APPLICATION OF APPLE POMACE FOR FUNGAL CULTIVATION

Högskoleingenjörsutbildning i kemi
Inriktning Bioteknik

Jonatan Viitala
Bill Floberg Karlsson

Program: Kemiingenjör - tillämpad bioteknik
Svensk titel: Användning av Äpplepressmassa för svampodling
Engelsk titel: Application of Apple pomace for Fungal Cultivation

HÖGSKOLAN I BORÅS

Utgivningsår: 2019
Författare: Jonatan Viitala och Bill Floberg Karlsson
Handledare: Akram Zamani
Examinator: Jorge Ferreira
Nyckelord: Äppelpressmassa, svamp, Apple pomace, filamentous fungi, *Rhizomucor* biomass, ethanol

---

SAMMANFATTNING

Äppelpressmassa är en solid biprodukt, producerad genom att pressa och trycka miliontals ton äpple i bland annat juiceindustrier. Den kvarstående massan motsvarar 25-30 % av äpplet och består utav skal, frön och fruktkött. Denna råvara har många tillämpningar då den har ett högt kolhydrat- och vätskeinnehåll. Detta examensarbete utvärderade användningen av äppelpressmassa från Herrljunga cider för att odla en filamentös svamp i syfte att producera biomassa och etanol. Massan blev utsatt för olika förbehandlingar för att extrahera så mycket socker som möjligt. Olika satser gjordes genom att blanda äppelpressmassa med vatten i olika förhållanden (g äppelpressmassa per g vatten) och olika vattentemperaturer. Äppeljuice producerades genom att filtrera blöt massa med ett fint tyg. Suspensioner gjordes genom att tillsätta vatten till massan och inte blanda det (icke-homogent). En annan variant gjordes genom att blanda äppelpressmassa och vatten med en mixer (homogent). Några utav äppeljuicesatserna pH justerades till 5.5 för att se hur det påverkade svamptillväxten. Satserna flyttades till Erlenmeyer flaskor, steriliserades och ympades med den filamentösa svampen *Rhizomucor*. Erlenmeyer flaskorna flyttades till ett skakvattenbad för att jäsa i 72 timmar. Prover togs var 24:e timma för att se socker- och etanolkoncentrationerna ändras. Detta analyserades med hjälp utav en HPLC. Resultaten visade att suspensionssatserna inte presterade bra jämfört med äppeljuicesatserna. Detta misstänks bero på att innehållet var väldigt visköst och hade lågt syreinnehåll för svampen att kunna växa. Att justera pH till 5.5 för äppeljuicesatserna visade sig inte vara bra, de presterade sämre jämfört med justerade satser. Förbehandlingen med en äppelpressmassa till vatten förhållandet på 1 g äppelpressmassa/1 g kallt vatten producerade mest biomassa med ett utbyte av 9.7 g torr biomassa per kg torr äppelpressmassa. För etanolproduktion hade förbehandlingsmetoden med en äppelpressmassa till vatten förhållandet på 1 g äppelpressmassa/1 g varmt vatten högst utbyte med 11.2 g etanol per kg torr äppelpressmassa.

ABSTRACT

Apple pomace is a solid by-product acquired from pressing and crushing millions of tons of apple in juice-industries. It represents 25-30 % of the original fruit and consists of peels, seeds and pulp. This raw material has multiple applications due to its high carbohydrate and moisture content. This bachelor thesis evaluated the use apple pomace acquired from Herrljunga cider for the cultivation of a filamentous fungus to produce biomass and ethanol. Different pretreatment strategies were applied to the apple pomace to extract as much sugars as possible. Several batches were made by mixing pomace and distilled water at different ratios (g pomace per g water) and different water temperatures. Apple juice was produced by filtering soaked pomace using a fine fabric. Apple pomace suspensions were made by adding pomace and water without mixing it (non-homogenised) and homogenised suspensions by mixing with a kitchen blender. Some apple juice batches were pH adjusted to 5.5 to investigate the effect on the fungal growth. The batches were put in Erlenmeyer flasks, sterilised and inoculated with the fungal strain *Rhizomucor* that has been isolated from Indonesian leaves used for tempe preparation. The Erlenmeyer flasks were incubated in a water shake for 72 h. Samples were taken every 24 h to follow sugar and ethanol concentrations. The samples were analysed by HPLC (High-Performance Liquid Chromatography). The results showed that apple pomace suspension did not perform well compared to the apple juice since the suspension was too viscous and lacked oxygen for the fungus to grow properly in the solution. The apple juice did show a significant improvement compared to the suspension, however pH adjustment to 5.5 had a negative impact on the fungal growth. Cold pre-treatment with an apple pomace to water ratio of 1 g pomace /g water produced the most biomass, with a yield of 9.7 g biomass per kg dry apple pomace. For ethanol production, an apple pomace to water ratio of 1 g pomace /g water using hot water had the highest yield of 11.2 g ethanol per kg dry apple pomace.

