Master Thesis

Department of Food Technology, Faculty of Engineering LTH

Author: Lucas Lissner

Supervisor: Åsa Håkansson

Examiner: Lars Nilsson

LUNDS UNIVERSITET

Non-alcoholic beer with maltose negative yeast strain Saccharomycodes ludwigii

Abstract

Non-alcoholic beer is gaining more attention in the brewing industry due to the negative health and economic consequences of alcohol consumption, even smaller microbreweries are showing interest in the production of non-alcoholic beer. Producing non-alcoholic beer has been restricted to the large breweries due to the special equipment needed and the high investment costs, but by using biological methods the process can be adapted to a traditional brewing setup. This involves using the special maltose negative yeast strain *Saccharomycodes ludwigii* and changed mashing step. The pure strain DBVPG 3010 has shown promising results in a screening study, producing high levels of desirable sensory compounds to mask the off-flavors usually a consequence of limited fermentation. This yeast will be compared with the commercially available yeast *S. ludwigii* WSL-17.

The aim of this master thesis is to develop a method for producing non-alcoholic beer customized for traditional brewery equipment and to test the produced beers in a sensory evaluation to judge the potential of the method. One of the primary objectives was to develop a method to produce a starter culture with adequate cell concentration and ensuring viability.

The results show that a suitable starter culture can be made using malt extract fortified with fructose with a magnetic stirrer and calculating cell concentration with a microscope and Burker chamber with methylene blue straining. The sensory evaluation gave positive results, from both expert panel and difference test, indicating that *S. ludwigii* DBVPG 3010 has high potential in small scale non-alcoholic beer production.

Preface

Non-alcoholic beer is get more popular as awareness increases about the negative health effects of alcohol consumption. Even smaller breweries, microbreweries are getting interested. Some challenges remain for the microbreweries when producing non-alcoholic beer, this master thesis aims to develop a method to produce non-alcoholic beer that is adapted to the equipment restrictions of a tradition brewing plant.

This master thesis has been made in cooperation with the microbrewery Lundabryggeriet AB, located in Torna Hällestad, Lund. The practical work has been done in the Department of Food Technology at The Faculty of Engineering, LTH.

Working with beer with a scientific approach has for a long time been a personal dream, and one of the reasons I picked this education. I want to thank the people involved in envisioning this dream:

Åsa Håkansson at the Department of Food Technology, for supervision, motivation, and a never-ending positivity.

Bo Bergenståhl at Lundabryggeriet AB, for raw materials, good talks, and brewer's perspective.

Elin Oscasson and Pamela Rosari Canaviri Paz at the Department of Food Technology, for always helping with everything.

Lars Nilsson at the Department of Food Technology, for helping with the sensory evaluation and feedback during the thesis.

Populärvetenskaplig sammanfattning

Användning av den maltosnegativa jäststammen *Saccharomycodes ludwigii* för tillverkning av alkoholfri öl, metodutveckling.

I detta projekt har en metod utvecklats som ger mikrobryggerier möjligheten att tillverka alkoholfri öl.

I takt med att fler blir medvetna om alkoholens negativa effekter, ökar intresset för alkoholfri öl, även hos väldigt små bryggeriet, så kallade mikrobryggerier. Då det krävs speciell utrustning och stora investeringar för att producera alkoholfri öl, har den stora majoriteten av mindre byggerier bara haft möjligheten att producera lättöl.

Metoden utnyttjar en speciell jäststam, *Saccharomycodes ludwigii*, som till skillnad från vanlig öljäst inte kan omvandla maltos till etanol. Eftersom maltos är den dominerande sockerarten i vört (maltbaserad sockerlösning som jäses till öl) kan man reducera alkoholhalten ner till en femtedel bara genom användandet av jästen. Genom att designa ett recept med mindre total sockermängd kan den slutliga alkoholhalten i ölet reduceras till under 0.5%. Två av de större utmaningarna med att använda denna speciella jäst är att det kan vara svårt att få jästen att växa och jäsa ordentligt och att det slutliga ölet blir för sött på grund av maltos som finns kvar.

Eftersom det är lite socker jästen kan använda för tillväxt är det viktigt att använda en starterkultur som har en tillräckligt hög cellkoncentration för en lyckad jäsning, det är också av vikt att ha en metod för att kunna kontrollera cellkoncentrationen och hur stor andel av cellerna som är levande. För att undvika att det slutliga ölet blir för sött kan man modifiera vissa av ölbryggningens steg, i detta projekt har mäskningen (här blandas vatten med krossad malt och sockret löses ut i vätskan under uphettning) modifierats för att minska bildningen av maltos. Istället främjar ändringen bildningen av längre kolhydratkedjor som är mindre söta än maltos och istället bidrar med smak och fyllighet till det slutliga ölet.

Resultaten visar att en ölbryggare med relativt enkla metoder och utrustning kan producera en starterkultur med kontroll över cellkoncentration och aktivitet som sedan kan användas för att jäsa ett alkoholfritt öl med bra sensoriska egenskaper.

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1. Introduction

1.1 Introduction

Beer is the most consumed alcoholic beverage in the world (Hager et al., 2014). Excessive consumption of alcohol is related, not only to negative health effects, but also substantial economic losses (Cdc.gov, 2018). The increasing awareness about the negative health effects of alcohol seem to increase the interest in low-alcoholic and non-alcoholic beer. This is starting to interest the smaller breweries, craft breweries. For some of the smaller breweries the equipment requirements confine them to make low-alcoholic beer (<2.25%) instead of non-alcoholic beer (<0.5%) (Systembolaget.se, 2018). The methods to produce alcohol free beer can be roughly divided into two groups, physical and biological methods, as seen in Figure 1.1:

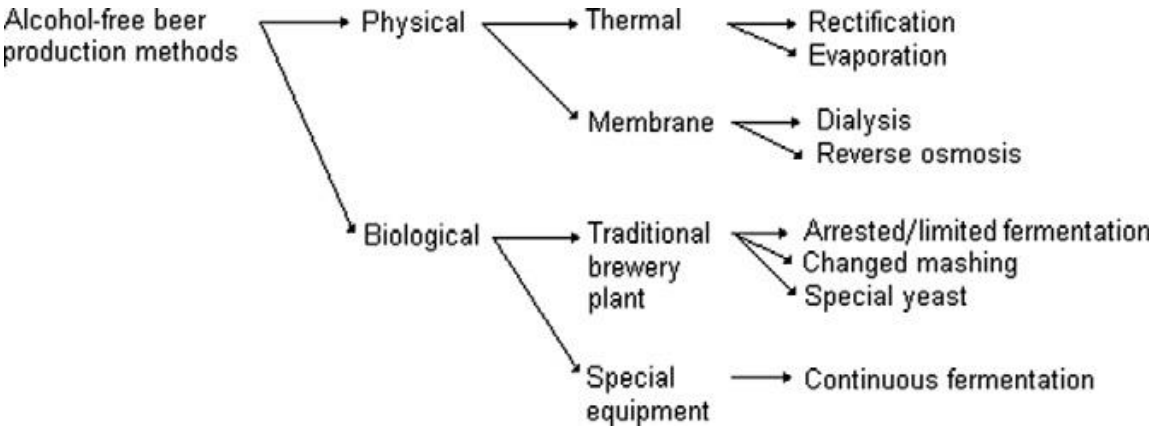


Figure 1-1. A breakdown of the different methods used to produce non-alcoholic beer (Brányik et al., 2012).

The physical removal methods are usually gentle and produce a beer with good sensorial quality, but these methods require special equipment and thus, extra investments (Brányik et al., 2012). On the other hand, the biological methods usually require less special equipment (except continuous fermentation) and therefore less investments. The downside of the biological methods is usually the reduction in sensory quality, especially related to worty off flavors and excessive sweetness that are the consequence of using limited or arrested fermentation (Brányik et al., 2012).

