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Abstract

Brännland Cider operates outside Umeå, in the northern part of Sweden. They produce ice cider and hard cider since 2012. Today the business produces approximately 100 000 bottles per year. Apple must is concentrated by freezing, then fermented to a level of ethanol to 7-13 vol% and at least 13° brix residual sugar. The yeast running the fermentation must stand a high osmotic pressure. Different yeasts are naturally present on the apples. The aim was to spontaneously ferment five different sorts of apple concentrate and examine the presence of yeast and bacteria, and isolate and identify yeasts from each sample. The results showed presence of *Saccharomyces cerevisiae, Metschnikowia pulcherrima, Hanseniaspora osmophila, Pichia membranifaciens* och *Metschnikowia rubicola* and/or *Metschnikowia leonuri*. The three first-named would be interesting for production of ice cider. The two last-mentioned are newly discovered and more literature is required to deduce the possible usage for ice cider. To be able to use the isolated yeasts, more analyses are necessary.

Keywords: Ice cider, spontaneous fermentation, cider, yeast

Sammanfattning

Brännland Cider har sin verksamhet utanför Umeå och gör iscider och cider av svenska äpplen sedan 2012. Idag producerar de cirka 100 000 flaskor om året. Iscider är fryskoncentrerad äppelmust som fermenteras till 7-13 vol% alkohol och minst 13° brix restsötma. Jästen som används behöver tåla högt osmotiskt tryck. Olika sorters jäst finns naturligt på äpplena. Denna jäst skulle kunna vara intressant för produktion av iscidern. Syftet med rapporten var att spontanjäsa fem olika sorters äppelmust och undersöka antal jäst och bakterier, samt isolera och identifiera jäst från dessa. Resultaten visade närvaro av *Saccharomyces cerevisiae, Metschnikowia pulcherrima, Hanseniaspora osmophila, Pichia membranifaciens* och *Metschnikowia rubicola* och/eller *Metschnikowia leonuri*. De tre förstnämnda skulle kunna vara intressanta för produktion av iscider. De två sistnämnda finns för lite litteratur för att dra slutsatser om användning. För att kunna använda jästerna som isolerats behövs fler undersökningar genomföras.

Nyckelord: iscider, spontanfermentering, vildjäsning, cider, jäst

Preface

This thesis is submitted in fulfilment of the requirements for the bachelor degree in Agriculture food science programme at Swedish university of agriculture, Uppsala. The project was conducted by Daniel Eriksson and Ella Råhlén in collaboration with Brännland Cider, during spring of 2019. The degree comprises 15 credits, corresponding to ten weeks of full-time studies.

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Abbreviations

DF = During fermentation
EF = End of fermentation
PF = Pre-fermentation

1 Introduction

1.1 Apples in Sweden

1.1.1 Production and consumption

The average yield of Swedish apple orchards, during the last two decades, has been 16 tonnes/hectare. The total farming area is 1500 hectare, nearly 90% of the total area can be found in Skåne. Increasing demand of Swedish produced apples has driven the production to an all-time high. In the year 2016, a quarter of the Swedish market consisted of Swedish produced apples. From the year 2000 to 2015, average intake was 11 kg per capita per year. (Jordbruksverket 2017)

A selection of storage tolerable varieties and modern storage solutions allows to have Swedish apples available all year round. An Ultra Low Oxygen (ULO) – storage lowers the concentration of oxygen in the room substantially, while the carbon dioxide concentration increases. This slows the ripening process, thus enables consumption of fresh apples out of season. Temperature control is also a control point to minimize the risk of spoilage. (ibid.)

1.1.2 Profitability

During the last two decades, the average output price of Swedish apples has increased by nearly 50%. In 2017 the output price was 8,50 SEK/kg. Though farmers still struggle with balancing the books, as the overall production cost has risen with 68% from 1995 to 2016. (ibid.).

An orchard with a conventional farming area of 20 hectare will have an average production cost of 8,16 SEK/kg. This includes farming, storage and packaging. The output price compared to the production cost gives an indication on the difficulties with running an apple orchard. (Ascard et al. 2010)

Due to low output prices of apples for consumption, one could consider the possibility to process the apples into a refined product. Cider is a commonly known beverage, usually made of apples. However, regulations for cider in Sweden states the minimum amount of fermented apple or pear juice to 15% of the total volume (LIVSFS 2005:11). To name the product "apple cider", the total volume of the added juice must be at least 50% apple juice. This means an apple cider could consist of eight percent fermented apple juice. It is also allowed to add sugar, additives and water.