TABLE OF CONTENTS

SAMMANFATTNING 2

ABSTRACT 3

1. INTRODUCTION 1

1.1 Background 2

1.2 Purpose 4

2. MATERIAL AND METHOD 5

Material: 5

1. Preparation of apple pomace for fermentation 5

1.1. Extraction of water-soluble fractions and their application for fungal fermentation 5

1.2. Preparation of apple pomace water mixture and their application for fungal fermentation 6

1.3. Adjusting pH of water soluble fractions and their use for fungal fermentation 6

2. Preparation of agar plates 7

3. Inoculation of fungi, fermentation, and sampling. 7

3.1. Harvesting of Biomass 8

4. Analysis of ethanol and sugar concentration 8

5. Determining ethanol and biomass yield. 9

3. RESULTS 9

3.1. Biomass yield from water soluble fractions 9

3.2. Biomass yield from water soluble fractions with adjusted pH 10

3.3. Biomass concentration from water insoluble fractions 11

3.4. Ethanol yield and sugar consumption from water soluble fractions 11

4. DISCUSSION 15

4.1 Future work 16

5. CONCLUSIONS 17

6. REFERENCES 1

1. INTRODUCTION

Apple pomace is a solid by-product that is produced in quantities in millions of tonnes, commonly produced from juice industries acquired by crushing and pressing apples, representing 25% to 30 % of the fruit. It mostly consists of peels, seeds and pulp. It has a high moisture content of around 70-75 % and a high biochemical oxygen demand and chemical oxygen demand [1]. The country that produce the most apples is China and stands for more than half of world production. Other large producers are the USA, Turkey and Poland [2]. In 2014, the total apple production of the world was 84,000,000 tons and 25-30 % is estimated to have been processed for mostly juice production. At the same year, the European Union harvested 13,000,000 tons of apples and around 1,000,000 tons of apple pomace was produced per year [3]. In Sweden, around 20,000 tons of apples are produced every year (2010) [4].

Raw apple pomace has limitations, it uses up large amounts of storage in apple processing plants due to its high water content and is susceptible to spoilage and microbial growth. Because of this, apple pomace is often regarded as waste and is disposed of in landfills, but it is also used as animal feed despite its low nutritional value [5]. Apple pomace in landfills can be a problem due to its acidic nature and prevents nearby seeds to germinate. Ruminant animals that eat apple pomace might be poisoned by alcohol that is produced by fermented apple pomace in their rumen [2].

A solution could be to dry and mill the apple pomace to reduce the density and become more economically sustainable in both storage and transportation [5]. Apple pomace used to be dried out in the sun, however, the apple pomace will be darkened due to enzymatic or oxidative browning and will lose its value as human food fortification. Therefore it is important to dry apple pomace enough to still be a useful resource; however, this could have a varying cost depending on how much energy is spent and the end-use [1].

In the industry, apple pomace is used to extract pectin with mineral based acids such as H_2SO_4, HNO_3 and HCl. A common yield ranges between 10-15 % dry weight pectin of dry weight apple pomace. The pomace that has been subjected to pectin extraction often leaves an acidic waste and could be a potential pollutant. Jing Luo, et al. (2019) considered the use of AA (acetic acid) to extract pectin in industrial use as an alternative to mineral acids. A stainless-steel system was used to adjust different variables such as AA concentration, temperature and time.

The highest yield of pectin, 19.6 % dry weight pectin compared to the dry weight of apple pomace was achieved with an AA concentration of 10 % (w/w), 100 °C for 110 minutes [6].

Apple pomace could be used as a substrate to produce useful resources through fermentation due to its high soluble sugar content (glucose, fructose and sucrose). It consists of sugar polymers such as cellulose and hemicellulose. It also contains lignin which is a polyaromatic compound that covers the sugar polymers. A study by Gabriel S. L. et al. (2013), presented an analysis of typical sugar fractions, varying depending on apple cultivars, such as 24-31 % glucose, 67-73 % fructose and roughly 3 % sucrose [7]. Dry apple pomace consists of 56 % carbohydrates (15.6 % soluble carbohydrates, 21.2 % cellulose and 14.8 % hemicellulose). The remaining composition is 18.5 % lignin and small amounts of water, proteins, fats and ash [3].

1.1 Background

When using apple pomace as a substrate it is important to extract as much sugar as possible. The high lignin and pectin content in apple pomace form a network that traps the sugars. To break this barrier, several pre-treatment methods can be used, one of which utilizes an acid or alkali substance to free the sugar polymers and increase the overall yield [8]. Several studies considered the use of apple pomace to produce Bio-H_2, Bioethanol, Biobutanol and citric acid [3, 5, 8-10].

Apple pomace was used as a substrate to produce Bio-H_2 via activity of a bacterial consortium from a river sludge through anaerobic digestion. The pomace was either pre-treated with sulphuric acid or ammonia liquor for disruption of the lignin and hemicellulose matrix to free cellulose and free sugars. In this study, only free sugars from apple pomace were investigated. Several factors affect the H_2 production such as pH, temperature and substrate concentration. The concentration of apple pomace has a significant impact on how much H_2 can be produced. Having a higher concentration resulted in lower yields since bacteria might produce alcohol and lactate instead of H_2 and volatile fatty acids (VFA), at higher H_2 partial pressure. Higher concentrations would also increase the production of VFAs that will decrease the pH and inhibit growths. Therefore, the optimal concentration for apple pomace was determined to be 15 g/l (g Apple pomace /l water), fermented at 37 °C and a pH of 7.0 resulted in a CHYm (maximum cumulative H_2 yield) of 101.08 ml/g TS (total solids) [5]. A similar study investigated production of H_2 with enzymatic hydrolysis. The apple pomace was pre-treated with hydrochloric acid, sulphuric acid or ammonia liquor at different concentrations. Apple pomace

was hydrolysed with cellulase at a pH of 5.0 and a substrate concentration of 20 g/l (g pomace / l water) to determine the optimal hydrolysis time, temperature and cellulase dosage. The results show that the highest CHYm was 134 ml/g TS for apple pomace pre-treated with ammonia liquor and hydrolysed for 48h at 45°C and cellulase dosage of 12.5 mg/g TS. The substrate concentration was 15 g/l (AP/W), fermented at 37°C and pH 7.0 [9].