1.2 Aim and objectives

The aim of this master thesis is to develop a method for the production of a non-alcoholic beer that can be used by craft brewers with traditional brewing equipment. The project is made in cooperation with a local microbrewery, Lundabryggeriet AB (Torna-Hällestad, Lund), and will employ a combination of the two biological methods that can be used in a traditional brewing plant, changed mashing and special yeast. The special yeast is *Saccharomycodes ludwigii* and is used due to its complete or partial inability to convert maltose and maltotriose into ethanol (Brányik et al., 2012). Two different cultures of *S. ludwigii* will be used, WSL-17 (Hefebank Weihenstephan, Germany) and DBVPG 3010 (DBVPG Industrial yeast collection, Italy). WSL-17 is a commercially available yeast used to produce non-alcoholic beers. DBVPG 3010 is a pure strain culture used successfully in non-alcoholic beer experiments. One earlier project has been done in cooperation with Lundabryggeriet AB with a similar aim (Chiron et al., 2017). One problem they had, was to reach a sufficient cell concentration in their starter culture and thus, one the objectives of this project will be to develop a method to produce a starter culture with sufficient cell concentration. Different media, growth parameters and methods for

analyzing cell concentration will be investigated. The other objective is to compare the beers produced with each other and with beer from Lundabryggeriet AB in a triangle test and evaluated with an expert panel.

2. Theory

2.1 Beer Brewing

Beer is commonly based on barley or wheat malt. The grain is first allowed to sprout to activate the enzymes in the grain, the process, called malting, is then stopped by heating/drying (kilning). The grain is now called malt and can be roasted or toasted to produce darker or caramelized malts. This process is most often done before the grain arrives to the brewery. The malt contains a compartment called the endosperm that contains starch and some sugars. The malt needs to be milled to crack open the protective woody shell that covers the endosperm to release the starch, see figure 2-1. Commonly the milling is done in rollers, rough cylinders that crush the malt grains between them. To aid mashing and clarification of the wort the malt should not be crushed to finely (Barth, 2013).

Figure 2-1. A schematic chart of the beer brewing process not including the malting step (ABC News, 2018).

The crushed malt, also called grist, is mixed with heated water in the next step, called the mash in, and mixed thoroughly to expose the grist to water. The mashing steps then follows in three different part, gelatinization, liquefication and saccharification. Gelatinization occurs when the starch absorb water and swell. Second part is liquefication where long starch polysaccharides, amylose and amylopectin, are broken down into smaller parts, e.g. dextrins and maltotriose, increasing the solubility of starch in water. The process is catalyzed by one of the enzymes activated when the grains sprouted, alpha-amylase. The other important enzyme activated by

sprouting is beta-amylase and is active during the saccharification part where shorter polysaccharides are split into disaccharides, maltose (Barth, 2013).

Temperature choice during mashing determines the activity of the enzymes and thus the properties of the wort and the final beer. Higher mash temperature (63-70 ^{o}C) is the optimum for alpha-amylase, while lower temperature (55-65 ^{o}C) favors beta-amylose (Mosher and Tranham, 2017) [a]. A beer mashed at a lower temperature will have higher amounts of fermentable sugars and will contribute to a stronger and drier beer while mashing at a higher temperature will favor the formation of dextrins instead of maltose. This will be employed in this thesis, where the mash temperature will be held at 70 ^{o}C to reduce the formation of maltose and favor the formation of dextrins. Because maltose can't be fermented by *S. ludwigii*, the disaccharide will only contribute with unwanted sweetness. Further, maltose is sweeter than dextrin (Nutrients Review, 2018) while dextrins instead contribute to the body and mouthfeel of the beer (Mosher and Tranham, 2017) [a]. For this reason, a high amount of Carahell Malt or "Dextrin Malt" will be used for the recipe (see appendix 8.1). After the malting process (sprouting of barley grains) the Dextrin Malt is exposed to a "stewing" where the grains are exposed to moisture and starch conversion temperatures (66-71 ^{o}C). This starts the enzymatic activity and by choosing the right temperature the maltster can favor the formation of dextrins (Brew Your Own, 2018). Using this type of malt will increase the amount of dextrins in the wort and thus, further reduce the amount of maltose.

The mashing step is completed by a "mash out" at a higher temperature (>70 ^{o}C) that will denature the enzymes and stabilize the wort. The wort is then separated from the remaining malt. Brewers may use this step to clear the wort by circulating it though the grain bed. Hot water can then be sprayed on the grain bed to wash out more sugars (Barth, 2013).

In the next step, the wort is brought to a boil and hops are added to add bitterness, taste, and aroma to the beer. Depending on when during the boil the hops are added, different type of flavors will be added to the finished beer. An addition of hops in the beginning of the boil will contribute with bitterness, an addition midway through the boil will add the hops "taste" while a late addition close to the finish of the boil will add hop aroma. The boil is also used to sterilize the wort and to denature proteins. Finally, the hops are separated from the liquid in a whirlpool and the wort is cooled before fermentation (Barth, 2013).

2.2 Fermentation

All ethanol percentages (%ABV) mentioned in this report will henceforth have the unit volume ethanol per volume beer/liquid (v/v). The fermentation step will be the second crucial step for this project, by using the yeast *S. ludwigii* instead of the common brewer's yeast *Saccharomyces cerevisiae* (ales) or *Saccharomyces pastorianus* (lagers), the conditions of the fermentation is changed. The usual brewer's yeast can metabolize a range of sugars but the main sugars available to the yeast in wort are glucose, maltose and maltotriose. During aerobic conditions the brewer's yeast will metabolize glucose and oxygen into carbon dioxide and energy, the energy used for reproduction. With anaerobic conditions the yeast cells must use a different metabolic pathway that result in the production of ethanol, carbon dioxide and energy. This pathway does not yield as much energy and thus, yeast reproduction is limited to aerobic conditions (Mosher and Tranham, 2017) [b].

Strains of *S. ludwigii* have been shown to have a complete or partial inability to ferment maltose and maltotriose (Yang, L. et al., 2011). In 1992 Narziß et al. fermented 11.5 oP wort at 20 ^{o}C for 5 days and reported slow attenuation (rate of sugar consumption), the significant impact of wort acidification on the production of sensory by-products and the masking effect of these by-products on the usual wort-like off-flavors of beers produced by limited fermentation. They

further recommended the usage of a weaker wort, from 11.5 oP to 7.5 to get an ethanol content of 0.45%. A slight wort-like off-flavor was however constant.

In a study, six different strains of *S. ludwigii* and five different strains of *Zygosaccharomyces rouxii* were used in fermentation trials to determine their suitability for production of low-alcoholic beer (De Francesco et al., 2015). They used a wort with 12 kg of extracts/100 liters of wort which translates to 12 ^{o}P (Craft Beer & Brewing Magazine, 2018). Analysis were made on the production of ethanol and sensory by-products after fermentation at 20 ^{o}C in 10 days. The results showed that the *S. ludwigii* produces less ethanol and more of the favorable sensory by-products compared to *Z. rouxii*. They concluded that the strain *S. ludwigii* DBVPG 3010 was most suitable to produce alcohol-free beer, at least in micro-fermentations, and that further experiments should be made with the yeast, but at a larger pilot-scale. (De Francesco et al., 2015).