1.1.3 Quality

In recent years, the popularity of smaller cider producers with higher quality products has increased. More commonly known producers are Brännland Cider, Pomologik and Särtshöga Vineyard. A high quality product could give an increased output price for the raw material. For an apple farmer, the increased output price may support the survival of the orchard.

Traditionally, apples used for cider production would have different sensorial attributes. A desired cider apple should be relatively low in acidity and have enough bitterness to give the distinct cider flavour with enough sugar content allowing fermentation to reach desired ethanol levels. (Ekholm et al. 2017)

1.2 Apple varieties

Apple varieties for cider production from France, England and Spain ripen late (Ekholm et al. 2017). The cold and early autumn in Sweden is therefore problematic for these varieties. Typical Swedish varieties can instead be used for production of ice cider. Apple varieties used in this thesis were Ingrid Marie, Cox's Orange, Rubinola, Lobo, Kim and Mutsu.

1.2.1 Ingrid Marie

The apple most Swedes associate with Swedish apple production is Ingrid Marie. It most likely derives from Cox's Orange since both sorts are alike. The origin of Ingrid Marie is though unknown. In a backyard of a Danish school in 1910, the teacher K. Madsen found an apple tree and named it after his daughter. The variety entered Sweden twenty years later and was, and still is, one of the most popular apples for both professional and hobby growers. (Svensson 2005)

1.2.2 Cox's Orange

In 1825, nine Ribston seeds were set by the brewer R. Cox in Buckinghamshire, England. One turned out to be Cox's Orange. During the second half of the 19th century the variety spread to France, Germany and finally Sweden. Cox's Orange dominated the orchards for decades and is still today a popular fruit. (ibid.)

1.2.3 Rubinola

The apple originates from Czech Republic and is a relatively new variety. The yellow and red fruit is a crossbreed between Rubin and Prima. Rubinola is suitable for ecological growing and is already a common fruit in the grocery stores. (Tord Erikssons Frukt n.d.)

1.2.4 Lobo

The colour of Lobo resembles the colour of Snow white's deep red apple. The popular fruit was discovered in Canada late 1900s from a seed from McIntosh. Thirty years later, the aromatic variety entered Sweden and is still a popular both in gardens and in orchards. (Svensson 2005)

1.2.5 Kim

Kim was produced in Balsgård, Sweden, in 1946 through a crossbreed between Cortland and Ingrid Marie. The red apple with fresh and enjoyable taste is commonly grown by professional growers in Sweden. (ibid.)

1.2.6 Mutsu

A green and slightly blushing apple named Mutsu originates from a crossbreed between Golden delicious and Indo in Japan, in the 1930s. The apple got its name from the Japanese emperor Mutsuhoti and came to Sweden forty years later. (ibid.)

1.3 Ice cider

There are few apple ice cider studies described in the literature. However, more research about ice wine and wine can be found. The raw material of Ice wine is frozen grapes from the vine which are pressed frozen and fermented. Ice wine has different legislations in different countries, the sugar content varies between 26°-

35° brix and the minimum alcohol content varies between 5.5% to 7% (w/v). (Mencarelli and Tonutti 2013)

Ice cider and ice wine are similar regarding the environment for the yeast. Therefore, literature about Ice wine was a resource when searching for information about ice cider.

1.3.1 Regulations

Apple ice cider is a fermented concentrated apple juice, with at least 30° brix sugar content, pre fermentation. According to Quebec government regulations, the sweet must have to be obtained with natural cold. This limits the production locations to areas with long and cold winter months. According to the regulations it is not allowed to add alcohol, sugar, colourants, flavours or preservatives. The alcohol content should be between 7-13% (w/v) and the residual sugar minimum 13° brix. (Government Québec 2019)

1.3.2 Extraction

There are two methods to obtain the must, cryo-concentration and cryo-extraction. The cryo-concentration, the more used method, begins when the outside temperature has dropped to a consistent -15°C. Apples are pressed and the must is transferred to containers and left outside. By freezing, the higher density of the sugars will sediment to the bottom of the vessel leaving the water at the top thus allowing the extraction of the high brix must. Approximately a quarter of the original must volume is fermented. Cryo-concentration contributes to a steadily achieved quality and there is a lower risk of not having a finished product. (Kirkey and Braden n.d.)