Production of bioethanol with apple pomace as substrate was investigated using enzymatic hydrolysis of the apple pomace and fermented with the baker's yeast *Saccharomyces cerevisiae*. Apple pomace was subjected to enzymatic hydrolysis using cellulase, pectinase and hemicellulase to breakdown lignocellulosic materials into useful free sugars such as glucose. Two pre-treatments were tested with either sulphuric acid or calcium oxide to investigate the impact on the production of bioethanol along with enzymatic hydrolysis. However, it yielded similar results to non-pre-treated pomace with enzymatic hydrolysis and was not used for fermentation. The fermentation had a metabolic yield of 92 % (g ethanol/g sugar) and a yield of 13.4 % (g ethanol/g pomace) [8].

Apple pomace was used as a substrate to produce Bio-butanol with *Clostridium beijerinckii* CECT 508 through anaerobic digestion. Several physicochemical pre-treatments were tested to determine which kind of treatment would result in the most extraction of free sugars and cellulosic material for enzymatic hydrolysis. The physicochemical pre-treatments consist of auto hydrolysis, different acids, alkalis, organic solvents and surfactants. The results showed that alkalis did not release as much sugars compared to the other pre-treatments and thus they were not further tested. Those that performed the best, from highest to lowest extracted sugars were HNO_3, PEG 6000 and acetone. HNO_3 recovered the most sugars with an efficiency of 76 % compared to the lowest one with 38 % for acetone. During fermentation in apple pomace pre-treated with HNO_3 the bacterium consumed 6 ± 1 % of the extracted sugars and therefore produced little Butanol. The author believes that it might be due to it having the highest production of inhibitors during pre-treatment and might have prevented enzymatic hydrolysis. Thus, the highest butanol concentration of 9.11 g/l was produced from PEG 6000 surfactant with a sugar consumption of 91 %. The yield was 0.28 g butanol per g total sugars consumed [3].

Dried and grounded apple pomace was used to inoculate *Aspergillus niger* BC_1 in a packed-bed reactor for production of citric acid. Several parameters during fermentation were observed such as aeration rate, bed height, particle size of the apple pomace and moisture content. The

author observed that having a higher aeration rate decreased the overall citric acid yield (g citric acid/ kg dry apple pomace). Having a higher bed height, particle size and moisture content provided a higher citric acid yield. The highest yield of citric acid was 124 g citric acid per 1 kg dry apple pomace, provided from the conditions of 0.81 l/min aeration rate, a bed height of 10 cm, particle sizes between 0.6-2.33 mm and a moisture content of 78% (weight water/weight pomace) [9].

1.2 Purpose

The purpose of this bachelor thesis was to evaluate if the by-product apple pomace can be used for the cultivation of a filamentous fungus. The evaluation was carried out by monitoring ethanol and biomass concentrations during fungal cultivation. Different factors were investigated including pretreatment strategy and need of pH adjustment. The apple pomace was kindly provided by Herrljunga cider. Herrljunga cider is a local brewer producing cider and must in Sweden, the company started in 1911.

2. MATERIAL AND METHOD

Material:

Apple pomace was provided by Herrljunga cider, while the following chemicals were purchased from common suppliers: D-glucose anhydrous (Fisher chemical), Peptone Special (Sigma-Aldrich), Agar bacteriological (Scharlau), Sulphuric Acid (2 % w/w, prepared beforehand), Sodium hydroxide (1 M, prepared beforehand).

1. Preparation of apple pomace for fermentation

1.1. Extraction of water-soluble fractions and their application for fungal fermentation

Water soluble fractions were extracted from apple pomace through the following process: apple pomace and distilled water were mixed together in 2000 ml beakers, according to Table 1 below. The contents were mixed with a gloved hand to let the water cover as much apple pomace as possible. For cold treatments at room temperature, the beakers were covered with an aluminium foil to prevent moisture loss and left overnight. The warm samples were boiled for 30 minutes and cooled to room temperature.

Table 1, pomace and water ratios for apple juice growth medium.

Batch	Temperature	Mass apple pomace	Mass water	Mixing method
A	Room	400 g	200 g	Manually
B	Room	200 g	200 g	Manually
C	Boiling	400 g	200 g	Manually
D	Boiling	200 g	200 g	Manually

Apple juice was extracted from the soaked pomace by pressing it through a fabric, afterwards the juice was filtered a second time with the same fabric to remove small particles. The volume of apple juice was measured and 50 ml juice was moved to 100 ml Erlenmeyer flasks which were closed with a cotton plug and aluminium foil on top. The juice was autoclaved for 20 minutes at 121 °C with a Systec Autoclave. The batches were made in duplicate. The remaining apple juice was stored in centrifuge tubes and put in a freezer for further use. The filtered apple pomace was weighted and then dried in an oven at 70 °C to measure the dry weight. This was to evaluate the remaining water content in the apple pomace.

1.2. Preparation of apple pomace water mixture and their application for fungal fermentation

Apple pomace suspensions were prepared through the following process: Batches E and F were prepared according to Table 2 in a non-mixed manner (not homogenised), and added to 250 ml Erlenmeyer flasks, closed with a cotton plug and aluminium foil on top, while Batches G and H were mixed together with a kitchen blender to form a homogenised smoothie. Similarly, to E and F, around 100 g of the homogenised suspension were transferred to 250 ml Erlenmeyer flasks and closed with a cotton plug and aluminium foil on top.

Table 2, pomace and water ratios for apple pomace suspension growth medium.

