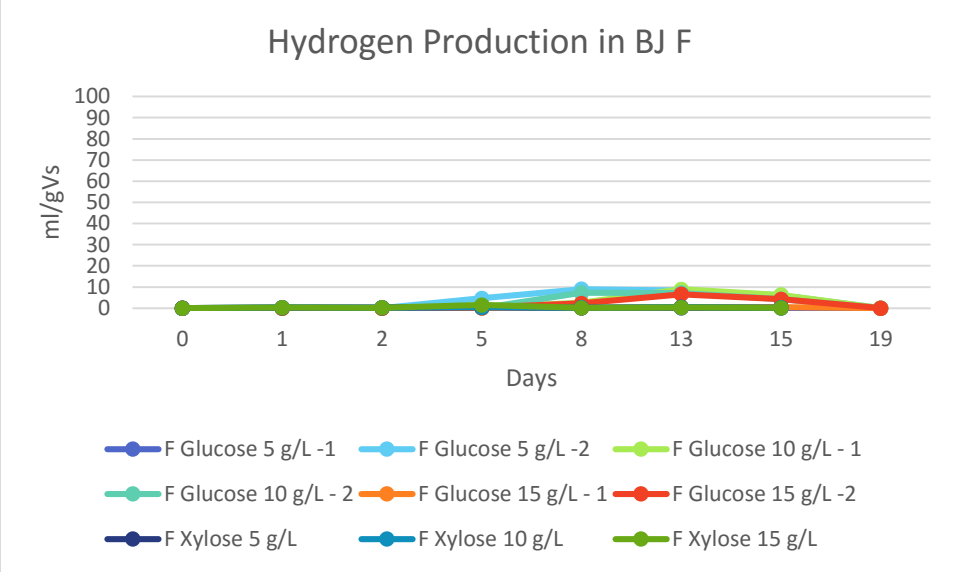

Figure 17: The Hydrogen production over time in the BJ F vials

In figure 18 and figure 19 the results from the samples inoculated with *L. salivarius* be observed. For all of the glucose samples it would seem that more glucose is consumed than added, this is due to an over addition of the initial glucose. All of the samples also consumes lactic acid, however the amount of butyrate produced is rather small compared to what is obtained from the BJ F experiment. The lack of butyrate production could be contributed to the fact that the fermentation only lasted 5 days. This does however suggest that there might be a conversion step between lactic acid being consumed the butyric

being produced. The consumption of lactic acid was lower in BJ F samples, but the butyrate production was much higher. L. salivarius had a large amount of Hydrogen production compared to what was seen in BJ F, so it could be that the lactic acid in the *L. salivarius* samples was converted to Hydrogen instead of butyric acid. It is also possible that the Hydrogen production is a metabolic conversion step, first lactic acid converts to Hydrogen and then the Hydrogen to butyrate. It is unlikely though since the BJ F samples, which had the highest amount of butyrate also produced very little Hydrogen. Since *L. salivarius* is a homolactic fermentative bacteria it is safe to conclude that the production of butyrate is to be contributed to a mixed culture. For the xylose samples the consumed xylose also seems to match the added amount of xylose. There was also some glucose consumed, suggesting that the xylose source has been contaminated with glucose. While it is possible that some glucose leftovers stem from either the inoculum or was already in the brown juice nothing like that showed up in the BJ E and BJ F samples meaning that contamination is more likely. The glucose can also be seen in the xylose control samples (see figure 19) further supporting the theory since the *L. salivarius* and the control samples were prepared together. The xylose samples also consumed some lactic acid though not as much as seen in the glucose samples. The three xylose samples produced approximately the same amount of butyrate and the production was similar to what was observed in glucose samples. The final pH for the glucose samples were rather high for the 5 g/L glucose sample and then declined through 10 g/L glucose and 15 g/L glucose. This suggest that there was some initial lactic acid production in some of the samples, which was then consumed again. The xylose samples followed the same pattern for final pH though the final PH observed in 5 g/L xylose was lower than observed in 5 g/L glucose. The Hydrogen production in the samples were quite varied, as can be seen in figure 20, from 10 ml/gVS to almost 80 ml/gVS. The xylose samples had a higher Hydrogen production than the glucose samples with the lowest, 5 g/L xylose, at 40 ml/gVS Hydrogen and the highest, 15 g/L xylose, at 75 ml/gVS. None of the samples have the Hydrogen plateauing so it is likely that if the fermentation had continued the Hydrogen production would also have continued.