Cryo-extraction starts with leaving the apples in the trees and let them freeze during the autumn. Apples are harvested when the temperature has dropped to subzero for several consecutive days. The extended time on the trees has left the apples with a distinct taste and a lower amount of water. When pressing the apples, a high brix juice is obtained. (ibid.)

The cryo-extraction could lead to a more developed flavour and higher price of the final product, though it comes with a vastly increased risk of not having a final product. This method depends on the weather and that the apples are resistant to pests and diseases. The apples need to be transferred quickly post-harvest, due to thawing. The pressing facility needs to withstand low temperatures to minimize the risk of spoilage. (ibid.)

1.4 Yeast in ice cider

Yeast has been used for centuries, mainly due to its capability to ferment carbohydrates. *Saccharomyces cerevisiae* is the prevalent yeast species regarding fermenting carbohydrates. The species can ferment sugars, commonly six-carbon molecules, into carbon dioxide and ethanol. A vast amount of strains have been isolated during the years, each having different characteristics. (Sicard and Legras 2011)

The usage of appropriate yeast strains when fermenting apple ice cider is of utmost importance. The yeast strain has shown to affect the fermentation rate, ethanol production, glycerol production and formation of volatile acids such as acetic acid. These affect the sensory characteristics of the final product. (Erasmus, Cliff & van Vuuren 2004)

Commonly used commercial yeast strains are K1-V1116 and EC-1118, which are *Saccharomyces cerevisiae* strains (Pickering 2006)

The yeast must endure a high osmotic stress, due to the high sugar content. To cope with the osmotic stress the yeast increases its production of glycerol. The glycerol works as an osmolyte that balances the osmotic pressure on the cells. Though, increased production of glycerol has been linked to a decreasing production of ethanol and increased concentration of acetic acid. (Pigeau et al. 2007)

Higher inoculum levels of yeast are usually needed compared to fermenting table wine. The higher inoculum levels are most likely needed due to the sugar content (Kontkanen et al. 2004).

The fermentation rate tends to abate before the wanted amount of sugars has been fermented, leaving the product with a high residual sugar concentration. It could be desired to add nutrients, assimilable nitrogen, to facilitate fermentation. (Ishmayana, Learmonth & Kennedy 2011)

1.5 Spontaneous fermentation

Since the juice is not pasteurized, the indigenous yeasts of the apples could be used to ferment the juice. The complex variety of non-*Saccharomyces* and *Saccharomyces* spp. affects the final product, in terms of contributing with different metabolites. Metabolites are such as esters, terpenoids, acetic acid and glycerol. It affects the final product in terms of sensory and chemical composition. (Jolly et al. 2014)

During the early stages of fermentation, the non-*Saccharomyces* yeasts increase in number. Though, as the fermentation continues and the ethanol levels rise, *Saccharomyces* prevails. The non-*Saccharomyces* yeasts inability to remain alive is due to their oxidative metabolism and sensitivity to high ethanol concentrations. (Combina et al. 2005)

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However, studies have shown that after a grape must is inoculated with industrial yeast there are still none-*Saccharomyces* spp. that survives a longer period during the fermentation. Thus, there is a possibility that they contribute to the final product. (Heard and Fleet 1985)

A combination of non-S*accharomyces* yeast and S*accharomyces* spp. could limit the amount of ethanol production but still have the sensorial attributes of the product, hence restricting the negative effects of a high ethanol concentration, such as decreased aroma perception and health problems. (Contreras et al. 1014)

Studies show that different species of *Candida*, *Hanseniaspora*, *Pichia* and *Metschnikowia* have been found in early stages of wine fermentation (Lee et al. 2011; Raymond Eder et al. 2017). *Hanseniaspora* spp. is often used to enhance apple flavour in cider and has been linked to production of volatile compounds. These metabolites are considered as "fruity", thus having a significant impact on the final product. Often found species are *Hanseniaspora uvarum, Hanseniaspora osmophila* and *Hanseniaspora occidentalis* (de Arruda Moura Pietrowski et al. 2012).