Batch	Temperature	Mass apple pomace	Mass water	Mixing method
E	Room	10 g	90 g	None
F	Room	20 g	80 g	None
G	Room	100 g	900 g	Blender
H	Room	200 g	800 g	Blender

Similarly, to the water-soluble fractions, the batches were made in duplicates. These flasks were sterilised for 20 minutes at 121 °C using a Systec Autoclave.

1.3. Adjusting pH of water soluble fractions and their use for fungal fermentation

The remaining apple juice from Batches A and B mentioned earlier was prepared for pH adjustment. In 100 ml Erlenmeyer flasks, 50 ml of A and B were transferred to their respective flasks. The pH was measured using a pH-meter under stirring to ensure a homogenous solution when adding 1 M NaOH to increase the pH to 5.5 from the average initial pH 4.24. When the samples have reached the pH to around 5.5, the flasks were sealed with cotton plugs and aluminium foil to be sterilised for 20 minutes at 121 °C in a Systec Autoclave. This was done in duplicates.

Batches I and I5.5 were prepared by mixing with a gloved hand, 400 g of apple pomace and 100 g water (Table 3) to a 2000 ml beaker. The mixture was covered in an aluminium foil to prevent moisture loss and was left to stay overnight. The following day, the mixture was filtered similarly to A-D mentioned earlier. The volume of apple juice was measured and divided into

two 50 ml batches called I and I5.5. Batch I will be a non-pH adjusted control for the pH adjusted sample I5.5. Sample I5.5 was pH adjusted to 5.5 similarly to sample A5.5 and B5.5 using 1 M NaOH. The flasks were sealed with cotton plugs and aluminium foil to be sterilised for 20 minutes at 121 °C in a Systec Autoclave. This was done in duplicates.

Table 3, pomace and water ratios for pH adjusted apple juice growth medium.

Batch	Temperature	Mass apple pomace	Mass water	Mixing method
A 5.5	Room	400 g	200 g	None
B 5.5	Room	200 g	200 g	None
I	Room	400 g	100 g	Manually
I 5.5	Room	400 g	100 g	Manually

2. Preparation of agar plates

Agar was prepared by mixing 4.0 g Glucose, 0.8 g peptone and 3.4 g agar with 200 ml distilled water in a 400 ml beaker. The pH was adjusted to 5.5 using Sulphuric Acid (2 % w/w). The contents were stirred with a magnetic stirrer to ensure homogeneity before autoclavation. The agar mixture was autoclaved in Systec Autoclave at 121 °C for 20 minutes.

The sterilised agar mixture was poured onto petri dishes under aseptic conditions and left at room condition to solidify. After solidifying, a spore suspension was prepared by adding 20 ml autoclaved distilled water to a plate of *Rhizomucor* fungus, spores were scraped from the plate using a spreader. Afterwards, the spore suspension was poured from the plate to a sterile centrifuge tube. This was all done under aseptic conditions.

The spore suspension was used to inoculate the agar plates, 0.1 ml spore suspension was spread with a spreader on each plate under aseptic conditions. The plates were placed in an oven at 30 °C for 4 days to grow.

3. Inoculation of fungi, fermentation, and sampling.

A spore suspension was prepared by adding 20 ml autoclaved distilled water to one of the previously prepared agar plates, spores were scraped from the plate using a spreader. Afterwards, the spore suspension was poured from the plate to a sterile centrifuge tube. This was all done under aseptic conditions. The sugar extract was inoculated by adding 1 ml spore

suspension per 50 ml extract. The Erlenmeyer flasks were shaken lightly and a 1 ml per 50 ml extract sample was taken.

The fungi was allowed to grow for 72 hours in a shake water bath at 35 °C and 100 rotations per minute, with samples taken every 24 hours. The sampling was conducted under aseptic conditions by transferring 1 ml liquid from the Erlenmeyer flask to a 1.5 ml Eppendorf tube. The sample was then centrifuged using a Heraeus Fresco 21 centrifuge at 10 G for 10 minutes. The supernatant was then transferred to another 1.5 ml Eppendorf tube and placed in a freezer for further HPLC analysis.

3.1. Harvesting of Biomass

On the third day of growth, after the last sampling, the contents in the Erlenmeyer flasks were separated with a kitchen sieve and a beaker under it. A spoon was used to push out the remaining liquid from the biomass into the beaker.

The liquid collected in the beaker was measured using a graduated cylinder and later transferred to a 50 ml Eppendorf tube to centrifuge at 5000 x g for 10 minutes using a Heraeus Megafuge 8. The supernatant was removed, and the solids were collected, weighted, dried and weighted again to determine the mass of suspended solids. The remaining biomass that was collected in the sieve was washed two to three times with distilled water, dried at 70 °C and weighted.

4. Analysis of ethanol and sugar concentration

The samples that were collected through the entire experiment were analysed using HPLC (High-Performance Liquid Chromatography) to determine sugar and ethanol concentrations. The HPLC was using a hydrogen-ion based ion-exchange column (Aminex HPX-87H, Bio-Rad, Hercules, CA, USA) at 60°C, using 0.6 ml/min of 0.5 mM H_2SO_4 solution as an eluent and an RI detector. The samples were centrifuged using a Heraeus Fresco 21 centrifuge at 10 G for 10 minutes to try to remove as much solids as possible. Around 1ml of each sample was transferred to a glass vial. Since the volume of water insoluble fraction samples were too small, 0.3 ml from samples E-H were extracted to their own Eppendorf tube along with 0.9 ml of distilled water. Similarly, to the previous samples, they were centrifuged at 10 G for 10 minutes and 1ml was transferred to each individual vial.