2.3 Starter Culture

When fermenting beer, it is important for both attenuation and sensory qualities to use a yeast culture of sufficient cell concentration. The amount of yeast cells added to the wort before fermentation is called pitch rate, in the unit cells/ml of wort. One earlier project, using the same yeast as in this thesis with the same aim, experienced troubles with fermentation. This was likely due to the quality if the yeast culture, in the aspect of pitching rate but maybe also due to contamination (Chiron et al., 2017). To archive a successful fermentation a pitching rate of around $6*10^6$ cells/ml seems to give satisfactory results (Jiang et al., 2017) (Wyeastlab.com, 2018). To reach the pitching rate the growth media needs to be considered. The cultures are delivered on YPD (yeast extract peptone dextrose) media and this was chosen as further growth media. The standard media contains glucose or dextrose, glucose was used for this thesis, but later exchanged for fructose to try increase the growth rate (Sohrabvandi et al., 2010).

2.4 Sensory Quality in Beer

When designing a beer recipe, it is important to consider the flavor profile of the finished beer. The different ingredients can contribute with different flavors, but one important flavor balance is between sweetness and bitterness. The malt adds body and fullness to the beer by the addition of dextrins but can also add sweetness (maltose) and toasty, caramelized, even bitter and burnt flavors, depending on the type of malting treatment. The other main ingredient is hops. One key role of hops is to add bitterness, taste and aroma to balance the sweetness from the malt. Using additions of roasted/scorched malt together with a small amount of hops is one way to combine the bitterness from both malt and hops to create a balanced beer, which is made in the case of stouts and porters. A beer made with little or no bitterness might be perceived as sweet and cloying while a beer made with too much hops might be perceived as harsh or thin.

The third ingredient that may affect the taste of the beer fundamentally is the yeast. The production of alcohols and other important sensory by-products will affect the flavor of the beer. The level of attenuation can affect the perceived sweetness of the beer, different types of yeast together with different fermentation temperatures may create different beer styles (ale and lager) and the amount of produced alcohols can contribute to creating different beers. Some of the more extreme cases include imperial stouts, barley wines and some Belgian strong ales, where ethanol percentages may reach more than 12%.

3. Materials and method

3.1 Culture purity and viability

Two cultures of *S. ludwigii* were used for this master thesis, DBVPG 3010 (Industrial Yeast Collection DBVPG, Italy) on slant agar and a commercially available culture, WSL-17 (Hefebank Weihenstephan GmbH, Germany) in a liquid culture. The two yeasts were plated on TSA (Sigma-Aldrich), VRBD (Sigma-Aldrich), ROGOSA (Sigma-Aldrich) and YPD (Sigma-Aldrich) agar with 0.1 ml of culture to determine purity and cell concentration.

All YPD media was made using the following recipe, 1000 ml:

10 g yeast extract

20 g bacterial peptone

20 g glucose/fructose

15 g agar (if used for plates)

1000 ml distilled water

From the YPD agar random colonies were picked and plated on new YPD agar plates to allow for cell harvest for DNA extraction, more than half of the colonies were picked. When growth was archived the plates were stored in a fridge (5 ^{o}C). The cells were harvested and suspended in autoclaved millQ water, the cells were then shaken for 30 minutes with glass beads to break the cell structure and finally the tubes were centrifuged and stored in a fridge until used. Later, the tubes were boiled to improve the extraction.

3.2 Polymerase Chain Reaction (PCR)

PCR is used to increase the DNA concentration in the extracted samples. This should improve the results when the samples are sent for sequencing, which is done to control strain purity of the cultures. A PCR-master mix is made with the following recipe per sample:

18.375 µl distilled water

2.5 µl Top Taq buffer

0.5 µl dNTP

0.5 µl primer 1

0.5 µl primer 2

0.125 Top Taq polymerase

Where the primer was either ENV1/ENV2 for bacteria or ITSF1/ITS4 and NL1/NL4 for yeast. The master mix was made on ice and before the reaction, 2.5 µl of extracted sample was added to the above recipe. The PCR was then run for 25-35 cycles and stored cold.

3.3 Gel electrophoresis

The gels were made with 0.75 grams of agarose and 50 ml of TAE-buffer, boiled 1 minute, and left to cool before being poured into gel tray with well combs in place. The gel is left to solidify in room temperature, 20 minutes. 2.5 µl of sample from PCR is mixed with 1 µl of loading buffer and pipetted into the wells of the gel, the electrophoresis is then run for 60 minutes.

3.4 Reaching sufficient pitch rate

The first trial was made using YPD media with glucose. 200 ml of media was inoculated with 1 ml of the original yeast samples. The WSL-17 directly from the original sample and the DBVPG 3010 from the first growth in liquid media, made directly after receival. These cultures are now called culture #1 and will be used for the future growth trials.

The next growth trial was made with 450 ml of YPD media containing fructose as sugar source. Inoculated with 50 ml of culture #1 and grown stationary for 96 hours in an incubator at 24 ^{o}C. The third growth trial was made with 450 ml of YPD media containing fructose as sugar source. Inoculated with 50 ml of culture #1 and grown with a stirrer plate (300 rpm) for 96 hours in room temperature (see section 4.2 for further information). Brix was monitored to follow the growth. YPD agar plates were used to determine the cell concentration (CFU) after each growth trial.

The agar plates do not seem to offer a good growing media for the yeast and a different method to determine the cell concentration was suggested. Using a Burker chamber for counting the cells gives instant results and can be used to closely follow the growth of a starter culture. With the use of methylene blue, the viability of the starter culture can be approximated. The methylene blue had to be ordered and thus, the results are without known viability. By taking samples during obvious active growth the assumption was made that all the cells visible were alive.

WSL-17 culture #1 used to inoculate two 400 ml liquid medias, liquid YPD with fructose and liquid malt extract reinforced with fructose (40 g dry malt extract (Muntons spray malt, Humlegården), 8 g fructose and 400 ml distilled water). These were grown with magnetic stirrer at 300 rpm for 18 hours, 400 ml of new media was then added to allow the growth to continue, but also to enable the sampling of actively growing culture. Two samples are taken from the active culture at two various places in the culture and 5 µl is pipetted from each into the Burker chamber. Four squares are counted, and an average value is calculated.

Using equation 1, the number of cells in the sample can be calculated. See appendix 8.2 for detailed calculations.

$$\frac{cells}{\mu l} = \frac{cells\ counted}{area\ counted\ (mm^2) * chamber\ depth\ (mm) * dilution\ of\ sample} \quad (1)$$

3.5 Brewing

Using the same ingredients as Lundabryggeriet's Redig Lättöl, Weyermann's German Pale Ale Malt Organic, German Carahell Malt Organic and German Sauer (Acid) Malt Organic. New Zeeland Hallertauer (Wakatu) hop pellets and Cascade hop pellets were used for the hop addition. Humlegårdens's Protafloc and Calcium Sulfate (Brewferm) was used as fining and water adjustment respectively. *S. cerevisiae* SafAle S-04 (Fermentis) dry yeast was used for the reference beer, for the other yeasts, see section 3.1. Water was taken directly from the tap.

For mashing and boiling, a 20 L Speidel Braumeister programmable brewery was used together with weighing scales, malt grinder, sampling equipment, Metrohm 744 pH meter, whisk and hydrometer. The mashing was according to the following steps:

Table 3.1. Mashing and boiling steps with times and temperatures.

Name	Description	Step Temperature	Step Time
Mash Step	Add 25,00 l of water at 70,0 C	70,0 C	70 min
Mash Step	Add 0,00 l of water at 75,0 C	75,0 C	10 min
Boil	Hops added 15 and 5 min before the end of the boil	102.0 C	70 min

See complete recipe in appendix 8.1

3.6 Fermentation

The wort was diluted with water to the desired original gravity 1.028 (7º Brix) and transferred while above 90 ºC to 12 cleaned (6 replicates for each yeast) 1 L glass bottles (IKEA) with plastic airlocks and cooled for micro fermentations, intended for ethanol measurements. The remaining 30 liters of wort was split on three 30 L plastic fermentation buckets with lids and plastic airlocks and cooled for pilot-scale fermentations. Before pitching the yeasts, the wort was brought to fermentation temperature, around 18 ºC, and vigorously shaken to oxidize the wort. The yeast was pitched according to the pitch rates in section 4.2 and fermented during 10 days at 18 ±1 ºC.