Pichia spp., *Metschnikowia* spp. and *Candida* spp. can produce acetaldehyde, acetic acid and acetate esters. These are metabolites that affect the final product in terms of sensorial and chemical properties. *Pichia* spp. and *Candida* spp. are both aerobic and can be found on the surface during fermentation, if air is not removed. After bottling, *Candida* spp. and *Pichia* spp. may produce unwanted sediment. Therefore, they are regarded as indicators of insufficient good manufacturing practices. Commonly found *Pichia* spp. are *Pichia membranifaciens, Pichia kluyveri* and *Pichia terricola*, it is possible to find *Metschnikowia pulcherrima*, *Candida lambica* and *Candida stellata* (Jolly et al. 2014). (Campaniello and Sinigaglia 2017)

1.6   Spoilage and Pathogens

Pathogens and mainly spoilage microorganisms must be taken into consideration, since the apple juice is not pasteurized the risk increase.

A possible pathogen in ice cider is Enterohaemorrhagic *Escherichia coli* (EHEC) serotype O157:H7. The bacteria survives pH levels down to 3.6 and a_w 0.95. It is causing diarrhoea and in worst case it is fatal. (Adams, Moss & McClure 2016)

It is not likely to have EHEC in the beverage because of the harsh environment with low a_w and pH. Pre fermentation, ice cider has at least 30° brix according to the legislations in Quebec. 40° brix has the a_w of 0.939 and 22° brix has a_w 0.983 (Erasmus, Cliff & van Vuuren 2004). Together with good hygiene, the low pH and the ethanol, the risk for the pathogen is basically negligible.

Furthermore, spoilage yeasts can be a problem for the quality of the product. Cloudiness, biofilms or sediments can ruin the look of the product. Flocculation can

appear when *Zygosaccharomyces bailii, S. cerevisiae* or *Saccharomycodes ludwigii* are present. Film on the surface can be produced by *Candida boidinii, Candida intermedia, Candida pseudointermedia, Candida pseudolambica, Candida tropicalis, Pichia occidentalis, Pichia kudriavzevii,, Wickerhamomyces anomalus, Kregervanrija fluxuum, Pichia mandshurica, Pichia membranifaciens* and *Brettanomyces spp.*

In high-sugar juices, more than 35% w/w, all spoilage yeasts float and form a surface film due to the density of the juice causing the yeast cells to float. The spoilage yeasts can be undetected in an industrial tank, floating on the surface. (Stratford 2006)

Off-flavours are unsolicited and some yeasts that spoil the ice cider visually, also spoil the product with unwanted tinges. *Pichia* species, like *Pichia membranifaciens* can give a yeasty aldehyde tinge. *Saccharomycodes ludwigii* produce high concentrations of acetoin and acetaldehyde. *Kloeckera apiculata* form high levels of esters and volatile acids, and *Brettanomyces bruxellensis* has a tinge of mouse, sweaty, leathery flavour. (ibid.)

1.7 Brännland Cider

Brännland Cider is located in Vännäsby, outside Umeå in northern Sweden. Former working with software, Andreas Sundgren Graniti founder and CEO, changed course to production of cider based on Swedish apples, with no additives. The tradition of making cider did not exist in Sweden for preconceived notions about the apples' quality. As pioneers, knowledge was to be found outside the cider community and with knowledge about wine, new ideas were born. After a couple of years of testing, it was concluded that the cold climate and the Swedish apples were suitable for production of ice cider.

The first 500 bottles of ice cider were released in 2012, and 2017 the vintage was 100 000 bottles. Not only ice cider, but also regular hard cider is produced. Last season, 170 tonnes of conventionally grown apples from Norrland to Skåne were pressed. The ice cider is sold all over Sweden and exported to a dozen countries. The beverages have won both national and international awards.

The mindset of Brännland is to never settle and search for new possibilities to improve. To develop the ice cider and strengthen the position on the market, regarding a unique product, a unique yeast could be beneficial for the product. By claiming the yeast as a product of their own, the ice cider catches an additional value for the consumers.

1.8 Aim and boundaries

The aim of this bachelor thesis was to examine the presence of yeast and bacteria in spontaneously fermented ice cider. Isolation and identification of present yeast to enable the direct usage of the yeast for production of ice cider.

2 Method

2.1 Sample preparation

Apples from different orchards were pressed in December 2018 and the juice was left to freeze outside. To extract the high brix juice, the juice was partially thawed repeatedly until 20-25% were extracted. The extracts were then stored in -18°C until start of fermentation.