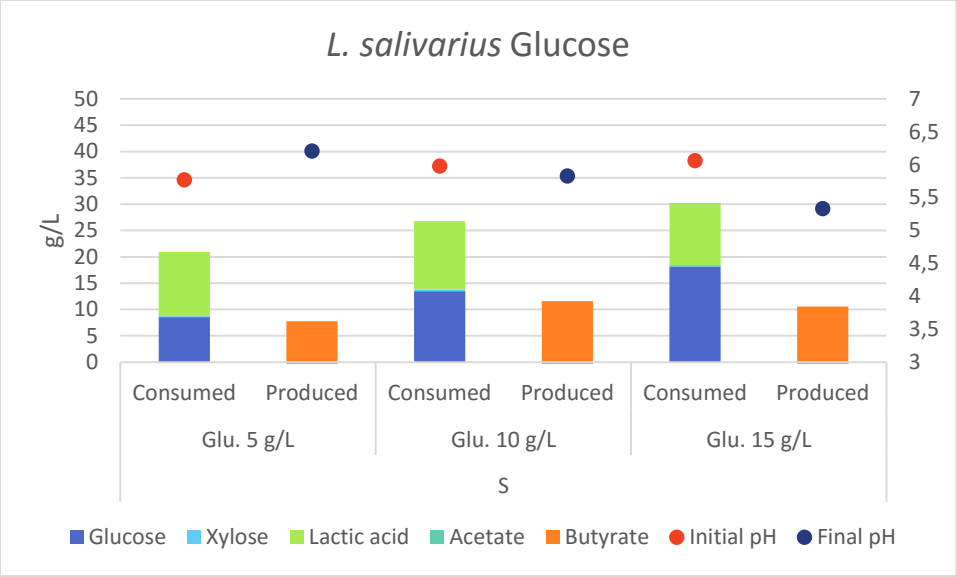

Figure 18:The production and consumption in L. salivarius glucose samples

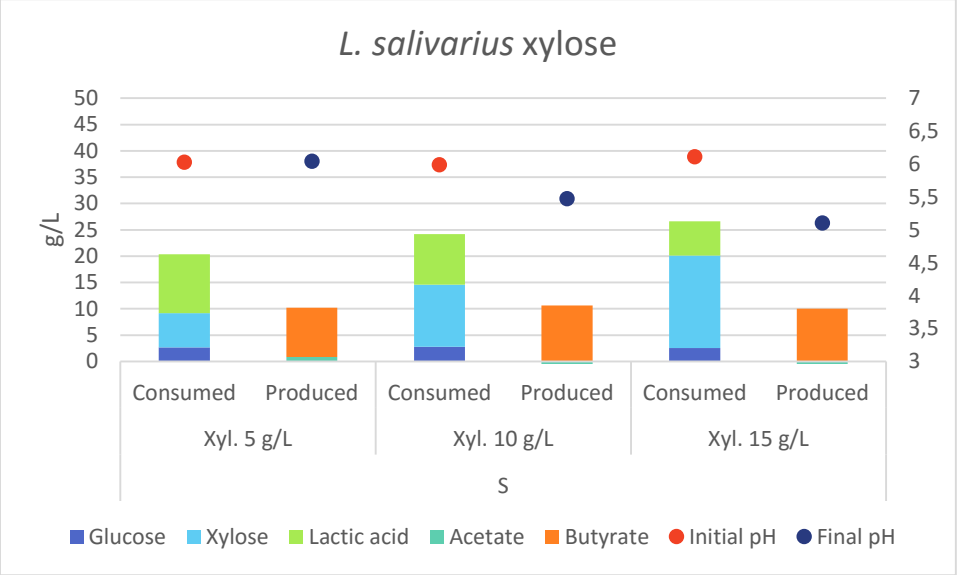

Figure 19:The production and consumption in L. salivarius xylose samples

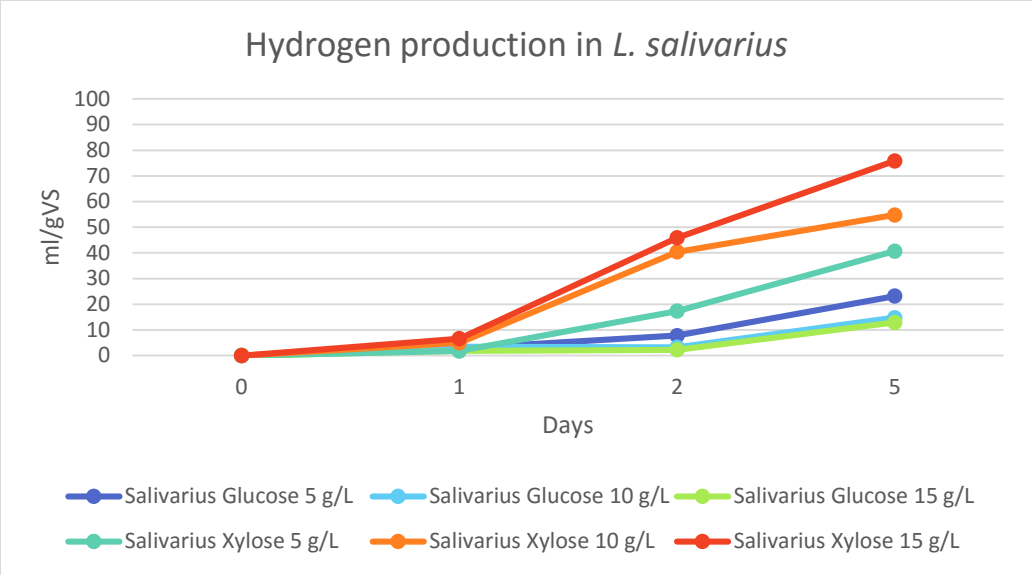

Figure 20: The Hydrogen production over time in the L. salivarius samples

The control samples results seen in figure 21 and figure 22 shows results very similar to the ones seen in *L. salivarius* further confirming that the mixed culture dominates the inoculated bacteria. The Hydrogen production in the control samples, figure 23, are similar to the Hydrogen production in *L. salivarius* 15 g/L xylose, 10 g/L glucose and 15 g/L glucose are almost identical to their counterparts inoculated with *L. salivarius.* The other xylose sample were slightly lower and the last glucose sample were slightly higher than seen in the *L. salivarius* samples. L. salivarius and the control samples both had a lot of Hydrogen production whereas the BJ F had very little Hydrogen production. BJ F on the other hand had a lot of butyrate production while there was very little Hydrogen production which does suggest that the Hydrogen production prevents butyrate production. However, the few samples from BJ F that had a little Hydrogen production were also the ones with high butyrate production. There are two other possibilities for the phenomenon, one is that butyrate production has an inhibitory effect on Hydrogen production. But this should mean that the samples with no Hydrogen should have a lot of butyrate, unless there is another factor involved. The other one is that butyrate can be consumed to produce Hydrogen. This option is a definite possibility, however there is no clear reason as to why only some of the would then consume the butyrate.

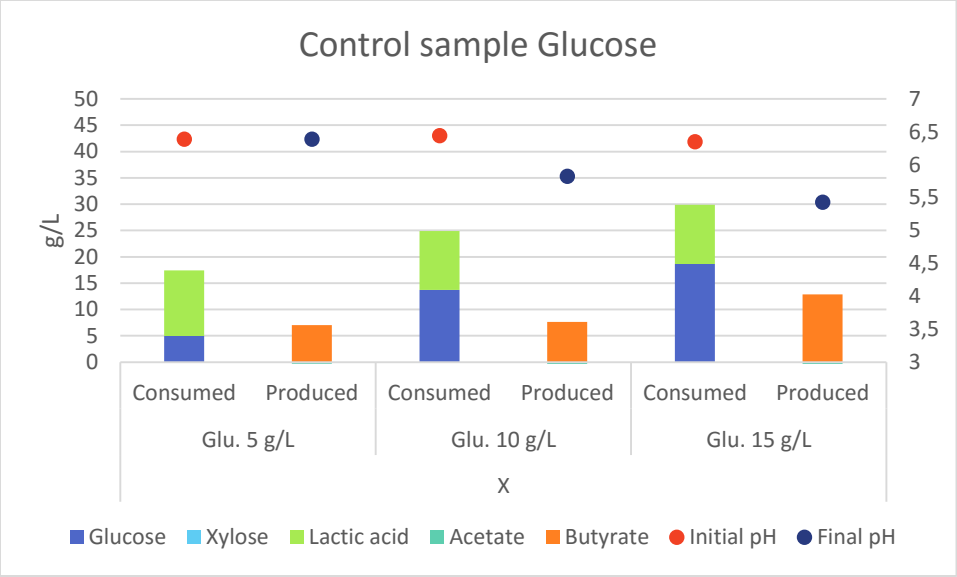

Figure 21:The production and consumption in glucose control samples

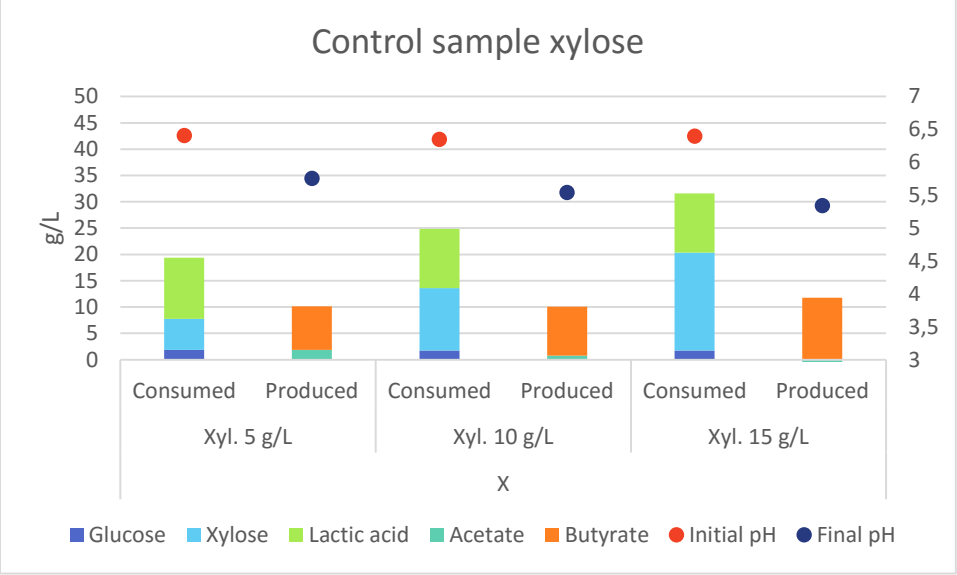

Figure 22: The production and consumption in xylose control samples

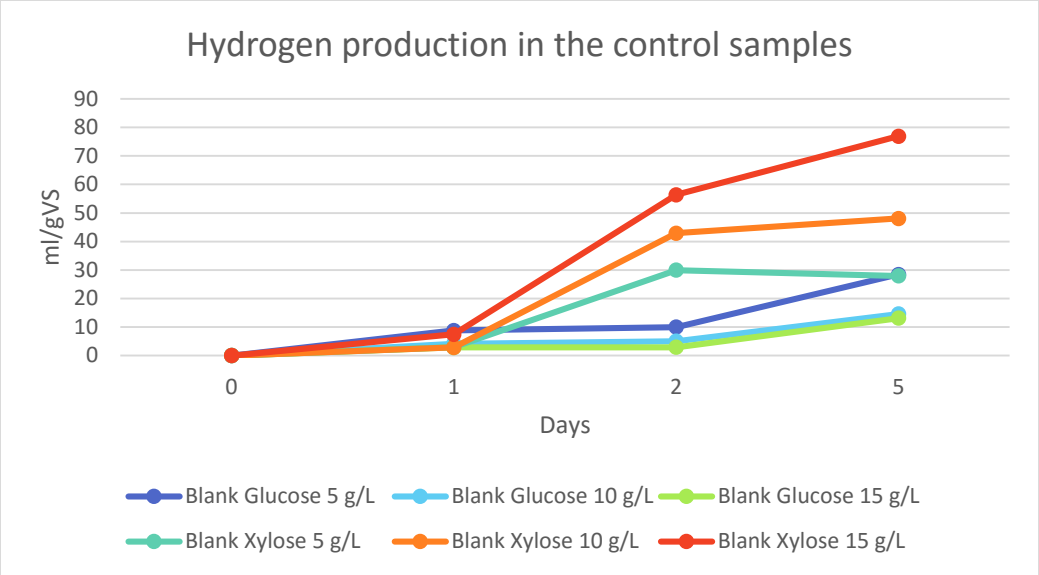

Figure 23: The Hydrogen production in the control samples

The fact that butyrate has been produced in much higher concentration than the lactic acid suggest that it would be more feasible to change the production objective from lactic acid to butyrate. So instead of optimizing for a lactic acid fermentation the process should be optimized for butyrate production. This change in process might also give better results considering that it appears to be the natural fermentation of the brown juice. The butyrate fermentation does however take quite a bit longer than the lactic acid fermentation so further experimentation is needed to determine the exact potential. Also if this production is to be continued it is important to determine the behavior of the mixed culture, because currently the behavior is all over the place. BJ E seems to have dominated over the mixed culture and thus resulted in very little in terms of products, or the fermentation stopped after initial lactic acid production for no clear reason. The BJ F have a similar problem in some of the samples while in others a lot of butyrate is produced. In the *L. salivarius* and the control samples a lot of Hydrogen is produced while the butyrate production is limited. While the different length of fermentation time probably does some difference there are just too much different microbial behavior for that alone to be a factor. Lactic acid could probably be produced without being consumed if the brown juice was sterilized so if lactic acid is to remain the intended product then alternative sterilization methods should probably be investigated. It should also be considered shifting to a continuous fermentation, since the lactic acid production has so far appeared to be inhibited.

9 PRESS CAKE FERMENTATION

9.1 PURPOSE & OVERVIEW

When adding a Carbon source to the brown juice it would be preferable if it was a biological waste product instead of an industrially made sugar, since the former would be the cheaper biomass. For this reason, the press cake was tested as a Carbon source for the experiment. The press cake is a waste product from the OrganoFinery, thus it would be advantageous if it could be repurposed as a feedstock for lactic acid production. Due to a sudden limitation in the brown juice available This experiment was performed in conjunction with another student, hence there had to be some compromises with the setup to satisfy the needs of both people involved. For this reason, the efficiency of different ratios between the brown juice and the biomass were tested and not all of the vials were pH adjusted, furthermore the pH adjusted vials did not have a control due to the lack of brown juice. Due to the time constriction, the results from the Carbon addition experiment (see chapter 8) had not been obtained not at the beginning of this experiment. This is part of the reason why the samples were inoculated despite the results from the former experiment showing the likelihood of the results coming from a mixed culture. The other reason is due to the need of the other student involved with the experiment. It is suspected that the press cake could contain fungal spores, so the samples are flushed to minimize the risk of fungal growth, in addition the other party involved requested anaerobic conditions as part the setup.