With the assumption that most of the carbon dioxide leaves during fermentation, the ethanol content could be calculated using equation 2, based on the change in specific gravity (a unit to measure the change in density of a liquid).

$$\%ABV = (OG - FG) * \frac{\frac{M(CH_3CH_2OH)}{M(CO_2)}}{\rho(CH_3CH_2OH)} * 100 = 131 - 25 * (OG - FG) \qquad (2)$$

Where $\%ABV$ is the percentage of alcohol in volume, OG and FG are the original and final gravities, $M(CH_3CH_2OH)$ and $M(CO_2)$ are the molar mass of ethanol and carbon dioxide and $\rho(CH_3CH_2OH)$ is the density of ethanol.

3.7 Bottling and pasteurization

The beer was transferred to 19 L kegs, pressurized with CO_2 and carbonated at 4 oC for 48 hours. Using a counter pressure bottle filler and a CO_2 supply, the carbonated beer was filled on 0.5 L sterilized glass bottles (in oven, 150 ºC, 120 minutes) and immediately capped. The bottles were pasteurized at 67 ºC in 8 minutes, cooled in an ice bath, labelled, and stored at 4 ºC. Maturation at 4 ºC for 4 days before sensory evaluation.

3.8 Sensory analysis

Two triangle tests were designed to compare the beer fermented with *S. ludwigii* with beer produced by Lundabryggeriet AB and a reference beer brewed with *S. cerevisiae* in the pilot hall at the faculty. All the beers included in the test were brewed using the same recipe, Lundabryggeriet's Redig Lättöl, see appendix 8.1

Some bottles were saved and delivered to Lundabryggeriet AB for evaluation by an expert panel, the comments can be seen in appendix 8.4. These are hedonic and discuss in depth the flavor aspects of the non-alcoholic beer, also in comparison with Lundabryggeriet's Redig Lättöl.

3.9 Statistical analysis

A sensory evaluation with two triangle tests was designed to compare three different beers. Test 1 contains the non-alcoholic beer and the Redig Lättöl brewed with the same equipment as the

non-alcoholic beer, in the pilot hall. Test 2 contains the non-alcoholic beer and the commercially available Redig Lättöl brewed by Lundabryggeriet AB. The results from the sensory evaluation were evaluated in Excel using chi-square distribution according to equation 3. The probability of 1/3 to pick the right sample by chance, was used to calculate the expected number of answers. For full calculation, see appendix 8.2. The null-hypothesis is that the participants can identify the different sample.

$$\chi^2 = \frac{(O-E)^2}{E} \quad (2)$$

Where *O* is the observed number of correct answers and *E* is the expected number of correct answers.

4. Results

4.1 Yeast culture purity and growth

The results from the agar plates were promising, no contaminations were found, further the viability seemed high. A colony forming unit (CFU) count was made on two plates after the cultures were grown for 24 hours at 25 ^{o}C in a shaking water bath, the average is showed in table 4.1.

Table 4.1. Cell concentration after 24 hours of growth using a shaking water bath in liquid YPD media with glucose.

Yeast	Dilution	CFU (average)	Cell concentration (CFU/ml)
DBVPG 3010	10^5	225	$2.25*10^7$
	10^6	28	$2.8*10^7$
WSL-17	10^6	301	$3.01*10^8$
	10^7	61	$6.1*10^8$

4.2 Starter culture

To reach a good pitch rate the yeast was cultured in liquid YPD media. The first trial was done using glucose as sugar source, the yeasts were plated on YPD agar media after 48 and 72 hours of stationary growth but this unfortunately gave no growth at all, maybe due to too much dilution and an insufficient inoculation (1 ml). A modification to the media was now made, changing the sugar source from glucose to fructose. The second growth trial was also made with longer growth time, the results can be seen in table 4.2. It should be noted that due to the low CFU of the results in table 4.2 and table 4.3, these results are not considered trustworthy, but merely an indication of the growth. To monitor the fermentation, Brix values were measured during growth.

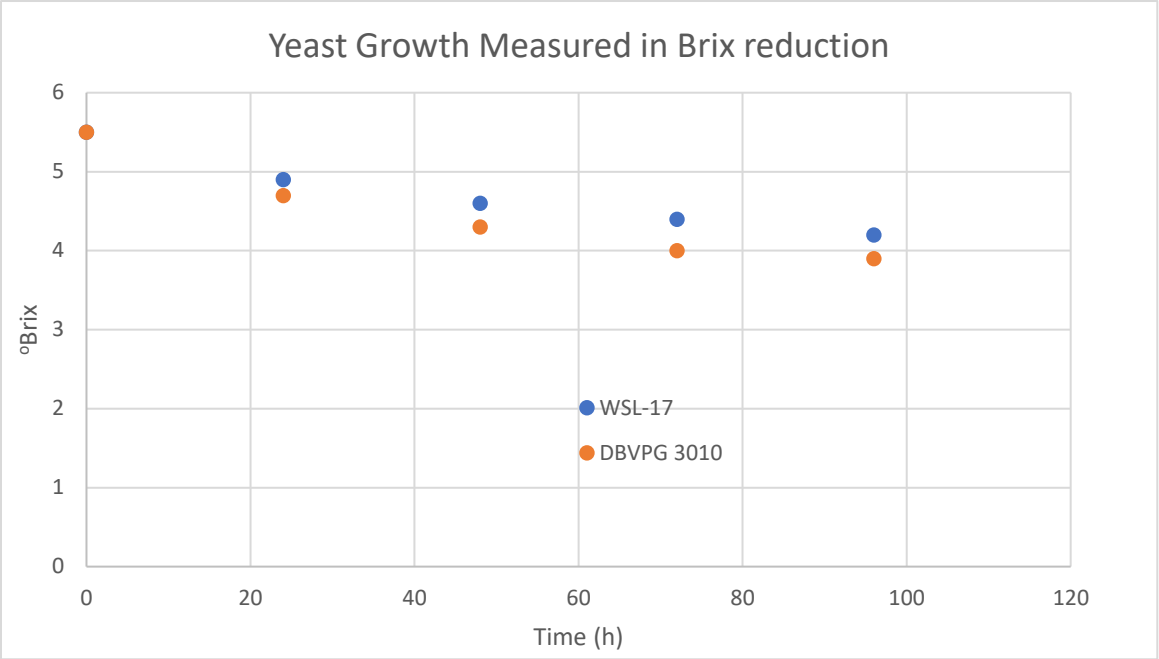


Figure 4-1. Showing the reduction in Brix to monitor the yeast growth over the course of 96 hours of stationary growth. The change in Brix is plotted against the time (h).

Table 4.2. Cell concentration after 96 hours of stationary growth using YPD agar media containing fructose.

Yeast	Dilution	CFU (average)	Cell concentration (CFU/ml)
DBVPG 3010	10^7	2.5	$2.5*10^7$
	10^8	0	0
	10^9	0	0
WSL-17	10^7	2	$2.0*10^7$
	10^8	1.5	$1.5*10^8$
	10^9	0.5	$5.0*10^8$

To increase the availability of oxygen to the yeast the cultures were now grown with a magnetic stirrer at 300 rpm. The previous cultures have all been grown in an incubator at 24 ºC, but the continuous stirring (and probably the increased growth rate) caused an increased temperature in the incubator with temperatures reaching above 30 ºC around the first 18 hours. The cultures were thus moved to room temperature where the temperature in the cultures stabilized around 23-24 ºC. The cultures were kept at these conditions for the remaining growth period. The first Brix measurement was made after 48 h, see results in figure 4.2. The following cell count can be seen in table 4.3.