Five juices were provided by Brännland Cider; Ingrid Marie, Cox's Orange, Kim, Lobo + Rubinola and Mutsu. They were incubated in sterile 100 ml Erlenmeyer flasks with aluminium foil on top at 25°C and swirled twice a day.

2.2 Selective media

The selective media for isolation of yeasts were yeast extract peptone dextrose (YPD; Yeast extract 10 gl^{-1}, peptone 20 g l^{-1}, dextrose g l^{-1}, agar 15 g l^{-1}) agar with chloramphenicol (100 mg l^{-1}). For bacteria, the media was tryptone glucose meat extract agar (TGEA, Merck, KGaA., Darmstadt, Germany) with delvocid (100 mg l^{-1}). For dilution series a saline solution NaCl 9 g l^{-1} was used.

Bacteria and yeast were cultivated on selective medium before, during and after fermentation. The TGEA plates, were divided into eight sections and on each section droplets of 10 µl were dropped from undiluted to 10^{-7} dilution before inoculation. During and after fermentation dilution from zero to 10^{-3} were cultivated. The TGEA plates were incubated in 30°C, 2-3 days.

For YPD the first and last cultivation were as droplets from ten times dilution to 10^{-8}, while samples from during fermentation were spread on separate plates from 10^{-4} to 10^{-9}, and then incubated in 25°C for 2-3 days. All cultivations were performed in triplicates both for YPD and TGEA.

2.3 Samples

During fermentation, samples were taken day six of fermentation, as well as at the end of fermentation. The end was defined as when no gas production was observed.

After incubation on YPD, re-streaks of five randomly selected colonies were performed. They were incubated in 25 °C for 2 days.

To ensure random selection, the colonies were numbered and if many colonies were on the plate, the plates were first divided into three sections, which in turn were chosen by a random generator from gallerit.se. Five of the numbered colonies were chosen by the generator and re-streaked.

2.4 Identification

Part of one colony were picked up with a sterile tooth pick, and dissolved in auto-claved water. The identification of yeast was performed by sequencing a PCR-fragment generated with the primers NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') for the region D1/D2 in the large ribosomal subunit (Kurzman and Robnett 1997). For amplification, DreamTaq Green 2x Master mix containing buffer, Taq, dNTPs and loading dye was also used.

The initial denaturation programme for the PCR was 94 °C 2 minutes, this was changed to 95 °C for 5 minutes, to break the cell walls. The PCR programme was initial denaturation 95°C for 5 min, denaturation 94°C for 30 s, annealing 50°C for 30 s, extension 72°C for 1 min, final extension 72°C for 10 min and hold at 16°C. The step denaturation to extension was repeated for 35 cycles.

Electrophoresis was performed with gel containing 1% agarose in 0.5xTBE with GelRed (Gelred (GelRedTM NucleicAcid Gel Stain, 10,000× in Water; Biotium, Hayward, USA) and the buffer 0.5xTBE (Tris ultrapure 53,91g l^{-1}, Boric acid 27,52 g l^{-1} and EDTA x Na_2 3,72 g l^{-1}).

The DNA fragments were sent for sequencing to Macrogen Europe (Amsterdam, Netherlands) and identified using BLAST on the databases National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Westerdijk Fungal Biodiveristy Institute's Pairwise sequence alignment (http://www.cbs.knaw.nl).

2.5   High-performance liquid chromatography (HPLC)

The machine used, Agilent 1100 (Agilent Technologies) combined with a Rezex-ROA-Organic Acid H+ (Phenomenex) colon. The colon was set to 60 °C and the mobile phase was 5 mM sulfuric acid with a flow of 0,6 ml/min. Juice samples were analysed concerning glucose, ethanol and methanol.

The standards for glucose, ethanol and methanol were mixed to the concentrations of 0.1, 1, 5 and 10 g l^{-1}. The samples were diluted ten times with 0.1 M HCl, centrifuged (13k x g for 60 sec) and filtered into the HPLC-vial via a 0.2 µl filter.

3   Results

3.1   Fermentation

After three days of fermentation all juices showed a visible sign of gas production. Ingrid Marie showed no sign of gas production after seven days of fermentation. Both Kim and Mutsu ended fermentation after 21 days. Lobo+Rubinola finished after 34 days, and Cox's Orange showed no gas production after further two more days.