5. Determining ethanol and biomass yield.

To know how much dry biomass was used per batch, several samples were collected during the experiment. The mass of the wet apple pomace after filtration was weighted, dried and weighted again. The difference in weight (g dry / g wet) determines the dry content of the pomace. This will be used to approximate how much dry pomace is used per batch. Apple pomace concentration per batch was calculated by dividing the amount of apple pomace (g) with the total extracted volume (l). To know how much equivalent wet pomace is used per sample, the concentration was multiplied with the volume of sample (l). Lastly, to determine the equivalent dry pomace per sample, the dry content of the pomace was multiplied with the equivalent wet volume.

The mass (g) of ethanol and biomass was calculated by multiplying their final concentration with the sample volume. To determine the ethanol and biomass yield, their corresponding mass (g) of a sample was divided with the equivalent dry weight pomace (kg) of the same sample.

3. RESULTS

3.1. Biomass yield from water soluble fractions

Water soluble fractions were extracted from soaked pomace by filtration with a fine fabric. Apple juice was inoculated with *Rhizomucor* and grown in Erlenmeyer flasks for 72 hours at 35 °C in a water shake bath. Biomass was separated from the growth suspension and the dry weight was measured. The biomass concentration was determined from the amount dry biomass per liter broth.

It was observed during filtration that the soaked apple pomace still had a large amount of water content remaining. The yields could be improved by optimizing the filtration method. The biomass separated easily from the broth, at first it was brown, very soft and contained a lot of moisture. By washing it and pushing out the water it became stiffer and contracted until it formed a nice small white-greyish ball.

Using an apple pomace to water ratio of 1 g/g (9.7 g ethanol/g dry apple pomace) gave higher biomass yields than 2 g/g (7.7 g ethanol/g dry apple pomace) and 4 g/g (4.0 g ethanol/g dry apple pomace) for both water temperature indicating that a lower pomace to water ration could

potentially improve yields. The use of hot water treatment gave no higher biomass yield compared to cold water treatment and was in fact lower, this can be seen in Table 4.

3.2. Biomass yield from water soluble fractions with adjusted pH

To study the effects of the pH, the apple pomace extract obtained using 1 and 2 g pomace/g water was subjected to a pH adjustment process (at 5.5) before the cultivation. Two new batches with a pomace to water ratio of 4 g/g were prepared using cold water, one was adjusted to pH 5.5 while the other was kept as a reference. Water soluble fractions had a pH adjustment, 1 M Sodium hydroxide was added dropwise to the apple juice until the pH was around 5.5.

It was observed that the final pH regardless if it was adjusted or not always stayed at around 3.5. Adjusting the pH was not beneficial as seen by the lower yield compared to the non-pH adjusted samples as seen on 1 g/g going from a yield of 9.7 g/kg to 7.06 g/kg. The reason might be due to the larger change of pH during fermentation compared to the non-changed samples as seen in Table 5.

Table 4: Biomass yields from water soluble fractions with different apple pomace to water ratios. A (2 g P /g W), B (1 g P/g W) and I (4 g P/g W) were extracted with room temperature water. C (2 g P /g W) and D (1 g P /g W) were extracted with boiling water. A5.5 (2 g P /g W), B5.5 (1 g P /g W) and I5.5 (4 g P /g W) were extracted with room temperature water and had their pH adjusted to 5.5

Biomass yields for pomace to water ratios at different temperatures and pH adjustments (g biomass/ kg dry apple pomace)							
A	B	C	D	I	B5.5	A5.5	I5.5
7.70 ± 0.28	9.70 ± 0.13	4.47 ± 0.15	9.52 ± 0.15	4.00 ± 0.02	7.06 ± 0.26	6.45 ± 0.31	3.48 ± 0.71

Table 5: pH before adjustment, pH after adjustment and pH after fermentation for water soluble fractions with different apple pomace to water ratios. A5.5 (2 g P /g W), B5.5 (1 g P /g W) and I5.5 (4 g P /g W) were extracted using room temperature water and had their pH adjusted to 5.5. I (4 g P /g W) was extracted using room temperature water.

Extraction condition	pH before adjustment	Adjusted pH	pH after fermentation
B5.5	4.26	5.45	3.65
A5.5	4.22	5.49	3.50
I5.5	4.23	5.50	3.51
I	4.23	-	3.53

3.3. Biomass concentration from water insoluble fractions

Apple pomace suspensions were made by mixing apple pomace and distilled water with a kitchen blender. A non-mixed suspension was also made. Similarly mentioned earlier, the batches were inoculated, fermented and the biomass was separated.

It was observed that the biomass was hard to separate from the growth suspension. A lot of apple pomace bits were stuck onto the biomass. It was also seen that the fungi started to grow on top of the broth for the thicker F (4g W/g P) and H (4g W/g P). This could indicate that the fungi was not happy due to the lack of oxygen in the suspension. The results were deemed too inconsistent due to the large amount apple pomace solids stuck on the biomass and not further investigated (Table 6).

Table 6, biomass concentration for water insoluble fractions with different water to apple pomace ratios. E (9 g W /g P) and F (4 g W/g P) were prepared without blending. G (9 g W/g P) and H (4 g W/g P) were prepared with blending.

Concentration of biomass for apple suspensions (g dry biomass / l water)			
E	F	G	H
7.74 ± 0.71	24.34 ± 2.94	7.85 ± 0.17	16.63 ± 1.80

3.4. Ethanol yield and sugar consumption from water soluble fractions

Sample sizes of 1 ml were taken every 24 h from the growth medium during the fermentation. The samples were transferred to glass vials and analysed by the HPLC.