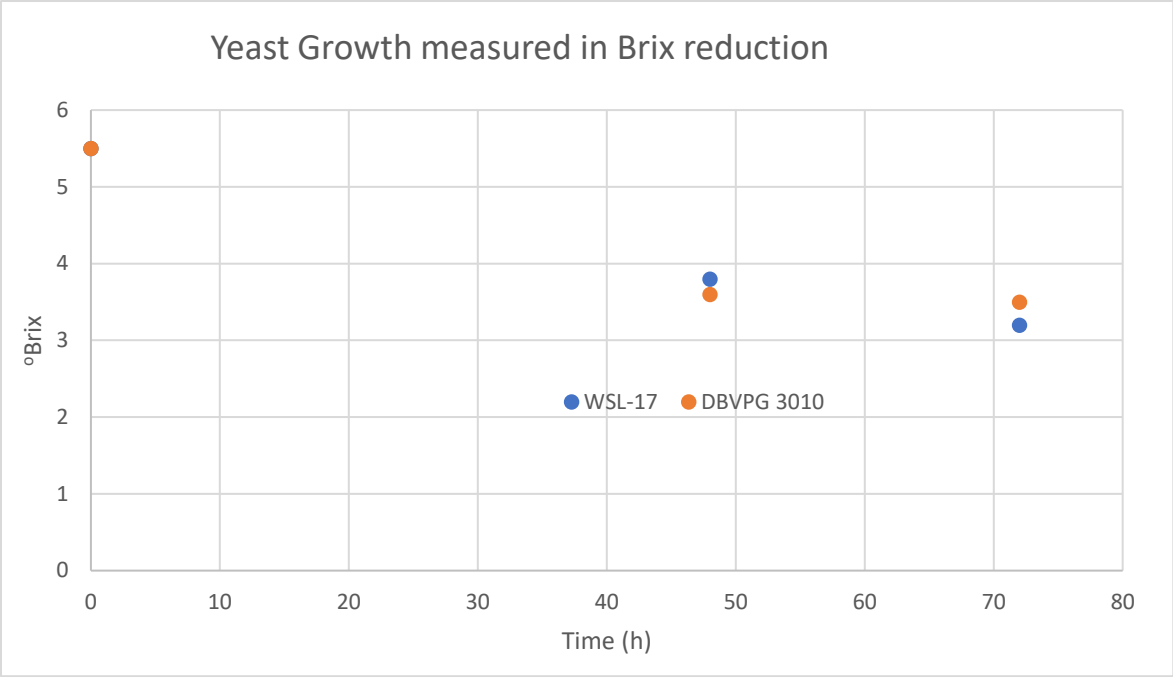


Figure 4-2. Showing the reduction in Brix to monitor the yeast growth using a magnetic stirrer for 72 hours. The change in Brix is plotted against the time (h).

Table 4.3. Cell concentration after 96 hours of growth using a magnetic stirrer and YPD media containing fructose.

Yeast	Dilution	CFUs (average)	Cell concentration (CFUs/ml)
DBVPG 3010	10^7	2.5	$2.5*10^7$
	10^8	0	0
	10^9	0	0
WSL-17	10^7	2	$2.0*10^7$
	10^8	1.5	$1.5*10^8$
	10^9	0.5	$5.0*10^8$

Using a Burker chamber with a microscope finally gave results showing the yeast growth. After 24 hours 200 ml of new media was added and after 18 more hours, 200 ml more. Four hours after the last media addition, the cell concentration of WSL-17 in malt extract media was approximately $1.6*10^9$ cells/ml while in YPD media, approximately $1.3*10^9$. The culture with YPD media was clumped together and the concentration in the Burker chamber varied strongly. This indicates two things, the samples needed to be diluted for future analysis, but also that the YPD media might make the yeast grow too fast. Flocculation might indicate that the fermentation is slowing and that the yeast might have lowered viability (Wyeastlab.com, 2018). It was decided to use malt extract for the final starter culture to give more control over the viability.

4.3 Pitch rate and fermentation

The above described method was repeated for both yeasts to make the starter cultures for the first fermentation trial with a few changes. The second addition of media was made after 18 hours and with 400 ml of media to increase the viability of the yeast at the time of pitching. The yeast can grow 4-5 hours until growth is apparent.

At the brew day this procedure was followed and concentrations from that count was as follows:

DBVPG 3010: $1.5*10^9$ cells/ml

WSL-17: $1.7*10^9$ cells/ml

Methylene blue was still not available and to compensate for the unknown viability the pitch rate was increased from $6*10^6$ cells/ml to $8.5*10^6$ cells/ml for WSL-17, and to $7.4*10^6$ cells/ml for DBVPG 3010.

Fermentation was monitored by measuring specific gravity and by day four, final gravity was reached at 1.024 for DBVPG 3010 and approximately 1.025 for WSL-17. The fermentation of WSL-17 failed or was infected and had to be discarded. Using the calculations in equation 2 this gives an ethanol content of 0.42 % for the beer made with DBVPG 3010. It should be noted that this method does not have high accuracy and a difference in ±0.001 might change the ethanol content to unacceptable levels (>0.5 %).

The micro fermentation tests seemed successful considering the change in specific gravity which followed the same pattern as the pilot fermentations. Unfortunately, no ethanol measurement could be done, because no machinery (gas chromatography) was available.

4.4 Sensory evaluation

The analysis showed that the null-hypothesis had to be rejected for test 1, the participants could not separate the non-alcoholic beer from the Redig Lättöl made with the same equipment. For test 2 the null-hypothesis could not be rejected, the participants could taste the difference between the non-alcoholic beer and Lundabryggeriet's Redig Lättöl.

Participants were asked to specify the reason for their choices and try to describe the difference between the samples. Some comments that were more common about the non-alcoholic beer include; sweeter, not as fruity, more/different aftertaste, a smell and taste of honey, more hoppy (aroma) and more bitter. They were also asked to pick a favorite sample out of all tried. In total, the non-alcoholic beer was the most common favorite. 14 out of 26 participants preferred the non-alcoholic beer, see appendix 8.3.

The expert panel found the non-alcoholic beer overall likable, with a slight sweetness but with an absent tone of dried fruit that did occur in Redig Lättöl, see appendix 8.4. They further commented on the higher bitterness and dryness in Redig Lättöl compare to the non-alcoholic beer. A difference in hop aroma was detected, both in taste and smell, and a worty smell was noticed in the non-alcoholic beer, probably due to the pasteurization.

5. Discussion

5.1 Original culture purity and viability

The first plate culturing indicated that both cultures were without infections and with high viability, unfortunately these were the first and only successful plate counts. The PCR and gel-electrophoresis experiments gave no results, probably due to faults in the chemicals used for the PCR. Unfortunately, nothing can be said about the strains of *S. ludwigii* in WSL-17 or the strain purity of DBVPG 3010.

5.2 Culture growth and pitch rate

The initial culture growth trials showed the advantage of using fructose as sugar source, in accordance with the results of Sohrabvandi et al. and the dependency of oxygen for growth. This indicates that to produce a culture with proper pitch rate these two factors are important. Using agar plates to grow *S. ludwigii* does not seem to be a good method for the determination of cell concentration. The results from these trials were inconsistent, even if growth was achieved. Using the Burker chamber counting method seems to be better suited for this yeast.