9.2 MATERIALS & METHODS

9.2.1 Chemicals & Solutions

Red clover brown juice, NaOH (2M), HCl (1M), MilliQ water, EtOH (70%), H_2SO_4 (2 M), H_2PO_4 (17 %).

9.2.2 Equipment

High Pressure Liquid Chromatograph Dionex Ultimate 3000, Centrifuge Hettich zentrifugation Mikro 200, Centrifuge Eppendorf MiniSpin plus, Incubator Formascientific CO_2 water jacketed incubator, pH meter Infolab WTW series pH 720, Gas chromatograph PerkinElmer precisely Clarus 400 Gas Chromatograph, SRI 310 gas chromatograph, 0.0001 g balance.

9.2.3 Material

Glass bottles (500 ml), pipettes (30-300 µl, 50 ml), automatic pipette, pipette tips, rubber stoppers, cramps, needles, syringe, 0,45 µm syringe filters, falcon tubes, pH strips, Eppendorf tubes (1,5 ml), chromatograph vials, 200 µl insertion tubes, pH strips.

9.2.4 Press cake experiment:

10 g of press cake was weighted into each 500 ml bottle. Brown juice was added so that the concentration in press cake was 5% in 6 vials, 10% in 6 vials and 15% in 6 vials, (200 ml, 100 ml and 67 ml brown juice respectively). The vials were divided into 3 sets of 6, each set containing 2 vials of each ratio. For one set the pH was adjusted to 6.5 with 2 M NaOH and inoculated with *L. salivarius.* Another set was only inoculated *L. salivarius* and the final set was neither inoculated not pH adjusted. The inoculum was 2 % of total volume. The bottles were all flushed with N_2 for creating anaerobic conditions. All vials were incubated at 37°C for 21 days. A 1.5 ml sample were taken for HPLC and VFA was taken, Hydrogen and pH were measured at 1, 2, 3, 4, 8, 11, 14 and 21 days. HPLC and VFA were prepared with slight differences from the procedure described in chapter 4. 450 µl sample was mixed with 50 µl acid (H_2SO_4 (2 M) for HPLC and H_2PO_4 (17 %) for VFA). The samples were centrifuged at 10000 rpm for 10 minutes and filtered through a 0.45 µm syringe filter into the 200 µl insertion tube. The insertion tube was inserted in the chromatograph vials, closed and send for analysis.

9.3 Results and Discussion

Figure 24 shows the change in concentration during the fermentation for the pH adjusted 5% sample. The graph shows that no glucose is released during the fermentation or at least it is in very small amounts no xylose either. The same can be observed with the pH adjusted 10% and pH adjusted 15% samples in figure 25 and figure 26 respectively. This most likely means that the bacteria in the brown juice are unable to break down the press cake for Carbon. This means that lactic acid consumption begins almost immediately which can be observe in all of the figures and butyric acid is also produced almost as soon as lactic acid starts being consumed. The pH 6.5 5% reaches the highest butyrate concentration after 11 days at around 35 g/L. After that the concentration falls again as butyrate is consumed. There is no clear tendency towards what is produced from the butyrate which means that it is entirely possible that the butyrate is consumed simply for cell maintenance. From the results it also appears that butyrate is continued to be produced after the lactic acid is consumed albeit at a much slower rate. The lactic acid appears to be fully consumed after three days, however butyrate is as previously mentioned being produced until day 11. Besides the butyrate production there is some minor production of other VFAs, acetate reaches around 10 g/L at the end of the fermentation while some iso-valerate also seems to be produced and then consumed again, at the highest it reaches around 5 g/L. While it is not exactly a large amount compared to butyrate it is noteworthy due to the fact that earlier experiments had no production of any other VFA other than butyrate and acetate.

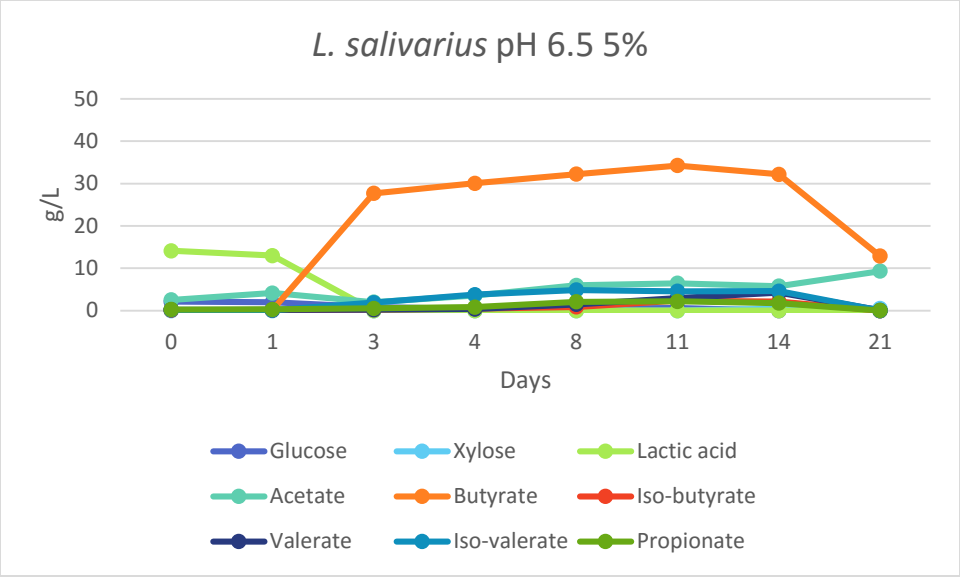

Figure 24: The change in compound concentration over time for L. salivarius at pH 6.5 with 5% press cake

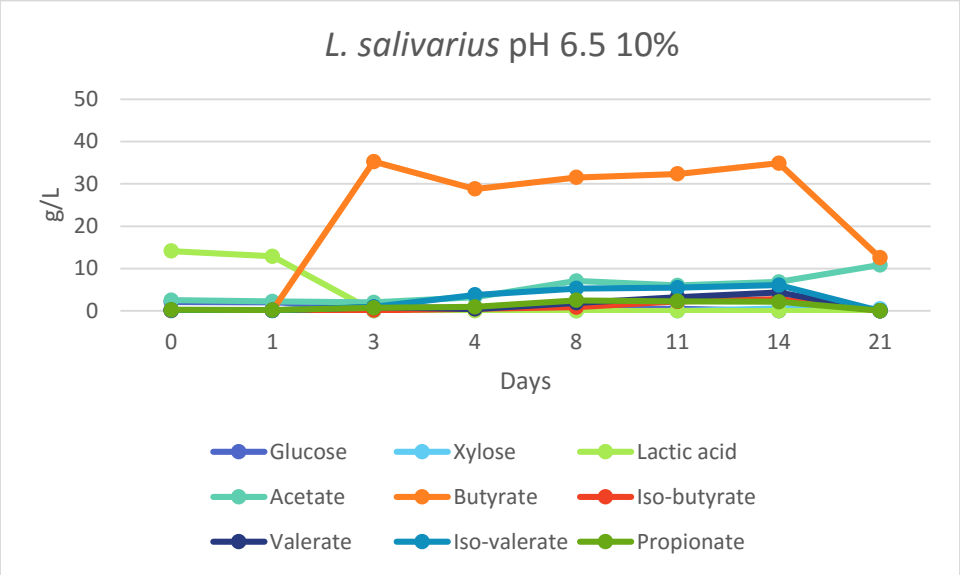

Figure 25: The change in compound concentration over time for L. salivarius at pH 6.5 with 10% press cake

The samples with *L. salivarius* pH 6.5 10%, figure 25, had a pattern quite similar to the what was seen in figure 24. Lactic acid was consumed after three days and the butyrate was produced between day one and day three. The highest butyrate concentration was obtained after 3 days at 35 g/L. Following that there was a slight consumption, before the butyrate increased again at and was back at approximately 35 g/L after 14 days. Following that the butyrate starts being consumed again and in large amounts. The acetate had a slow but steady ascent to 10 g/L after 21 days iso-valerate production reached its peak at 6 g/L after 14 days. There is also some increased valerate production only slightly less than the iso-valerate.

In figure 26 the results of *L. salivarius* pH 6.5 15% can be observed. These results have some slight differences from the other results, they do however also share several similarities. Consumption of lactic acid was much slower in this sample and the lactic acid was not fully consumed until day 8. Consequently, the butyrate production was also much slower than in the other two samples. A slight butyrate production began after 3-4 days, however it was between day 4 and day 8 that the production significantly increased. The highest butyrate concentration was reached after 14 days and was 45 g/L, the highest reached in any experiment performed in this report. It seems unlikely that the increased butyrate in this sample had anything to do with the ratio of brown juice and press cake. Since there appeared to be no release of free sugars then it is unlikely that the press cake functions as a substrate. It appears as though the lactic acid is the only compound that is consumed and all the sample shave lactic acid in equal amounts. A preparation mistake for all the 15% sample was made and these sample received approximately three times the amount of inoculum as the other samples (6% instead of 2%). This should however had led to a faster consumption, not a slower one. In addition, the results of the fermentations are not the results of a *L. salivarius* metabolism, which means it was probably a mixed culture.