During sugar extraction with hot water, it was noted that water evaporates significantly (145 ml from hot extraction compared to 293 ml in cold extraction) which might have influenced the yields.

As a general trend for all samples, ethanol concentrations peaked at 48 hours and decreased at 72 hours. The fungi preferred to consume glucose as opposed to the high amount of sugar mix (mostly fructose) present in the solution according to Figure 1-3. The highest ethanol yield was 11.22 g ethanol per kg dry apple pomace obtained using an apple pomace to water ratio of 1g P/g W and hot water while the second highest ethanol yield was 10.53 g ethanol per kg dry

apple pomace using an apple pomace to water ratio of 2 g P/g W and cold water. The only visible trends were that pH adjusted samples gave lower ethanol yields.

Table 7, Ethanol yields from water soluble fractions with different apple pomace to water ratios. A (2 g P /g W), B (1 g P/g W) and I (4 g P/g W) were extracted with room temperature water. C (2 g P /g W) and D (1 g P /g W) were extracted with boiling water. A5.5 (2 g P /g W), B5.5 (1 g P /g W) and I5.5 (4 g P /g W) were extracted with room temperature water and had their pH adjusted to 5.5

Ethanol yields for pomace to water ratios at different temperatures and pH adjustments (g biomass/ kg dry apple pomace)							
A	B	C	D	I	B5.5	A5.5	I5.5
10.53 ± 0.02	6.56 ± 0.28	6.85 ± 0.78	11.22 ± 0.17	8.85 ± 0.13	3.41 ± 0.06	8.25 ± 0.20	4.58 ± 0.05

Figure 1, Multiple graphs showing the ethanol and sugar profile for the fermentation. A (2 g P /g W), B (1 g P/g W) and I (4 g P/g W) were extracted with room temperature water.

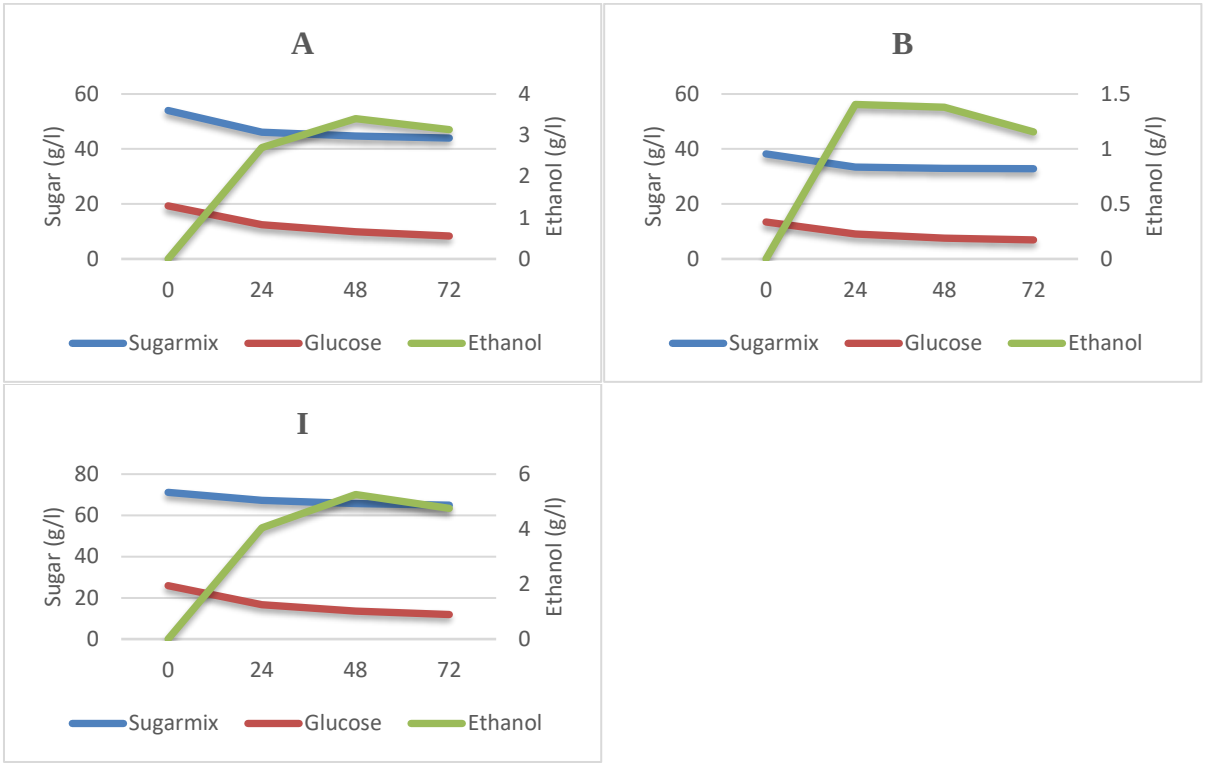


Figure 2, Multiple graphs showing the ethanol and sugar profile for the fermentation. C (2 g P /g W) and D (1 g P /g W) were extracted with boiling water.