5.3 Fermentation

The fermentation went faster than expected, 4 days was needed for the reduction in specific gravity. Unfortunately, the fermentation with WSL-17 failed/was infected, the smell was (very) unappealing, especially when compared to the successful fermentation of DBVPG 3010. The fast fermentation is noteworthy because it is easier to incorporate into the usual brewing schedule of Lundabryggeriet AB, longer fermentation time would affect the production capacity/rate. The fermentation time is not in accordance with neither the results reported by De Francesco et al. (10 days) or Narziß et al. (5 days). The long fermentation times could be related to pitching rates, unfortunately unknown for the studies. It would be interesting to investigate the impact of pitch rates on both fermentation times and flavor of the beer. A higher pitch rate might shorten the fermentation time but perhaps also produce more desirable sensory by-products. The dependency of oxygen on cell reproduction was shown in the culture growth experiments and proper oxidizing the wort before fermentation might further increase the sensory qualities of the beer. Little literature exists about this yeast, unfortunately no more comparisons can be made with existing results.

The micro fermentations seemed successful based on the specific gravity measurements made on some bottles, but nothing can be said since no ethanol analysis could be made. For any further experiments, it is highly recommended to make sure that equipment is not only available but also functioning and fit for the application.

Ethanol content was instead calculated to 0.42 % based on the measured changes in specific gravity. Due to the low accuracy of that value, the ethanol content might be higher than 0.5 % and thus the beer is no longer considered legally non-alcoholic. This is an issue for this project, but a more serious problem for a micro-brewery because they might not have the funds to invest in equipment with the accuracy to ensure a legal non-alcoholic beer.

5.4 Sensory evaluation

Choosing Lundabryggeriet's Redig Lättöl as a reference beer has both benefits and downsides. Since the Redig Lättöl recipe is the basis for the non-alcoholic beer, comparing these two beers

(especially the Redig Lättöl made in the pilot hall) would show the difference the fermentation has on the sensory qualities of the beer. The difference in ethanol content is not the only reason, but also the difference in remaining sugars. The sugars not converted to ethanol remain in the beer, imparting sweetness and body. Some noticed this, but the result of the first triangle test show that statistically, there is no significant difference in taste. In comparison, participants could taste the difference between non-alcoholic beer and Lundabryggeriet's Redig Lättöl in the seconds triangle test.

The fact that these two triangle tests are different indicates that the difference in production (pilot scale vs micro-brewery) affects the taste significantly. That some people preferred the non-alcoholic beer is an interesting side note, the increased sweetness/body and "intensity" of the taste was preferred instead of the more light and dry reference beer. However, this is not a statistically significant result, but could be an indication that the increased body of the non-alcoholic beer might be used to create a beer with more intense flavor and definitively something to be considered for future works.

The downside of using the Redig Lättöl as a reference beer is if the brewer wants to produce a beer that can compete with regular beer (starköl). This could be one of the prospects of a non-alcoholic beer with a more intense flavor.

It should finally be noted that even though the inadequate accuracy of the ethanol content analysis is a factor that might affect the taste of the beer and should be considered when interpreting the results, the big downside is the legal aspect. If the brewery can't comply with the regulations, it does not matter how good the beer tastes.

5.5 Expert panel

The first note about the slight sweetness of the non-alcoholic beer was in accordance with the comments from the triangle tests. It is promising that the expert panel, just as many of the participants of the triangle test, perceived this as a positive sensory quality. This suggests, that instead of trying to counter the sweetness with further development, the beer can be developed around this sensory quality. As discussed above, this could be one way to compete with a regular beer.

The second note is about the difference in bitterness and dryness. Both these factors are probably due to the difference in sweetness, since sweetness will balance "mask" the bitterness. It is also suggested that this could be because of the difference in equipment and thus, possible difference in the yield of sensory compounds from the hops. The difference detected in hop aroma in smell and taste could also be related to this, but as suggested, might also be related to the different yeasts.

Finally, the worty smell, not clearly described by the triangle test participants (a few mentioned a "different smell"), which is likely due to the pasteurization, is an issue, but might be solved with better pasteurization process. No mention of a worty off-flavor was made, an improvement compared with what was reported by Narziß et al. Maybe this could be due to the increased production of desirable sensory by-production association with DBVPG 3010 (De Francesco et al., 2015).

6. Conclusions

One of the objectives of this project was to produce a starter culture with adequate cell concentration and the results show that with the right conditions, *S. ludwigii* can be made to reproduce rapidly with simple methods and equipment. The results show that using malt extract reinforced with fructose as media, with magnet stirrer plate, is the recommended method. Further, it is possible to easily control and ensure cell concentration and viability using a microscope with Burker chamber and methylene blue.

The sensory evaluation shows that offering consumers a non-alcoholic alternative to low-alcoholic beer should be possible using the yeast strain *S. ludwigii* DBVPG 3010. Due to the failed fermentation of WSL-17, further experiments should be conducted to compare the two different cultures. The expert panels comments were to an extent in line with the comments from the participant of the triangle test, with a few specific comments that could be related to the difference in the scale of the brewing.

More fermentation experiments should be made to investigate the impact of pitch rates and wort oxygenation on fermentation times and sensory qualities. Experiments should also be made on the beer recipe, eg. increasing bitterness to balance the increased sweetness/body of the beer. The effect of different types of hops could also be investigated, also suggested by the expert panel.

Hedonic sensory evaluation could be used to determine whether the panelists prefer the fullness of the non-alcoholic beer and to compare it with regular beers (starköl).

7. References

Articles

Brányik, T., Silva, D., Baszczyński, M., Lehnert, R. and Almeida e Silva, J. (2012). A review of methods of low alcohol and alcohol-free beer production. Journal of Food Engineering, 108(4), pp. 493-506.

De Francesco, G., Turchetti, B., Sileoni, V., Marconi, O. and Perretti, G. (2015). Screening of new strains of Saccharomycodes ludwigii and Zygosaccharomyces rouxii to produce low-alcohol beer. *Journal of the Institute of Brewing*, 121(1), pp.113-121.

Hager, A., Taylor, J., Waters, D. and Arendt, E. (2014). Gluten free beer – A review. *Trends in Food Science & Technology*, 36(1), pp.44-54.

Jiang, Z., Yang, B., Liu, X., Zhang, S., Shan, J., Liu, J. and Wang, X. (2017). A novel approach for the production of a non-alcohol beer (≤0.5% abv) by a combination of limited fermentation and vacuum distillation. *Journal of the Institute of Brewing*, 123(4), pp.533-536.

Narziß, L., Miedaner, H., Kern, E., and Leibhard, M. (1992) Technology and composition of non-alcoholic beers. Processes using arrested fermentation, Brauwelt Int. 4, 396–410.

Sohrabvandi, S., Razavi, S., Mousavi, S. and Mortazavian, A. (2010). Characteristics of different brewer's yeast strains used for non-alcoholic beverage fermentation in media containing different fermentable sugars. *Iranian Journal of Biotechnology*, 8(3), pp.178-185.

Yang, L., Hong, L., Jin-Hua, D. (2011). Non-alcoholic Beer Production by Saccharomycodes ludwigii[J]. Food Science, 32(15), pp. 186-190

Books

Barth, R. (2013). *The chemistry of beer*. 1st ed. Hoboken, New Jersey: Wiley, pp.17-31.

Mosher, M. and Tranham, K. (2017). *Brewing Science: A Multidisciplinary Approach*. 1st ed. Springer, a: pp. 176-177, b: pp. 303

Websites

ABC News. (2018). *Beer science*. [online] Available at: http://www.abc.net.au/news/2016-08-17/beer-science/7749158 [Accessed 30 May 2018].

Brew Your Own. (2018). *Dextrin Malts - Brew Your Own*. [online] Available at: https://byo.com/mr-wizard/dextrin-malts/ [Accessed 12 Feb. 2018].