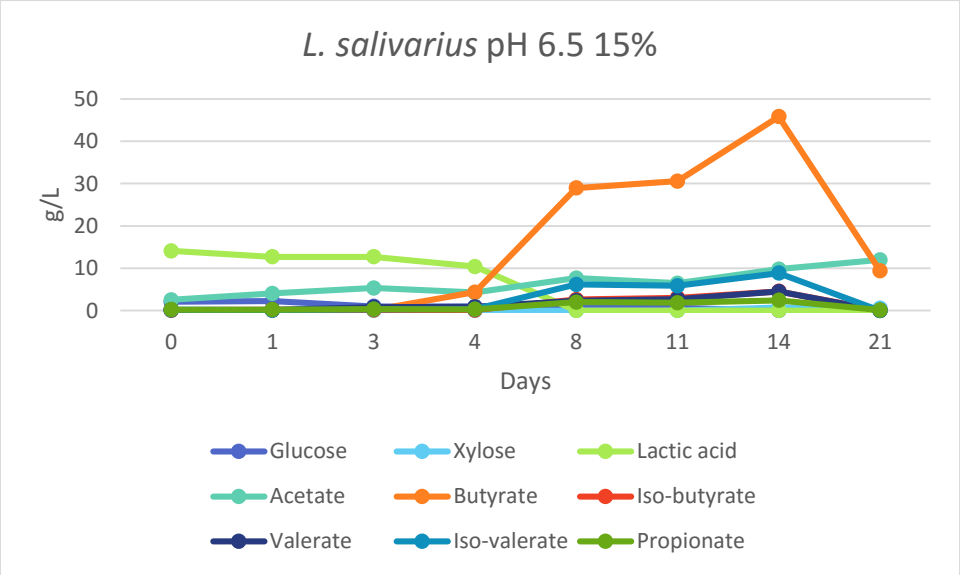

Figure 26: The change in compound concentration over time for L. salivarius at pH 6.5 with 15% press cake

In figure 27 and figure 28 results can be observed for the 10% press cake hydrolysis and the 10% press cake with bacteria addition (see appendix 12.5 for the 5% and 15% samples). The results observed shows almost identical behavior for the samples. After 11 days of no activity the lactic acid is completely consumed by day 14, however there is no indication as to what is produced from the lactic acid in either sample. The *L. salivarius* 15% press cake does start a butyrate production between day 14 and day 21, the

concentration is just above 10 g/L. There is also a slight increase in acetate in both samples, but it is only at approximately 5 g/L. The lack of activity clearly indicates that lower pH functions as an inhibitor on the bacteria in the green juice. That the lactate finally does begin to be consumed suggests that the inhibition mostly affects the bacteria by prolonging the lag phase before fermentation can occur. This would however mean that some kind of production should happen, and with one exception it does not. That could of course simply mean that the production is also delayed by the inhibition. If the lactic acid was simply consumed for maintaining cell growth in harsh conditions, then the consumption should have been more gradual. When the lactic acid is consumed there should also have been an increase in the pH (see figure 29), for some of the samples that is true (*L. salivarius* 5% and L. *salivarius* 10% raises to approximately 5.7 and 5.3 respectively), while the pH of the other samples remains consistent throughout the fermentation at approximately 4.3. The lactic acid concentration in the brown juice should have been the reason for the low pH and its consumption should have raised the pH.

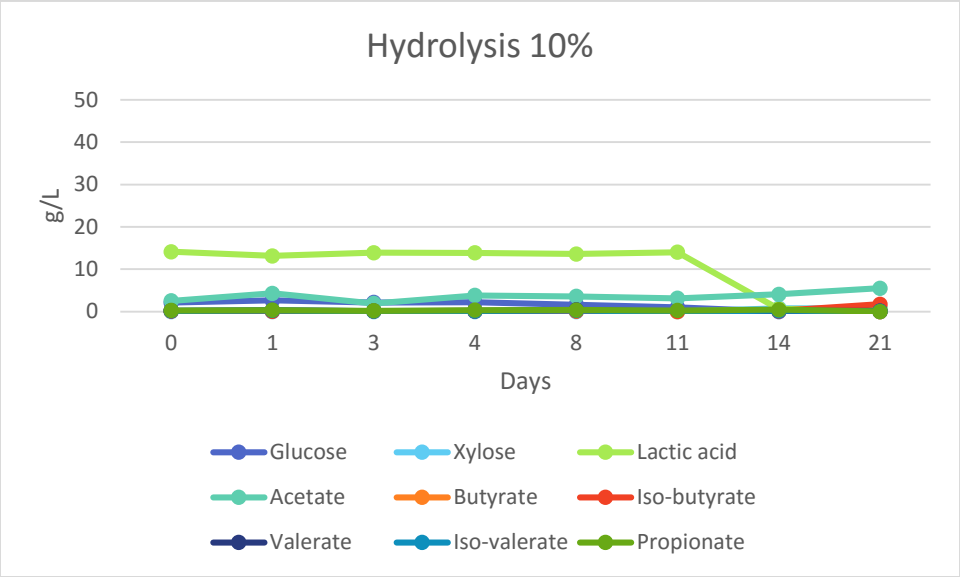

Figure 27: The change in compound concentration for the 10% press cake sample with no bacterial addition

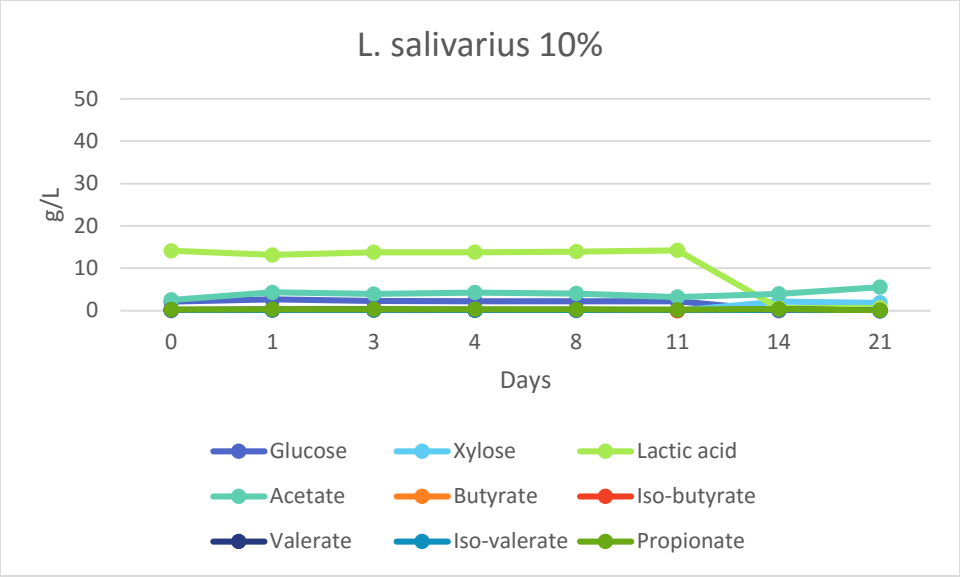

Figure 28: The change in compound concentration during the fermentation for the samples with 10% press cake and a bacterial inoculation

From figure 29 the change in pH of the samples during the fermentations can be observed. For the pH adjusted samples 5% and 10% the pH increased as the lactic acid was consumed and the butyrate production has begun. Following that the pH steadily decreases independently of the butyrate production and any other determined compound until it reaches the starting value of the unadjusted pH vials. The 15 % pH adjusted vial has very little pH change in pH during the fermentation during the fermentation which is rather odd considering the massive production of butyrate and the lactic acid consumption. It could be that since the consumption and production happens simultaneously there is no significant change in pH. The lack of change in the 15 % pH adjusted sample and several of the other samples could possibly be contributed the buffering capacity of the brown juice. The initial pH was not measured so the first pH values in the graph is after 24 hours of fermentation. The vials with pH adjusted to 6.5 all had a value at around 5,5 instead, meaning that either initial fermentation in the first 24 hours have caused a large drop in pH or the inoculum has lowered the pH as was seen in chapter 6. The initial pH observed in chapter 7 is around 6 for pretty much all the samples, despite them being adjusted to 6.5. It means that the initial pH observed in the pH adjusted samples of this experiment was likely a combination of both the previously mentioned factors. The pH of brown juice when not adjusted is 4.2 (see chapter 4), which is consistent with what is observed for the unadjusted samples.

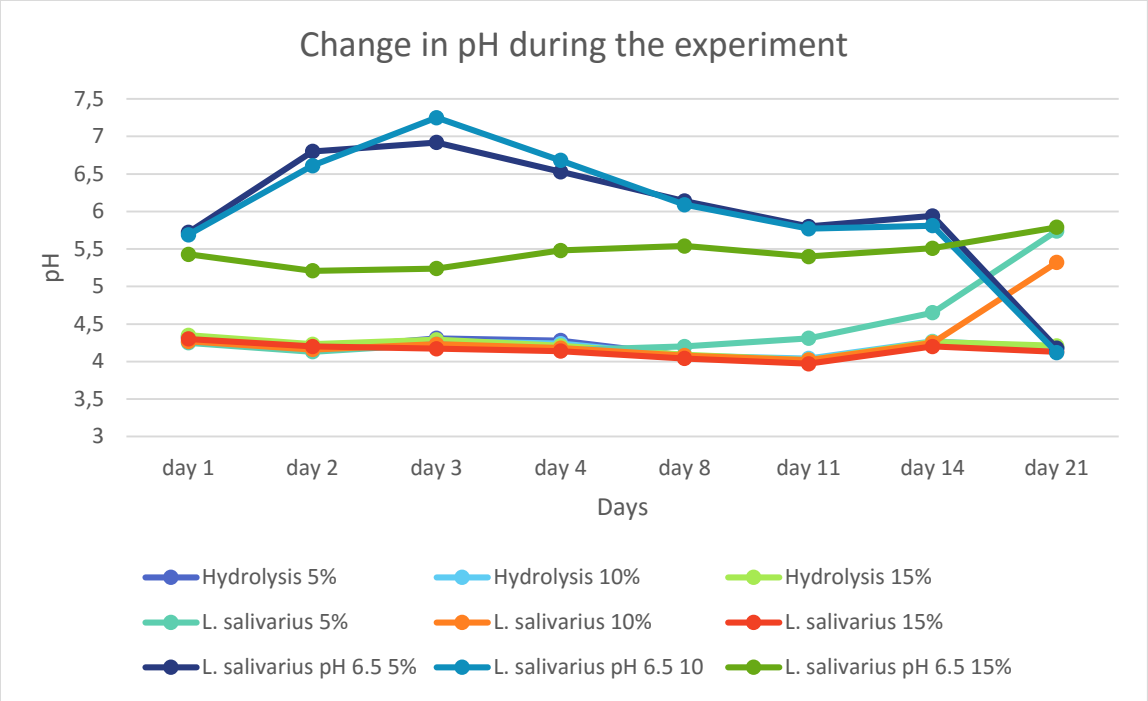

Figure 29: The change in pH in the samples during the fermentation time

Figure 30 shows the Hydrogen production for all of the samples. Only three of the samples had any significant Hydrogen production, these were the pH adjusted samples. *L. salivarius* pH 6.5 5% obtained the highest gas production after 3 days at 40 ml/gVS. The Hydrogen seems to follow a pattern of being produced, then consumed and then produced again throughout the fermentation. There is no clear pattern connecting the Hydrogen production to either the lactic acid consumption nor to the production and consumption of butyrate. There is also no connecting pattern to be spotted between the Hydrogen production and the change in pH. However, since the inoculated bacteria are not the bacteria responsible for this fermentation, but rather the mixed culture already in the brown juice, it is possible that the production of Hydrogen is occurring independently of the butyrate production. *L. salivarius* 6.5 5% showed methane production at the end of the experiment, suggesting that the results obtained is part of methane production metabolism. Part of the methane production consists of the formation of VFAs and