All five juices showed an increase in yeast cfu during fermentation (Table 2) compared to the yeast cfu before fermentation (Table 1). The yeast cfu dropped when the fermentation was finished (Table 3). The bacterial cfu were only above the detection level in Cox´s Orange during the fermentation (Table 2). The seventh day of fermentation, Lobo+Rubinola showed a visible film on the flask and a precipitation.

Table 1. *Colony forming units (log cfu) of yeast and bacteria before start of fermentation.*

Juice	Yeast	Bacteria
Ingrid Marie	5.16 ± 0.12	< 4.00
Cox's Orange	$< 4.00^a$	< 4.00
Kim	4.30 ± 0.00	< 4.00
Lobo + Rubinola	4.20 ± 0.17	< 4.00
Mutsu	4.24 ± 0.34^b	< 4.00

[a]All three replicates below detection level (10 000 cfu/ml) $\log_{10} 4.0$. [b]n=2 only had to readable plates. Values are presented as mean (n=3) $\log_{10}$ cfu/ml $\pm$ std deviation.

Table 2. *Colony forming units (log cfu) of yeast and bacteria during fermentation.*

Juice	Yeast	Bacteria
Ingrid Marie	7.93±0.04	< 2.00[a]
Cox's Orange	7.36±0.02	2.26±0.24
Kim	7.99±0.07	< 2.00[a]
Lobo + Rubinola	7.38±0.05	< 2.00[a]
Mutsu	7.66±0.16	< 2.00[a]

[a]All three replicates below detection level (100 cfu/ml) $\log_{10}$ 2.0. Values are presented as mean (n=3) Log_{10} cfu/ml ± std deviation.

Table 3. *Colony forming units (log cfu) of yeast and bacteria after the end of fermentation.*

Juice	Yeast	Bacteria
Ingrid Marie	7.80±0.18	< 2.00[a]
Cox's Orange	5.65±0.16	< 2.00[a]
Kim	7.14±0.13	< 2.00[a]
Lobo + Rubinola	6.73±0.26	< 2.00[a]
Mutsu	< 2.00[a]	< 2.00[a]

[a]All three replicates below detection level (100 cfu/ml) $\log_{10}$ 2.0. Values are presented as mean (n=3) Log_{10} cfu/ml ± std deviation.

3.2   Identification of yeast species

A total of 25 yeasts were isolated and 20 were identified, see Table 4. In Cox Orange, *M. pulcherrima* and *M. rubicola* or *M. leonuri* were identified. The Lobo and Rubinola juice showed presence of *M. pulcherrima* and *P. membranifaciens*. Only *H. osmophilia* was identified in the Mutsu juice. *S. cerevisiae* colonies were isolated from the juice made of Ingrid Marie apples. There was no match for the sequences of Kim due to poor quality of the sequence.

Table 4. *Yeasts identified from the juices.*

Juice	Yeast
Ingrid Marie	*Saccharomyces cerevisiae* (5)
Cox's Orange	*Metschnikowia pulcherrima* (4) *Metschnikowia rubicola/ Metschnikowia leonuri* (1)
Kim	N.D
Lobo + Rubinola	*Metschnikowia pulcherrima* (4) *Pichia membranifaciens* (1)
Mutsu	*Hanseniaspora osmophila* (5)

Numbers indicate number identified of totally five colonies. N.D = Not detected.

3.3 High-performance liquid chromatography (HPLC)

See the results from the HPLC in table 5. The levels of glucose were reduced during the fermentation. The concentration of ethanol rose during fermentation. For all samples the highest concentration was measured when fermentation was finished with a range between 35 g l^{-1} and 84 g l^{-1}. Methanol was found in Ingrid Marie and Kim in low concentration.

Table 5. *Concentration of glucose, ethanol and methanol (g l^{-1})*

Juice	Glucose	Ethanol	Methanol
Ingrid Marie PF	110	-	-
Ingrid Marie DF	30	70	3
Ingrid Marie EF	20	75	3
Cox's Orange PF	120	-	-
Cox's Orange DF	110	7.8	-
Cox's Orange EF	72	69	-
Kim PF	91	-	-
Kim DF	55	37	5
Kim EF	13	84	2
Lobo + Rubinola PF	112	-	-
Lobo + Rubinola DF	85	8	-
Lobo + Rubinola EF	41	35	-
Mutsu PF	112	-	-
Mutsu DF	74	52	-
Mutsu EF	67	57	-