Craft Beer & Brewing Magazine. (2018). *The Oxford Companion to Beer Definition: Plato gravity scale*. [online] Available at: https://beerandbrewing.com/dictionary/NpUFIRRVLp/plato-gravity-scale/ [Accessed 14 Feb. 2018].

Nutrients Review. (2018). *Sweeteners: Relative Sweetness, Calories, Glycemic Index - Nutrients Review*. [online] Available at: http://www.nutrientsreview.com/articles/sweeteners.html [Accessed 12 Feb. 2018].

Systembolaget.se. (2018). Om alkoholfri öl | Systembolaget. [online] Available at: https://www.systembolaget.se/fakta-och-nyheter/fakta-om-dryck/alkoholfritt/alkoholfri-ol/ [Accessed 12 Feb. 2018].

Wyeastlab.com. (2018). *Pitch Rates | Wyeast Laboratories*. [online] Available at: https://wyeastlab.com/pitch-rates [Accessed 27 Mar. 2018].

Wyeastlab.com. (2018). *Clarification / Flocculation | Wyeast Laboratories*. [online] Available at: http://www.wyeastlab.com/clarificationflocculation [Accessed 24 May 2018].

Reports

Chiron, C., Longoni, T., You, Q. and Yuan, Q. (2017). *Alcohol-free beer*. Lund: Lund University. Supervisor: Lars Nilsson

8. Appendix

8.1 Recipe and mash schedule

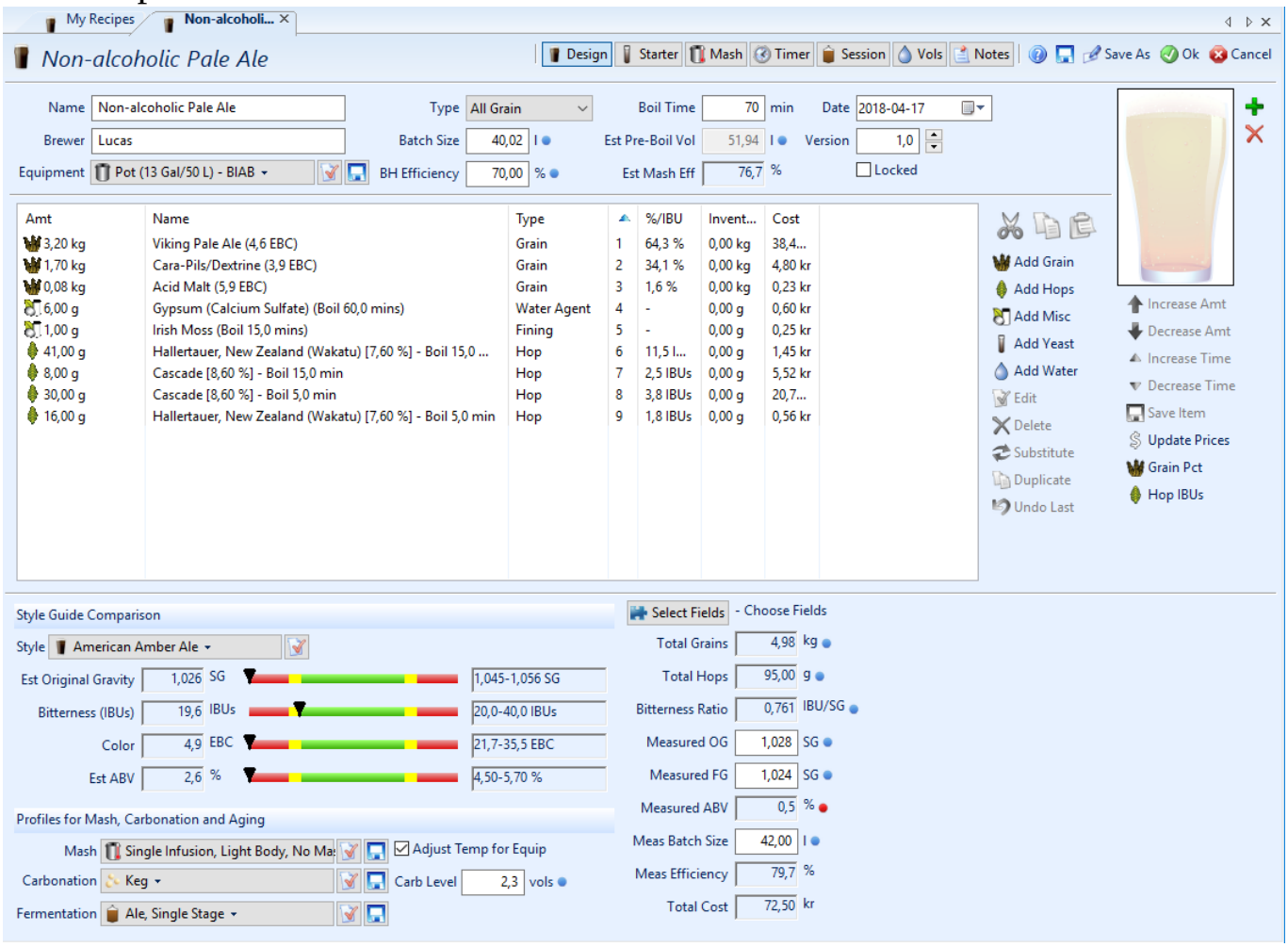
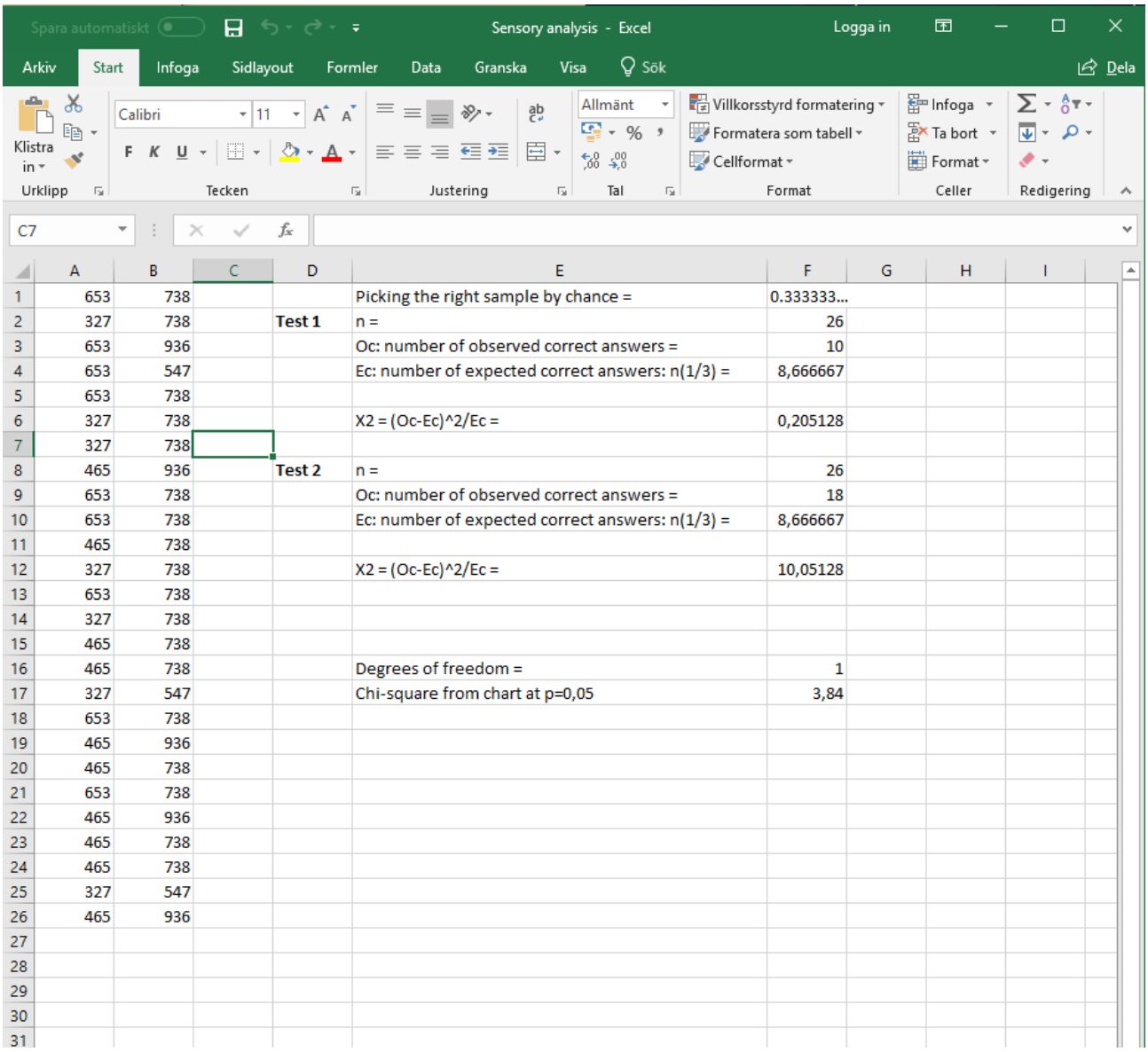