Hydrogen production (Al Seadi et al. 2008). This does however not explain the Hydrogen loss occurring during the fermentation.

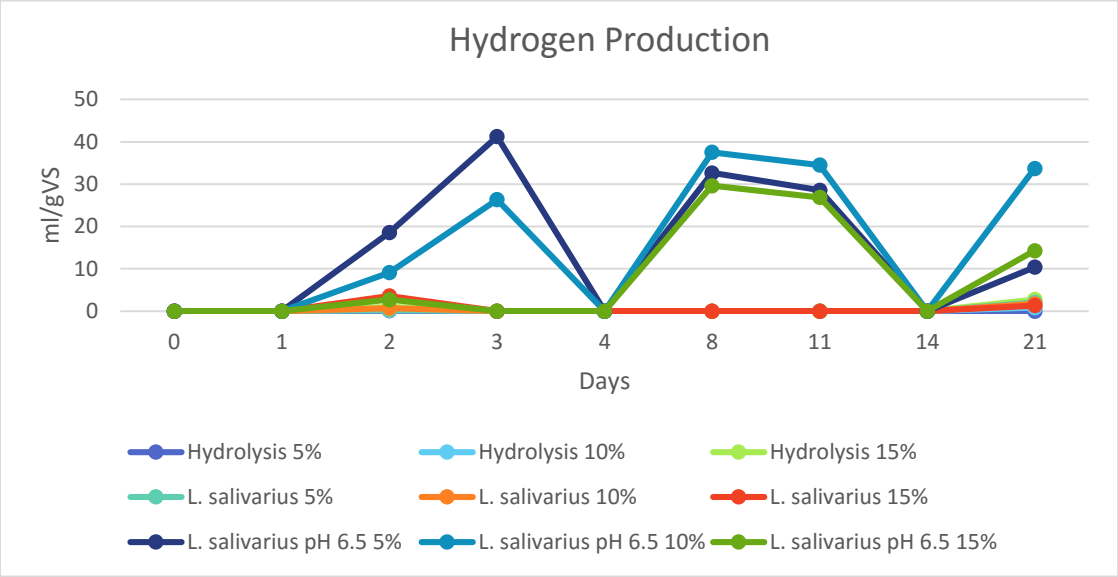

Figure 30: The Hydrogen production in the samples using press cake as a Carbon source

It is obvious that rather than having potential for lactic acid production the brown juice has a good talent for lactic acid production. The butyrate production might increase if more Carbon source was made available to the bacteria. While the press cake did not appear to be degraded by the bacteria in the brown juice it is possible that an enzymatic hydrolysis prior to fermentation could lead to a release of free sugars. The fermentation time should probably be shortened since all of the butyrate producing samples had a significant decrease in butyrate between day 14 and day 21.

10 Conclusion and Future Prospects

In this thesis the potential and optimal conditions for lactic acid in brown juice was determined and evaluated. From both green juice and brown juice several bacteria were isolated and screened for lactic acid production in green juice. *L. salivarius*, which was used as a positive control proved to be the best lactic acid producer when compared to the isolated bacteria. Little difference was seen with the aerobic and anaerobic conditions leading to the working conditions being aerobic.

Increasing the pH of the green juice proved gave varied results depending on which bacteria was used, but the best lactic acid producer, *L. salivarius*, preferred the higher pH range with the highest concentration of lactic acid being obtained at pH 6.5. The lactic acid concentration obtained at this pH was 21.5 g/L.

The bacteria isolated from the brown juice and used for determination of condition were identified. All of the bacteria were all as different strains of *P. pentosaceus*.

When switching the fermentation media from green juice to brown juice it was determined that sterile conditions could not be obtained by autoclaving without destroying the brown juice as a fermentation media. This led to the fermentation being performed under nonsterile conditions. Glucose and xylose were used as Carbon source added in the brown juice. While the bacteria in the brown juice proved to be fully capable of consuming the added sugar, lactic acid was not produced in amounts similar to what was produced in green juice. In fact the lactic acid was often actually consumed, making the brown juice unsuitable for lactic acid production. While brown juice proved to be unsuitable for lactic acid production, there appeared to be a great for butyrate production in the brown juice. Almost 40 g/L of butyrate was produced in the brown juice. Under the nonsterile conditions the inoculated cultures overall did not appear to be able to compete with the mixed culture in the brown juice. For the future it would be beneficial to conclusively determine whether the inoculations of a pure culture do any difference for the butyrate production. It might also be worth examining if lactic acid production could be performed in brown juice if another sterilization method was found and the fermentation was switched to continuous.

Press cake was utilized also utilized as a Carbon source but with no obvious success, no free sugars seemed to be released from the press cake during the fermentation. Lactic acid was however still consumed and butyrate was still produced. While the ratio between press cake and brown juice did not appear to do any difference for the production, the pH proved to be crucial. The samples with no pH adjustment produced absolutely nothing while the pH adjusted samples had a butyrate concentration of 34-45 g/L. For future prospect there are several options. The optimal conditions for butyrate production should be determined and the most profitable fermentation time should also be determined. It would also be good to repeat the experiment comparing it with a control sample on equal conditions. It should also be determined if the addition of Carbon source could lead to further butyrate production and if switching fermentation mode could be effective. On a different note it could also prove worthwhile to determine the press cakes potential as a biomass if it is properly pretreated, for instance with an enzyme hydrolysis.

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12 APPENDIX:

12.1 CHAPTER 5

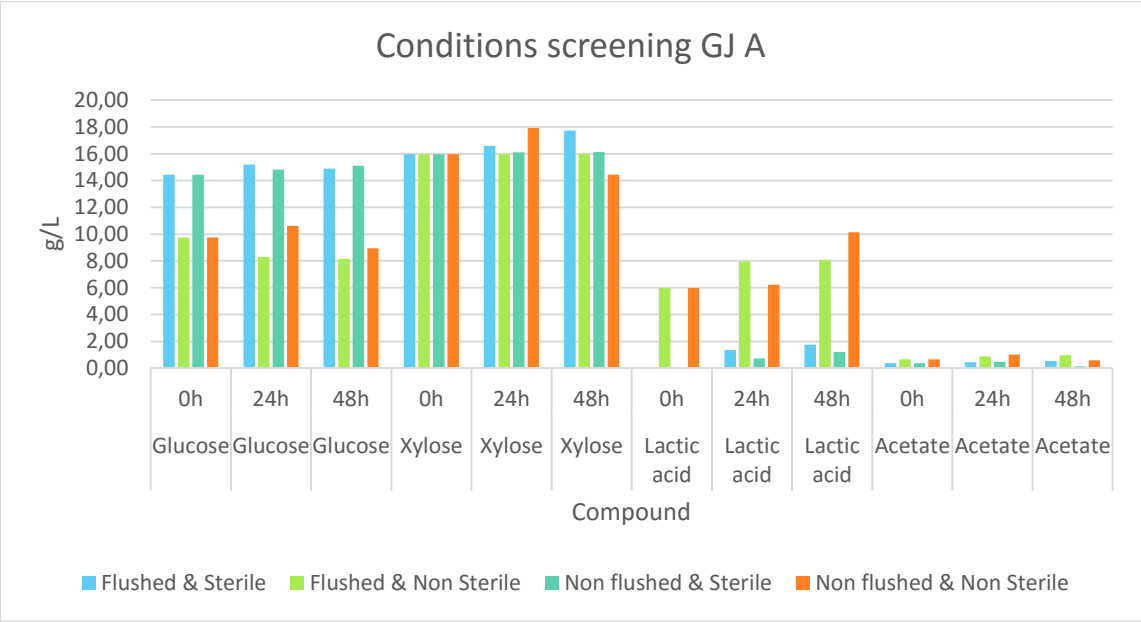

Figure 31: The consumption and production in GJ A under aerobic, anaerobic, sterile and nonsterile conditions