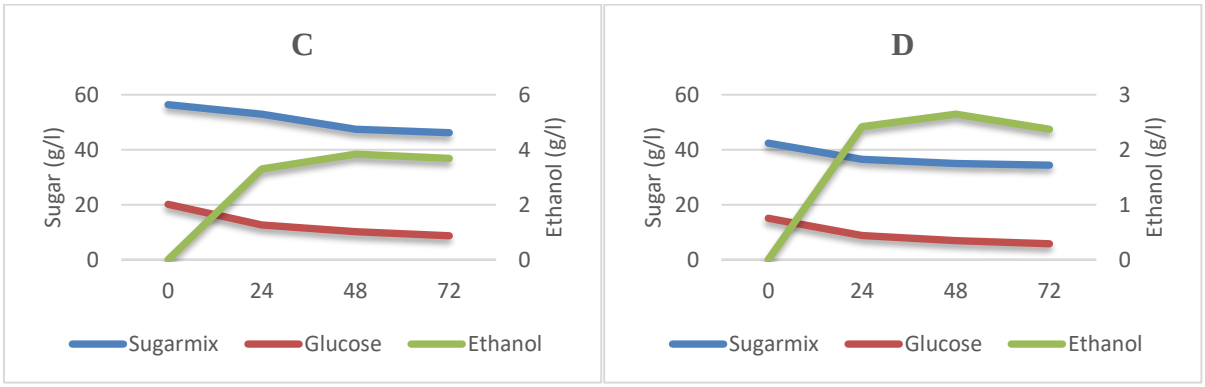
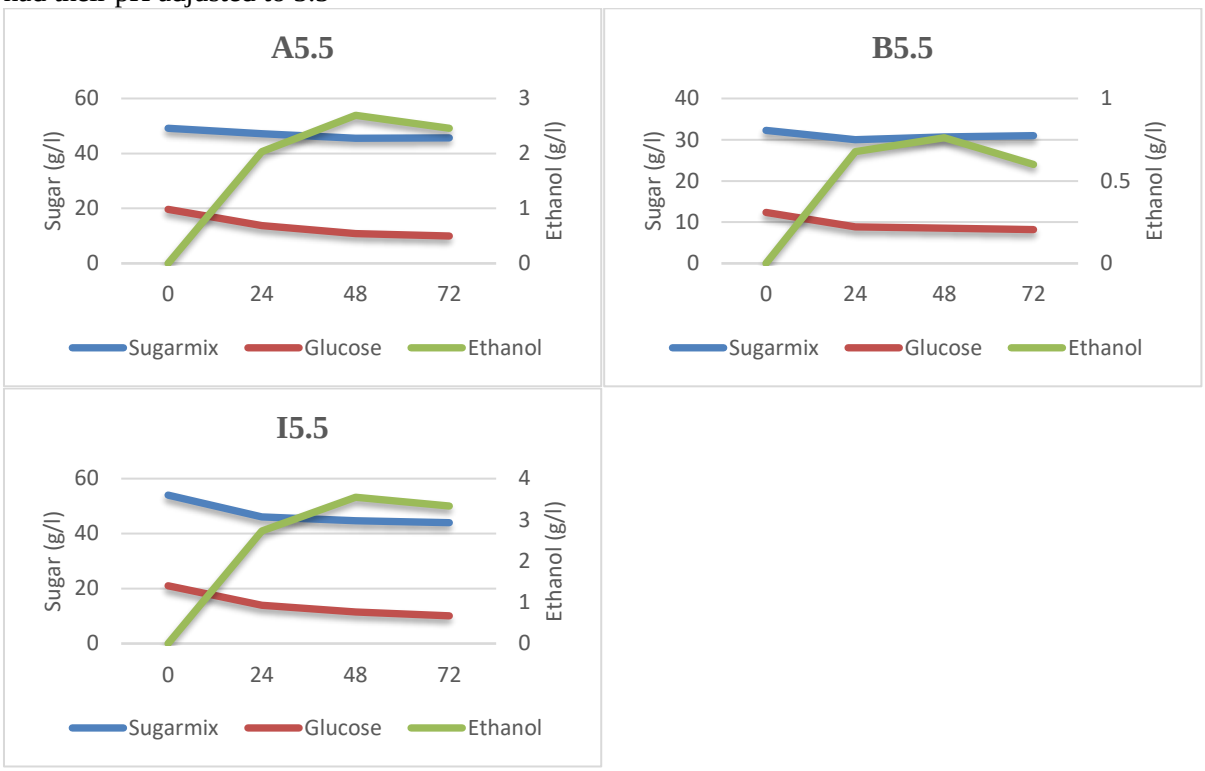


Figure 3, Multiple graphs showing the ethanol and sugar profile for the fermentation. A5.5 (2 g P /g W), B5.5 (1 g P /g W) and I5.5 (4 g P /g W) were extracted with room temperature water and had their pH adjusted to 5.5



4. DISCUSSION

Starting with water soluble fractions, the highest biomass yields was obtained using an apple pomace to water ratios of 1 g P/g W with cold water followed by 1 g P/g W with hot water. This might be due to the water not covering the entire apple pomace when extracting using higher apple pomace to water ratios. Comparing Figure 1 and 2 shows that using hot water for the extraction does not contribute to that much higher glucose concentrations, but the ethanol concentrations increases significantly, although we do not have an explanation for such observation. The cost of heating might however make this a less applicable approach. Further research should focus on lowering the apple pomace to water ratios while maintaining high yields. All glucose was not consumed so increasing the fermentation time might also result in higher yields or add nutrients to the medium to help the fungus to keep consuming sugars.

Cold water extraction was deemed more interesting for the follow-up tests with pH adjustments. Apple pomace extracts with apple pomace to water ratios of 1, 2 and 4 g/g were prepared and pH was adjusted to 5.5. However, adjusting the pH to 5.5 decreased the biomass and ethanol production. This is unlike in other research which had a small increase in biomass and ethanol yields when cultivating *Rhizopus oryzae* in a solid-rich wastewater stream from a wheat-starch plant [10]. The ethanol production decreased. This might be due to the pH being less constant during the fermentation, leading to less production since the fungus is always adjusting. The yield could improve by having a constant pH of 5.5 with a bioreactor since the low pH could’ve stopped the fungal growth.