PF= Pre-fermentation, DF= During fermentation and EF= End of fermentation

4 Discussion

The aim of this bachelor thesis was to isolate and identify indigenous yeast of spontaneously fermented ice cider. The isolated yeasts could later be used as a starter culture, to ferment whole batches of ice cider. The yeast should be able to withstand high ethanol concentrations and complete fermentation in a feasible amount of time. The identified yeast species were *Metscnikowia pulcherrima, M. rubicola/ M. leonuri, Pichia membranifaciens, Hanseniaspora osmophila* and *Saccharomyces cerevisiae.*

The HPLC only measured levels of glucose, however fructose, sucrose and glucose are the main sugars that are present in apples. These are all fermentable sugars and that would explain the ethanol production. The ethanol production exceeded the theoretical limit if only glucose was present. (Ackermann, Fisher & Amando 1992)

4.1 Isolated yeasts and cfu

The colony forming units increased in all juices, compared to before fermentation (Table 1) and during fermentation (Table 3). The fermentation was conducted in temperatures fitting most yeasts, hence the increase. After fermentation, the number of cfu decreased in all samples. The CFU went below the detection limit in the Mutsu juice after fermentation (Table 3). The reason could be a too long time period between end of fermentation and spreading. The detection level of the spreading before start of fermentation was low due to small droplets and the lowest dilution spread was ten times diluted.

Isolated yeast from these spontaneously fermented apple ice ciders does not necessarily originate from the apples. It is possible that the sample has been contaminated. The contamination could have occurred during the pressing, storage or during the laboratory work.

4.1.1 *Metschnikowia pulcherrima*

M. pulcherrima is a species that has been found in numerous studies on spontaneously fermented wine (Clemente-Jimenez et al. 2014; Combina et al. 2005; Valles et al. 2007). It has an impact on the concentration of different aromas e.g. thiols, esters and terpenes. Single culture fermentation has shown an ethanol production of maximum 4% (w/v) (Zohre and Erten 2002). It also exhibited poor fermentation rate, leaving a high amount of residual sugar (ibid.). That study was, however, performed with regular grape juice and not a high brix juice. Table 5 shows low usage of glucose and low ethanol production in the early stages of fermentation, regarding the juices of where *M. pulcherrima* was isolated. This further indicates poor fermentation abilities of this yeast. However, in both juices after fermentation, see Table 5, the ethanol increased, possibly, due to growth of *S. cerevisiae*. The traits of *M. pulcherrima* gives an indication that it may not be suited for single culture usage in a fermentation of ice cider.

However, Lallemand has a FLAVIA™ on the market. FLAVIA is a pure culture of *M. pulcherrima* that has been isolated from nature in Chile. Lallemand claims that, together with an appropriate *S. cerevisiae* strain it should impact on the expression of terpenes and thiols due to its enzymatic activity. (Lallemand 2019) Eventually the isolate of *M. pulcherrima* from the Cox´s Orange or the Lobo and Rubinola juice could be used in a similar way.

4.1.2 *Metschnikowia leonuri* and *Metschnikowia rubicola*

M. leonuri only differs in a few nucleotides from *M. rubicola*, though enough to not be considered as a divergent member of *M. rubicola.* Such difference could make it difficult to tell them apart when only using one pair of primers for the enumeration step. *M. leonuri* differs from *M. rubicola* in sequences for ITS, EF-1α and RPB2. They are similar in the D1/D2 regions, hence it is problematic to distinguish them when only using D1/D2 primers. Both can ferment glucose and sucrose and have only little ethanol tolerance. These are two relatively newly described species, hence the small amount of information about them. (Kurtzman et al 2018)

It is therefore difficult to say if the species would be appropriate as a single culture in ice cider fermentation. To determine the strain, the PCR would be repeated with different primers.

4.1.3 *Pichia membranifaciens*

P. membranifaciens was isolated from the Lobo and Rubinola concentrates. During the isolation, ethanol concentration was lower than 1% (w/v) in the juice (Table 5).

P. membranifaciens belongs to the genus *Pichia*, which can be found on insects, soil, water and fruits (Santos et al. 2009). *P. membranifaciens* is regarded as an opportunistic species, that causes spoilage by mistakes in the manufacturing process (Stratford 2006). It is resistant against preservatives and tolerant to ethanol (Kurtzman et al 2011).