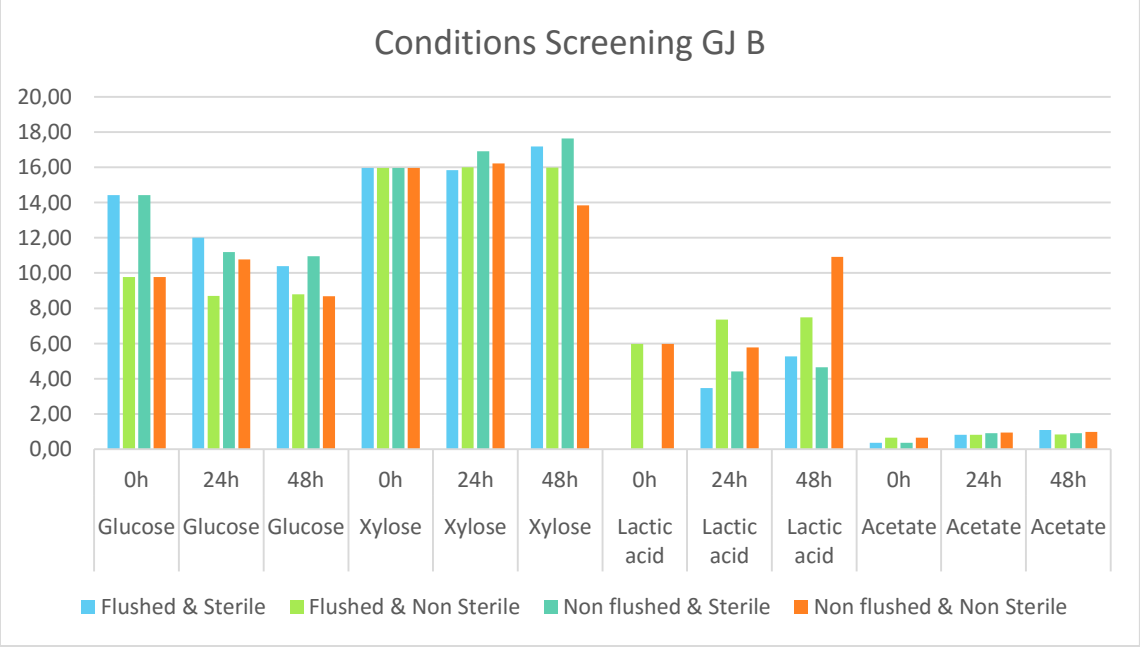

Figure 32: The consumption and production in GJ B under aerobic, anaerobic, sterile and nonsterile conditions

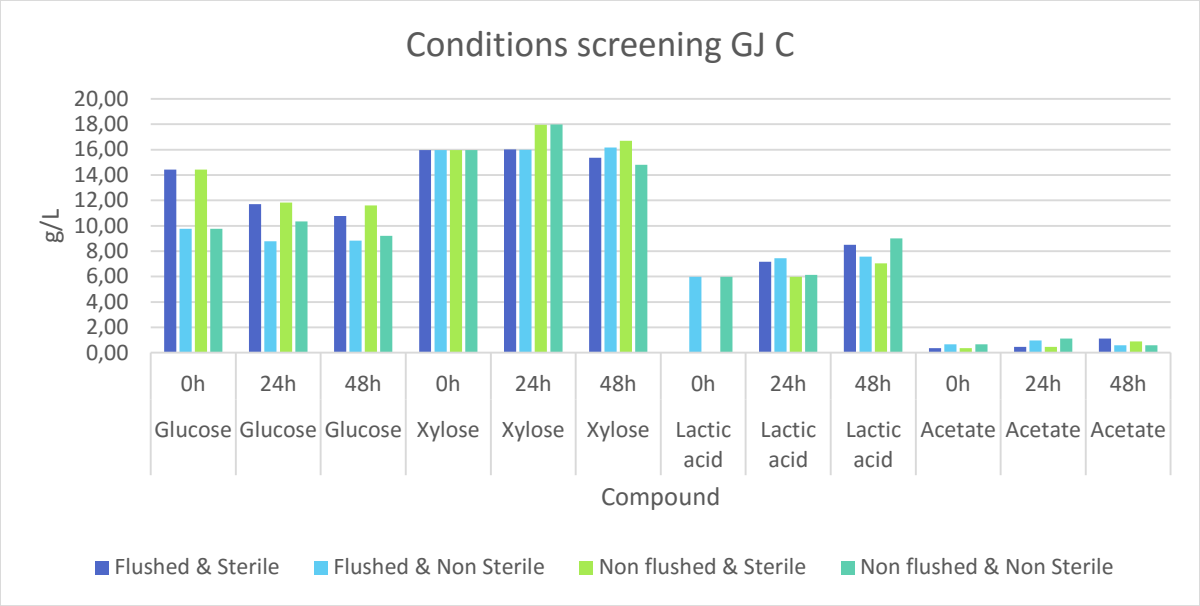

Figure 33: The consumption and production in GJ C under aerobic, anaerobic, sterile and nonsterile conditions

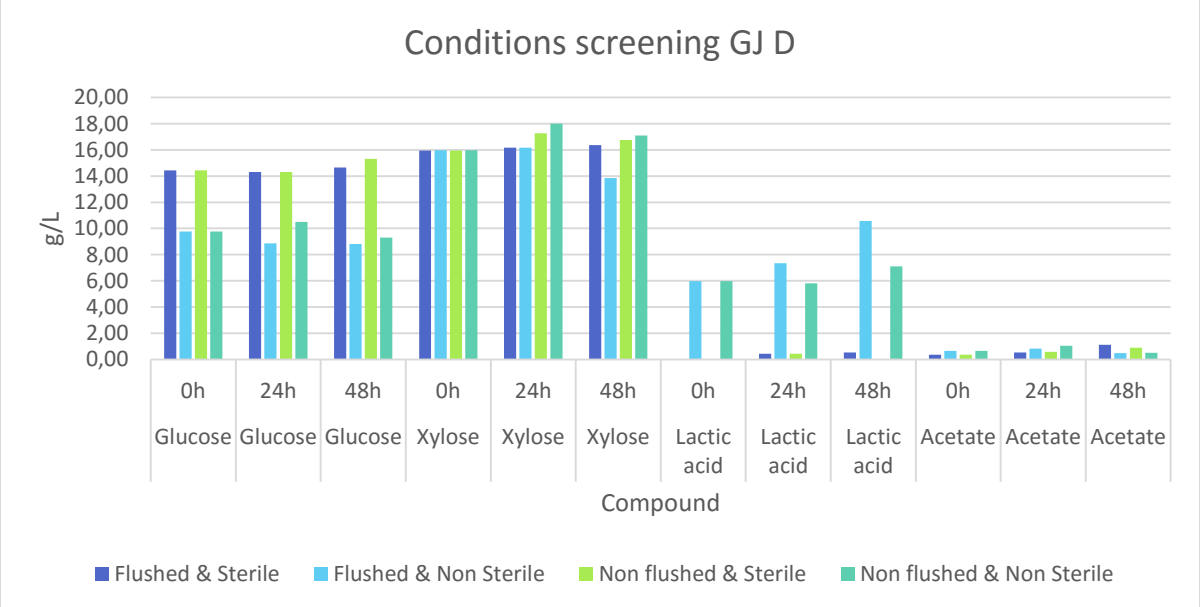

Figure 34: The consumption and production in GJ D under aerobic, anaerobic, sterile and nonsterile conditions

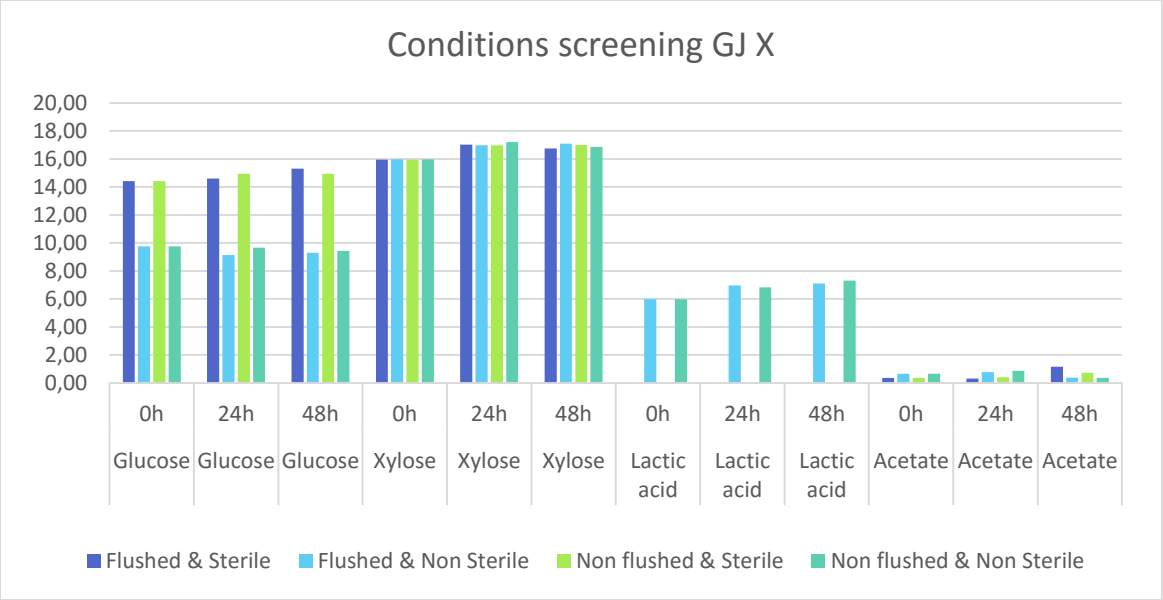

Figure 35: The consumption and production in the control sample under aerobic, anaerobic, sterile and nonsterile conditions