Figure 8.1. Showing the recipe and the mashing/boiling schedule in the program BeerSmith. It should be noted that malts in this recipe are different from the malts used, but only by name. It also shows the alcohol content as estimated by the program based on the reduction in specific gravity.

8.2 Calculations

Showing the full calculation for the last counting of DBVPG 3010 before pitching:

$$\frac{37}{0{,}0025\ (mm^2) * 0{,}1\ (mm) * 10} = 1{,}48 * 10^6\ \frac{cells}{\mu l} = 1{,}48 * 10^9\ \frac{cells}{ml}$$



	A	B	C	D	E	F	G	H	I
1	653	738			Picking the right sample by chance =	0.333333...			
2	327	738		**Test 1**	n =	26			
3	653	936			Oc: number of observed correct answers =	10			
4	653	547			Ec: number of expected correct answers: n(1/3) =	8,666667			
5	653	738							
6	327	738			X2 = (Oc-Ec)^2/Ec =	0,205128			
7	327	738							
8	465	936		**Test 2**	n =	26			
9	653	738			Oc: number of observed correct answers =	18			
10	653	738			Ec: number of expected correct answers: n(1/3) =	8,666667			
11	465	738							
12	327	738			X2 = (Oc-Ec)^2/Ec =	10,05128			
13	653	738							
14	327	738							
15	465	738							
16	465	738			Degrees of freedom =	1			
17	327	547			Chi-square from chart at p=0,05	3,84			
18	653	738							
19	465	936							
20	465	738							
21	653	738							
22	465	936							
23	465	738							
24	465	738							
25	327	547							
26	465	936							
27									
28									
29									
30									
31									

Figure 8.2. Shows the statistical analysis made in Excel. The results from test 1 are not statistically significant and the null-hypothesis has to be rejected, the non-alcoholic beer could not be separated from the pilot scale Redig Lättöl. The result from test 2 shows that the null-hypothesis can't be rejected and that the participants could differentiate the non-alcohol beer from the Redig Lättöl brewed by Lundabryggeriet AB.

8.3 Raw data from triangle test

Table 8.1. The complete result from the triangle tests. Each participant has tried two triangle tests, where the preferred samples are as follows: 1 = Redig Lättöl on brewed on pilot scale, 2 = non-alcoholic beer made with DBVPG 3010, 3 = Lundabryggeriet's Redig Lättöl, large scale.

Participant	Test	Correct answer	Comments	Preferred sample
1	1	Yes	Less sweet aromas, drier	2
	2	Yes	More bitterness, more hoppy aroma, sweeter	2
2	1	No	Less bitter	
	2	Yes	More intense flavor	2
3	1	Yes	Hoppy, almost a first taste of candy	1,2
	2	Yes	Fruity flavor, other tasted weird	2
4	1	No	-	
	2	Yes	It was different	2
5	1	Yes	Bitter, de andra var vattniga	
	2	Yes	Bättre smak	2
6	1	No	Not so bitter in the after taste like the others. Small difference in color	
	2	No	Not so sweet like the others	2
7	1	Yes	Less bitter	
	2	Yes	Less fruity	2
8	1	No	Smelled different and and tasted deeper, more "earthy"	2,2,1
	2	Yes	Lighter taste, not as fruity as the two others	
9	1	No	-	2
	2	Yes	-	
10	1	No	Slightly sweeter	2,1
	2	Yes	Much fresher taste	
11	1	Yes	Lite syrligare, lite mer smak (mindre blaskig lager-känsla), inte lika söt som de två andra.	
	2	Yes	Lite humligare (blommigare?), lite längre besk eftersmak, något mindre syrlig	
12	1	Yes	The aftertaste feels more prominent in the other two	
	2	No	The taste is more intense	3
13	1	No	Sweet sour taste, not as sour as the rest	2
	2	Yes	Bitter sweet taste, the other ones were more waterish	
14	1	No	Slightly more watery, more instant taste, a bit more acidic?	2
	2	No	A bit more bread taste, less watery/fuller taste	
15	1	No	-	
	2	No	-	
16	1	No	Bäst smak överlag, främst eftersmaken blommigare än de övriga	2
	2	No	Mest karaktär, mest smak	
17	1	No	More "watery" taste	
	2	Yes	Other initial taste and feeling	
18	1	No	Random	
	2	Yes	Random	
19	1	No	More bitter	
	2	Yes	More fruity	
20	1	No	-	
	2	Yes	-	
21	1	Yes	Different mouthfeel, slightly different smell. I associate with stale lager	
	2	No	Slightly different mouthfeel and perhaps a bit less sour/citrusy	
22	1	No	More malty taste, sweeter	
	2	Yes	Bit more sweet, rounder (?) taste	2
23	1	Yes	Higher bitterness and hop aroma, lower sweetness and not the smell of honey the other had	
	2	No	-II-	

24	1	Yes	Less body, slightly more acidic	
	2	No	Different aftertaste	
25	1	Yes	It tasted better	
	2	Yes	A bit sweeter	
26	1	No	Less strong taste	
	2	Yes	Different aftertaste	

8.4 Comments from expert panel

Cited from mail by Bosse Bergenståhl, in Swedish:

"*Alla noterade en låg sötma som fungerar bra i dina båda öl. Aromerna och smakintrycken från malten skiljer sig dock rätt mycket åt från vår jäst jämfört med den jäst du använder. I Redig Lättöl finns rätt mycket toner av torkad frukt som inte återfinns i de båda vi testade här i Hällestad.*
Alla noterade också att Redig är bittrare och torrare än de båda ölen du presenterade.

Vi noterade en viss vörtighet i doften från den pastöriserade ölen, denna fanns inte i den opastöriserade varianten. Frågan är var gränsen går för att undvika vörtighet vid pastörisering. Annars var ölet mycket behagligt.

Noterade dock att humlen framträder på ett annat vis i Redig, detta kan ju till viss del bero på vattenskillnader, men jag tror det mer är beroende av jästens olika karaktär, citrustonerna från cascadehumlen var svåra att förnimma i dina varianter, men vi får nog högre utbyte av både arom- och bitterämnen i vårt bryggverk med den kokare vi har.

Definitivt en jäst som är möjlig att använda i produktion dock. Frågan är dock vilken variant man ska välja.
Frågan är också hur olika humletyper och andra parametrar slår i ett alkoholfritt öl."