It produces a surface film, which is characteristic for spoilage yeasts in liquids with high sugar concentration. It gives a haze in the product. Off-flavours are also produced, with phenolic, ester and acidic undertones. (Stratford 2006)

Hence, it would seem inappropriate to use *P. membranifaciens* as a single culture, since it is a spoilage yeast.

One strain of *P. membranifaciens* has been shown to produce a killer toxin. That toxin has been linked to inhibit growth of *Brettanomyces* spp. in wine production. The same study did not find any *Saccharomyces* spp. that were sensitive to the toxin, allowing a possibility to direct use the toxin and still be able to ferment the product. (Santos et al. 2009)

4.1.4 *Hanseniaspora osmophila*

Hanseniaspora spp. is an often encountered genus in grape microflora (Giorello et al. 2019). *H. osmophila* (or *H. vineae* (Jolly et al. 2014)) have been found in studies regarding spontaneously fermented wine (Valles et al. 2007; Jolly et al. 2014). It is supposedly suppressed when the ethanol concentration increases (Assis et al 2014). The isolated *H. osmophila* were present in the Mutsu juice. The HPLC (Table 5) analysis shows the usage of glucose is relatively low and so is the production of ethanol. The isolated *H. osmophila* may not be of any use in a single culture inoculation to ferment apple ice cider.

However, studies have shown that wine produced with *H. osmophila* and *S. cerevisiae* could be of more complex sensorial attributes compared with wine yeast fermented wines (Giorello et al. 2019; Lleixà et al. 2016; Zhang et al. 2018).

In the study by Medina et al. 2013, a co-fermentation with *H. osmophila* and *S. cerevisiae* was conducted with the aim to study correlation between the use of *H. osmophila* and increase of aroma compounds. The study showed an increase of acetate esters and ethyl esters. A co-fermentation resulted in a 17-time increase in concentration of 2-phenylethyl acetate ester, which contributes with aromas such as fruity, flowery and honey. The increase was compared with fermentation with only *S. cerevisiae*.

Another study showed the same correlation between the usage of wine co-fermented with *H. osmophila* and *S. cerevisiae*. In the same study, a sensory analysis was performed in which a fruitier aroma was observed in the co-fermented wine

compared to single culture with *S. cerevisiae*. However, no differences were obtained regarding the overall quality of aromas. (Viana et al. 2009)

The *H. osmophila* isolated from the Mutsu juice could therefore be of interest for a co-fermentation regarding apple ice cider.

4.1.5 *Saccharomyces cerevisiae*

S. cerevisiae was isolated and identified in the Ingrid Marie juice. *S. cerevisiae* is commonly known not to be the dominant genus pre-fermentation. It vastly increases in cfu during the period of fermentation.(Assis et al. 2014)

As shown in Table 5, in a period of a week it seems that most of the glucose had been used. The ethanol concentration almost met the required levels for regulations of ice cider. This could give an indication of the usefulness of this yeast, since the fermentation rate and ethanol production seems reasonable. The impact of yeast strain could be of importance and affect the final product in terms of ethanol, aromas and fermentation rate (Erasmus, Cliff & van Vuuren 2004).

4.1.6 Kim

The sequences from isolates in Kim did not give any matches on the used databases, due to poor quality. The results from the gel electrophorese gave clear results on that the procedure went successful. It is difficult to point out what went wrong with the sequences. One possibility could be that the PCR contained more than one template. The PCR samples were transferred to Eppendorf tubes, it could be a possible source of contamination. The HPLC results in Table 5, indicates decrease of glucose and increase of ethanol. This is the desired outcome of usage of yeast in this fermentation. It is not possible to further describe any abilities of the indigenous yeast from the Kim juice.

5 Conclusion

The time to finish the fermentation varied 27 days between the fastest and slowest juice. In all juices, levels of glucose decreased while ethanol increased. Three out of the identified yeasts, *S. cerevisiae, H. osmophila* and *M. pulcherrima*, could suit the production of ice cider. Based on the literature study, these yeasts have the potential for production of ice cider.

Based on results from HPLC, the indigenous yeasts from Kim could meet the production requirements. However, further studies are required to determine the yeasts potential.

M. leonuri and *M. rubicola* need more specific sequencing to differentiate them, as well as more literature about the yeast are necessary to describe its usefulness in production.

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