The fungus grew on the surface of low water to pomace mediums when cultivated with water insoluble fractions. Observations during fermentation showed that the liquid had limited movement due to the viscous nature from the large amount of insoluble mass. Therefore, it is suspected that the fungus did not thrive due to a lack of oxygen in the broth. Support for this suspicion was found in literature study by Gibbs, P.A., et al., (2000), where the effects of high viscosity on filamentous fungi was discussed [11]. Having higher aeration provided from an airlift bioreactor could potentially help.

The results show that *Rhizomucor* prefers to consume glucose over fructose. This is in accordance with research done by Satari, B., et al., (2016), where free sugars from citrus waste containing mostly glucose and fructose was used for cultivation of *Mucor indicus* and *Rhizopus*

oryzae. The fructose remained intact during the cultivation in the shake flaks while glucose was completely consumed [12]. Due to the high amount of remaining fructose present in the broth, more research could be carried out to optimize the fructose consumption including e.g. longer cultivation time or different fungal strains.

4.1 Future work

Several suggestions for future work were thought about during this research project. The goal was to see how much biomass can be produced from apple pomace as a sugar source for a filamentous fungus. The amount of biomass could be improved by the following steps.

- Use lower apple pomace to water rations for water soluble fractions.
- Adding nutrients to medium for better sugar consumption.
- Having higher aeration for apple pomace suspension samples.
- Having a constant pH of 5.5 with a bioreactor.
- Increase the duration for fermentation to longer than 3 days.
- Improve the filtration method and extract more juice from the pomace to increase the overall yield.

5. CONCLUSIONS

In conclusion, water soluble fractions acquired by pre-treating apple pomace with cold distilled water for 24 h with an apple pomace to water ratio of 1g P/1g W, produced the most biomass per kg dry apple pomace. Heat treatment and pH adjustment had no positive impact on biomass production since both decreased during fermentation. The fungus *Rhizomucor* sp. preferred consuming glucose over fructose during the growth. The general trend was that lower apple pomace to water ratios resulted in higher yields.

The highest ethanol yields were acquired by pre-treating apple pomace with boiling distilled water for 30 minutes with an apple pomace to water ratio of 1g P/1g W. Since the yield was similar to that of cold water, heat-treatment might not be applicable due to energy costs. The yields for hot water treatment might not be accurate due to evaporation during extraction. More research should be made to improve several aspects of this methodology to extract more sugars and increase the overall yields for biomass and ethanol production.

6. REFERENCES

1. Shashi Bhushan, K.K., Madhu Sharma, Bikram Singh & P. S. Ahuja, *Processing of Apple Pomace for Bioactive Molecules.* Critical Reviews in Biotechnology, 2008. **28**(4): p. 285-296.
2. Cargnin, S.T. and S.B. Gnoatto, *Ursolic acid from apple pomace and traditional plants: A valuable triterpenoid with functional properties.* Food Chemistry, 2017. **220**: p. 477-489.
3. Hijosa-Valsero, M., A.I. Paniagua-García, and R. Díez-Antolínez, *Biobutanol production from apple pomace: the importance of pretreatment methods on the fermentability of lignocellulosic agro-food wastes.* Applied Microbiology and Biotechnology, 2017. **101**(21): p. 8041-8052.
4. Jönsson, E., *Apple pomace in Southern Sweden, current uses and potential for new products* 2010, Sveriges Lantbruksuniversitet. p. 33.
5. Feng, X., et al., *Biohydrogen production from apple pomace by anaerobic fermentation with river sludge.* International Journal of Hydrogen Energy, 2010. **35**(7): p. 3058-3064.
6. Luo, J., Y. Xu, and Y. Fan, *Upgrading Pectin Production from Apple Pomace by Acetic Acid Extraction.* Applied Biochemistry and Biotechnology, 2019. **187**(4): p. 1300-1311.
7. Gabriel, L.S., et al., *Multivariate Analysis of the Spectroscopic Profile of the Sugar Fraction of Apple Pomace.* BRAZILIAN ARCHIVES OF BIOLOGY AND TECHNOLOGY, 2013. **56**(3): p. 439-446.
8. Magyar, M., et al., *Conversion of apple pomace waste to ethanol at industrial relevant conditions.* Applied Microbiology and Biotechnology, 2016. **100**(16): p. 7349-7358.
9. Hui Wang, J.W., Zhong Fang, Xiaofang Wang abd Huaiyu Bu, *Enhanced bio-hydrogen production by anaerobic fermentation of apple pomace with enzyme hydrolysis.* International Journal of Hydrogen Energy, 2010. **35**(15): p. 8303-8309.
10. Souza Filho, P.F., A. Zamani, and M.J. Taherzadeh, *Edible Protein Production by Filamentous Fungi using Starch Plant Wastewater.* Waste and Biomass Valorization, 2018.
11. Gibbs, P.A., R.J. Seviour, and F. Schmid, *Growth of Filamentous Fungi in Submerged Culture: Problems and Possible Solutions.* Critical Reviews in Biotechnology, 2000. **20**(1): p. 17-48.
12. Satari, B., et al., *Co-Production of Fungal Biomass Derived Constituents and Ethanol from Citrus Wastes Free Sugars without Auxiliary Nutrients in Airlift Bioreactor.* International journal of molecular sciences, 2016. **17**(3): p. 302-302.

HÖGSKOLAN
I BORÅS

Besöksadress: Allégatan 1 · Postadress: 501 90 Borås · Tfn: 033-435 40 00 · E-post: registrator@hb.se · Webb: www.hb.se