12.2 CHAPTER 6

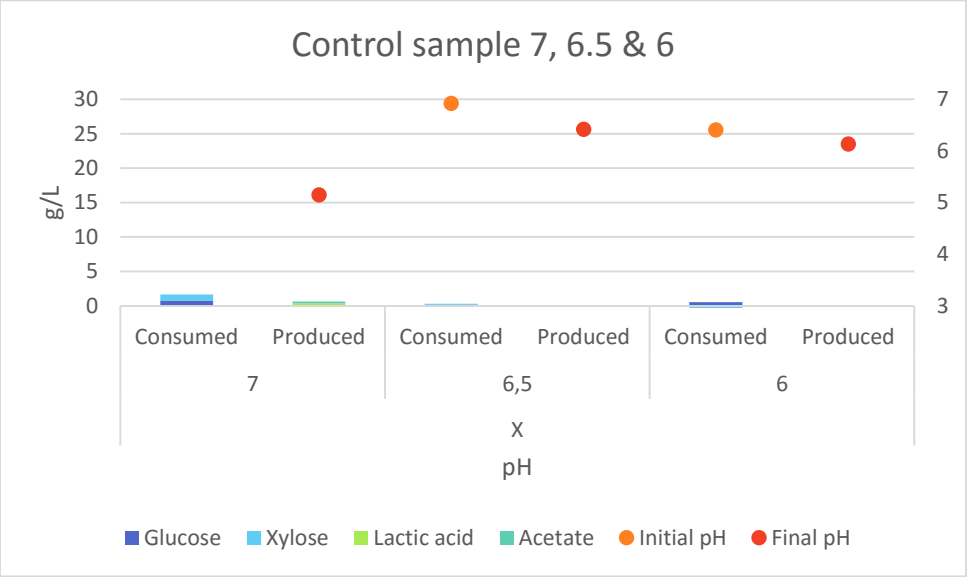

Figure 36: The consumption and production in the control samples with pH 7. 6.5 and 6

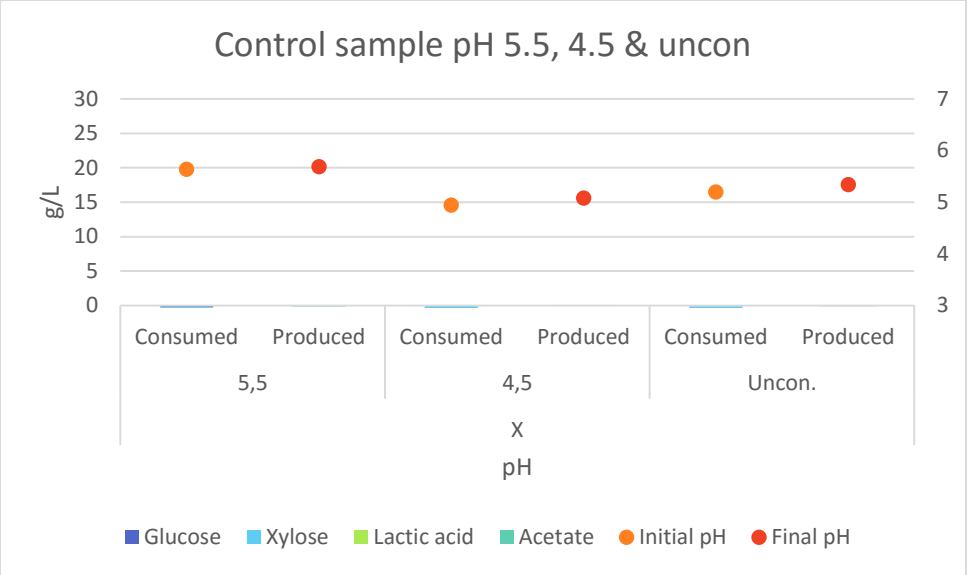

Figure 37: The consumption and production in the control samples with pH 7. 6.5 and 6

12.3 CHAPTER 7

12.3.1 Mastermix recipe per 50 µl:

33 µl Sigma H_2O

5 µl 10 x Buffer

1 µl dNTP Mix (10 mM of each)

4 µl fD1* (10 µM)

4 µl rD1* (10 µM)

1 µl RUN polymerase

12.3.2 Sequences

A-1:

GTGGCATGCACGTTGTTACGACTTCACCCTAATCATCTGTCCCACCTTAGACGGCTAGCTCCTAAAAGGTTACCCCA
CCGGCTTTGGGTGTTACAAACTCTCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGC
ATGCTGATCCGCGATTACTAGCGATTCCGACTTCGTGTAGGCGAGTTGCAGCCTACAGTCCGAACTGAGAATGGTT
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A-2:

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E-1:

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CATGCTGATCCGCGATTACTAGCGATTCCGACTTCGTGTAGGCGAGTTGCAGCCTACAGTCCCAACTGAAAATGGT
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E-2:

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F-1:

NNNNN

F-2:

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12.4 CHAPTER 8

12.4.1 Graphs

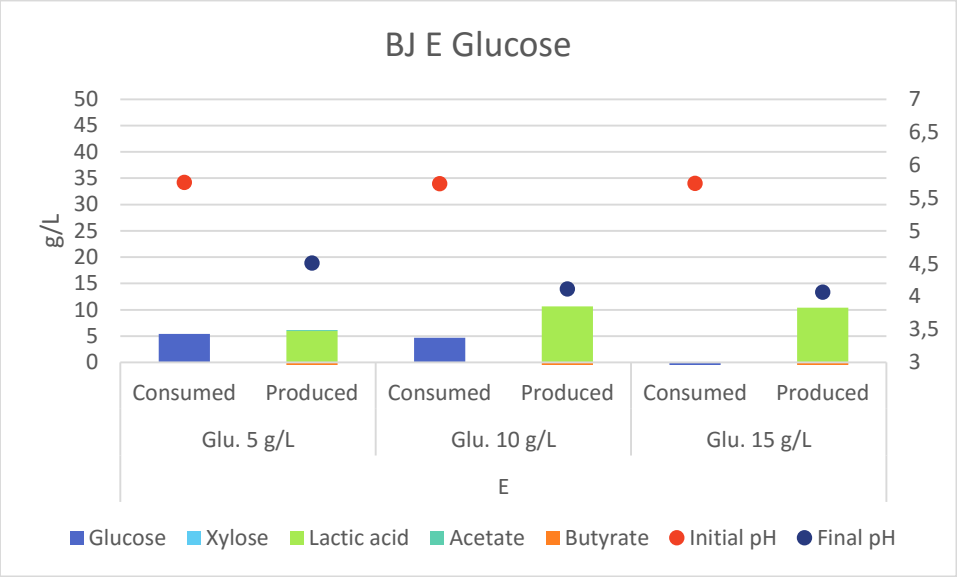

Figure 38: Consumption and production in the BJ E glucose samples

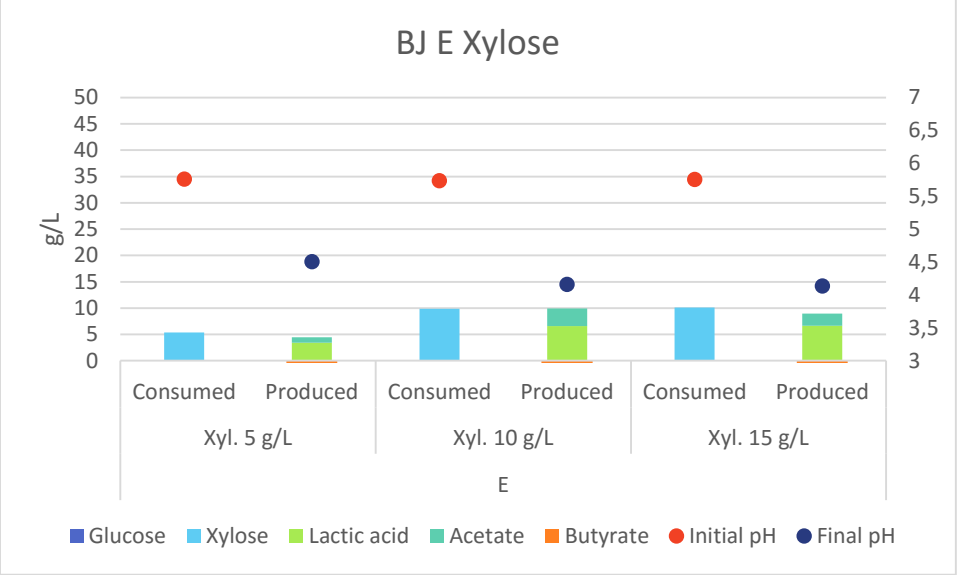

Figure 39: The consumption and production in the BJ E xylose samples

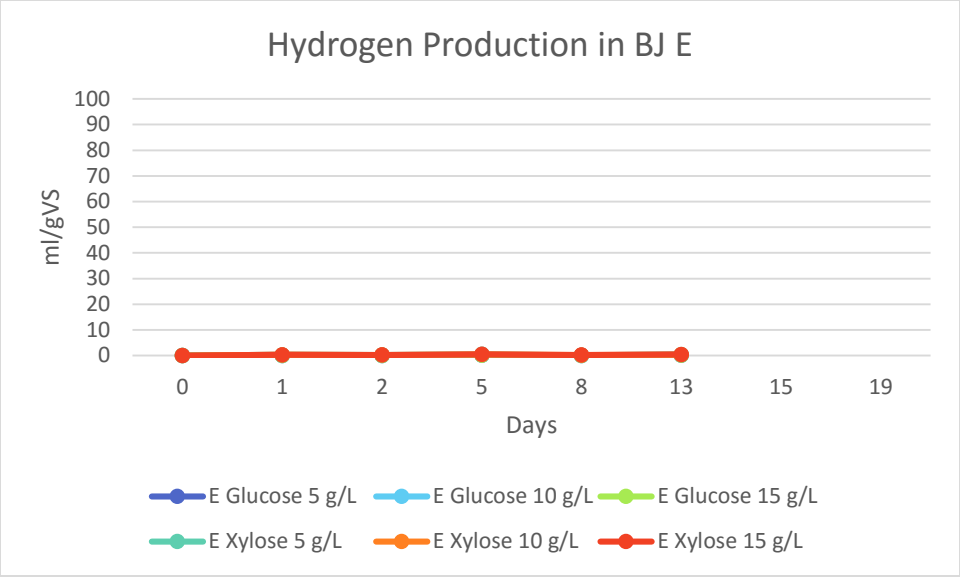

Figure 40: The Hydrogen production in BJ E

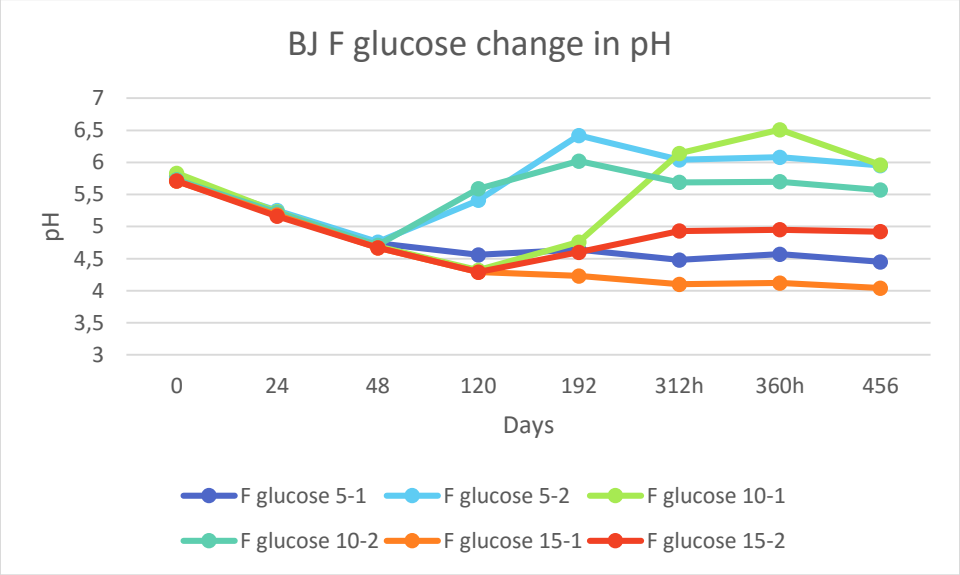

Figure 41: The change in pH over time for the BJ F samples

12.5 CHAPTER 9

12.5.1 Graphs

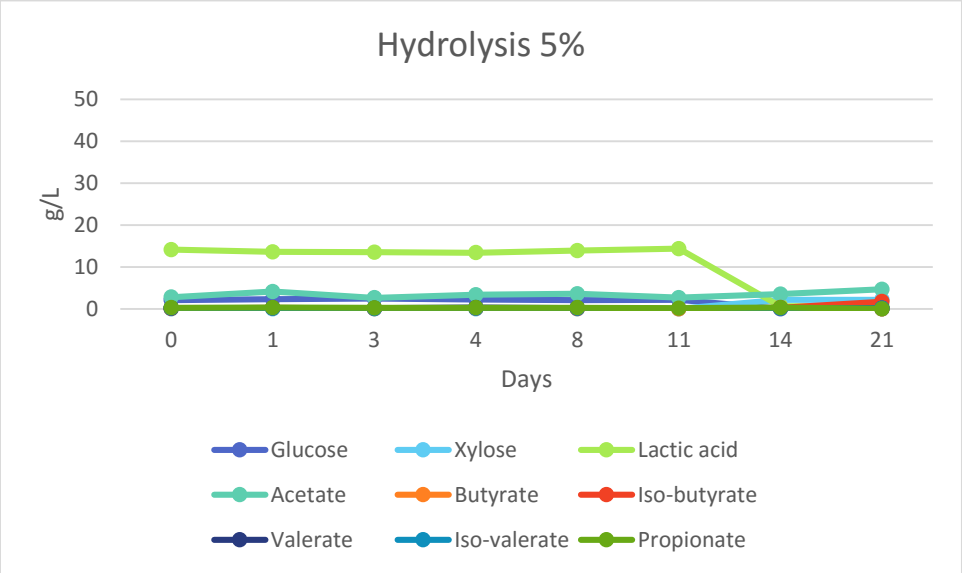

Figure 42: The consumption and production in the 5% press cake with no bacterial addition

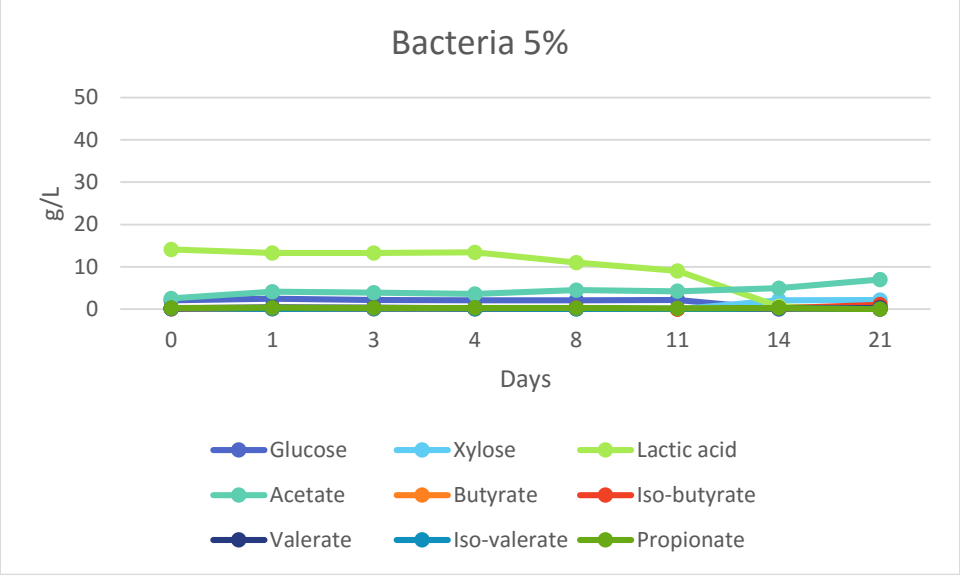

Figure 43:The consumption and production in the 5% press cake with bacterial addition

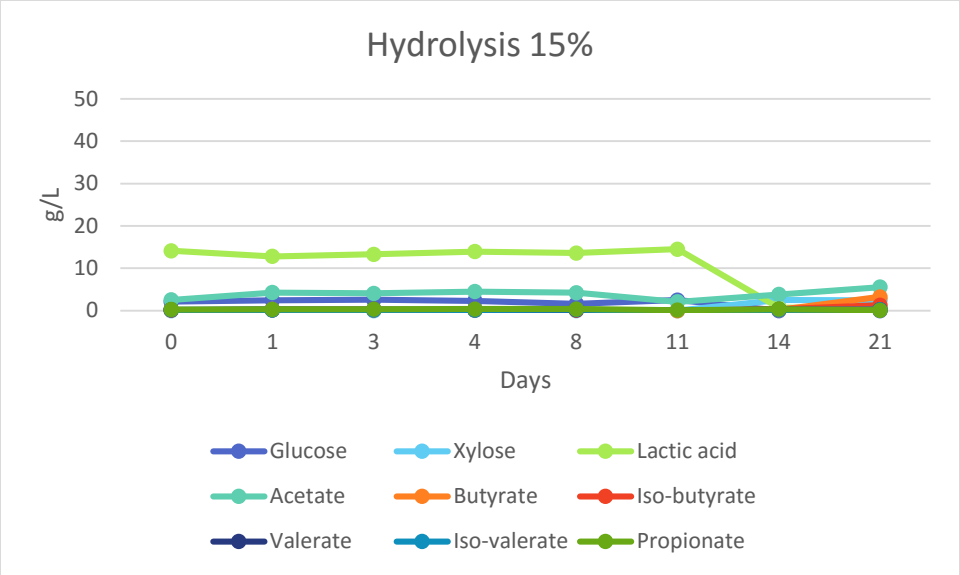

Figure 44:The consumption and production in the 15% press cake with bacterial addition

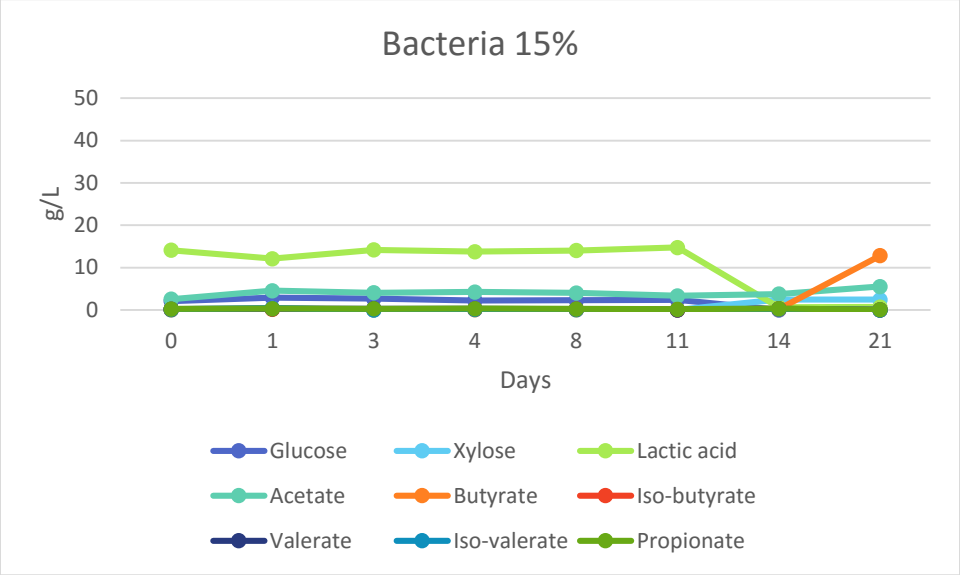

Figure 45:The consumption and production in the 15% press cake with no bacterial addition